UNIVERSIDAD PRIVADA TELESUP

FACULTAD DE INGENIERÍA Y ARQUITECTURA

ESCUELA PROFESIONAL DE INGENIERÍA INDUSTRIAL Y COMERCIAL

TESIS

CONCENTRACIÓN DE DESINFECTANTES QUÍMICOS Y SU RELACIÓN CON LA HIGIENIZACIÓN DE LECHUGA EN EL LABORATORIO DE LA UNIVERSIDAD PRIVADA TELESUP 2018.

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DEDICATORIA

A Dios por haberme permitido llegar hasta este punto, además de su infinita bondad y amor.

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RESUMEN

La investigación tuvo como finalidad evaluar métodos de desinfección para lechuga (*Lactuca sativa*), por lo que el estudio se enfocó en evaluar por medio de análisis microbiológico la efectividad de los métodos de desinfección para hortalizas comercializados actualmente en el mercado nacional y partiendo de los resultados obtenidos se evaluaron métodos de desinfección químicos: químicos no convencionales y no químicos a diferentes concentraciones y tiempos de acción.

Para el desarrollo de la fase experimental de la investigación se tomó como punto de muestreo el mercado mayorista de Santa Anita y para evaluar la efectividad de los métodos de desinfección se realizaron análisis microbiólogos para determinar la reducción o inhibición de microorganismos tales como: Coliformes totales, Coliformes fecales, *Escherichia coli* (E. coli) y *Salmonella*.

Tomando los resultados de los análisis microbiológicos, así como los límites permisibles para el uso de métodos químicos de desinfección, según las fuentes bibliográficas consultadas, se procedió a determinar los métodos adecuados para desinfectar las hortalizas objeto de análisis en la investigación.

Palabras Claves: Concentración, desinfectantes químicos, higienización.

ABSTRACT

The purpose of the research was to evaluate disinfection methods for lettuce (*Lactuca sativa*), so the study focused on evaluating, through microbiological analysis, the effectiveness of disinfection methods for vegetables currently marketed in the national market and based on the results obtained chemical, non-conventional and non-chemical chemical disinfection methods at different concentrations and times of action were evaluated.

For the development of the experimental phase of the research, the Santa Anita wholesaler market was taken as a sampling point and to evaluate the effectiveness of the disinfection methods, microbiological analyzes were carried out to determine the reduction or inhibition of microorganisms such as: Total coliforms, Fecal coliforms, Escherichia coli (E. coli) and Salmonella.

Taking the results of the microbiological analyzes, as well as the permissible limits for the use of chemical disinfection methods, according to the consulted bibliographic sources, it was proceeded to determine the adequate methods to disinfect the vegetables object of analysis in the investigation.

Key Words: Concentration, chemical disinfectants, sanitization

ÍNDICE DE CONTENIDOS

ÍNDICE DE TABLAS

ÍNDICE DE FUGURAS

INTRODUCCIÓN

La investigación se desarrolló debido a que en la actualidad los cambios socioculturales han multiplicado la demanda de alimentos de consumo fácil y rápido. El factor determinante de las nuevas tendencias del consumo es el creciente interés por alimentos sanos, seguros, libres de aditivos, es decir, productos frescos o con características similares a los frescos y obtenidos de forma respetuosa con el medio ambiente. Si a esto, se añade el aumento en el poder adquisitivo, el resultado es una creciente demanda de frutas y hortalizas procesadas en fresco. Éstas necesitan un tiempo mínimo de preparación y poseen las mismas características nutricionales del producto entero del cual proceden.

En la actualidad se revisó la seguridad y potencialidad del ozono para la industria alimentaria y se presentó generalmente reconocido como seguro (GRAS) para las aplicaciones en contacto con los alimentos (U.S. FDA, 1997). Esta declaración de GRAS se sometió a la Food and Drug Administration (FDA).

Investigaciones y aplicaciones comerciales demuestran que el ozono puede reemplazar a desinfectantes tradicionales proporcionando otros beneficios. La ventaja más importante de este gas es que es efectivo a bajas concentraciones (0,01 ppm o menos) y durante períodos de exposición muy cortos. Este gas permite la reutilización, recirculación del agua empleada en el lavado de frutas y hortalizas tras una previa filtración. Elimina el color, olor y turbidez del agua al reducir las cargas orgánicas. La principal función que se le atribuye es su acción microbicida, inhibiendo bacterias como *Escherichia coli, Listeria monocytogenes* y otros patógenos alimentarios muchos más rápido que los desinfectantes tradicionales como el cloro que forma residuos químicos en el agua sobrante que recaen en el medio ambiente o la formación de compuestos cancerígenos como trihalometanos (THM) y cloraminas.

En términos generales éstas son las razones que han impulsado para realizar la investigación con el propósito de determinar si existe o no influencia entre las concentraciones en los diferentes desinfectantes químicos y la higienización de la lechuga

La hipótesis planteada fue las concentraciones de desinfectantes químicos se relacionan significativamente con la higienización de la lechuga.

El método general utilizado en la investigación fue el método científico.
El objetivo general planteado en la investigación fue determinar la relación de las concentraciones de desinfectantes químicos y la higienización de la lechuga.

El contenido del informe está estructurado en cuatro capítulos, de la siguiente manera:

CAPÍTULO I, Contiene el planteamiento del problema, abarcando la caracterización de la problemática, formulación del problema, objetivos de la investigación, justificación e importancia y delimitación del problema de la investigación

CAPÍTULO II, Guarda relación con el desarrollo del marco teórico, comprendiendo los antecedentes de la investigación, teoría científica que fundamente el estudio, y el marco teórico conceptual.

CAPÍTULO III, Abarca la parte metodológica de la investigación, en la que se incluye el tipo y nivel, el método y diseño de investigación, población y muestra, procedimientos de la investigación, técnicas e instrumentos de recolección de datos, técnicas de análisis y procesamiento de datos.

CAPÍTULO IV, Detalla el análisis e interpretación de los resultados las discusiones de la relación de las concentraciones de desinfectantes químicos y la higienización de la lechuga.

Finalmente se ha establecido las respectivas conclusiones y recomendaciones obtenidas.

I. PROBLEMA DE INVESTIGACIÓN

1.1. Planteamiento del problema

Las enfermedades transmitidas por alimentos (ETA) son producidas por la ingestión de alimentos o agua contaminados con agentes químicos o microbiológicos en cantidades tales que afectan la salud del consumidor a nivel individual o en grupos de población. La contaminación puede deberse a la deficiencia en el proceso de elaboración, manipulación, conservación, transporte, distribución o comercialización de alimentos y agua, las cuales pueden clasificarse en infecciones o intoxicaciones alimentarias sin incluir las reacciones de hipersensibilidad a los alimentos.

Las enfermedades de transmisión alimentaria abarcan un amplio espectro de dolencias y constituyen un problema de salud pública creciente en todo el mundo. Se deben a la ingestión de alimentos contaminados por microorganismos o sustancias químicas. La contaminación de los alimentos puede producirse en cualquier etapa del proceso que va de la producción al consumo de alimentos (“de la granja al tenedor") y puede deberse a la contaminación ambiental, ya sea del agua, la tierra o el aire.

La manifestación clínica más común de una enfermedad transmitida por los alimentos consiste en la aparición de síntomas gastrointestinales, pero estas enfermedades también pueden dar lugar a síntomas neurológicos, ginecológicos, inmunológicos y de otro tipo. La ingestión de alimentos contaminados puede provocar una insuficiencia multiorgánica, incluso cáncer, por lo que representa una carga considerable de discapacidad, así como de mortalidad. Fuente: Carga mundial de enfermedades de transmisión alimentaria: estimaciones de la OMS. 2015.

Una vez que el producto es cosechado, comienza de inmediato la senescencia, haciéndolo más sensible al deterioro microbiano. El grado y la velocidad del incremento de la población de microorganismos depende del producto y las condiciones de almacenamiento. El deterioro es realmente causado por sólo una pequeña proporción de la microbiota inicialmente presente y un tipo específico

de alteración se desarrolla bajo las condiciones normales de almacenamiento a temperaturas apropiadas. Los factores que influyen sobre la microbiota dominante determinan la clase de deterioro y son la contaminación inicial, las propiedades del sustrato, las condiciones ambientales y las características de los microbios.

Todos los vegetales poseen una microbiota residente que subsiste con pequeñísimas cantidades de carbohidratos, proteínas y sales inorgánicas disueltas en el agua exudada o condensada sobre la superficie del hospedante. Otros factores importantes son la contaminación a partir del suelo, el agua, los animales domésticos y salvajes, y la extensión del contacto durante la cosecha con las superficies sucias de las cosechadoras y contenedores.

La microbiota dominante sobre las hortalizas recién cosechadas es muy variable. Está constituida por bacterias gramnegativos como *Enterobacter, Pantoea y Pseudomonas*, pero las partes que crecen cerca o dentro del suelo contienen bacterias Gram positivas, por ejemplo, *Bacillus, Paenibacillus, Clostridium* y organismos corineformes. Sobre la superficie de algunas verduras se propagan los *Leuconostoc* y *Lactobacillus*. Muchos de estos organismos son pectinolíticos o celulíticos y originan el reblandecimiento característico de las podredumbres blandas, por ejemplo, *Pectobacterium carotovorum*.

Para asegurar la calidad e inocuidad de las hortalizas es necesario minimizar la contaminación de los productos con microorganismos patógenos que puedan afectar la salud del consumidor. A su vez, es de suma importancia, reducir al máximo el inóculo de patógenos vegetales que puedan afectar la calidad del producto durante el almacenamiento postcosecha.

Existen varios métodos para reducir la flora superficial de frutas y hortalizas. Cada método tiene ventajas y desventajas dependiendo del tipo de producto y del proceso. En general los métodos usados se basan en procesos físicos y/o químicos. Entre los métodos físicos podemos mencionar la remoción mecánica, los tratamientos térmicos, y la irradiación. Los métodos químicos involucran el uso de agentes químicos como desinfectantes superficiales.

1.2. Formulación del problema

1.2.1. Problema general.

¿Cómo se relacionan las concentraciones de desinfectantes químicos y la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup 2017?

1.2.2. Problemas específicos.

¿Cómo se relaciona la concentración de ácido láctico en la higienización de lechuga en el laboratorio de la Universidad Privada Telesup 2017?

¿Cómo se relaciona la concentración de ozono en la higienización de lechuga en el laboratorio de la Universidad Privada Telesup 2017?

¿Cómo se relaciona la concentración de hipoclorito de sodio en la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup 2017?

1.3. Justificación y aportes del estudio

La inocuidad de los alimentos es sumamente importante ya que impacta tanto la economía como la salud pública.

La falta de inocuidad en la producción y manipulación de alimentos es uno de los factores que da origen a la proliferación de enfermedades transmitidas por alimentos (ETA's), las cuales han ocasionado graves repercusiones en la salud e inclusive la muerte de una cantidad significativa de personas a nivel mundial.

Los problemas de inocuidad pueden también afectar la economía de los países latinoamericanos, dado que en la mayoría de estos las exportaciones de productos agrícolas, principalmente frutas y hortalizas, constituyen una parte importante de sus ingresos.

Por lo anteriormente descrito es importante enfatizar que el manejo de la inocuidad de los alimentos es un proceso igualmente importante que el manejo de la calidad de los mismos. Usualmente, se realiza a base de la aplicación de programas donde se integran:

La higiene, mediante la aplicación de buenas prácticas agrícolas y/o buenas prácticas de manufactura

La estandarización de procedimientos mediante la escritura de procedimientos operativos estándar de saneamiento

El control de los peligros. El control puede consistir, dependiendo de la medida de control que se use, en reducir, prevenir o eliminar los peligros

Cuando en la cadena alimentaria se ha identificado un peligro que necesita ser controlado y se tiene una medida de control para dicho peligro, es posible la aplicación de sistemas de aseguramiento de la calidad muy conocidos como: Buenas prácticas agrícolas (BPA), Buenas Prácticas de Manufactura (BPM), Análisis de Peligros y Puntos Críticos de Control (HACCP).

Actualmente la aplicación de sistemas de aseguramiento de la inocuidad en un elemento de competitividad y reconocimiento por parte de los consumidores, los cuales cada vez son exigentes en cuanto al cumplimiento de requisitos y estándares de calidad e inocuidad.

Por lo anterior, el propósito de este estudio es evaluar la eficiencia de desinfección de tres sustancias en coliformes presentes en hojas de lechuga (*Lactuca sativa*). Nosotros esperamos que los desinfectantes a base de ozono, que es uno de los desinfectantes menos utilizados, en comparación con el cloro y el ácido láctico, sea la que presente la mayor eficiencia para eliminar *Escherichia coli* y *Salmonella sp* en muestras crudas de lechuga.

1.4. Objetivos de la Investigación

1.4.1. Objetivo general.

Determinar la relación de las concentraciones de desinfectantes químicos y la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup 2017.

1.4.2. Objetivos específicos.

- Determinar la relación de la concentración de ácido láctico en la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup 2017.
- Determinar la relación de la concentración de ozono en la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup 2017.
- Determinar la relación de la concentración de hipoclorito de sodio en la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup 2017.

II. MARCO TEÓRICO

2.1. Antecedentes de la investigación

2.1.1. Antecedentes nacionales.

Maldonado O. (2014). “Efecto del tiempo de exposición con ozono gaseoso y tiempo de almacenamiento sobre las características fisicoquímicas, recuento de mohos y levaduras y aceptabilidad general de arilos de granada (*Punica Granatum L*.) mínimamente procesada”.

Se evaluó el efecto del tratamiento de ozono gaseoso y tiempo de almacenamiento sobre las características fisicoquímicas, recuento de mohos y levaduras y aceptabilidad general en arilos de granada mínimamente procesada. Se llegó a las siguientes conclusiones:

Se determinó efecto significativo del tratamiento de exposición a ozono gaseoso y tiempo de almacenamiento sobre las características fisicoquímicas, recuento de mohos y levaduras y aceptabilidad general en arilos de granada mínimamente procesada.

El tratamiento con 20 minutos de exposición a ozono gaseoso presentó el mayor color (L*, a*), contenido de antocianinas totales; así como, la menor pérdida de peso, contenido de solidos solubles en arilos de granada mínimamente procesada.

El tratamiento con 20 minutos de exposición a ozono gaseoso mostró el menor recuento de mohos y levaduras a los 12 días de almacenamiento a 5 °C en arilos de granada mínimamente procesada.

El tratamiento con 20 minutos de exposición a ozono gaseoso obtuvo la mayor aceptabilidad general en arilos de granada a los 8 días de almacenamiento.

Segura M. (2013). “Efecto del ozono en las características fisicoquímica, microbiológica y colorimétrica de la lechuga (*Lactuca sativa L*.) mínimamente procesada’’. Universidad Nacional del Centro del Perú. Facultad de Ciencias Aplicadas. Escuela Académico Profesional de Ingeniería Agroindustrial. Tesis para optar el título profesional de Ingeniero Agroindustrial.

La investigación tuvo como objetivo evaluar la influencia de las concentraciones en los diferentes tiempos de contacto de agua ozonizada en las características fisicoquímicas, microbiológicas y colorimétricas de la lechuga mínimamente procesada. Llegó a las siguientes conclusiones:

1. La lechuga fresca tuvo un contenido de humedad de 95.02%, pH 6.29 y % de acidez titulable de 0.1229 (expresado en ácido cítrico).
2. La lechuga fresca tuvo una carga microbiana de coliformes totales 8.0 x 102 UFC/g, mohos 4.3 x103UFC/g y levaduras de 4.3 x103UFC/g.
3. El mejor tratamiento optimo según el desarrollo experimental fue el t2 que fue a una concentración de un 1ppm por 9 min. ya que cumplió con una mayor cromaticidad C* (32.12) y una menor coordenada a* (-10.76) frente a todos los tratamientos planteados, siendo estos últimos importantes para la pureza del color verde y a la vez cumplió con todos los requisitos microbiológicos según la norma técnica peruana Nº071-MINSA/DIGESA, 2008.
4. La composición del % de humedad y fisicoquímica del tratamiento óptimo (t2) a una concentración de un 1ppm por 9 min es de: humedad 96.27%; pH 6.63 y de acidez titulable 0.0825%. En el recuento microbiológico fue de: coliformes totales <10 UFC/g; mohos <10 UFC/g y levaduras <10 UFC/g. En la característica colorimétrica fue de: cromaticidad (C*) 32.12 y la coordenada a* -10.76.
5. La caracterización del % de humedad y fisicoquímica del tratamiento óptimo (t2) a una concentración de un 1ppm por 9 min. almacenados a 5 °C fue de: humedad a los cero días 96.27%, al quinto día 95.27 % y al décimo día 91.20 %; pH a los cero días 6.63, al quinto día 6.63 y al décimo día 6.85 y en él % de acidez titulable a los cero días 0.0825%, al quinto día 0.065% y al décimo 0.0591%. En el recuento microbiológica fue de: coliformes totales a los cero días <10 UFC/g al quinto día <10 UFC/g y al décimo día <10 UFC/g; mohos a los cero días <10 UFC/g, al quinto día <10 UFC/g y al décimo día 3.0 x 102UFC/g y levaduras del cero al día 10 fue <10 UFC/g. En la característica colorimétrica fue de: cromaticidad (C*) a los cero días 32.12, al quinto día 33.86 y al décimo día 35.79 y coordenada a* a los cero días -10.76, al quinto día -11.54 y al décimo día -11.45 respectivamente.

Muñoz S. (2005). “Frecuencia de enterobacterias en verduras frescas de consumo crudo expendidas en cuatro mercados de Lima Metropolitana”. Universidad Nacional Mayor de San Marcos. Tesis para optar el título profesional de Médico Veterinario. Lima. Perú,

Luego de realizar el presente estudio cuya finalidad fue analizar el grado de contaminación fecal mediante la determinación de coliformes fecales y *E. coli* Tipo I (Típico), así como evaluar la presencia de *Salmonella*, en tres de las verduras de mayor consumo crudo en nuestro medio, es decir la lechuga (*Lactuca sativa*), la col (*Brassica oleracea*) y la espinaca (*Spinacea oleracea*), al momento de ser expendidas en los mercados mayoristas más importantes (4) de Lima Metropolitana y adquiridas por el consumidor, se llegó a las siguientes conclusiones:

1) Los mercados 1 y 3 presentaron los mayores porcentajes (22.2% y 64.4%) de verduras contaminadas con coliformes fecales, en niveles que excedieron los límites máximos recomendados según la ICMSF (100) y el MINSA (10), respectivamente.
2) La espinaca fue la verdura que más excedió los límites máximos recomendados para coliformes fecales establecidos por la ICMSF y el MINSA, seguida de la lechuga y finalmente la col.
3) En el 18.9% y 56.7% del total de verduras muestreadas en el presente estudio, se detectó la presencia de coliformes fecales en niveles que excedieron los límites máximos recomendados según la ICMSF (100) y el MINSA (10), respectivamente.
4) El mercado 4 presentó un 2.2% de verduras contaminadas con *E. coli* Tipo I (Típico) en niveles que excedieron el límite máximo recomendado según la ICMSF y el MINSA (10). Dicho porcentaje correspondió a una muestra de espinaca.
5) En el 0.6% del total de verduras muestreadas en el presente estudio, se detectó la presencia de *E. coli* Tipo I (Típico) en niveles que excedieron el límite máximo recomendado según la ICMSF y el MINSA (10).
6) El mercado 2 presentó el mayor porcentaje de verduras contaminadas con *Salmonella spp* (20%).
7) La verdura más contaminada con *Salmonella spp*. fue la col, seguida de la

espinaca y finalmente la lechuga.

8) El 10% del total de verduras muestreadas en el presente estudio estuvieron contaminadas con *Salmonella spp*.

Los mercados 3 y 4 fueron los que presentaron las verduras con el mayor porcentaje de aceptabilidad total; mientras que el mercado 2 obtuvo el mayor porcentaje de verduras con la calificación de rechazable. El mercado 4 fue el único que presentó un porcentaje de verduras con la calificación de aceptabilidad provisional.

2.1.2. Antecedentes internacionales.

Baptista J. (2010).” Respuesta de distintos cultivares de mini lechuga (*Lactuca sativa L*.) a diversas fisiopatías y a la acumulación de nitratos en hojas durante tres ciclos, con tres soluciones nutritivas y en dos modalidades de cultivo”. Universidad Politécnica de Valencia. Tesis para optar el grado de Master en producción vegetal y ecosistemas agroforestales. Valencia. España.

Conclusiones.

Como era previsible existen diferencias entre cultivares para el peso medio de las piezas comercializables de minilechugas, de manera que en todos los ciclos, el mayor valor lo obtuvo Cherry en coincidencia con lo observado en otros trabajos (Ferriol et al., 2009). En términos generales también se observó que este parámetro era menor con la solución menos rica en N (S1), aunque este efecto sólo se manifestó de manera clara en el segundo ciclo (ciclo 2).

En cultivo bajo invernadero y en todos los ciclos ensayados, el calibre fue mayor que al aire libre, lo que podría explicarse a causa de la mayor integral térmica propiciada por el invernadero, lo que induciría un mayor desarrollo. En los ciclos 2 y 3 se constató una interacción solución x modalidad de cultivo por la que, al aire libre, el mayor peso medio de las plantas comercializables se obtenía con la solución con un mayor contenido en nitratos (S3). En esta modalidad de cultivo (aire libre) en el que la incidencia de fisiopatías fue menor, se registraron los mayores valores de producción comercial y porcentaje de plantas comerciales.

Tanto en invernadero como al aire libre los menores pesos medios comercializables se dieron en el ciclo 1 y los mayores en el ciclo 3, lo que redundó en una menor producción comercial en el ciclo 1 respecto a los ciclos 2 y 3, aunque tanto en este parámetro como en el porcentaje de plantas comerciales se detectó una interacción entre ciclos y modalidad de cultivo de forma que las diferencias eran mayores en invernadero. Al margen de la hipotética influencia de los distintos factores ambientales, debe reseñarse que las plantas trasplantadas en el ciclo 1 adolecían de falta de uniformidad y eran de menor vigor que las de los ciclos 2 y 3, por problemas de manejo en la fase de semillero en bandejas.

Como ya ha sido constatado en estudios anteriores, la susceptibilidad varietal es un factor muy importante en el desarrollo de las distintas fisiopatías estudiadas (Maroto, 1997) y en el caso particular del “tipburn” ha sido corroborado en otros tipos de lechuga (Ryder and Waycott, 1998; San Bautista et al., 2003).

En estos experimentos y en los tres ciclos estudiados, especialmente en los dos últimos, Cherry se ha mostrado como el cultivar menos susceptible a esta fisiopatía, mientras

Como era previsible existen diferencias entre cultivares para el peso medio de las piezas comercializables de minilechugas, de manera que, en todos los ciclos, el mayor valor lo obtuvo Cherry en coincidencia con lo observado en otros trabajos (Ferriol et al., 2009). En términos generales también se observó que este parámetro era menor con la solución menos rica en N (S1), aunque este efecto sólo se manifestó de manera clara en el segundo ciclo (ciclo 2).

En cultivo bajo invernadero y en todos los ciclos ensayados, el calibre fue mayor que al aire libre, lo que podría explicarse a causa de la mayor integral térmica propiciada por el invernadero, lo que induciría un mayor desarrollo. En los ciclos 2 y 3 se constató una interacción solución x modalidad de cultivo por la que, al aire libre, el mayor peso medio de las plantas comercializables se obtenía con la solución con un mayor contenido en nitratos (S3). En esta modalidad de cultivo (aire libre) en el que la incidencia de fisiopatías fue menor, se registraron los mayores valores de producción comercial y porcentaje de plantas comerciales.

Tanto en invernadero como al aire libre los menores pesos medios comercializables se dieron en el ciclo 1 y los mayores en el ciclo 3, lo que redundó en una menor producción comercial en el ciclo 1 respecto a los ciclos 2 y 3, aunque tanto en este parámetro como en el porcentaje de plantas comerciales se detectó una interacción entre ciclos y modalidad de cultivo de forma que las diferencias eran mayores en invernadero. Al margen de la hipotética influencia de los distintos factores ambientales, debe reseñarse que las plantas trasplantadas en el ciclo 1 adolecían de falta de uniformidad y eran de menor vigor que las de los ciclos 2 y 3, por problemas de manejo en la fase de semillero en bandejas.

Como ya ha sido constatado en estudios anteriores, la susceptibilidad varietal es un factor muy importante en el desarrollo de las distintas fisiopatías estudiadas (Maroto, 1997) y en el caso particular del "tipburn" ha sido corroborado en otros tipos de lechuga (Ryder and Waycott, 1998; San Bautista et al., 2003).

En estos experimentos y en los tres ciclos estudiados, especialmente en los dos últimos, Cherry se ha mostrado como el cultivar menos susceptible a esta fisiopatía, mientras que Etna se comportó como el cultivar más proclive al desarrollo de "tipburn", lo que coincide con lo observado en otros experimentos (Torres et al., 2009a)**.**

Velázquez K. (2011)." Balance de macronutrientes en el sistema Agua, suelo y cultivo sometido a riego" Universidad Nacional Autónoma de México. Tesis para obtener el grado de maestría en Ingeniería Ambiental-Agua. México.

Conclusiones.

Al caracterizar el suelo, antes de ser irrigado por los diferentes tipos de agua, la concentración de P, Mg y Ca es baja. Sin embargo, la concentración es media de N y K de acuerdo con las propiedades de fertilidad que debe contar un suelo (NOM021-SEMARNAT-2000). Así también, el suelo presenta alto porcentaje de materia orgánica lo cual es importante para la retención de humedad.

Que el contenido de macronutrientes en el agua residual tratada no presenta variaciones importantes. Por lo tanto, el sistema aplicado de tratamiento no afecta

el contenido de nutrientes, lo que significa que el agua tratada mantiene además de que cumple con la calidad microbiológica requerida, e implica que existe más disponibilidad de estos nutrientes en el suelo que pueden contribuir a mejorar la productividad de los cultivos.

Que en cuanto al balance de masa en el sistema agua, suelo y cultivo la concentración de Ca y Mg, en todos los tipos de agua que se emplearon hay una acumulación en el suelo; en el caso de la concentración del P hay pérdidas en el suelo final debido a la baja concentración inicial en los diferentes tipos de agua; la concentración de K permaneció casi constante. Al comparar el agua potable con nutrientes y agua residual tratada la concentración de N en el suelo presenta una acumulación en ambos casos, lo que significa que adicionan nutrientes al igual que un agua potable a la que se le añadió fertilizante comercial.

Que, al evaluar el efecto de los diferentes tipos de agua de riego en el contenido de macronutrientes en el suelo y tejido vegetal, el cultivo irrigado con agua potable con nutrientes mostró mayor contenido de P con un 64.70% en comparación con el agua residual tratada. El contenido de N y K en el cultivo irrigado con agua residual cruda y TPAO3 + F + Desinfección no hay variaciones, lo que indica que es una ventaja irrigar con aguas residuales tratadas, ya que el contenido nutricional se conserva en la lechuga italiana (Lactuca sativa L) de acuerdo a la hipótesis que se propuso en este trabajo el agua residual tratada sí aportó al suelo los macronutrientes necesarios suelo para el crecimiento del cultivo lechuga (Lactuca sativa L).

Salinas C. (2013). “Introducción de cinco variedades de lechuga (Lactuca sativa L.) En el barrio Santa Fe de la Parroquia Atahualpa en el cantón Ambato”. Para optar el título de Ingeniero Agroindustrial. Ambato. Ecuador.

Conclusiones.

La variedad de lechuga HM 1 (V1), fue una de los que mejores resultados reportó en las condiciones ambientales de la parroquia Atahualpa del cantón Ambato, al presentar el mayor crecimiento en altura de planta a los 45 días del trasplante (18,89 cm), como también a los 60 días (19,59 cm). Los repollos reportaron el mejor

desarrollo en diámetro ecuatorial (17,28 cm), consiguiéndose así mismo el mayor peso (307,98 g), ubicándose en la escala de solidez de muy sólidos, siendo éstos muy compactos y muy consistentes, por lo que reportó los más altos rendimientos (18,31 tm/ha), siendo la variedad que mejor se adaptó a la zona de estudio. Por otro lado, las plántulas estuvieron listas para el trasplante a los 29,00 días, siendo los tratamientos que menor porcentaje de emergencia experimentaron (84,67%); por lo que es una alternativa para el productor de lechuga del centro del país, con los cual se dotará de repollos bien conformados y sólidos, al superar en varias variables a la variedad testigo Great Lakes.

Con respecto a la variedad HM 5 (V5), el crecimiento y desarrollo de las plantas fue muy aceptable, al presentar la tercera mejor altura de planta 30 días (10,16 cm) y la segunda mayor altura de a los 60 días (19,53 cm), los pesos de los repollos fueron relevantes con el segundo mejor valor (284,43 g), siendo estos sólidos, superando en varias variables a la variedad testigo Great Lakes. El porcentaje de emergencia fue de 92,33% y los días al trasplante de 28,00 días, por lo que es una alternativa para los productos de lechuga, con la cual conseguirán buenos resultados, en las condiciones de la parroquia Atahualpa del cantón Ambato.

La variedad de lechuga Great Lakes (V6), considerada testigo, reportó buenos resultados, con el tercer mejor peso del repollo (255,18 g), ubicándose en la categoría de sólidos. El porcentaje de emergencia fue de 91,67%. Por otro lado, fue la variedad más tardía al trasplante (30,67 días), consecuentemente fue la variedad más tardía a la cosecha de los repollos (81,00 días)

En relación a la variedad HM 3 (V3), se observó que fueron las plantas que mayor crecimiento y desarrollo reportaron a los 30 días (10,27 cm). Los repollos se ubicaron en la escala de solidez en sólidos. El porcentaje de emergencia fue de 96,00% y fue una de las variedades más tardías al trasplante 30,00 días.

En cuanto a la variedad HM 2 (V2), reportó el porcentaje de emergencia del 87,67%), siendo trasplantada a los 28,67 días de la siembra. Fue la variedad con menor crecimiento y desarrollo de los repollos, con el menor diámetro ecuatorial (14,88 cm). Por otro lado, los repollos se ubicaron en la escala de solidez de moderadamente sólidos.

En referencia a la variedad HM 4 (V4), fue la más precoz al trasplante (26,67 días), consecuentemente fue la más precoz a la cosecha de los repollos (73,00 días). El porcentaje de emergencia fue de 94,33%. Por otro lado, el crecimiento y desarrollo de las plantas fue el menor, con la menor altura de planta 30 días (8,54 cm), como a los 45 días (16,72 cm) y a los 60 días (17,62 cm). Los repollos reportaron el menor peso (214,90 g), por lo que registraron los más bajos rendimientos (10,55 tm/ha). Los repollos se ubicaron en la escala de moderadamente sólidos.

Del análisis económico se concluye que, el tratamiento de la variedad HM 1 (V1), alcanzó la mayor relación beneficio costo de 0,25, en donde los beneficios netos obtenidos fueron 0,25 veces lo invertido, siendo desde el punto de vista económico el tratamiento de mayor rentabilidad.

Campos R. (2008), comparó la longitud de las plántulas y el porcentaje de germinación de semillas de lechuga (*Lactuca sativa L*) irrigadas con aguas residuales tratadas mediante la combinación de métodos antes mencionada. Encontró que las plántulas irrigadas con aguas desinfectadas con ozono presentaban un crecimiento ligeramente menor de 25mm de longitud, aunque mostraban mayor porcentaje de germinación de 86% que las semillas irrigadas con agua de la red suplementada con fertilizantes de 30mm de longitud y 84% de germinación. Adicionalmente observó que las semillas irrigadas con agua desinfectada con cloro presentaron el efecto contrario, es decir un mayor crecimiento de plántulas de 30mm de longitud, pero bajos porcentajes de germinación del 70%.

Un aspecto que resta por evaluar de este método de tratamiento y su aplicación en la producción de agua residual tratada para la agricultura es lo relativo al seguimiento de los nutrientes que esta aporta, así como su comportamiento en los suelos irrigados y el aprovechamiento por los cultivos; de ahí la importancia de la presente tesis. Higienización de lechugas en que se utilizó ozono para higienizar en uva, cebolla.

2.2. Bases teóricas de la variable independiente: Desinfectantes químicos.

2.2.1. Desinfectantes.

Los desinfectantes, son sustancias químicas que tienen como fin disminuir o eliminar el número de microorganismos que se encuentran en áreas que pueden entrar en contacto con los alimentos. Los procesos de desinfección, por su parte, pueden llegar a ser más efectivos si se lleva a cabo una limpieza completa del equipo o de la superficie que se va a desinfectar, debido a que la materia orgánica que puede estar presente es capaz de reducir la capacidad biocida de los desinfectantes, debido a su efecto diluyente (Marriott, 2003). Para lograr una buena limpieza y desinfección en las instalaciones es necesario conocer las diferentes formas de contaminación para que de esta manera se pueda implementar un sistema de control y prevención adecuado (Guevara, 1999).

Los desinfectantes deben seleccionarse teniendo en cuenta el tipo de microorganismo que se desea eliminar, el tipo de producto que se elabora y el material de las superficies que entran en contacto con el producto utilizado (Ascenzi. L, 1996). Por otra parte, los desinfectantes varían en su composición química y actividad, por esto, existen en el mercado productos con mayor concentración, lo que asegura una rápida y eficaz acción. Además del tipo de condiciones a nivel de su composición, se deben tener en cuenta diferentes factores físico-químicos que en algún momento pueden llegar a afectar la eficacia de los desinfectantes, como lo son:

- Tiempo de exposición: La carga microbiana y la diversa sensibilidad de la población bacteriana al desinfectante, debido a la edad, formación de esporas y otros factores fisiológicos determinan el tiempo requerido para que el desinfectante sea eficaz.
- Temperatura: Aumentar la temperatura favorece la velocidad de destrucción de los microorganismos.
- pH: La actividad de los desinfectantes tiene lugar dentro de una zona concreta de pH, por lo que dicha actividad puede verse influida por cambios

relativamente pequeños de pH.

- Limpieza del Equipo: algunos compuestos clorados, yodados y otro tipo de desinfectantes pueden reaccionar con los compuestos orgánicos de la suciedad que no hayan sido eliminados, ya que una limpieza deficiente puede reducir la eficacia de un desinfectante.
- Dureza del Agua: Los compuestos de amonio cuaternario son incompatibles con sales de calcio y magnesio, por lo que no deben usarse en combinación con aguas duras. A medida que aumenta la dureza del agua, decrece la eficacia de estos desinfectantes.
- Adherencia Bacteriana: La adherencia de ciertos microorganismos a ciertas superficies sólidas supone una mayor resistencia al cloro (Marriott, 2003)

Es importante considerar, además, ciertos parámetros que se deben tener en cuenta y las condiciones necesarias que debe cumplir un desinfectante, que va a ser utilizado en la industria procesadora de alimentos:

- Destruir rápidamente los microorganismos, siendo igual de eficaces con las bacterias Gram positivas que con las Gram negativas. Deben destruir la mayoría de las esporas fúngicas, siendo también conveniente la destrucción de las esporas bacterianas.
- Ser suficientemente estables en presencia de residuos orgánicos y si fuera necesario, en presencia de aguas duras.
- No ser corrosivos ni dar color a ninguna superficie.
- Ser inodoros o no desprender olores desagradables.
- No ser tóxicos, ni irritantes a los ojos o a la piel.
- Fácilmente solubles en agua y arrastrables por enjuagado.
- Deben ser estables durante mucho tiempo en forma concentrada y durante menor tiempo en formas diluidas.
- Económicamente competitivos y al emplearlos presentar una buena relación costo/efectividad (Forsythe y Hayes, 2002).

2.2.2. Dimensiones de los desinfectantes químicos.

2.2.2.1 Ácido láctico.

El ácido láctico es un compuesto muy versátil utilizado en la industria alimenticia, química, farmacéutica, textil, la agricultura, alimentación animal entre otros (Requena et al., 1995; Marín, 2011).

Fue descubierto en 1780 por el químico sueco Scheele, quien lo aisló de la leche agria, fue reconocido como producto de fermentación por Blondeaur en 1847 y tan solo en 1881 Littlelon inicia la fermentación a escala industrial. El ácido láctico tiene un carbono asimétrico lo cual da lugar a actividad óptica. Existen dos isómeros ópticos, el D (-) láctico y L (+) láctico y una forma racémica constituida por fracciones equimolares de las formas L (+) y D(-). A diferencia del isómero D(-) la configuración L(+) es metabolizada por el organismo humano. Tanto las dos formas ópticamente activas como las formas racémicas se encuentran en estado líquido, siendo incoloros y solubles en agua. En estado puro son sólidos altamente higroscópicos de punto de fusión bajo, el cual es difícil de determinar debido a la extrema dificultad de producirlo anhidro, es por esta razón que se manejan rangos de 18 -33°C (Sudirerp, 1995, Parés y Juárez, 1997). Las propiedades físico química se muestran en la Tabla 1.

Tabla 1.
Propiedades físico química del ácido láctico

Fórmula	$C_3H_6O_3$
Peso molecular	90,08
Índice de refracción	14,414
Punto de fusión	L(+) y D(-) 52,8 a 54°C
Punto de ebullición	125 – 140°C
Gravedad específica	1206
Calor de combustión	3616cal/g
Viscosidad	40,33 $mNsm^{-2}$
Densidad	1,249
Constante dieléctrica	22

Adaptado de Dean (1987)

El ácido láctico puede ser obtenido por vía química o biotecnología. La producción Química, está basada en la reacción de acetaldehído con ácido cianhídrico (HCN) para dar lactonitrilo, el cual puede ser neutralizado a ácido láctico; otro tipo de reacción se basa en la reacción a alta presión del acetaldehído con monóxido de carbono y agua en presencia de ácido sulfúrico como catalizador.

Nogales, et al 2012 demuestra efectividad del ácido láctico en el agua del lavado, donde se sometieron fresas procesada se envasaron en bandejas de polipropileno termoselladas utilizando atmósfera modificadas pasivas.

a. Concentración química del ácido láctico.

El ácido láctico tiene efectos bacteriostáticos y bactericidas; soluciones acuosas de ácido láctico sirven para descontaminar (Woolthuis y Smulders, 1985). El uso de ácido láctico es limitado a concentraciones altas, ya que estas influyen en la calidad del producto. El ácido láctico puede alterar el color de la carne fresca bovina en donde hay sangre, lo cual se puede prevenir con duchas de agua para la remoción de la sangre (Edwards y Fung, 2006).

Se han realizado estudios a concentraciones de 2 a 4% y con temperaturas de 32 a 55°C, donde se reportan diferentes reducciones y se concluye que está relacionado a la inoculación usada en estos estudios. Por ejemplo, Castillo et al. (2001) reportaron reducciones de 4 log UFC para bacterias aeróbicas mesófilas, Salmonella y E. coli en trozos de carne inoculadas con cepas y Gill y Badoni (2004) encuentra reducciones menores, con 0,8 a 1,2 Log UFC en canales con contaminación natural.

Los ácidos orgánicos aumentan la vida media del producto modificando la atmósfera del empaque del producto porque aumentan la fase no esporulada del microorganismo (Greer y Dilts, 1995).

La acción antimicrobiana se atribuye al cambio de pH del medio, la desnaturalización de las proteínas y afecta el funcionamiento de la membrana celular por medio del ión lactato en el ciclo energético de los microorganismos (Lopez et al., 2002).

Las desventajas de este químico es el efecto corrosivo sobre los equipos de aspersión. Se ha demostrado que el uso a temperaturas elevadas aumenta este efecto. Además, crece la preocupación de microorganismos acido resistentes (Dickson, 1991).

La acción bactericida del ácido láctico sobre las bacterias Gram negativas, especialmente el grupo de las Enterobacterias se debe a que produce una desorganización de la capa de lipopolisacáridos presentes en la superficie de la membrana externa, los cuales se encargan de la permeabilidad de la barrera de la misma (Helander et al., 1997). Al ser hidrosoluble, el ácido láctico tiene acceso al periplasma bacteriano a través de las proteínas “porinas” de la membrana exterior (Ockerman et al., 1974). Su forma no disociada penetra la membrana citoplasmática, produciendo disminución del pH intracelular y una ruptura de la fuerza protón motriz transmembrana (Ojeda y Vásquez, 2009).

2.2.2.2 Ozono.

El ozono (O_3) es un poderoso agente oxidante usado como desinfectante (Rice, 1986). Además de ser un agente germicida de varios microorganismos el

ozono es más eficaz que el cloro para la desinfección o destrucción de virus y bacterias.

La solubilidad de ozono es un factor limitante que afecta progresivamente al proceso de ozonación. El ozono al descomponerse en solución acuosa forma radicales libres peróxidos de hidrógeno y de hidróxido respectivamente (HO2 y HO) los cuales tienen gran poder oxidante, y además de desaparecer rápidamente, pueden reaccionar con impurezas; tales como sales metálicas, materia orgánica, iones hidrogeno e hidroxilos presentes en la solución. Estos radicales libres formados por la descomposición del ozono son aparentemente las principales especies reactivas (White, 1999).

2.2.2.2.1 Mecanismos de desinfección asociados con el uso del ozono.

El ozono, como ya se mencionó, es un oxidante muy fuerte con un alto poder germicida de virus, bacterias, protozoarios. Los mecanismos asociados a la desinfección con cloro incluyen (Singer et al., 1989):

- La oxidación o destrucción directa de la pared de la célula con la salida de componentes celulares.
- Las reacciones con los subproductos radicales de la descomposición del ozono.
- El daño a los componentes de los ácidos nucleicos (purinas y pirimidinas).
- La ruptura de los enlaces de carbono-nitrógeno.

En general se cree que las bacterias son destruidas debido a la oxidación de su protoplasma, dando como resultado la desintegración de la pared de la célula (fisuramiento o lisis de la célula).

La eficacia de la desinfección depende de la susceptibilidad de los organismos a ser tratados, del tiempo de contacto y de la concentración de ozono. La desinfección con ozono es el método utilizado en varios países del mundo, en de 1983 había 54 planta de tratamiento en Estados Unidos y dos plantas en Francia. El Japón, existían 74 plantas de tratamiento y en Alemania existía una planta piloto,

en algunos complejos japoneses estaba siendo empleado para purificar las aguas negras para el reuso en jardines decorativos (Singer et al., 1989). La desinfección con ozono se utiliza generalmente en plantas de tamaño mediano o grande una vez que el agua residual haya recibido por lo menos tratamiento secundario. Además de la desinfección, otro uso común del ozono en el tratamiento del agua residual es el control de malos olores. La desinfección con ozono es un método poco utilizado en los Estados Unidos aun cuando en Europa esta tecnología ha tenido una amplia aceptación por varias décadas. El tratamiento con ozono tiene la capacidad de lograr niveles más altos de desinfección en comparación con el cloro o la luz ultravioleta; sin embargo, los costos de inversión, así como los gastos de mantenimiento no son competitivos con las alternativas disponibles. Por lo tanto, el ozono es utilizado con poca frecuencia, principalmente en casos especiales en los cuales otras alternativas no son efectivas.

2.2.2.2.2 Ventajas y desventajas de la desinfección con ozono.

Tabla 2.
Ventajas y desventajas de la desinfección con ozono

Ventajas	Desventajas
El ozono es más eficaz que la utilización del cloro para la desinfección o destrucción de virus y bacterias.	La baja dosificación puede no desactivar efectivamente algunos virus, esporas o quistes.
El proceso de ozonización utiliza un período corto de contacto (aproximadamente de 10 a 30 minutos).	El proceso de ozonización es una tecnología más compleja que la cloración o la desinfección con luz ultravioleta, por lo cual se requieren equipos complicados y sistemas de contacto eficientes.
No existen residuos peligrosos que necesiten ser removidos después del proceso de ozonización porque el ozono se descompone rápidamente.	El ozono es muy reactivo y corrosivo, requiriendo así de materiales resistentes a la corrosión tales como el acero inoxidable.
Después del proceso de ozonización, los microorganismos no crecen nuevamente, a excepción de aquellos protegidos por las partículas en la corriente de agua residual,	El proceso de ozonización no es económico para las aguas residuales con altas concentraciones de sólidos suspendidos (SS), demanda bioquímica del oxígeno (DBO), demanda química del oxígeno, o carbono orgánico total.
El ozono es generado dentro de la planta, existiendo así muy pocos problemas de seguridad industrial asociadas con el envío y el transporte.	El ozono es extremadamente irritante y posiblemente tóxico, así que los gases de escape que salen de la cámara de contacto deben ser destruidos para evitar que los trabajadores estén expuestos a ellos.
El proceso de ozonización eleva la concentración de oxígeno disuelto (O. D.) del efluente. El incremento O. D. puede eliminar la necesidad de re-aeración y también puede incrementar el nivel de O. D. en la corriente de agua receptora	El costo del tratamiento puede ser relativamente alto en cuanto a la inversión de capital y la demanda de energía eléctrica.

Adaptado de EPA 1999 c

a. Concentración química del ozono

La concentración de una solución nos indica la cantidad de soluto presente

en una cantidad de solución.

Si tenemos una solución, el soluto estará presente en una determinada proporción con respecto al solvente. Esa proporción no cambiará a menos que se adicione más soluto o más solvente. En consecuencia, la concentración permanece constante. Es importante notar que la concentración es una propiedad intensiva.

El método de desinfección por ozonización consiste en agregar cantidades suficientes de ozono lo más rápidamente que sea posible, de manera que satisfaga la demanda y mantenga un residuo de ozono durante un tiempo suficiente para asegurar la inactivación o destrucción de los microorganismos.

La demanda de ozono en la mayoría de los sistemas de abastecimiento de agua suele ser mayor a la del cloro, debido a su gran potencial de oxidación. Los procesos de desinfección por ozono normalmente tratan de mantener un residual mínimo de 0,4 a 0,5 ppm después de 10 a 20 minutos de contacto con el agua.

El mecanismo de desinfección en la ozonización se basa en el alto poder del ozono como oxidante protoplasmático general. Esta condición convierte al ozono en un eficiente destructor de bacterias y la evidencia sugiere que es igual de efectivo para atacar virus, esporas y quistes resistentes de bacterias y hongos.

A diferencia del cloro, la capacidad desinfectante del ozono no depende tanto de su período de retención en el agua (aunque esto tiene un efecto), sino más bien de la dosis suministrada (en la fórmula C x T prima entonces el valor de “C”). Esto se debe a que su alto potencial oxidante produce gran inestabilidad del ozono, incluso en el agua destilada, lo que quiere decir que quedará ozono remanente y por un corto tiempo solo cuando toda la materia con alta capacidad de oxidación haya sido oxidada. En caso contrario, es posible que no se haya satisfecho completamente la demanda de ozono. Dada su escasa permanencia, es compresible entonces la importancia de determinar adecuadamente la demanda de ozono y la dificultad que reviste determinar el residual que asegure una desinfección completa.

La primera experiencia en desinfección de agua mediante ozono se remonta

a 1886. Diferentes autores, y de hecho se admite, que un agua que mantiene una concentración de ozono residual superior a 0,4 mg/L. durante un periodo de tiempo no inferior a 4 minutos aseguran una desinfección adecuada, incluso en estas condiciones se asegura la eliminación de los virus de la poliomielitis.

En algunas variedades es muy efectiva una ozonización intermitente, por ejemplo, en las manzanas. La dosificación en estos casos oscila entre 1 a 3 p.p.m/v.

Para evaluar la eficiencia del ozono como desinfectante en la eliminación de V. cholerae O1 fenotipo rugoso, esta bacteria se inoculó en el agua residual tratada alcanzando una densidad bacteriana en el orden de 106 a 109 UFC/100 mL, esta se determinó con la escala nefelométrica de Mac Farland conforme a la densidad encontrada por Orta et al., 2000 de 3 X 106 UFC/100 mL para V. cholerae en agua residual y a los índices reportados para coliformes fecales (Jiménez y Chavez 2000) en México.

2.2.2.3 Hipoclorito de sodio.

El cloro es uno de los desinfectantes más utilizado en la industria alimenticia. Se utiliza para el tratamiento del agua potable, de procesamiento y lavado, equipos y otras superficies. (Richardson et al., 1998).

El cloro es un germicida eficaz contra carga microbiana, su acción germicida depende de la concentración empleada, pH, temperatura, contenido de materia mineral y orgánica.

La eficacia de la desinfección con cloro es dependiente de la temperatura, pH, grado de mezclado, tiempo de contacto, presencia de sustancias que intervienen en proceso y concentración de microorganismos que requieren ser destruidos (Rojas et al., 2002).

En varias investigaciones sobre desinfección con cloro realizadas con mayor interés en la eliminación de bacterias durante los años cuarenta a los setenta, proporcionó observaciones sobre la manera en que el cloro mata a estos microorganismos.

La exposición al cloro parece causar alteraciones físicas, químicas y bioquímicas en la pared de la célula. De esa manera destruye la barrera protectora de la célula, con lo que interfiere con las funciones vitales y se produce la muerte del microorganismo. Una posible secuencia de los eventos durante la cloración sería:

(1) la eliminación de la barrera suministrada por la pared de la célula mediante reacciones del cloro con determinados sitios en la superficie de la célula,

(2) la liberación de elementos constitutivos celulares vitales,

(3) la terminación de las funciones asociadas con la membrana y

(4) la terminación de las funciones celulares. Durante el transcurso de esta secuencia de eventos, el microorganismo muere, lo que significa que ya no es capaz de crecer ni causar enfermedad alguna.

Uno de los problemas que presenta el cloro es que se combina con las sustancias orgánicas naturales que pueden estar presentes en el agua para formar trihalometanos (THMs), entre ellos cloroformo, que es carcinógeno: Puesto que los THMs no se eliminan por métodos de tratamiento convencionales, el agua que se va a clorar debe estar libre de sustancias orgánicas naturales, en caso contrario, se debe utilizar otro desinfectante (Cáceres, 1990).

2.2.2.3.1 Ventajas y desventajas de la desinfección con cloro.

El cloro es un desinfectante que tiene ciertas limitantes en términos de salud y seguridad, pero al mismo tiempo tiene un largo historial como un desinfectante efectivo (EPA, 1999a)

Tabla 3.
Ventajas y desventajas de la desinfección con cloro

Ventajas	Desventajas
La cloración es una tecnología establecida	El cloro residual, aún a bajas concentraciones, es tóxico a los organismos acuáticos y por ello puede requerirse la descloración
En la actualidad la cloración es más eficiente en términos de costo que la radiación UV o la desinfección con ozono (excepto cuando la descloración y el cumplimiento con requisitos de incendios son requeridos	Todas las formas de cloro son muy corrosivas y tóxicas. Como consecuencia, el almacenamiento, el transporte y el manejo presentan riesgos cuya prevención requiere normas más exigentes de seguridad industrial.
El cloro residual que permanece en el efluente del agua residual puede prolongar el efecto de desinfección aún después del tratamiento inicial, y puede ser medido para evaluar su efectividad.	El cloro oxida ciertos tipos de materiales orgánicos del agua residual generando compuestos más peligrosos (tales como los metanos trihalogenados [MTH]).
La desinfección con cloro es confiable y efectiva para un amplio espectro de organismos patógenos	Los niveles de sólidos disueltos se incrementan en el agua efluente.
El cloro es efectivo en la oxidación de ciertos compuestos orgánicos e inorgánicos.	El cloro residual es inestable en presencia de altas concentraciones de materiales con demanda de cloro, por lo cual pueden requerirse mayores dosis para lograr una desinfección adecuada.
La cloración permite un control flexible de la dosificación.	Algunas especies parásitas han mostrado resistencia a dosis bajas de cloro, incluyendo los occisitos de *Cryptosporidium parvum*, los quistes de *Entamoeba histolytica* y *Giardia lamblia*, y los huevos de gusanos parásitos.
El cloro puede eliminar ciertos olores molestos durante la desinfección.	Se desconocen los efectos a largo plazo de la descarga de compuestos de la descloración al medio ambiente.

Adaptado de EPA 1999 a

a. Concentración química del hipoclorito de sodio.

La concentración de una solución nos indica la cantidad de soluto presente en una cantidad de solución.

Si tenemos una solución, el soluto estará presente en una determinada proporción con respecto al solvente. Esa proporción no cambiará a menos que se adicione más soluto o más solvente. En consecuencia, la concentración permanece constante. Es importante notar que la concentración es una propiedad intensiva.

El cloro fue uno de los primeros sistemas de desinfección de canales usados en la industria cárnica y se ha demostrado la reducción en el recuento de microorganismos con agua clorada a 200 y 500 ppm. Desafortunadamente estos niveles de cloro no son permitidos en la industria de alimentos y concentraciones bajas no son efectivas. El agua clorada a 200 ppm tiene reducciones de 1,5 a 2,3 Log en aerobios mesófilos en la superficie de carcasas (Kotula et al., 1964).

El Cloro a 20-50 ppm fue incluido en la lista permitida por FSIS en 1995. En EU y Australia están prohibidos los niveles por encima de 10 ppm. Es aprobado por la USDA junto con clorito de sodio acidificado, fosfato trisódico, lactoferricina b y ácidos orgánicos (Stopforth et al., 2004).

La aspersión de químicos desinfectantes ayuda a disminuir el peligro potencial en el crecimiento que puede ocurrir por cambios en la temperatura y tiempo insuficiente de las canales en cuartos fríos y/o transferencia de canal a canal (Jeremiah, 2007).

Las desventajas en el uso del cloro son los olores intensos, corrosión de equipos, irritación de los ojos, posible formación de cloraminas.

Se aconsejan niveles hasta 200 ppm, nivel máximo permitido en los Estados Unidos, donde está patentado por Chlor-Chill™ para el tratamiento de canales de vacuno, cerdo y oveja sin que se detecten malos olores (James et al., 1992).

El mecanismo de acción del cloro se ha reportado ser por inhibición de la oxidación de la glucosa, porque la célula pierde la capacidad de respirar (Albrich et al., 1981), ya que inhibe el succinato deshidrogenasa deteniendo el flujo de electrones a oxígeno, realizado por el citocromo. Además de la inactivación de proteínas de membrana comprometidas en la replicación de ADN. Albrich et al. (1981) proponen el mecanismo de acción por disfunción metabólica, con el

agotamiento de nucleótidos de adenina. Varios estudios coinciden en que los efectos antimicrobianos se llevan a cabo en la membrana celular (Rosen et al., 1987).

2.3. Bases teóricas de la variable dependiente: Higienización de la lechuga

2.3.1. Cultivo de lechuga.

La lechuga (*Lactuca sativa L*.), es originaria de las costas del sur y sureste del Mar Mediterráneo, desde Egipto hasta Asia Menor. Los egipcios le comenzaron a cultivar 2400 años antes de esta era y se supone que la utilizaban para extraer aceite de la semilla y para forraje; en pinturas encontradas en tumbas egipcias aparecen plantas que asemejan lechugas romanas o tipo Cos, con hojas alargadas y terminadas en puntas (Mallar, 1978).

2.3.1.1 . Clasificación botánica.

Tabla 4.
Clasificación botánica de la lechuga

Reino	:	Plantae
División	:	Macrophyllophita
Sub división	:	Magnoliophytina
Clase	:	Paenopsida 27
Orden	:	Asterales
Familia	:	Astereaceae
Género	:	Lactuca
Especie	:	Sativa
Nombre científico	:	*Lactuca sativa L*.
Nombre común	:	Lechuga

Recuperado de Mallar, 1978

2.3.2. Higienización.

“El objetivo de la higiene de los alimentos es el estudio de métodos para la producción, preparación y presentación de alimentos sanos y capaces de mantener una buena calidad (Hobbs, 1986, p. 1)

La sanidad se considera una ciencia porque busca proteger la salud humana y está relacionada con factores físicos, químicos, biológicos y microbiológicos que constituyen el ambiente; sin embargo, algunos microorganismos causan desperdicios y enfermedades, mientras que muchos otros son benéficos en el procesamiento y preparación de alimentos. De esta manera la sanidad se usa para mantener alimentos frescos, seguros y atractivos durante un periodo razonable de tiempo; además de que su correcta implementación es esencial para proteger la reputación del establecimiento y asegurar que los productos, equipo y utensilios sean tanto de alta calidad, como libres de toda contaminación (Minor & Cychi, 1984).

2.3.3. Dimensiones de la higienización de la lechuga.

2.3.3.1. Carga microbiana.

La carga microbiana se define como la estimación cuantitativa del número de microorganismos viables en, o sobre, un producto médico antes de la esterilización.

Cualquier proceso de esterilización permitirá la destrucción de un número limitado de microorganismos.

Un proceso de esterilización se considera eficaz cuando hay menos de una posibilidad en 1 millón de encontrar una unidad no estéril en un producto previamente esterilizado.

Comúnmente, se reconoce que el Nivel de Garantía de la Esterilidad estándar es 10E-6.

La reducción de la carga microbiana en el dispositivo médico antes de ser esterilizado garantizará que el proceso de esterilización sea capaz de lograr un nivel de esterilización eficiente. Dado que el empaque hace parte de este dispositivo médico, Arjowiggins Healthcare ha puesto en marcha un control periódico de carga microbiana sobre las envolturas. En la industria, el control del nivel de carga microbiana en los dispositivos médicos y en los sistemas de empaque es crucial: es una base para determinar la cantidad de agente esterilizante que debe ser

utilizado (esterilización gamma). En CSSD, las buenas prácticas recomiendan realizar controles periódicos de la carga microbiana del medio ambiente (aire, agua, superficie, dispositivos médicos antes de ser empacados). Como consecuencia de ello, es necesario reducir al máximo la contaminación potencial a causa de la materia prima**.**

2.3.3.2. Unidad formadora de colonia (UFC).

En microbiología, la unidad formadora de colonias (UFC) es una unidad de medida que se emplea para la cuantificación de microorganismos, es decir, para contabilizar el número de bacterias o células fúngicas (levaduras) viables en una muestra líquida o sólida.

2.3.3.2.1 Patrón de crecimiento de microorganismos.

La fase tardía es el inicio de su crecimiento, en esta fase los microorganismos tratarán de adaptarse al ambiente en un tiempo aproximado de veinte a treinta minutos, provocando una disminución en su población. A continuación, ocurre la fase logarítmica, la cual presentará un rápido crecimiento en el número de microorganismos como consecuencia de su reproducción.

Posteriormente se presentará la fase estacionaria, como su nombre lo indica, los microorganismos son limitados en su crecimiento alcanzando un punto de equilibrio sobre su reproducción por causa de los factores ambientales. La competencia de los microorganismos por espacio y nutrientes para sobrevivir ocasiona una reducción en su población dando como resultado la fase de muerte acelerada. Finalmente, la fase de muerte reducida se presenta cuando la muerte de los microorganismos comienza a desacelerarse, volviéndose constante (Marriot, 1997)

2.3.3.2.2 Factores adecuados para su crecimiento.

Simultáneamente, los microorganismos necesitan tener ciertas condiciones favorables o factores del ambiente para provocar su crecimiento. De acuerdo a la Secretaría de Salud (1994), estas condiciones son conocidas como CHATTO

(comida, humedad, acidez, tiempo, temperatura y oxígeno).

2.3.3.2.3 Humedad.

Sin excepción, todos los microorganismos necesitan como factor indispensable el agua para su crecimiento. El vapor de agua se condensa en los alimentos, equipo, paredes y techos, por lo tanto, la mayoría de las superficies presentan las condiciones favorables para el crecimiento de microbios; pero si el aire es seco o tiene una humedad relativamente baja, los microbios tienen menos posibilidad para crecer.

2.3.3.2.4 Acidez.

El valor de pH muestra qué tan ácido o alcalino es un producto; su rango varía de 0.0 a 14.0. Un pH de 7.0 se considera como neutro, cualquier pH que se encuentre por abajo del neutro se conoce como ácido, y alcalino si se encuentra por arriba de éste. La mayoría de los microorganismos prefieren un pH neutro para su desarrollo, como ejemplo se encuentran las levaduras, que crecen mejor en un ambiente ácido y más favorable aún, si el pH se encuentra entre 4.0 y 4.5; por el contrario, las bacterias consiguen un óptimo crecimiento si el pH es más cercano al neutral (The Educational Foundation of National Restaurant Association, 1992).

2.3.3.2.5 Temperatura.

La temperatura varía de mínima a máxima para un crecimiento óptimo dependiendo del tipo de microorganismo que se trate. De acuerdo con Longree & Armbuster (1996), pequeños cambios de temperatura pueden causar deterioro en alimentos, así como distintas enfermedades causadas por su consumo. Aunque se habla de distintas temperaturas óptimas para cada tipo de microorganismo, existe un rango que se conoce como la zona de peligro de la temperatura.

Desde el punto de vista de la Secretaria de Turismo (1996), la zona de peligro se define como el rango de temperatura en el cual las bacterias se desarrollarán con mayor facilidad en los alimentos, ocasionando serios problemas en la salud.

Esta zona comprende de 4º C a 60º C.

2.3.3.2.6 Tiempo.

Actúa al momento de la temperatura debido a que el tiempo total de alimentos potencialmente peligrosos expuestos a la zona de peligro no debe exceder de dos horas. Por lo mismo, es importante que cuando los alimentos sean calentados o enfriados se mantengan fuera de la zona de peligro, realizando un procedimiento adecuado (Lundberg & Walker, 1993).

2.3.3.2.7 Oxígeno.

Algunos microorganismos necesitan oxígeno para crecer, otros crecen sólo en ausencia de oxígeno y muchos más crecen sin importar la existencia de dicho elemento. Los microorganismos aeróbicos son aquellos que requieren de oxígeno para subsistir, por el contrario, aquéllos que no lo necesitan son conocidos como microorganismos anaeróbicos. Además, existen también los microorganismos facultativos que se caracterizan por su crecimiento en presencia o ausencia de dicho factor, tal es el caso de los lactobacilos (Nacional Restaurant Association, 1995).

a. Conteo bacteriano de la carga microbiana.

La prueba de carga microbiana determina el número total de microorganismos viables en, o sobre, un dispositivo médico y se evalúa de acuerdo con la norma ISO 11737. Con base a esta norma se ha determinado el tipo de carga microbiana en nuestra envoltura y se ha desarrollado y validado un método de extracción. Los tipos de microorganismos sobre las envolturas vigilados por Arjowiggins son: Bacterias aeróbicas, Levaduras y mohos.

2.4. Definición de términos básicos

Ácido Láctico:

El ácido láctico, o su forma ionizada, el lactato (del lat. lac, lactis, leche), también conocido por su nomenclatura oficial ácido 2-hidroxi-propanoico o ácido α-hidroxi-

propanoico, es un compuesto químico que desempeña importantes roles en varios procesos bioquímicos, como la fermentación láctica.

Concentración:

Desde la perspectiva de la química, concentración es una noción que describe a la relación, asociación o proporción que se puede establecer al comparar la cantidad de soluto (es decir, de sustancia capaz de disolverse) y el nivel de disolvente (es decir, la sustancia que logra que el soluto se disuelva) presentes en una disolución.

Desinfección:

Se denomina desinfección a un proceso físico o químico que mata o inactiva agentes patógenos tales como bacterias, virus y protozoos impidiendo el crecimiento de microorganismos patógenos en fase vegetativa que se encuentren en objetos inertes.

Desinfectantes:

Un desinfectante es un producto que permite eliminar las bacterias, los virus o los microorganismos. La utilización de un desinfectante permite limitar o, incluso, hacer desaparecer completamente, los riesgos de contaminación de una enfermedad.

Higienización:

Técnica que reduce el número de patógenos hasta niveles aceptables para la salud pública. El proceso puede realizarse sobre substratos diversos (habitaciones, alimentos, ropa, etc.) y mediante distintos procedimientos (agentes químicos, tratamientos térmicos, etc.).

Hipoclorito de Sodio:

El hipoclorito de sodio (cuya disolución en agua es conocida como lejía) es un compuesto químico, fuertemente oxidante de fórmula NaClO. Contiene cloro en estado de oxidación +1, es un oxidante fuerte y económico.

Ozono:

El Ozono es un gas que normalmente se encuentra en la atmósfera, su fórmula química es O3, lo que significa que cada molécula de ozono está formada por tres átomos de oxígeno. Este gas tiene un color azulado y desprende un olor característico fácilmente reconocible.

PPM:

Partes por millón (ppm) es una unidad de medida con la que se mide la concentración. Determina un rango de tolerancia. Se refiere a la cantidad de unidades de una determinada sustancia (agente, etc) que hay por cada millón de unidades del conjunto. Por ejemplo, en un millón de granos de arroz, si se pintara uno de negro, este grano representaría una (1) parte por millón.

Sanitización:

Es el proceso por el cual se realiza una reducción sustancial del contenido microbiano, hasta un nivel de seguridad, sin que se llegue a la desaparición completa de microorganismos patógenos, sin producir algún tipo de infección.

UFC:

En microbiología, la unidad formadora de colonias (abreviadamente, UFC) es una unidad de medida empleada en la cuantificación de microorganismos, es decir, el número de bacterias o células fúngicas (levaduras) viables en una muestra líquida o sólida.

III. MÉTODOS Y MATERIALES

3.1 Hipótesis de la investigación

3.1.1 Hipótesis general.

Las concentraciones de desinfectantes químicos se relacionan significativamente con la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup 2018.

3.1.2 Hipótesis específicas.

H1. La concentración de ácido láctico se relaciona significativamente con la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup 2018.

H2. La concentración de ozono se relaciona significativamente con la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup 2018.

H3. La concentración de hipoclorito de sodio se relaciona significativamente con la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup 2018.

3.2 Variables de estudio.

3.2.1 Definición conceptual.

3.2.1.1. Variable independiente.

Desinfectantes químicos: Los desinfectantes, son sustancias químicas que tienen como fin disminuir o eliminar el número de microorganismos que se encuentran en áreas que pueden entrar en contacto con los alimentos. Los procesos de desinfección, por su parte, pueden llegar a ser más efectivos si se

lleva a cabo una limpieza completa del equipo o de la superficie que se va a desinfectar, debido a que la materia orgánica que puede estar presente es capaz de reducir la capacidad biocida de los desinfectantes, debido a su efecto diluyente (Marriott, 2003). Para lograr una buena limpieza y desinfección en las instalaciones es necesario conocer las diferentes formas de contaminación para que de esta manera se pueda implementar un sistema de control y prevención adecuado (Guevara, 1999).

3.2.1.2. Variable dependiente.

Higienización de la lechuga: "El objetivo de la higiene de los alimentos es el estudio de métodos para la producción, preparación y presentación de alimentos sanos y capaces de mantener una buena calidad (Hobbs, 1986, p. 1)

3.2.2 Definición operacional.

Operacionalización de la variable

Tabla 5.
Operacionalización de las variables

Variables	Dimensiones	Indicadores	Unidades de medida
Variable Independiente:	ácido láctico	Concentración química	%
Concentración de Desinfectantes químicos	Ozono	Concentración química	ppm
	Hipoclorito de sodio	Concentración química	ppm
Variable dependiente: Higienización de la lechuga	Carga microbiana	Conteo bacteriano	UFC

3.3 Tipo y Nivel de investigación

3.3.1 Tipo de investigación.

De acuerdo con Hernández, Fernández y Baptista (2010) la investigación cumple

dos propósitos básicos:

- La Investigación básica, que es la que realiza conocimientos y teorías
- La investigación aplicada, que es la que soluciona problemas prácticos.

La presente investigación es Aplicada. Ya que trata de resolver la influencia de los desinfectantes químicos sobre la higienización de la lechuga.

3.3.2 Nivel de investigación.

El nivel es explicativo por que explica el comportamiento de una variable en función de otras, por ser estudios de causa – efecto requieren control y debe cumplir otros criterios de causalidad. Hernández, Fernández y Baptista (2010)

3.4 Diseño de la investigación.

Se utilizará el diseño Cuasi Experimental con pre y post test, con un grupo experimental y un grupo de control. (Hernández, Fernández y Baptista, 2010 pág. 58)

El esquema es: **G1 01 X 02**

G2 03 – 04

Dónde:

G1 = Grupo experimental

G2 = Grupo control

X = Variable Independiente

01 y 03 = Información pre - prueba

02 y 04 = Información post - prueba

3.5 Método de investigación

El método es hipotético deductivo, que es una descripción del método científico. Es el procedimiento o camino que sigue el investigador para hacer de su actividad una

práctica científica. Sampieri (2006, p.147)

3.6 Población y Muestra de estudio

3.6.1 Población.

Para Hernández Sampieri, "una población es el conjunto de todos los casos que concuerdan con una serie de especificaciones" (p. 65). Es la totalidad del fenómeno a estudiar, donde las entidades de la población poseen una característica común la cual se estudia y da origen a los datos de la investigación. Para esta investigación la población estará delimitada por el mercado mayorista de Santa Anita.

3.6.2 Muestra.

"La muestra es, en esencia, un subgrupo de la población. Es un subconjunto de elementos que pertenecen a ese conjunto definido en sus características al que se le llama población (H. Sampieri, citado por Balestrini 2001 Pág. 141). La muestra utilizada para el presente trabajo de investigación estará representada por la especie *Lactuca sativa* variedad arrepollada (escarola)

3.6.3 Muestreo.

En el muestreo probabilístico, "cada elemento de la población tiene una posibilidad conocida de ser seleccionada para la muestra" (Kinnear y Taylor, 1998 p.404) Esto se debe a que mediante reglas matemáticas se logra un muestreo de iguales posibilidades para todos, el cual es llamado muestreo aleatorio simple. La presente investigación se hará con un procedimiento de muestreo probabilístico, puesto que se escogerá al azar muestras de lechugas en el mercado mayorista de verduras de Santa Anita.

Se utilizará la siguiente fórmula:

$$n = \frac{Z^2 pq}{e^2}$$

Donde:

Z = nivel de confianza

p = .5

q = .5

e = porcentaje de error

3.7 Materiales, equipos y reactivos

3.7.1 Materiales de laboratorio.

- ✓ Vaso de precipitación de 100 – 250 ml.
- ✓ Pipeta de 1ml, 5 ml.
- ✓ Placas Petrifilm 3M para el Recuento de E. coli y Coliformes Totales
- ✓ Mechero de bunsen.
- ✓ Pinzas.
- ✓ Frascos de muestreo 200 ml.

3.7.2 Equipos.

- Autoclave.
- Incubadora a 35ºC 0.5ºC
- Balanza.
- Contadores de colonias.
- Campana de flujo laminar**.**

3.7.3 Medios de cultivo y reactivos.

- ✓ Agua destilada.
- ✓ Alcohol yodado al 1%.
- ✓ Solución salina fisiológica.
- ✓ Hipoclorito de sodio al 25, 50 ppm.
- ✓ Agua ozonizada 1,5 y 2 ppm.
- ✓ Ácido láctico al 2% y 4%.

3.8 Técnicas e instrumentos de recolección de datos

3.8.1 Técnicas de recolección de datos.

a. Selección de la muestra:

Se realizó una evaluación de las lechugas de los comerciantes del mercado mayorista de Santa Anita.

b. Toma de muestra:

Se recolectaron 5 unidades de lechuga de los comerciantes del mercado mayorista y estos fueron elegidos al azar.

c. Acondicionamiento de las muestras:

Las muestras elegidas fueron deshojadas y puestas en bolsas de polietileno y acondicionadas en una caja conservadora (tecnoport) con hielo para su posterior transporte a la cuidad de Lima.

d. Transporte de la muestra:

La muestra fue transportada a una temperatura de 5 °C. a la ciudad de Lima para su posterior desinfección y análisis microbiológico.

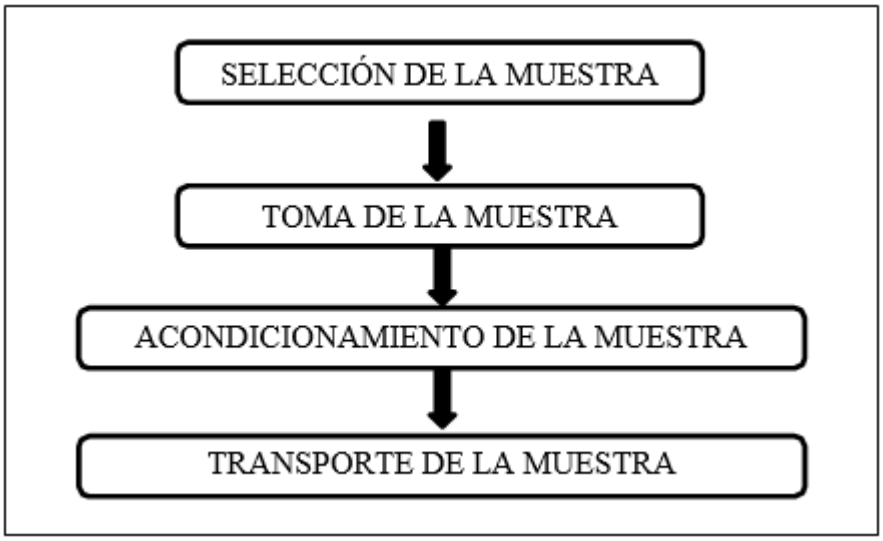


Figura 1. Diagrama de flujo del método de toma de muestras propuesto.

3.9 Metodología para la desinfección de las lechugas

3.9.1 Descripción del método de desinfección propuesto.

- **Acondicionamiento de muestras**

  Se acondicionaron las muestras en lugares limpios para evitar una mayor contaminación.

- **Lavado 1**

  Se realizó esta operación con el objetivo de eliminar las impurezas que naturalmente se encuentran en las lechugas post cosecha, tales como tierra, estiércol, partículas extrañas, pajas, parásitos, etc. El lavado simple es para todas las muestras y se realizó con agua tratada libre de cloro por 5 min.

- **Desinfección**

  En esta operación se sumerge:

  Tratamiento 1:

  Se realizó en una solución de agua con hipoclorito de sodio a 25 ppm por 2 minutos.

  Se realizó en una solución de agua ozonizada al 1,5 ppm por 2 minutos

  Se realizó en una solución de agua ácido láctico al 2% por 2 minutos

  Tratamiento2:

  Se realizó en una solución de agua con hipoclorito de sodio a 50 ppm por 2 minutos.

  Se realizó en una solución de agua ozonizada al 2 ppm por 2 minutos.

  Se realizó en una solución de agua ácido láctico al 4% por 2 minutos.

- **Lavado 2**

Las muestras aplicadas después de la desinfección con los tratamientos fueron enjuagadas con 500 ml de agua tratada libre de cloro por 5 min.

- **Aireado**

  Las muestras de lechuga se airearon con la finalidad de eliminar el agua y evitar un deterioro mayor de las muestras.

- **Almacenamiento**

  Las muestras se almacenaron en bolsas de polipropileno estériles y selladas herméticamente a una temperatura de 5 °C, para su posterior análisis.

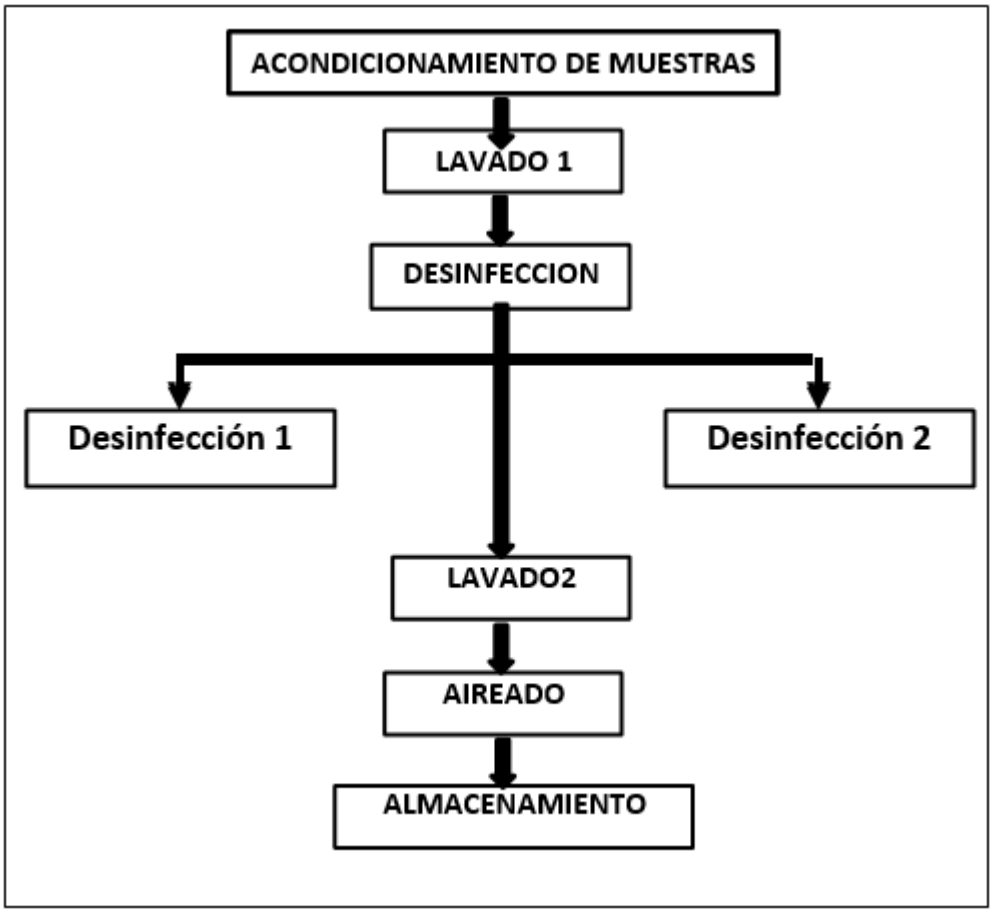


Figura 2. Diagrama de flujo de la desinfección de las muestras de lechuga.

3.9.2 Acondicionamiento de las muestras para análisis.

1. Pesar 10 g de muestra en un contenedor estéril adecuado.
2. Aforar la muestra con Solución Salina Fisiológica al 1% (Volumen total a trabajar debe ser 100 ml).
3. Agitar la mezcla.
4. Analizar la muestra

3.9.3 Descripción del Método para el recuento de Coliformes totales y *Escherichia coli* mediante la técnica petrifilm. Método recomendado por AOAC (2009)

1. Colocar la placa Petrifilm para el recuento de E. Coli sobre una superficie plana y lisa.
2. Levante la lámina superior y con la pipeta perpendicular dispense 1 ml. De la muestra en el centro del botón de la lámina.
3. Deje caer la lámina hacia abajo sobre la muestra, evite la formación de burbujas de aire.
4. Coloque el esparcidor plástico con el lado plano hacia abajo sobre el centro de la lámina. Presione cuidadosamente sobre el centro y distribuye cuidadosamente la muestra. Distribuya el inoculo en la placa petrifilm sobre el área de crecimiento antes de que se forme un gel. No deslice el esparcidor a través de la lámina.
5. Remueva el esparcidor de la placa y espera que se gelatinice la placa.
6. Incubar las placas caras arriba en pilas de hasta 10 placas. El tiempo de incubación y la temperatura varía según el método. Incubar a 35 ± 1°C durante 24 ± 2 horas.
7. Lecturar las placas petrifilm e intérprete.

3.9.4 Instrumentos de recolección de datos.

Se realizaron los análisis estadísticos con el apoyo del software STATGRAPHICS

16.1 para el procesamiento y análisis de datos de las comparaciones de muestras y el desarrollo de las relaciones factoriales.

3.10 Métodos de análisis de datos

La investigación cumple con el método Inductivo y Deductivo y los análisis de datos para la contrastación de las hipótesis se realizan con el comportamiento de las medidas de tendencia central como son las medias, desviación estándar y las medianas con las pruebas de T de Student, prueba de F, mediante sus ANOVA.

La Investigación Experimental, porque demuestra la relación causa efecto entre las variables las que comprobaron en los diseños estadísticos y con su optimización de la superficie de respuesta para el mejor tratamiento

La deducción va de lo general a lo particular. El método deductivo es aquél que parte los datos generales aceptados como valederos, para deducir por medio del razonamiento lógico, varias suposiciones, es decir; parte de verdades previamente establecidas como principios generales, para luego aplicarlo a casos individuales y comprobar así su validez.

La inducción va de lo particular a lo general. Empleamos el método inductivo cuando de la observación de los hechos particulares obtenemos proposiciones generales, o sea, es aquél que establece un principio general una vez realizado el estudio y análisis de hechos y fenómenos en particular.

La inducción es un proceso mental que consiste en inferir de algunos casos particulares observados la ley general que los rige y que vale para todos los de la misma especie.

3.11 Desarrollo de la propuesta de valor

Los impactos sobre la inocuidad de los alimentos en el área de la investigación establecida por la UPTELESUP propone esta metodología en base a las propuestas de métodos de desinfección planteadas en esta investigación, actuarán como medidas correctivas frente a la problemática de contaminación microbiológica presentada en la hortaliza en estudio, hasta que se logre sensibilizar

a la población sobre la importancia de producir y consumir alimentos inocuos y de calidad, no pueden pasar desapercibidos para los proyectos en general y para los proyectos de inocuidad alimentaria en particular.

Hoy en día existen muchas alternativas para el uso de productos de desinfección y cada vez vemos más opciones biodegradables o amigables con el medio ambiente, usando estos materiales no se contribuya a la contaminación medio ambiental.

3.12 Aspectos deontológicos

En la ordenación del desarrollo de la tesis se han cumplido la función sobre las actividades empíricas del trabajo han cumplido una triple función:

a) Fijar una serie de criterios, de carácter científico-funcional, para el desarrollo de los tratamientos para su operatividad y eficacia a las actividades ejercidas en el ámbito cubierto por las normas establecidas.
b) Se realizaron las orientaciones éticas para el ejercicio de los tratamientos y plasmarlas en los resultados previa constatación de las hipótesis

La tesis es de autoría propia, se ha aceptado las normas internacionales de citas y referencias para las fuentes consultadas por lo que la redacción es original y con citas de los autores respectivos.

Los datos que se presentaran en los resultados son reales, no han sido falseados, ni duplicados, ni copiados, por lo que los resultados que se presentarán en la tesis se constituirán en aportes a la realidad investigada y comprobada empíricamente; por lo que el informe final es de mi total responsabilidad en el marco de la ética personal.

IV. RESULTADOS

4.1 Criterios de selección de los métodos de desinfección

Para la realización de la investigación se utilizaron en primer lugar los métodos de desinfección químicos que actualmente se comercializan en los diferentes mercados y supermercados del país y que se encuentran elaborados a partir de los siguientes componentes activos:

a) Hipoclorito de sodio: 25 ppm – 50 ppm
b) Ozono: 1.5 ppm – 2 ppm
c) Ácido láctico: 2% - 4 %

Con tiempos de contactos: 2 – 4 – 6 – 8 – 10 min

4.2. Reporte de las medidas biométricas de las lechugas

La Tabla 6 reporta las medidas biométricas de las lechugas antes de ser higienizadas

Tabla 6.
Medidas biométricas de promedio de 100 lechugas

Diámetro (cm.)	Alto (cm.)	Peso (g)	Densidad global (Kg /m^3)	Forma Biométrica
56,79	17,48	196,35	449, 59	Esférica

4.3. Resultados de la carga microbiana de lechuga antes de ser Higienizadas.

La Tabla 7 reporta la carga microbiana de las lechugas antes de ser higienizadas

Tabla 7.
Resultado de análisis microbiológico de las frutas y hortalizas antes de ser Higienizadas

Muestra	Coliformes totales (N.M.P./d L)	Coliformes fecales (N.M.P./d L)
Lechugas	1.0×10^2	< 1

4.4. Resultados de la higienización de lechuga

Tabla 8.
Resultados de higienización de lechugas tratadas con agua ozonificada en diferentes tiempos de contacto

PRODUCTO	CONCENTRACIÓN DE OZONO (ppm)	TIEMPO DE CONTACTO (min)	PRESENCIA DE GERMENES VIABLES	ATRIBUTO SENSORIAL
LECHUGAS	1,5	2	+	Sin olor
	2,0	2	+	Sin olor
	1,5	4	+	Sin olor
	2,0	4	+	Sin olor
	1,5	6	+	Sin olor
	2,0	6	+	Sin olor
	1,5	8	+	Sin olor
	2,0	8	-	Sin olor
	1,5	10	-	Sin olor
	2,0	10	-	Sin olor

Tabla 9.
Resultados de higienización de lechugas tratadas con hipoclorito de sodio en diferentes tiempos de contacto

PRODUCTO	CONCENTRACIÓN DE HIPOCLORITO DE SODIO (ppm)	TIEMPO DE CONTACTO (min)	PRESENCIA DE GÉRMENES VIABLES	ATRIBUTO SENSORIAL
LECHUGAS	25	2	+	Sin olor
	50	2	-	Con olor cloro
	25	4	-	Con olor cloro
	50	4	-	Con olor cloro
	25	6	-	Con olor cloro
	50	6	-	Con olor cloro
	25	8	-	Con olor cloro
	50	8	-	Con olor cloro
	25	10	-	Con olor cloro
	50	10	-	Con olor cloro

Tabla 10.
Resultados de higienización de lechugas tratadas con ácido láctico en diferentes tiempos de contacto

PRODUCTO	CONCENTRACION DE ÁCIDO LÁCTICO (%)	TIEMPO DE CONTACTO (min)	PRESENCIA DE GERMENES VIABLES	ATRIBUTO SENSORIAL
LECHUGAS	2	2	+	Sin olor
	4	2	+	Sin olor
	2	4	+	Sin olor
	4	4	+	Sin olor
	2	6	+	Con olor
	4	6	+	Con olor
	2	8	+	Con olor
	4	8	-	Con olor
	2	10	-	Con olor
	4	10	-	Con olor

Procesamiento Estadístico de los Resultados

Evaluación organoléptica de los tratamientos óptimos de Ozono y Ácido láctico									
Concentración de Ozono 2 ppm x 8 minutos de contacto				Concentración de Ácido láctico 4 % x 8 minutos					
	Juez seleccionado								
Valor del atributo sensorial	1	2	3	4	5	6	7	8	9
Hay diferencia	2	2	2	1	2	2	2	1	2
No hay dieferncia	1	1	1	2	1	1	1	1	2
hay diferencia de los atributos : olor - sabor - frescura				hay diferencia de los atributos : olor - sabor - frescura					

Figura 3. *Comparación de los tratamientos óptimos de ozono y ácido láctico respecto a los atributos sensoriales de olor, sabor y frescura de acuerdo a la escala hedónica de clasificación*

a. Desarrollo de la prueba de hipótesis para análisis discriminativos

   Se aplicó la T de Student aplicada a comparación pareada simple, cuyos valores observados que se hallan para una suma de atributos que se hallan en este tipo de prueba pertenecen a variables discretas cuya distribución de sus valores tienen una distribución normal.

b. Cuadro de evaluación sensorial

Nombre del Juez :	Fecha:
Muestra evaluada:	Prueba:
Marque una opción por cada par de muests presentadas	

PAR DE MUESTRAS	HAY DIFERENCIAS	NO HAY DIFERENCIAS
A		
B		

Figura 4. Cuadro de evaluación sensorial

c. Desarrollo del procedimiento

- Planteamiento de la hipótesis:

  Hp: Si existen diferencias entre las muestras

  Ho: No hay diferencia entre las muestras

- Nivel de significación: 0.05
- Desarrollo de la prueba de Hipótesis
- Consideraciones:
  - Los datos siguen una distribución normal
  - Las muestras fueron elegidas aleatoriamente
- Criterios de decisión
  - Se acepta la Hp si T calculado ≤ T tabular
  - Se rechaza la Hp si T calculado > T tabular

d. Desarrollo estadístico de la evaluación sensorial

d.1. Comparación de Dos Muestras - Hay diferencia & No hay diferencia (No hay diferencia)

Muestra 1: Hay diferencia

Muestra 2: No hay diferencia

Selección de la Variable: No hay diferencia

Muestra 1: 9 valores en el rango de 1.0 a 2.0

Muestra 2: 9 valores en el rango de 1.0 a 2.0

Tabla 11.
Resumen Estadístico

	Hay diferencia	*No hay diferencia*
Recuento	9	9
Promedio	1.77778	1.22222
Desviación Estándar	0.440959	0.440959
Coeficiente de Variación	24.8039%	36.0784%
Mínimo	1.0	1.0
Máximo	2.0	2.0
Rango	1.0	1.0
Sesgo Estandarizado	-1.9839	1.9839

Curtosis Estandarizada	0.449906	0.449906

El cuadro anterior contiene el resumen estadístico que se trabajó para las dos muestras de datos de particular interés son el sesgo estandarizado y la curtosis estandarizada que pueden usarse para comparar si las muestras provienen de distribuciones normales; los valores de estos estadísticos fuera del rango de -2 a +2 indican desviaciones significativas de la normalidad, lo que tendería a invalidar las pruebas que comparan las desviaciones estándar, en este caso, ambos valores de sesgo estandarizado se encuentran dentro del rango esperado, ambas curtosis estandarizadas se encuentran dentro del rango esperado.

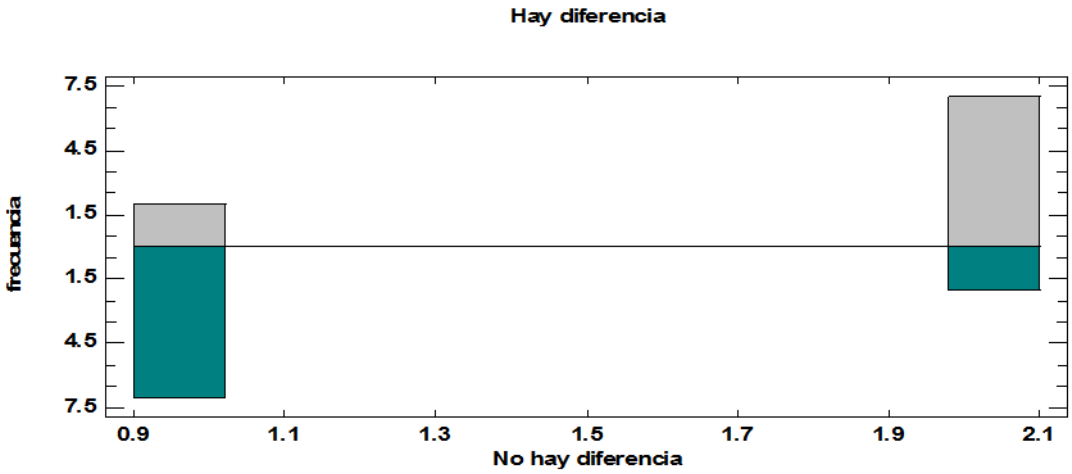


Figura 5. *Gráfico de las frecuencias de la comparación pareada simple*

d.2. Comparación de Medias

Intervalos de confianza del 95.0% para la media de Hay diferencia: 1.77778 +/- 0.338952 [1.43883; 2.11673]

Intervalos de confianza del 95.0% para la media de No hay diferencia: 1.22222 +/- 0.338952 [0.883271; 1.56117]

Intervalos de confianza del 95.0% intervalo de confianza para la diferencia de medias suponiendo varianzas iguales: 0.555556 +/- 0.440665 [0.11489; 0.996221]

- **Prueba t para comparar medias**

Hipótesis nula: media1 = media2

Hipótesis Alt.: media1 <> media2

Suponiendo varianzas iguales: t = 2.67261 valor-P = 0.016681

Se rechaza la hipótesis nula para alfa = 0.05.de tablas T tabular: 1.860

Se ejecutó la prueba t para comparar las medias de las dos muestras; se construyó intervalos, o cotas, de confianza para cada media y para la diferencia entre las medias encontrándose el interés particular el intervalo de confianza para la diferencia entre las medias, el cual se extiende desde 0.11489 hasta 0.996221; puesto que el intervalo no contiene el valor 0, existe una diferencia estadísticamente significativa entre las medias de las dos muestras, con un nivel de confianza del 95.0%.

Al realizarse la prueba T para evaluar hipótesis específicas acerca de la diferencia entre las medias de las poblaciones de las cuales provienen las dos muestras; en este caso, la prueba se ha construido para determinar si la diferencia entre las dos medias es igual a 0.0 versus la hipótesis alterna de que la diferencia no es igual a 0.0. Puesto que el valor-P calculado es menor que 0.05, se puede rechazar la hipótesis nula en favor de la alterna las que se puede observar en la figura siguiente de cajas u bigotes

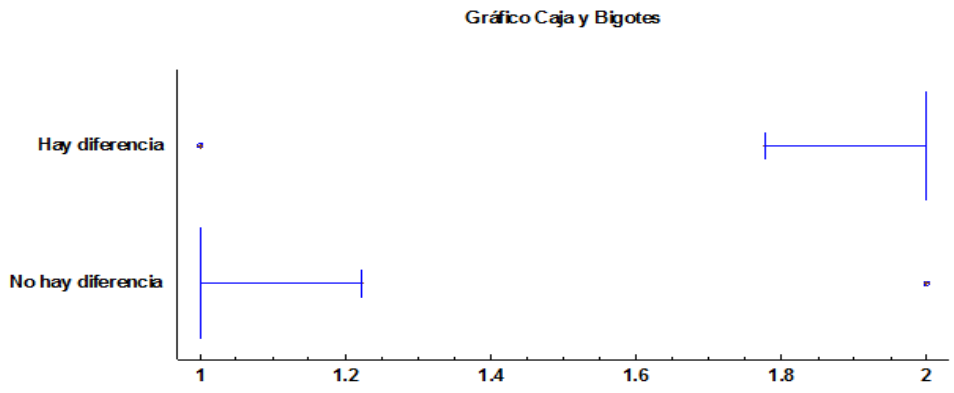


Figura 6. *Gráfico de cajas y bigotes de la prueba de Hipótesis de la evaluación sensorial*

Optimización de los valores de las concentraciones de Ozono respecto el tiempo de contacto y concentración de ozono

	Alto	Bajo
Concentracion de ozono	2.0 ppm	1.5 pp,
Tiempo de contacto	10 min	8 min

Figura 7. *Optimización de la concentración de Ozono*

a. Análisis del Experimento - Rendimiento

Tabla 12.
Efectos estimados para Rendimiento (Porcentaje)

Efecto	*Estimado*	*Error Estd.*	*V.I.F.*
promedio	96.6667	0.30429	
A: ppm de Ozono	1.33333	0.333333	1.0
B: Tiempo de contacto Ozono	1.33333	0.333333	1.0
AA	0.0	0.57735	1.0
AB+ bloque	-3.0	0.57735	2.0
BB	0.0	0.57735	1.0
bloque	1.66667	0.430331	1.66667
bloque	1.66667	0.430331	1.66667

La Tabla 12 muestra las estimaciones para cada uno de los efectos estimados y las interacciones, también se determinó error estándar de cada uno de estos efectos, el cual mide su error de muestreo, se debe indicar que el factor de inflación de varianza (V.I.F.) más grande, es igual a 2.0. Para un diseño perfectamente ortogonal, todos los factores serían igual a 1. Factores de 10 o más normalmente se interpretan como indicativos de una buena optimización.

a. Análisis de Varianza para Rendimiento

R-cuadrada = 99.6032 porciento

R-cuadrada (ajustada por g.l.) = 96.8254 porciento

Error estándar del est. = 0.408248

Error absoluto medio = 0.111111

Estadístico Durbin-Watson = 1.66667

Auto correlación residual de Lag 1 = 2.84356E-14

Tabla 13.
Análisis de Varianza para Rendimiento

Fuente	*Suma de Cuadrados*	*Gl*	*Cuadrado Medio*	*Razón-F*	*Valor-P*
A: ppm de Ozono	2.66667	1	2.66667	16.00	0.1560
B: Tiempo de contacto Ozono	2.66667	1	2.66667	16.00	0.1560
AA	0.0	1	0.0	0.00	1.0000
AB+bloque	4.5	1	4.5	27.00	0.1210
BB	0.0	1	0.0	0.00	1.0000
bloques	6.25	2	3.125	18.75	0.1590
Error total	0.166667	1	0.166667		
Total (corr.)	42.0	8			

La tabla 13 ANOVA particiona la variabilidad de Rendimiento en piezas separadas para cada uno de los efectos, entonces prueba la significancia estadística de cada efecto comparando su cuadrado medio contra un estimado del error experimental. En este caso, 0 efectos tienen una valor-P menor que 0.05, indicando que son significativamente diferentes de cero con un nivel de confianza del 95.0%.

El estadístico R-Cuadrada indica que el modelo, así ajustado, explica 99.6032% de la variabilidad en Rendimiento. El estadístico R-cuadrada ajustada, que es más adecuado para comparar modelos con diferente número de variables independientes, es 96.8254%. El error estándar del estimado muestra que la desviación estándar de los residuos es 0.408248. El error medio absoluto (MAE) de 0.111111 es el valor promedio de los residuos. El estadístico de Durbin-Watson (DW) prueba los residuos para determinar si haya alguna correlación significativa basada en el orden en que se presentan los datos en el archivo.

Tabla 14.
Coeficiente de regresión para Rendimiento

Coeficiente	*Estimado*
constante	-8.5
A: ppm de Ozono	56.6667
B: Tiempo de contacto Ozono	11.1667
AA	0.0
AB	-6.0
BB	0.0

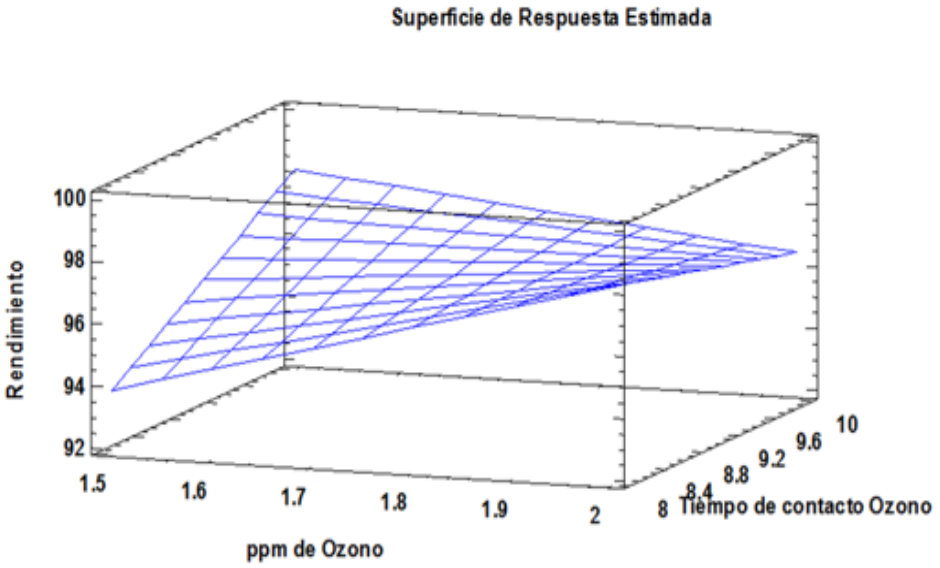
La ecuación del modelo ajustado es

Rendimiento = -8.5 + 56.6667*ppm de Ozono + 11.1667*Tiempo de contacto Ozono + 0.0*ppm de Ozono^2 - 6.0*ppm de Ozono*Tiempo de contacto Ozono + 0.0*Tiempo de contacto Ozono^2

Esta ecuación permite que se obtenga el rendimiento con sus respectiva grafica

Tabla 15. *Optimizar Respuesta*
Meta: maximizar Rendimiento
Valor óptimo = 98.1667

Factor	*Bajo*	*Alto*	*Óptimo*
ppm de Ozono	1.5	2.0	1.5
Tiempo de contacto Ozono	8.0	10.0	10.0



Rendimiento = -8.5 + 56.6667*ppm de Ozono + 11.1667*Tiempo de contacto Ozono + 0.0*ppm de Ozono^2 - 6.0*ppm de Ozono*Tiempo de contacto Ozono + 0.0*Tiempo de contacto Ozono^2

Figura 8. *Superficie de respuesta para la optimización del 98.1667 % del uso del Ozono*

Con estos valores se demuestra que el mejor tratamiento es del Ozono con un rendimiento de 98.1667 porciento.

V. DISCUSIÓN

5.1. Discusión del reporte de la medida biométrica de las lechugas

Estas medidas están dentro del rango de las medidas indicadas por Calzada (1 978).

5.2. Discusión del análisis microbiológico de las lechugas antes de ser higienizadas

La Tabla 7 muestra que todos los tratamiento tuvieron un contenido microbiológico no apto para el consumo humano y de los productos a higienizar la lechuga muestra hasta contenido de 1.2 x 102 N.M.P./dL coliformes totales y 1 N.M.P./dL coliformes fecales, esta carga se debe a que estas lechugas son expendidos con mucho contenido de tierra y material orgánico, todas las otras mostraron un comportamiento similar a algunas tiene un contenido de humedad alto, se debe indicar que algunas presentaron visualmente presencia de esporas negras.

5.3. Discusión los resultados de la higienización de lechugas

Al evaluar los resultados de las Tablas 8, 9 y 10 se observó que los tratamientos que presentaron mayor concentración de ozono mayores de 1,5 ppm y mayor tiempo de contacto reducen drásticamente el contenido de patógenos y coliformes totales; y solo se reportaron presencias de gérmenes viables que fueron eliminados por el mayor tiempo de contacto del agua ozonificada , el tiempo de 4 minutos resulto un valor óptimo para la eliminación de los germines viables y los coliformes, estos valores coinciden con lo que afirma Kim et al. (1 999).

VI. CONCLUSIONES

3.1 Conclusiones

- La relación de concentración óptima de desinfectante para la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup fue de 8 ppm de ozono en el agua con un tiempo de contacto de 10 minutos con un rendimiento de 98.16 porcientos.

- La relación de la concentración de ácido láctico es de 4 % y un tiempo de contacto de 8 minutos con ausencia de gérmenes viables, pero con atributos sensoriales no aceptable con respecto al olor en la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup.

- La relación de la concentración de ozono en la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup fue de 1.5 ppm en el agua con un tiempo de contacto de 10 minutos con ausencia de gérmenes viables y atributos sensoriales aceptables.

- La relación de la concentración de hipoclorito de sodio en la higienización de la lechuga en el laboratorio de la Universidad Privada Telesup fue de 25 ppm y tiempo de contacto de 2 minutos con ausencia de gérmenes viables, pero con atributos sensoriales no aceptables con respecto al olor.

VII. RECOMENDACIÓN

3.2 Recomendaciones

- Realizar investigaciones usando soluciones marinadas para la lechuga y evaluar su inocuidad y tiempo de vida útil.
- Realizar investigaciones de lechugas en refrigeración empacadas al vacío para evaluar su inocuidad y tiempo de vida útil.
- Investigar los procesos de congelación de lechuga para evaluar las características fisicoquímicas y calidad sensorial en sus usos comunes.
- Evaluar el efecto de exposición a ozono en el contenido de compuestos fenólicos totales, de la lechuga.
- Evaluar el efecto de exposición a ozono y el tiempo de almacenamiento en el contenido de flavonoides y actividad antioxidante de las hojas de lechuga.
- Determinar los grados de calidad en su uso gastronómicos de las variedades de lechugas producidas en el Perú.

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ANEXOS

Anexo 1: Matriz de consistencia

Matriz de Consistencia

"Concentración de desinfectantes químicos y su relación con la higienización de lechuga en el laboratorio de la UPT 2018"

PROBLEMA	OBJETIVOS	HIPÓTESIS	VARIABLES	METODOLOGÍA
PROBLEMA GENERAL ¿Cómo se relacionan las concentraciones de desinfectantes químicos y la higienización de la lechuga en el laboratorio de la UPT 2018? **PROBLEMAS ESPECÍFICOS** ¿Cómo se relaciona la concentración de ácido láctico en la higienización de lechuga en el laboratorio de la UPT 2018? ¿Cómo se relaciona la concentración de ozono en la higienización de lechuga en el laboratorio de la UPT 2018? ¿Cómo se relaciona la concentración de hipoclorito de sodio en la higienización de la lechuga en el laboratorio de la UPT 2018?	**OBJETIVO GENERAL** Determinar la relación de las concentraciones de desinfectantes químicos y la higienización de la lechuga en el laboratorio de la UPT 2018. **OBJETIVOS ESPECIFICOS** a. Determinar la relación de la concentración de ácido láctico en la higienización de la lechuga en el laboratorio de la UPT 2018. b. Determinar la relación de la concentración de ozono en la higienización de la lechuga en el laboratorio de la UPT 2018. c. Determinar la relación de la concentración de hipoclorito de sodio en la higienización de la lechuga en el laboratorio de la UPT 2018.	**HIPÓTESIS GENERAL** **H1:** Las concentraciones de desinfectantes químicos se relacionan significativamente con la higienización de la lechuga en el laboratorio de la UPT 2018. **HIPÓTESIS ESPECIFICAS** **H1:** La concentración de ácido láctico se relaciona significativamente con la higienización de la lechuga en el laboratorio de la UPT 2018. **H2:** La concentración de ozono se relaciona significativamente con la higienización de la lechuga en el laboratorio de la UPT 2018. **H3:** La concentración de hipoclorito de sodio se relaciona significativamente con la higienización de la lechuga en el laboratorio de la UPT 2018.	**VARIABLE INDEPENDIENTE** Concentración de desinfectantes químicos **DIMENSIONES** ácido láctico (%) Ozono (mg/h) hipoclorito de sodio (ppm) **VARIABLE DEPENDIENTE** Higienización de la lechuga **DIMENSIÓN** Carga microbiana (UFC)	**ENFOQUE** El método es cuantitativo **TIPO DE INVESTIGACIÓN** Aplicada **NIVEL DE INVESTIGACIÓN** Explicativo **DISEÑO** Cuasi Experimental El esquema es**:** **G1 01 X 02** **G2 03 – 04** G1 = Grupo experimental G2 = Grupo control X = Variable Independiente 01 y 03 = Información pre-prueba 02 04 = Información post-prueba

Anexo 2: Matriz de Operacionalización

Variable	Dimensiones	Indicadores	Unidades de medida
Variable Independiente: Concentración de Desinfectantes químicos	Ácido láctico	Concentración química	%
	Ozono	Concentración química	ppm
	Hipoclorito de sodio	Concentración química	ppm
Variable dependiente: Higienización de la lechuga	Carga microbiana	Conteo bacteriano	UFC

Anexo 3: Validación de Instrumentos

Normas AOAC

CERTIFICATE

By Authority Of
THE UNITED STATES OF AMERICA
Legally Binding Document

By the Authority Vested By Part 5 of the United States Code § 552(a) and Part 1 of the Code of Regulations § 51 the attached document has been duly INCORPORATED BY REFERENCE and shall be considered legally binding upon all citizens and residents of the United States of America. *HEED THIS NOTICE*: Criminal penalties may apply for noncompliance.

Document Name: AOAC: Official Methods of Analysis (Volume 1)

CFR Section(s): 9 CFR 318.19(b)

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THE EXECUTIVE DIRECTOR
OFFICE OF THE FEDERAL REGISTER
WASHINGTON, D.C.

17. Microbiological Methods

Wallace H. Andrews and James Messer, *Associate Chapter Editors*
Food and Drug Administration

(When preparing culture media, use distd or deionized H_2O such as Purified Water USP XXII, found to be free from traces of dissolved metals, and bactericidal or inhibitory compds. Use anhyd. salts unless otherwise specified.)

970.77 Cross Reference Tables

A. Methods for Examination of Foods

B. Methods for Examination of Organisms

Virus
Beef, ground **975.56**

EGGS AND EGG PRODUCTS

939.14 Sampling of Eggs and Egg Products

Microbiological Methods

Final Action

("Compendium of Methods for the Microbiological Examination of Foods," 2nd ed. Prepd by the APHA Intersociety/Agency Committee on Microbiological Methods for Foods. 1984. Marvin L. Speck, Ed., should be used as guide for further study of microorganisms obtained in culturing technics described.)

A. Equipment

(**a**) *Liquid eggs.*—Sampling tube or dipper, sterile sample containers with tight closures (pt (500 mL) Mason jars or friction top cans are most practical), alcohol, alcohol lamp or other burner, absorbent cotton, clean cloth or towel, and H_2O pail.

(**b**) *Frozen eggs.*—Elec. (high-speed) or hand drill with 1 × 16″ auger, hammer and steel strip (12 × 2 × 0.25″), or other tool for opening cans; tablespoon, hatchet or chisel, precooled sterile containers, etc., as in (**a**).

(**c**) *Dried eggs.*—Grain trier long enough to reach to bottom of containers to be sampled. Clean sample containers with tight closures (pt (500 mL) Mason jars or paperboard cartons), clean cloth or towel, and tablespoon.

B. Methods

Take samples from representative number of containers in lot, **925.29**. Sterilize sampling tube or dipper, auger, spoon, and hatchet by wiping with alcohol-soaked cotton and flaming over alcohol lamp or other burner. Between samplings, thoroly wash instruments, dry, and resterilize. Open and sample all containers under as nearly aseptic conditions as possible.

(**a**) *Liquid eggs.*—Thoroly mix contents of container with sterile sample tube or dipper, and transfer ca 400 mL (0.75 pt) to sterile sample container. Keep samples at <5° but avoid freezing. Observe and record odor of each container sampled as normal, abnormal, reject, or musty.

(**b**) *Frozen eggs.*—Remove top layer of egg with sterilized hatchet or chisel. Drill 3 cores from top to bottom of container: first core in center, second core midway between center and periphery, and third core near edge of container. Transfer drillings from container to sample container with sterile spoon. Examine product organoleptically by smelling at opening of fourth drill-hole made after removal of bacteriological sample. (Heat produced by elec. drill intensifies odor of egg material, thus facilitating organoleptic examination.) Record odors as normal, abnormal, reject, or musty. Refrigerate samples with solid CO_2 or other suitable refrigerant if analysis is to be delayed or sampling point is at some distance from laboratory.

(**c**) *Dried eggs.*—For small packages, take entire parcel or parcels for sample. For boxes and barrels, remove top layer with sterile spoon or other sterile instrument, and with sterile trier remove ≥3 cores as in (**b**). (Samples should consist of ca 400 mL (0.75 pt).) Aseptically transfer core to sample container with sterile spoon or other suitable instrument. Store samples under refrigeration or in cool place.

Ref.: JAOAC **22,** 625(1939).

940.36 Culture Media for Eggs and Egg Products

Microbiological Methods

Final Action

A. Standard Methods Media

(**a**) *Dilution water.*—To prep. stock soln, dissolve 34 g KH_2PO_4 in 500 mL H_2O, adjust to pH 7.2 with 1*N* NaOH (ca 175 mL), and dil. to 1 L with H_2O. To prep. buffered H_2O for dilns, dil. 1.25 mL stock soln to 1 L with boiled and cooled H_2O. Autoclave 15 min at 121°.

(**b**) *Buffered glucose broth (MR-VP medium).*—For Me red-Voges Proskauer (MR-VP) tests. Dissolve 7.0 g proteose peptone, 5.0 g glucose, and 5.0 g K_2HPO_4 in ca 800 mL H_2O with gentle heat and occasional stirring. Filter, cool to 20°, and dil. to 1 L. Dispense 10 mL portions into test tubes and autoclave 12–15 min at 121°. Max exposure to heat should be ≤30 min. Final pH, 6.9 ± 0.2.

(**c**) *Endo medium.*—Suspend 3.5 g K_2HPO_4, 10.0 g peptone, 20.0 g agar, and 10 g lactose in 1 L H_2O. Boil to dissolve, add H_2O to original vol., and clarify if necessary. Dispense in 100 mL portions and autoclave 15 min at 121°. Final pH, 7.4 ± 0.1. Before use, melt and add 0.25 g Na_2SO_3 and 1.0 mL filtered 5% alc. soln basic fuchsin.

(**d**) *Eosin methylene blue agar (Levine).*—Dissolve 10.0 g peptone, 2.0 g K_2HPO_4, and 15.0 g agar in 1 L H_2O. Boil to dissolve and add H_2O to original vol. Dispense in 100 or 200 mL portions and autoclave 15 min at 121°. Final pH, 7.1 ± 0.1. Before use, melt and to each 100 mL add 5 mL sterile 20% lactose soln, 2.0 mL 2% aq. Eosin Y soln, and 1.3 mL 0.5% aq. methylene blue soln.

(**e**) *Koser's citrate broth.*—Dissolve 1.5 g $NaNH_4HPO_4.4H_2O$, 1.0 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, and 3.0 g Na citrate.$2H_2O$ in 1 L H_2O. Dispense in 10 mL portions into test tubes and autoclave 15 min at 121°. Final pH, 6.7 ± 0.1.

(**f**) *Lactose broth.*—Dissolve on H_2O bath, with stirring, 3.0 g beef ext and 5.0 g polypeptone or peptone in 1 L H_2O. Add 5.0 g lactose. Dispense into fermentation tubes and autoclave 15 min at 121°. Max. exposure to heat should be ≤30 min. Final pH, 6.7 ± 0.2.

(**g**) *Plate count agar (tryptone glucose yeast agar).*—Suspend 5.0 g peptone-tryptone (pancreatic digest of casein), 2.5 g yeast ext, 1.0 g glucose, and 15.0 g agar in 1 L H_2O. Heat and boil until all ingredients are dissolved. Autoclave 15 min at 121°. Final pH, 7.0 ± 0.1.

(**h**) *Tryptophane broth.*—Dissolve by heating, with stirring, 10.0 g tryptone or trypticase in 1 L H_2O. Dispense in 5 mL portions into test tubes and autoclave 15 min at 121°. Final pH, 6.9 ± 0.2.

B. Other Media

(**a**) *Malt agar.*—Dissolve by boiling 30 g malt ext (Difco) and 15.0 g agar in 1 L H_2O. Autoclave 15 min at 121°. Just before use, melt malt agar and acidify with 85% lactic acid to pH 3.5. Do not reheat medium after addn of acid.

(**b**) *Milk protein hydrolysate glucose agar.*—BBL dehydrated, or prep. from 9.0 g milk protein hydrolysate, 1 g glucose, 15 g agar, and 1 L H_2O; adjust to pH 7.0. Autoclave 15 min at 121°, cool to room temp., and readjust pH to 7.0, if necessary.

(**c**) *Physiological salt soln.*—Dissolve 8.5 g NaCl in 1 L H_2O. Autoclave 15 min at 121° and cool to room temp.

(**d**) *Veal infusion agar.*—Mix 500 g ground lean veal and 1 L H_2O. Infuse overnight in refrigerator and strain thru cheesecloth without pressure. Dil. to original vol. with H_2O

and skim off any fat. Steam in Arnold sterilizer 30 min and filter thru paper. Add 10.0 g peptone (Difco), 5.0 g NaCl, and 15.0 g agar.

Steam in Arnold sterilizer to dissolve ingredients. Adjust to pH 7.6 and steam in Arnold sterilizer 15 min. Filter thru buchner with paper pulp mat, with suction. (Use egg albumen for clarification when necessary. Add fresh white of 1 egg previously beaten with 50 mL medium or its equiv. in desiccated egg white (1.5 g) to each L of medium before adjusting pH and after cooling to 50°. Shake thoroly to ensure soln of egg white. Let stand 20 min. Heat in Arnold sterilizer 15 min to coagulate egg white. Shake vigorously and reheat. Filter, adjust to pH 7.6, steam in Arnold sterilizer 15 min, and filter.)

Place 10 mL portions in test tubes or 80 mL portions into bottles. Autoclave 20 min at 121°; final pH, 7.4.

For hemolytic tests, cool melted agar to 45° and add 5% defibrinated horse, sheep, or rabbit blood prior to pouring plates (0.5 mL blood/10 mL medium).

940.37 Technics for Eggs and Egg Products

Microbiological Methods

Final Action

A. Preparation of Sample

(a) *Liquid eggs.*—Thoroly mix sample with sterile spoon or sterile mech. stirrer and prep. 1:10 diln by aseptically weighing 11 g egg material into sterile wide-mouth g-s or screw-cap bottle; add 99 g sterile diln H_2O, **940.36A(a)**, or sterile physiological salt soln, **940.36B(c)**, and 1 tablespoonful sterile glass shot. Thoroly agitate 1:10 diln to ensure complete soln or distribution of egg material in diluent by shaking each container rapidly 25 times, each shake being up-and-down movement of ca 30 cm, time interval not exceeding 7 sec. Let bubbles escape. Transfer representative portion from 1:10 diln for higher serial dilns as needed. Proceed as in **940.37B–F(a)**. Pour all plates and inoculate other media within 15 min after prepn of first diln to avoid growth or death of microorganisms.

(b) *Frozen eggs.*—Thaw frozen egg material as rapidly as possible to avoid increase in number of microorganisms present and at temp. low enough to prevent destruction of the microorganisms (≤45° for ≤15 min). (Frequent rotary shaking of sample container aids in thawing frozen material. Thawing temp. may be maintained by use of H_2O bath or bacteriological incubator.) Proceed as in (a).

(c) *Dried eggs.*—Thoroly mix sample with sterile spoon or spatula. Prep. 1:10 diln as in (a). If material is relatively insol. (stored samples), use 0.1*N* LiOH as diluent. Prep. serial dilns as in (a) and proceed as in **940.37B–F(b)**.

B. Plate Counts

Inoculate one set of petri plates with 1 mL portion of each suitable diln. Pour plates with tryptone glucose yeast agar or milk protein hydrolysate glucose agar previously cooled to 42–45°. Incubate inoculated plates 3 days at 32°. Count plates with aid of Quebec colony counter, if available. Express final results as number of viable microorganisms/g egg material.

C. Incidence of Coliform Group

(a) Inoculate 1.0 mL portions from suitable dilns of egg material into fermentation tubes of lactose broth. Incubate 24–48 hr at 35°. Streak eosin methylene blue or Endo medium plates from all lactose broth cultures showing gas production. Incubate plates 24–48 hr at 35°. Examine plates of differential media for colonies of microorganisms of coliform group. Record number of coliform bacteria/g egg material as reciprocal of highest diln showing pos. confirmation on differential media.

(b) *Biochemical reaction (optional).*—Inoculate from colonies of coliform types of bacteria appearing on differential agar plates to agar slants, **940.36A(g)** or **940.36B(b)**. Incubate 24 hr at 35°. Purify cultures for further study. Obtain IMViC biochem. reactions of purified cultures by following tests:

Kovacs test (indole production), **966.24(a)**;

Acid production in Me red indicator, **966.24(b)**;

Acetylmethylcarbinol production, **966.24(b)**;

Koser sodium citrate test (utilization of Na citrate as sole source of C), **966.24(c)**.

Note: Follow methods for biochem. reactions recommended in "Standard Methods for Examination of Water and Waste Water," 16th ed., 1985, American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1015 15th St NW, Washington, DC 20005.

D. Incidence of Hemolytic Staphylococci and Streptococci —Procedure

Inoculate petri plates with 1 mL portions of suitable dilns of sample. Pour plates with veal infusion agar contg 5% defibrinated horse, sheep, or rabbit blood (0.5 mL blood/10 mL medium). Cool agar to 45° and add blood just before pouring plates. Incubate plates 24 hr at 35°. Confirm presence of coccus types of microorganisms by microscopic examination of smears taken from representative colonies and stained by Gram method. Express final results as number/g.

E. Tests for Fungi—Procedure

Inoculate petri plates with 1 mL portions of suitable dilns of sample. Pour inoculated plates with malt agar, **940.36B(a)**, previously cooled to 42–45°. Incubate plates 5 days at 20° or at room temp., if 20° incubator is not available. Express final results as number of fungi/g egg material. Confirm yeast colonies by microscopic examination of smears stained by Gram method.

F. Direct Microscopic Counts

North aniline oil-methylene blue stain.—Mix 3.0 mL aniline oil with 10.0 mL alcohol, and slowly add 1.5 mL HCl with const agitation. Add 30.0 mL satd alc. methylene blue soln, dil. to 100.0 mL with H_2O, and filter.

(a) *Liquid and frozen eggs.*—Place 0.01 mL undild egg material on clean, dry microscopic slide and spread over area of 2 sq cm (circular area with diam. of 1.6 cm suggested). Let film prepn dry on level surface at 35–40°. Immerse in xylene ≤1 min; then immerse in alcohol ≤1 min. Stain ≥45 sec in North aniline oil-methylene blue stain (10–20 min preferred; exposure up to 2 hr does not overstain). Wash slide by repeated immersions in H_2O and dry thoroly before examination. Observe subsequent operations and precaution as in "Standard Methods for Examination of Dairy Products," 15th ed., 1985, American Public Health Association. Express final result as number of bacteria/g egg material (double microscopic factor, since 2 sq cm area is used).

(b) *Dried eggs.*—Place 0.01 mL of 1:10 or 1:100 diln of dried egg material on clean, dry microscopic slide and spread over 2 sq cm.

Note: 0.1*N* LiOH may be used as diluent and is preferred for samples that are relatively insol. Circular area with diam. of 1.6 cm is preferable. Addn of drop of H_2O to each film facilitates uniform spreading.

Proceed as in (a). Double microscopic factor, since area of 2 sq cm is used, and multiply count by 10 or 100, depending on whether film was prepd from 1:10 or 1:100 diln.

Ref.: JAOAC **36,** 91, 316(1953).

CHILLED, FROZEN, PRECOOKED, OR PREPARED FOODS, AND NUTMEATS

966.23 Microbiological Methods

First Action 1966
Final Action 1989

(For the detn of aerobic plate count, most probable number of coliform bacteria and *Escherichia coli*, and *Staphylococcus* in products such as frozen cooked meat, poultry, and vegetable products; cooked and/or breaded seafood; bakery products; salads; tree nut meats; and ingredients of food samples collected during sanitation inspections of food producing establishments, unless specific directions are given for that product.)

A. Media and Reagents

Ingredients and reagents used to prep. following media may be product of any manufacturer if comparative tests show that satisfactory results are obtained. Use pure carbohydrates suitable for biological use; ACS reagent grade inorg. chemicals; and dyes certified by "Biological Stain Commission" for use in media.

For convenience, dehydrated media of any brand equiv. to formulation may be used. Test each lot of medium for sterility and growth-promoting qualities of suitable organisms (e.g., inoculate media contg lactose with coliform bacteria, *Staphylococcus* media with *Staphylococcus*, etc.).

Det. pH before autoclaving with pH meter stdzd against std buffers, **964.24**. Adjust pH, when necessary, by adding 1*N* NaOH or 1*N* HCl so that stated final pH results after autoclaving.

Use sterile glass or plastic, 100 × 15 mm, petri dishes.

(**a**) *Plate count agar.*—*See* **940.36A(g)**.

(**b**) *Lauryl sulfate tryptose broth.*—Dissolve 20.0 g trypticase or tryptose (pancreatic digest of casein), 5.0 g NaCl, 5.0 g lactose, 2.75 g K_2HPO_4, 2.75 g KH_2PO_4, and 0.1 g Na lauryl sulfate in 1 L H_2O with gentle heat, if necessary. Dispense 10 mL portions into 20 × 150 mm test tubes contg inverted fermentation tubes 10 × 75 mm. Autoclave 15 min at 121°. Final pH, 6.8 ± 0.1.

(**c**) *Brilliant green lactose bile (BGLB) broth.*—Dissolve 10.0 g peptone and 10.0 g lactose in ca 500 mL H_2O. Add soln (pH 7.0–7.5) of 20 g dehydrated oxgall or oxbile in 200 mL H_2O. Dil. to 975 mL and adjust pH to 7.4. Add 13.3 mL 0.1% soln of brilliant green, and dil. to 1 L with H_2O. Filter thru cotton and dispense 10 mL portions into 20 × 150 mm test tubes contg inverted 10 × 75 mm fermentation tubes. Autoclave 15 min at 121°. Final pH, 7.2 ± 0.1.

(**d**) *Eosin methylene blue agar (Levine).*—*See* **940.36A(d)**.

(**e**) *Baird-Parker medium (egg tellurite glycine pyruvate agar, ETGPA).*—(*1*) *Basal medium.*—Suspend 10.0 g tryptone, 5.0 g beef ext, 1.0 g yeast ext, 10 g Na pyruvate, 12.0 g glycine, 5.0 g $LiCl.6H_2O$, and 20.0 g agar in 950 mL H_2O. Heat to bp with frequent agitation to dissolve ingredients completely. Dispense 95 mL portions into screw-capped bottles. Autoclave 15 min at 121°. Final pH, 7.0 ± 0.2 at 25°. Store ≤1 month at 4 ± 1°.

(*2*) *Enrichment.*—Bacto EY tellurite enrichment (Difco Laboratories) or prep. as follows: Soak fresh eggs ca 1 min in diln of satd $HgCl_2$ soln (1 + 1000). Aseptically crack eggs and sep. yolks from whites. Blend yolk and physiological saline soln, **940.36B(c)**, (3 + 7, v/v) in high-speed blender ca 5 sec. To 50 mL egg yolk emulsion add 10 mL filter sterilized 1% K tellurite soln. Mix and store at 4 ± 1°.

(*3*) *Complete medium.*—Add 5 mL warmed enrichment to 95 mL molten basal medium cooled to 45–50°. Mix well, avoiding bubbles, and pour 15–18 mL into sterile 100 × 15 mm petri dishes. Store plates at room temp. (≤25°) for ≤5 days before use. Medium should be densely opaque; do not use nonopaque plates. Dry plates before use by 1 of following methods: (*a*) in convection oven or incubator 30 min at 50° with lids removed and agar surface downward; (*b*) in forced-draft oven or incubator 2 hr at 50° with lids on and agar surface upward; (*c*) in incubator 4 hr at 35° with lids on and agar surface upward; or (*d*) on laboratory bench 16–18 hr at room temp. with lids on and agar surface upward.

(*4*) *Interpretation.*—Colonies of *S. aureus* are typically circular, smooth, convex, moist, 2–3 mm in diam. on uncrowded plates, gray-black to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone (ppt) and frequently with outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle. Occasional non-lipolytic strains may be encountered which have same appearance, except that surrounding opaque and clear zones are absent. Colonies isolated from frozen or desiccated foods which have been stored for extended periods are frequently less black than typical colonies and may have rough appearance and dry texture.

(**f**) *Trypticase (tryptic) soy broth with 10% sodium chloride.*—Add 95 g NaCl to 1 L of soln of 17.0 g trypticase or tryptose (pancreatic digest of casein), 3.0 g phytone (papaic digest of soya meal), 5.0 g NaCl, 2.5 g K_2HPO_4, and 2.5 g glucose. Heat gently if necessary. Dispense into 16–20 mm diam. tubes to depth of 5–8 cm. Autoclave 15 min at 121°. Final pH, 7.3 ± 0.2.

(**g**) *EC broth.*—Dissolve 20.0 g trypticase or tryptose (pancreatic digest of casein), 1.5 g Bacto bile salt No. 3 or bile salt mixt., 5.0 g lactose, 4.0 g K_2HPO_4, 1.5 g KH_2PO_4, and 5.0 g NaCl in 1 L H_2O. Dispense 8 mL into 16 × 150 mm test tubes contg inverted 10 × 75 mm fermentation tube. Autoclave 15 min at 121°. Final pH, 6.9 ± 0.1.

(**h**) *Brain-heart infusion.*—*See* **967.25A(r)**. Dispense into bottles or tubes for storage and autoclave 15 min at 121°.

(**i**) *Desiccated coagulase plasma (rabbit) with EDTA.*—Reconstitute according to manufacturer's directions. If not available, reconstitute *desiccated coagulase plasma (rabbit)* and add Na_2H_2EDTA to final concn of 0.1% in reconstituted plasma.

(**j**) *Tryptophane broth.*—*See* **940.36A(h)** but dispense in 10 mL portions.

(**k**) *Buffered glucose broth (MR-VP medium).*—*See* **940.36A(b)**. BBL Microbiological Systems, No. 11383, or equiv.

(**l**) *Koser's citrate broth.*—*See* **940.36A(e)**.

(**m**) *Butterfield's buffered phosphate diluent.*—(*1*) *Stock soln.*—Dissolve 34.0 g KH_2PO_4 in 500 mL H_2O, adjust to pH 7.2 with ca 175 mL 1*N* NaOH, and dil. to 1 L. Store in refrigerator. (*2*) *Diluent.*—Dil. 1.25 mL stock soln to 1 L with H_2O. Prep. diln blanks with this soln, dispensing enough to allow for losses during autoclaving. Autoclave 15 min at 121°.

B. Preparation of Sample

(Prep. all decimal dilns with 90 mL sterile diluent plus 10 mL previous diln unless otherwise specified. Shake all dilns 25 times in 30 cm arc. Pipets must accurately deliver required vol. Do not use to deliver <10% of their total vol. For example, to deliver 1 mL, do not use pipet >10 mL; to deliver 0.1 mL, do not use pipet >1 mL.)

(**a**) *Frozen and/or prepared foods.*—Use balance with capacity of ≥2 kg and sensitivity of 0.1 g to aseptically weigh 50 g unthawed (if frozen) sample into sterile high-speed blender jar. Add 450 mL diluent, (**m**)(*2*), and blend 2 min. (If necessary to temper frozen sample to remove 50 g portion, hold

≤18 hr at 2–5°.) Not >15 min should elapse from time sample is blended until all dilns are in appropriate media.

If entire sample consists of <50 g, weigh portion equiv. to $^1/_2$ sample and add vol. of sterile diluent required to make 1:10 diln. Total vol. in blender jar must completely cover blades.

(**b**) *Tree nut meat halves and larger pieces.*—Aseptically weigh 50 g sample into sterile jar. Add 50 mL diluent, (**m**)(*2*), and shake vigorously (50 times thru 30 cm arc) to obtain 10^0 diln. Let stand 3–5 min and shake just before making serial dilns and inoculations.

(**c**) *Nut meal.*—Aseptically weigh 10 g sample into sterile jar. Add 90 mL diluent, (**m**)(*2*), and shake vigorously (50 times thru 30 cm arc) to obtain 10^{-1} diln. Let stand 3–5 min and shake to resuspend just before making serial dilns and inoculations.

C. Aerobic Plate Count

Seed duplicate petri dishes in dilns of 1:10, 1:100, 1:1000, etc., using plate count agar, (**a**). Ordinarily 1:100 thru 1:10,000 are satisfactory. Place 1 mL appropriate diln in each plate, and add molten agar (cooled to 42–45°) within 15 min from time of original diln. Incubate 48 ± 2 hr at 35° and count duplicate plates in suitable range (30–300 colonies). If plates do not contain 30–300 colonies, record diln counted and note number of colonies found. Average counts obtained and report as aerobic plate count/g.

966.24 Coliform Group and *Escherichia coli* in Tree Nut Meats

Microbiological Method

Final Action 1971

Seed 3-tube most probable number (MPN) series into lauryl sulfate tryptose broth, (**b**), using 1 mL inocula of 1:10, 1:100, and 1:1000 dilns, with triplicate tubes at each diln. (For nut meats (halves and larger pieces), begin MPN detn with 10^0 diln; for nut meal, begin with 10^{-1} diln.) Incubate 48 ± 2 hr at 35° for gas formation as evidenced by displacement of liq. in insert tube or by vigorous effervescence when tubes are shaken *gently*. Examine tubes for gas formation at 24 and 48 hr intervals. Transfer, using 3 mm loop, from gassing tubes to BGLB, (**c**) (omit this transfer for tree nuts), and EC broth, (**g**), at time gas formation is noted.

Incubate BGLB broth 48 ± 2 hr at 35°. Using MPN Table **966.24**, compute MPN on basis of number of tubes of BGLB broth producing gas by end of incubation period. Report as MPN of coliform bacteria/g.

Incubate EC broth 48 ± 2 hr at 45.5 ± 0.05° in covered H_2O bath. Submerge broth tubes in bath so that H_2O level is above highest level of medium. Examine tubes for gas formation at 24 and 48 hr intervals. Streak gas-pos. tubes on Levine's eosin methylene blue agar plates, (**d**), and incubate plates 24 ± 2 hr at 35°.

Pick 2 or more well isolated typical colonies from Levine's eosin methylene blue agar plates and transfer to agar slants prepd from agar medium, (**a**). Incubate 18–24 hr at 35°. If typical colonies are not present, pick 2 or more colonies most likely to be *E. coli*. Pick ≥2 from every plate.

Transfer growth from plate count agar slants into following broths for identification by biochem. tests:

(**a**) *Tryptophane broth.*—Incubate broth, (**j**), 24 ± 2 hr at 35° and test for indole by adding 0.2–0.3 mL Kovacs reagent, **967.25B**(**a**), to 24 hr culture. Test is pos. if upper layer turns red.

(**b**) *MR-VP medium.*—Incubate medium, (**k**), 48 ± 2 hr at 35°. Aseptically transfer 1 mL culture to 13 × 100 mm test tube to test for acetylmethylcarbinol. Add 0.6 mL 5% alc. α-naphthol soln, 0.2 mL KOH soln (4 + 10), and few crystals of creatine. Shake and let stand 2 hr. Test is pos. if eosin pink develops. Alternatively, *see* **967.27D**(**c**)(*1*).

Incubate remainder of MR-VP medium for addnl 48 hr and test for Me red reaction by adding 5 drops Me red soln to culture. Test is pos. if culture turns red; neg., if yellow. (Prep. Me red soln by dissolving 0.1 g Me red in 300 mL 90% alcohol and dilg to 500 mL with H_2O.)

(**c**) *Koser citrate broth, (1).*—Incubate 96 hr at 35° and record growth as + or −.

(**d**) *Lauryl sulfate tryptose broth, (b).*—Incubate 48 ± 2 hr at 35°. Examine tubes for gas formation.

(**e**) *Gram stain.*—Perform Gram stain on 18 hr agar slant ("Standard Methods for the Examination of Water and Waste Water," 16th ed., 1985). Coliform organisms will stain red (neg.); Gram-pos. organisms will stain blue-black.

(**f**) *Classification.*—Classify biochem. types as follows:

Indole	*MR*	*VP*	*Citrate*	*Type*
+	+	−	−	Typical *E. coli*
−	+	−	−	Atypical *E. coli*
+	+	−	+	Typical Intermediate
−	+	−	+	Atypical Intermediate
−	−	+	+	Typical *Enterobacter aerogenes*
+	−	+	+	Atypical *Enterobacter aerogenes*

Table 966.24 Most Probable Numbers (MPN) per 1 g of Sample, Using 3 Tubes with Each of 0.1, 0.01, and 0.001 g Portions

Positive Tubes				Positive Tubes				Positive Tubes				Positive Tubes			
0.1	0.01	0.001	MPN	0.1	0.01	0.001	MPN	0.1	0.01	0.001	MPN	0.1	0.01	0.001	MPN
0	0	0	<3	1	0	0	3.6	2	0	0	9.1	3	0	0	23
0	0	1	3	1	0	1	7.2	2	0	1	14	3	0	1	39
0	0	2	6	1	0	2	11	2	0	2	20	3	0	2	64
0	0	3	9	1	0	3	15	2	0	3	26	3	0	3	95
0	1	0	3	1	1	0	7.3	2	1	0	15	3	1	0	43
0	1	1	6.1	1	1	1	11	2	1	1	20	3	1	1	75
0	1	2	9.2	1	1	2	15	2	1	2	27	3	1	2	120
0	1	3	12.	1	1	3	19	2	1	3	34	3	1	3	160
0	2	0	6.2	1	2	0	11	2	2	0	21	3	2	0	93
0	2	1	9.3	1	2	1	15	2	2	1	28	3	2	1	150
0	2	2	12	1	2	2	20	2	2	2	35	3	2	2	210
0	2	3	16	1	2	3	24	2	2	3	42	3	2	3	290
0	3	0	9.4	1	3	0	16	2	3	0	29	3	3	0	240
0	3	1	13	1	3	1	20	2	3	1	36	3	3	1	460
0	3	2	16	1	3	2	24	2	3	2	44	3	3	2	1100
0	3	3	19	1	3	3	29	2	3	3	53	3	3	3	>1100

Other groupings may appear; in such cases cultures are usually mixed. Restreak to det. their purity.

Compute MPN of *E. coli*/g, considering Gram neg., nonspore-forming rods producing gas in lactose and producing ++−− or −+−− IMViC patterns as *E. coli*.

Refs.: JAOAC **49,** 270, 276(1966); **51,** 865, 867(1968); **58,** 1154(1975).

977.27 Bacteria in Foods and Cosmetics

Spiral Plate Method

First Action 1977
Final Action 1981

A. Principle

Bacterial suspension from prepd sample of food or cosmetic is deposited continuously on surface of rotating agar plate. Resultant track on surface is in form of Archimedes spiral. Vol. is decreased while dispensing stylus moves from center to edge so that exponential relationship exists between vol. deposited and radius of agar. On incubation, colonies develop along lines where liq. was deposited. Counting grid is calibrated for sample vol. associated with different areas of agar. No. colonies per known area is counted and calcd to bacterial concn.

B. Apparatus

Spiral plating machine.—For use with 150 × 15 mm (100 × 15 mm may be used) petri dishes and adjusted to deliver total vol. of 0.035 mL/plate. Platform carrying plate is rotated at ca 50 rpm and is connected mech. to lead screw driving hollow syringe dispenser. Backflow syringe, 2-way valve, and vac. trap control loading and dispensing of sample, disposal of residual sample, and rinsing of system. Liq. is dispensed from backflow syringe thru thin wall Teflon tubing thru stylus to surface of agar plate. (Available com. from Spiral Systems Marketing, 4853 Cordell Ave, Suite A10, Bethesda, MD 20014.)

C. Plates

Pour 40–45 mL portions plate count agar, **940.36A(g)**, into 150 × 15 mm (100 × 15 mm may be used) petri dishes; let harden and dry to smooth, even surface.

D. Calibration of Spiral Counters

To det. vol. associated with different parts of counting grid, prep. 11 bacterial suspensions by dilg 1:1 from 10^6 to 10^3 cells/mL (use nonspreaders). Plate all dilns in duplicate by both **966.23D** and spiral plater, using same medium and incubator. Count spiral plates as in **977.27G** and divide by av. count/mL by **966.23C** to calc. vol. of counted grid area.

$$\text{mL in counted area} = \frac{\text{No. spiral colonies on area}}{\text{count/mL by } \mathbf{966.23C}}$$

E. Preparation of Samples

Weigh 50 g sample into sterile blender jar, add 450 mL diln H_2O, **940.36A(a)**, and blend 2 min. If necessary, let settle few min before removing portion of supernate for spiral plating. (Presence of particles may clog tubing.)

Liqs may be used directly or after dilg 1 + 9 with diln H_2O.

F. Operation

Check stylus tip angle by letting vac. hold microscope cover slip against face of stylus tip at 1 mm above platform. Cover slip should be parallel to rotating platform in all directions. Adjust angle if necessary. Check stylus at start position.

Clean stylus tip before use and between plating each sample by rinsing 1 sec with com. 5.25% NaOCl soln and then 1 sec with sterile H_2O. Identify 3 disposable polyethylene sample cups and fill with com. 5.25% NaOCl soln, sterile H_2O, and sample. Turn vac. filling valve to "on" and move sample holder into position under stylus tip. Lower stylus into NaOCl soln and lift out twice. Repeat with H_2O. Lower stylus into sample soln. Draw soln thru stylus until continuous column of liq. is present in tube above vac. filling valve. With tip of stylus still below surface of sample, close vac. valve. Raise stylus and move sample holder out of way.

Identify lid of agar plate and remove lid. Place dish on turntable, and lower stylus until tip rests freely on agar surface. Start app. and let rotate until stylus is lifted and app. stops automatically. Remove dish and replace cover. Incubate 48 ± 3 hr at 35 ± 1°.

After all samples have been plated, flush app. with NaOCl soln and H_2O. When not in use, leave filled with H_2O.

G. Counting Spiral Plates

Transparent viewing grid consists of 13.2 cm circle divided into 5 areas by 4 concentric circles equidistant along diam. (marked 1 (furthest) and 4 (nearest) to center) and into eight 45° wedges or octants, marked A thru H. Thus, each octant is subdivided by 4 arcs linearly equidistant from each other. Outer ring of 2 opposite octants (e.g., A and E) is further subdivided in half by arc in middle (marked $^1/_2$), and outer ring thus formed is divided in half by line toward center. Addnl lines are provided for use with 10 cm plates.

After incubation, center plate over grid. Choose any octant sector and count colonies from outer edge toward center until 20 colonies have been counted. Continue counting remaining colonies contained in segment in which 20th colony was observed. Record this count together with No. segment that included 20th colony (i.e., $^1/_2$, 1, 2, 3, or 4). Count opposite similar segment and add together. If 20 colonies are not contained in an octant, count all colonies on plate and designate as T (total). If total No. colonies counted exceeds 75 in completing count in segment contg 20th colony, count will generally be low because of coincidence error associated with crowding of colony. In this case, count circumferentially adjacent annular segments starting with sector 1 until ≥50 colonies are counted, and complete count of remaining colonies in segment in which 50th colony was observed.

Divide No. colonies counted (or sum of 2 sector counts) by corresponding vol. sectors counted in mL to obtain bacterial count/mL. Use as vol. that calcd for that sector(s) from calibration, **977.27D**, based on std plate count.

Refs.: JAOAC **60,** 807(1977); **64,** 408(1981).

986.32 Aerobic Plate Count in Foods

Hydrophobic Grid Membrane Filter Method

First Action 1986
Final Action 1987

A. Principle

Hydrophobic grid membrane filter (HGMF) uses membrane filter imprinted with hydrophobic material in grid pattern. Hydrophobic lines act as barriers to spread of colonies, thereby dividing membrane filter surface into sep. compartments of equal and known size. Number of squares occupied by colonies is enumerated and converted to most probable number value of organisms by using formula given below.

B. Apparatus, Culture Media, and Reagents

(**a**) *Hydrophobic grid membrane filter (HGMF).*—Membrane filter has pore size of 0.45 μm and is imprinted with

nontoxic hydrophobic material in grid pattern. ISO-GRID (available from QA Laboratories, Ltd, 135 The West Mall, Toronto, Ontario, Canada M9C 1C2) or equiv. meets these specifications.

(**b**) *Filtration units for HGMF.*—Equipped with 5 μm mesh prefilter to remove food particles during filtration. One unit is required for each sample. ISO-GRID (available from QA Laboratories, Ltd) or equiv. meets these specifications.

(**c**) *Pipets.*—1.0 mL serological with 0.1 mL graduations, 1.1 or 2.2 mL milk pipets are satisfactory. 5.0 mL serological with 0.1 mL graduations.

(**d**) *Blender.*—Waring Blendor, or equiv. multispeed model, with low-speed operation at 10 000–12 000 rpm, and 250 mL glass or metal blender jars with covers. One jar is required for each sample.

(**e**) *Vacuum pump.*—H_2O aspirator vac. source is satisfactory.

(**f**) *Manifold or vacuum flask.*

(**g**) *Peptone/Tween 80 (PT) diluent.*—Dissolve 1.0 g peptone (Difco 0118) and 10.0 g Tween 80 in 1 L H_2O. Dispense enough vol. into diln bottles to give 90 ± 1 mL or 99 ± 1 mL after autoclaving 15 min at 121°.

(**h**) *Tryptic soy-fast green agar (TSFA).*—15.0 g tryptone, 5.0 g phytone (or soytone), 5.0 g NaCl, 0.25 g fast green FCF (CI No. 42053), and 15.0 g agar dild to 1 L with H_2O. Heat to boiling. Autoclave 15 min at 121°. Temper to 50–55°. Aseptically adjust pH to 7.3 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use.

(**i**) *Tris buffer.*—1.0M. Dissolve 121.1 g tris(hydroxymethyl)amino methane in ca 500 mL H_2O. Adjust soln to desired pH with concd HCl and dil. to 1 L with H_2O. Store at either room temp. or 4–6°.

(**j**) *Acetate buffer.*—1.0M. Dissolve 60 mL glacial acetic acid in ca 500 mL H_2O. Adjust soln to desired pH with 5M NaOH and dil. to 1 L with H_2O. Store at 4–6°.

(**k**) *Amylase stock soln.*—Dil. 10 g α-amylase (Sigma Chemical Co., No. A1278 or equiv.) to 100 mL with tris buffer, pH 7.0. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material; then filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**l**) *Cellulase stock soln.*—Dil. 10 g cellulase (Sigma No. C0901 or equiv.) to 100 mL with acetate buffer, pH 5.0. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material; then filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**m**) *Diastase stock soln.*—Dil. 10 g diastase (Sigma No. A6880 or equiv.) to 100 mL with tris buffer, pH 7.0. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material; then filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**n**) *Hemicellulase stock soln.*—Dil. 10 g hemicellulase (Sigma No. H2125 or equiv.) to 100 mL with acetate buffer, pH 5.5. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material; then filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**o**) *Trypsin stock soln.*—Dil. 10 g trypsin (Difco No. 0153 or equiv.) to 100 mL with tris buffer, pH 7.6. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material; then filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**p**) *Lecithinase (phospholipase A_2) stock soln.*—Dil. com. enzyme soln (Sigma No. P9139 or equiv.) to 25 units/mL with tris buffer, pH 8.0. Filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**q**) *Pectinase stock soln.*—Use com. enzyme soln of pectinase from *Aspergillus niger,* contg 3–6 units/mg protein, dissolved in 40% glycerol (Sigma No. P5146 or equiv.). Filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**r**) *Protease stock soln.*—Use com. enzyme soln of protease from *Bacillus subtilis,* containing 7–15 units/mg protein (Biuret) in aq. soln (Sigma No. P8775 or equiv.) Filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

C. Sample Preparation

(**a**) *Liquid egg.*—Thoroly mix sample with sterile spoon or spatula and prep. 1:10 diln by aseptically weighing 11 g egg material into sterile wide-mouth g-s or screw-cap bottle; add 99 mL PT diluent, (**g**), and 1 tablespoon of sterile glass shot. Thoroly agitate 1:10 diln to ensure complete soln or distribution of egg material in diluent by shaking each container rapidly 25 times, each shake being up-and-down movement of ca 30 cm, time interval not exceeding 7 s. Let bubbles escape. Transfer representative portion from 1:10 diln for higher serial dilns as needed. If enzyme treatment is needed (see Table **986.32**), combine 5 mL of 1:10 diln with 1 mL enzyme stock soln. Incubate 20–30 min at 35–37° in H_2O bath. Correct for addnl diln factor by filtering 1.2 mL of enzyme-treated sample.

(**b**) *Other liquid samples.*—Mix contents of sample container thoroly. To prep. 1:10 diln, aseptically transfer 10 mL sample into 90 mL PT diluent, (**g**). Mix by shaking bottle 25 times thru 30 cm arc in 7 s. Transfer representative portions

Table 986.32 Enzyme Treatments for Foods[a]

Food	Enzyme
Skim milk	none
Raw milk	none
Fluid dairy products other than skim milk	trypsin
Ice cream: without stabilizers	trypsin
contg gums	hemicellulase
contg cellulose derivatives	cellulase
Spray-dried milks	trypsin
Cheeses	trypsin
Spray-dried cheese powders	cellulase or protease[b]
Sour cream	diastase
Yoghurt	trypsin
Butter	none
Margarine	none
Egg: liq. or powder	trypsin
Raw beef, pork, poultry	trypsin
Cooked meat or poultry	trypsin
Flour	none
Rice	none
Chocolate	amylase
Breakfast cereals	cellulase
Cake mixes	amylase
Fruit puree (e.g., fig paste)	pectinase
Raw vegetables	none
Lecithin	lecithinase
Food colorings	none
Gums	hemicellulase
Citrus juices	pectinase
Infant formula	trypsin
Sodium caseinate	protease
Nut meats	none
Shrimp	none
Oysters	trypsin

[a] Based on analysis of 1 mL of 1:10 diln. Foods tested at dilns of 1:100 or higher do not usually need enzyme treatment.

[b] Varies, depending on individual product.

from 1:10 diln for higher serial dilns as needed. If enzyme treatment is needed (see Table **986.32**), combine 5 mL of 1:10 diln with 1 mL enzyme stock soln. Incubate 20–30 min at 35–37° in H_2O bath. Correct for addnl diln factor by filtering 1.2 mL of enzyme-treated sample.

(**c**) *Whole egg powder.*—Thoroly mix sample with sterile spoon or spatula and prep. 1:10 diln by aseptically weighing 11 g egg material into sterile wide-mouth g-s or screw-cap bottle; add 99 mL PT diluent, (**g**), and 1 tablespoon of sterile glass shot. Thoroly agitate 1:10 diln to ensure complete soln or distribution of egg material in diluent by shaking each container rapidly 25 times, each shake being up-and-down movement of ca 30 cm, time interval not exceeding 7 s. Let bubbles escape. Transfer representative portion from 1:10 diln for higher serial dilns as needed. If testing 1:10 diln is necessary, prep. 1:100 diln and combine 10 mL of 1:100 diln with 1 mL trypsin stock soln, (**o**). Incubate 20–30 min at 35–37° in H_2O bath. Filter entire 11 mL vol. to test 1:10 diln.

(**d**) *Other foods.*—To prep. 1:10 diln, aseptically weigh 10 g sample into sterile blender jar. Add 90 mL PT diluent, (**g**), and blend 2 min at low speed (10 000–12 000 rpm). Transfer representative portion from 1:10 diln for higher serial dilns as needed. If enzyme treatment is needed (see Table **986.32**), combine 5 mL of 1:10 diln with 1 mL enzyme stock soln. Incubate 20–30 min at 35–37° in H_2O bath. Correct for addnl diln factor by filtering 1.2 mL of enzyme-treated sample.

D. Analysis

Select appropriate diln for analysis, depending on desired counting range. Ordinarily, 1:100 diln is satisfactory, producing counting range of 100/g or mL to 500 000/g or mL. Use 1:10 diln if very low counts are expected.

(See Figs **986.32A** and **986.32B**). Turn on vac. source. Place sterile filtration unit on manifold or vac. flask. Open clamp A. Rotate back funnel portion C. Aseptically place sterile HGMF on surface of base D. Rotate funnel forward. Clamp shut by sliding jaws L of stainless steel clamp over entire length of flanges B extending from both sides of funnel C and base D, and rotating moving arm K into horizontal (locked) position.

Aseptically add ca 15–20 mL sterile H_2O to funnel. Pipet required vol. of appropriate diln into funnel. Apply free end of vac. tubing E to suction hole F to draw liq. thru prefilter mesh G. Aseptically add addnl 10–15 mL sterile H_2O to funnel and draw thru mesh as before. Close clamp A to direct vac. to base of filtration unit and draw liq. thru HGMF.

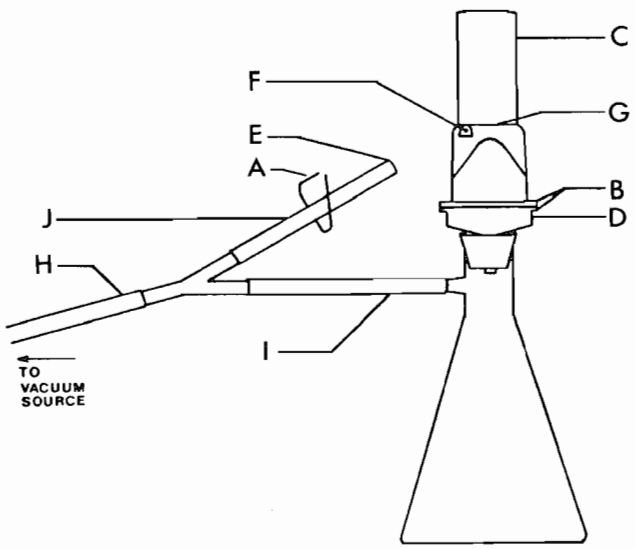


FIG. 986.32A—Filtration unit

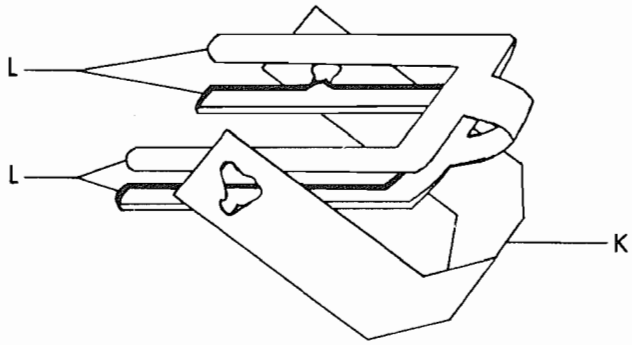


FIG. 986.32B—Filtration unit clamp

Open clamp A. Rotate moving arm K of stainless steel clamp into unlocked (ca 45° angle) position and slide jaws L off of flanges B. Rotate back funnel C. Aseptically remove HGMF and place on surface of pre-dried TSFA (**h**) plate. Avoid trapping air bubbles between filter and agar.

(**a**) *Raw milk, pasteurized milks and creams, and egg powders.*—Incubate 48 ± 3 h at 32°. Colonies will be various shades of green. Count all squares contg one or more colonies (pos. squares) except that if a single colony has clearly spread to adjacent squares, count it as one pos. square. Convert pos. square count to MPN with the formula, MPN = $[N \log_e (N/(N - x))]$, where N = total number of squares and x = number of pos. squares. Multiply by reciprocal of diln factor and report as MPN of total bacteria/g or mL.

(**b**) *Liquid egg.*—Incubate 3 days (72 ± 3 h) at 32°. Proceed as in (**a**).

(**c**) *All other foods.*—Incubate 48 ± 3 h at 35°. Proceed as in (**a**).

Ref.: JAOAC **69,** 671(1986).

988.18 Aerobic Plate Count

Pectin Gel Method

First Action 1988

A. Principle

Method uses pretreated petri plates contg thin "hardener" layer, and liq. medium contg nutrients with pectin as only gelling agent. Liq. medium, 12–15 mL, is poured into pretreated petri plate and undild or dild sample is added. Plate is rotated and rocked to mix sample and medium. Plates are then allowed to stand on level surface 30–40 min until medium solidifies. Total process is done at ambient temp. Plates are then incubated and counted.

B. Materials

Note: Before pectin base medium formulated from individual ingredients is used, comparability to commercially available medium must be demonstrated.

Pectin gel tubes and plates.—Pectin gel is available as sterile liq. in individual tubes contg sufficient gel to pour 1 plate. Use tubes of Redigel and pretreated petri plates (RCR Scientific, Inc., 206 W Lincoln Ave, Goshen, IN 46526), or equiv. that meets specifications.

To prep. plate count pectin gel from individual ingredients, suspend 5.0 g pancreatic digest of casein, 2.5 g yeast ext, and 1.0 g glucose, in 500 mL H_2O. Suspend 15 g low methoxyl pectin in 500 mL H_2O. Heat individual mixts until all ingre-

dients are dissolved. Autoclave solns 15 min at 121°. Combine nutrient and pectin solns and adjust pH to 7.0 ± 0.1. To prep. pretreated petri plates, prep. hardener layer mixt. of 1% agar with 0.02 $CaCl_2$ concn. Sterilize mixt. by autoclaving 15 min at 121°. Aseptically dispense 5 mL portions of mixt. into sterile petri plates.

C. Preparation of Samples

Prep. all decimal dilns with 90 mL sterile diluent (Butterfield's phosphate buffer) plus 10 mL of previous diln unless otherwise specified. Shake all dilns 25 times in 30 cm arc. Pipets must accurately deliver required vol. Do not use to deliver <10% of their total vol. For example, to deliver 1 mL, do not use pipet >10 mL; to deliver 0.1 mL, do not use pipet >1 mL.

(a) *Dairy products.*—Measure (or weigh) 11 mL (or g) sample and dil. in 99 mL Butterfield's diluent. For solid samples, blend 2 min at 10 000 to 12 000 rpm. Prep. addnl dilns so that total colonies/plate is in 25–250 range. Incubate plates 48 ± 3 h at 32 ± 1°.

(b) *Nondairy products.*—Weigh 50 g sample into 450 mL Butterfield's diluent and blend 2 min at 10 000 to 12 000 rpm. Prep. further dilns by dispensing 10 mL sample into 90 mL diluent so that total colonies/plate is in 30–300 range. Incubate plates 48 ± 2 h at 35 ± 1°.

D. Determination

(1) Lift lid of pretreated petri plate and pour liq. pectin gel from 1 tube (12–15 mL) into plate. Replace lid and swirl plate to cover bottom with pectin gel. Prep. number of plates needed for samples being run (duplicate plates for each diln). Plates *must* be used within 5 min after liq. pectin gel is poured.

(2) Add 1 mL inoculum (sample) to liq. pectin gel in petri plate. Touch pipet tip once to dry spot on inside wall of plate (above level of liq. pectin gel) after dispensing sample to rest point in pipet tip. *Immediately* rotate and rock plate to mix sample thoroly with pectin gel. Do not spill pectin gel over sides of plate. (*Note:* This step is primary difference in procedure between pectin gel and agar-based media. *Do not* add inoculum (sample) to pretreated petri plate and pour pectin gel over it. This would lock sample in one small area of plate without sepn of individual colonies.)

(3) Let inoculated plates stand on level surface until pectin gel is solid (ca 30–40 min), and then incubate 48 ± 2 h at 35 ± 1° for nondairy products and 48 ± 3 h at 32 ± 1° for dairy products.

(4) Count duplicate plates in suitable range (30–300 colonies for nondairy products, 25–250 colonies for dairy products). If plates do not contain proper range of colonies, record diln counted and note number of colonies found. Average counts obtained and report as aerobic plate count/g or mL.

Ref.: JAOAC **71,** 343(1988).

COLIFORMS

989.11 Coliforms in Dairy Products

Pectin Gel Method

First Action 1989

A. Principle

Method uses pretreated plates contg thin "hardener" layer and liq. medium contg nutrients with pectin as sole gelling agent. Liq. medium (10–12 mL) is poured into pretreated plate and undild or dild sample is added. Plate is rotated and rocked to mix sample and medium. Plates are then allowed to rest on level surface until medium solidifies. Then, 3–4 mL liq. medium is poured as overlay and allowed to solidify. Total process is done at ambient temp. Plates are then incubated and counted as for agar-based prepns.

B. Materials

Note: Pectin base medium may be formulated from individual ingredients; suitability for analysis must be demonstrated.

Pectin gel and plates.—Violet red bile (VRB) pectin gel is available as sterile liq. in individual units contg sufficient gel to pour 1 plate or in units to pour 8 plates. VRB Redigel and pretreated plates (RCR Scientific, Inc., 206 W Lincoln Ave, Goshen, IN 46526), or equiv., meet specifications of method.

To prep. VRB pectin gel from individual ingredients, suspend 7.0 g pancreatic digest of gelatin, 3.0 g yeast ext, 10.0 g lactose, 1.5 g bile salts No. 3, 5.0 g NaCl, 0.03 g neutral red, and 0.002 g crystal violet in 500 mL H_2O. Suspend 15 g low methoxyl pectin in 500 mL H_2O. Heat individual mixts until all ingredients are dissolved. Autoclave solns 15 min at 121°. Combine nutrient and pectin solns and adjust pH to 7.4 ± 0.2. To prep. pretreated petri plates, prep. hardener layer mixt. of 1% agar with 0.02 $CaCl_2$ concn. Sterilize mixt. by autoclaving 15 min at 121°. Aseptically dispense 5 mL portions of mixt. into sterile petri plates.

C. Preparation of Samples

To prep. dilns, measure (or weigh) 11 mL (or g) sample and dil. in 99 mL Butterfield's or 2% Na citrate diluent. For solid samples, blend 2 min at 10 000 to 12 000 rpm. Prep. addnl dilns so that total colonies/plate is in 25–250 range. Incubate plates 48 ± 3 h at 32 ± 1°. Shake all dilns 25 times in 30 cm arc. Pipets must accurately deliver required vol. Do not use to deliver <10% of their total vol. For example, to deliver 1 mL, do not use pipet > 10 mL; to deliver 0.1 mL, do not use pipet >1 mL.

D. Determination

(1) Lift lid of pretreated petri plate and pour ca 75% (10-12 mL) of liq. medium from tube into plate. (*Note:* Remove cap from each tube of liq. pectin gel medium as it is needed to pour plate.) Prep. number of plates, in duplicate, needed for samples being run. Replace lid and swirl plate to cover bottom with liq. medium. Plates must be used within 5 min.

(2) Add inoculum (sample) to liq. pectin gel in petri plate. Touch pipet tip once to dry spot on inside wall of plate (above level of liq. medium) after dispensing sample to rest point in pipet tip. *Immediately* rotate and rock plate to mix sample thoroly with pectin gel. Do not spill mixt. over sides of plate. (*Note:* This step is primary difference in procedure between pectin gel and agar-based media. *Do not* add inoculum (sample) to pretreated petri plate and pour pectin gel over it. This would lock sample in one small area of plate without sepn of individual colonies.)

(3) Let inoculated plates stand on level surface until pectin gel is solid, and then pour remaining medium (3–4 mL) from tube as overlay and let gel solidify. Incubate in same manner as for agar-based plates (24 ± 2 h at 32 ± 1°).

(4) After 24 h incubation, count all red or pink colonies. Report as coliforms/mL or g.

(5) Pick 5 colonies of each type present on each plate and transfer to brilliant green lactose bile broth fermentation tubes, **966.23A(c)**. Incubate 48 ± 3 h at 32 ± 1° and check for gas production, which is considered pos. for coliforms.

(6) If any picks from step 5 are neg. for gas production, adjust counts (step *4*) accordingly.

Ref.: JAOAC **72,** 298(1989).

986.33 Bacterial and Coliform Counts in Milk

Dry Rehydratable Film Methods

First Action 1986
Final Action 1988

A. Principle

Method uses bacterial culture plates of dry medium and cold H_2O-sol. gel. Undild or dild samples are added directly to plates at rate of 1.0 mL per plate. Pressure, when applied to plastic spreader placed on overlay film, spreads sample over ca 20 sq. cm growth area. Gelling agent is allowed to solidify and plates are incubated and then counted. Either pipet or plate loop continuous pipetting syringe can be used for sample addn for bacterial count analyses.

B. Apparatus

(**a**) *Std method plates.*—Plates contain std methods media nutrients, **940.36A(g)**, cold H_2O-sol. gelling agent coated onto film base, overlay film coated with gelling agent, and 2,3,5-triphenyltetrazolium chloride indicator. Circular growth area of single plate contains ca twenty 1 cm squares outlined on film base. Petrifilm Aerobic Count Plates® (available from Medical-Surgical Division/3M, 225-5S 3M Center, St. Paul, MN 55144) or equiv. meets these specifications.

(**b**) *Violet red bile plates.*—Plates contain violet red bile nutrients conforming to APHA standards as given in Compendium of Methods for the Microbiological Examination of Foods, 2nd ed., 1984 (American Public Health Association, 1015 15th St, NW, Washington, DC 20005), cold H_2O sol. gelling agent, and 2,3,5-triphenyltetrazolium chloride. Petrifilm VRB Plates® (available from Medical-Surgical Division/3M), or equiv. meets these specifications.

(**c**) *Plastic spreader.*—Provided with Petrifilm plates, consists of concave side and smooth flat side, designed to spread milk sample evenly over plate growth area.

(**d**) *Pipets.*—Calibrated for bacteriological use of plate loop continuous pipetting syringe to deliver 1.0 mL.

(**e**) *Colony counter.*—Std app., Quebec model preferred, or one providing equiv. magnification and visibility.

C. Analysis

(**a**) *Bacterial colony count.*—Use Petrifilm Aerobic Count Plates or equiv. plates. Place plate on flat surface. Lift top film and inoculate 1 mL sample onto center of film base. Carefully roll top film down onto inoculum. Distribute sample over prescribed growth area with downward pressure on center of plastic spreader device (recessed side down). Leave plate undisturbed 1 min to permit gel to solidify. Incubate plates 48 ± 3 h at 32° ± 1°.

In incubator, place plates in horizontal position, clear side up, in stacks not exceeding 10 units. Count plates promptly after incubation period. If impossible to count at once, store plates after required incubation at 0–4.4° for not >24 h. This should be avoided as a routine practice.

Use std colony counter for counting purposes. Magnifier-illuminator may also be used to facilitate counting. Colonies stain in various shades of red. Count all colonies in countable range (30–300 colonies).

To compute bacterial count, multiply total number of colonies per plate (or av. number of colonies per plate if counting duplicate plates of same diln) by reciprocal of diln used. When counting colonies on duplicate plates of consecutive dilns, compute mean number of colonies for each diln before detg av. bacterial count. Estd counts can be made on plates with >300 colonies and should be reported as estd counts. In making such counts, circular growth area can be considered to contain ca twenty 1 cm squares. To isolate colonies for further identification, lift top film and pick colony from gel.

(**b**) *Coliform count.*—Use Petrifilm Coliform Count Plates or equiv. plates. Proceed as in (**a**), but distribute sample over plate by using plastic spreader, flat side down. Incubate plates 24 ± 2 h at 32° ± 1°. Count as in (**a**), but count only red colonies that have one or more gas bubbles associated (within 1 colony diam.) with them. Count all colonies in countable range (15–150 colonies). Red colonies without gas bubbles are not counted as coliform organisms.

Ref.: JAOAC **69,** 527(1986).

989.10 Bacterial and Coliform Counts in Dairy Products

Dry Rehydratable Film Methods

First Action 1989

Method Performance:
AEROBIC COUNT
Chocolate milk:
s_r = 0.102; s_R = 0.177; RSD_r = 4.3%; RSD_R = 7.5%
Cheese:
s_r = 0.113; s_R = 0.117; RSD_r = 3.6%; RSD_R = 3.7%
Nonfat dry milk:
s_r = 0.151; s_R = 0.230; RSD_r = 4.5%; RSD_R = 6.9%
Evaporated milk:
s_r = 0.193; s_R = 0.198; RSD_r = 8.3%; RSD_R = 8.5%
Ice cream:
s_r = 0.180; s_R = 0.222; RSD_r = 6.9%; RSD_R = 8.5%

COLIFORM COUNT
Chocolate milk:
s_r = 0.164; s_R = 0.257; RSD_r = 9.2%; RSD_R = 14.4%
Cheese:
s_r = 0.221; s_R = 0.225; RSD_r = 10.4%; RSD_R = 10.6%
Nonfat dry milk:
s_r = 0.197; s_R = 0.151; RSD_r = 8.5%; RSD_R = 4.5%
Evaporated milk:
s_r = 0.200; s_R = 0.225; RSD_r = 13.0%; RSD_R = 13.0%
Ice cream:
s_r = 0.081; s_R = 0.131; RSD_r = 4.1%; RSD_R = 6.6%

A. Principle

Method uses bacterial culture plates of dry medium and cold H_2O-sol. gel. Undild or dild samples are added to plates at rate of 1.0 mL per plate. Pressure, when applied to plastic spreader placed on overlay film, spreads sample over ca 20 sq. cm growth area. Gelling agent is allowed to solidify and plates are incubated and then counted. Pipet, plate loop continuous pipetting syringe, or automatic pipet can be used for sample addn for bacterial count analyses.

B. Apparatus and Reagent

(**a**) *Aerobic count plates.*—Plates contain std methods media nutrients, **940.36A(g)**, cold H_2O-sol. gelling agent coated onto film base, overlay film coated with gelling agent, and 2,3,5-triphenyltetrazolium chloride indicator. Circular growth area of single plate contains ca twenty 1 cm squares outlined on film base. Petrifilm Aerobic Count Plates™ (available from Medical-Surgical Division/3M, 225-5S 3M Center, St. Paul, MN 55144) meet these specifications.

(**b**) *Coliform count plates.*—Plates contain violet red bile

nutrients conforming to APHA standards as given in Compendium of Methods for the Microbiological Examination of Foods, 2nd ed., 1984 (American Public Health Association, 1015 18th St, NW, Washington, DC 20005), cold H_2O-sol. gelling agent, and 2,3,5-triphenyltetrazolium chloride indicator. Petrifilm Coliform Count Plates™ (available from Medical-Surgical Division/3M) meet these specifications.

(c) *Plastic spreader.*—Provided with Petrifilm plates, consists of recessed side and smooth flat side, designed to spread sample evenly over plate growth area.

(d) *Pipets.*—Calibrated for bacteriological use, or plate loop continuous pipetting syringe to deliver 1.0 mL. Automatic pipet to deliver 1.0 mL may be used.

(e) *Colony counter.*—Std app., Quebec model preferred, or one providing equiv. magnification and visibility.

(f) *Dilution water.*—*See* **940.36A(a)**.

C. Sample Preparation

(a) For total plate counts: Aseptically prep. 1:10 diln (11 g/99 mL diln H_2O). Mix well and plate. Prep. addnl dilns as required. Ordinarily, 1:10 and 1:100 dilns are sufficient.

(b) For coliform counts:

(1) Cream, half-and-half, condensed mild, egg nog, cottage cheese, butter, margarine, and related products.—Make 1:5 diln (24.75 g/99 mL diln H_2O). Mix well and plate 1 mL on each of 2 plates. Multiply total of counts on 2 plates by 2.5 to obtain count/g.

(2) Sour cream, dips, and yogurt.—Proceed as in (*1*) except after diln, adjust pH to 6.6–7.2 with 1.0N NaOH (ca 0.1 mL/g sample).

(3) Buttermilk.—Make 1:10 diln (11 g/99 mL diln H_2O). Adjust pH to 6.6–7.2 with 1.0N NaOH (ca 0.1 mL/g samples). Mix well and plate 1 mL on each of 2 plates. Multiply total of counts on 2 plates by 5 to obtain count/g.

(4) Ice cream, sherbet, and mixes.—Hydrate dry-film plates with 1 mL sterile diln H_2O and allow at least 1 h for gel to solidify. Then, lift top film of prehydrated dry-film coliform count plate (gel will adhere to top film) and dispense 0.5 mL of 2:3 homogenate (10 g/5 mL diln H_2O) onto bottom film of each of 3 plates. Replace top film gently over sample. Add counts on the 3 plates to obtain count/g. Alternatively, plate 1 plate and multiply result by 3 to obtain count/g.

(5) Cheese.—Proceed as in (*1*). Do not use citrate buffer to homogenize sample.

(6) Chocolate milk.—Proceed as in (*1*).

D. Analysis

(a) *Bacterial colony count.*—Use dry-film aerobic count plates. Place plate on flat surface. Lift top film and inoculate 1 mL sample onto center of film base. Carefully roll top film down onto inoculum. Distribute sample over prescribed growth area with downward pressure on center of plastic spreader device (recessed side down). Leave plate undisturbed 1 min to permit gel to solidify. Incubate plates 48 ± 3 h at 32 ± 1°.

In incubator, place plates in horizontal position, clear side up, in stacks not exceeding 20 units. Count plates promptly after incubation period. If impossible to count at once after required incubation, store plates at 0–4.4° for not >24 h. This should be avoided as a routine practice.

Use std colony counter for counting purposes. Magnifier-illuminator may also be used to facilitate counting. Colonies stain in various shades of red. Count all colonies in countable range (25–250 colonies).

To compute bacterial count, multiply total number of colonies per plate (or av. number of colonies per plate if counting duplicate plates of same diln) by reciprocal of diln used. When counting colonies on duplicate plates of consecutive dilns, compute mean number of colonies for each diln before detg av. bacterial count. Estd counts can be made on plates with >250 colonies and should be reported as estd counts. In making such counts, circular growth area can be considered to contain ca twenty 1 cm squares. To isolate colonies for further identification, lift top film and pick colony from gel.

(b) *Coliform count.*—Use dry-film coliform count plates. Proceed as in (a), but distribute prepd sample over plate by using plastic spreader, flat side down. Incubate plates 24 ± 2 h at 32 ± 1°. Count as in (a), but count only red colonies that have one or more gas bubbles associated (within 1 colony diam.) with them. Count all colonies in countable range (15–150 colonies). Red colonies without gas bubbles are not counted as coliform organisms.

Ref.: JAOAC **72,** 312(1989).

978.23 Fecal Coliforms in Shellfish Growing Waters

Medium A-1 Method

First Action 1978
Final Action 1979

(Applicable to enumeration of fecal coliforms and also as presumptive test for *Escherichia coli* in shellfish growing waters)

A. Apparatus

(a) *Pipets.*—1.0 mL serological with 0.1 mL graduations and 10.0 mL with 0.1 mL graduations. Pipets conforming to APHA stds as given in "Standard Methods for the Examination of Dairy Products," 15th ed., 1985, American Public Health Association, 1015 15th St, NW, Washington, DC 20005, may also be used.

(b) *Incubator.*—Air, 35 ± 0.5°.

(c) *Water bath.*—Covered, circulating, 44.5 ± 0.2°.

(d) *Dilution bottles or tubes.*—Borosilicate glass, with glass or rubber stoppers or polyethylene screw caps equipped with Teflon liners.

B. Media

Note: Because geographical differences may affect performance of Medium A-1 method, det. comparability with LST-EC tube method prior to using Medium A-1. Moreover, this medium must be made from individual ingredients. Preformulated Medium A-1 is unacceptable.

(a) *Butterfield's buffered phosphate diluent.*—*See* **966.23A(m)**.

(b) *Medium A-1 broth.*—Dissolve 5 g lactose, 20 g tryptone, 5 g NaCl, and 0.5 g salicin in 1 L H_2O. Heat to dissolve ingredients, pipet in 1 mL Triton X-100 (Rohm & Haas Co.), and adjust pH to 6.9 ± 0.1. For 10 mL sample aliquots, prep. and use double strength medium. To achieve approx. same level of medium and inoculum in all tubes, dispense 10 mL portions of single strength broth into 150 × 18 mm tubes contg inverted fermentation vials; use 175 × 22 mm tubes contg inverted fermentation vials for double strength broth. Autoclave 10 min at 121°. Formation of flocculent ppt, particularly in double strength medium, is common and does not impair performance. Store in dark at room temp. and use within 7 days. Store dehydrated ingredients and/or medium under conditions that will prevent absorption of moisture.

C. Determination

Shake sample and each successive diln bottle vigorously using 25 complete up and down movements of ca 30 cm in 7 sec. Inoculate H_2O sample directly into tubes contg A-1 Medium in suitable decimal dilns using 3 or 5 tubes/diln with

Butterfield's buffered phosphate diluent. Place inoculated tubes into air incubator and incubate 3 hr at 35 ± 0.5°. Transfer tubes to H_2O bath and incubate 21 ± 2 hr at 44.5 ± 0.2°. Maintain H_2O level in bath above level of liq. in inoculated tubes. Presence of gas in inverted vial or of dissolved gas which can be removed by slight agitation of tube constitutes pos. test. Use std Most Probable Number (MPN) tables, Table **966.24** or Table **978.23**, to det. MPN values. Report results as fecal coliform MPN/100 mL sample.

Ref.: JAOAC **61,** 1317(1978).

983.25 Total Coliforms, Fecal Coliforms, and *Escherichia coli* in Foods

Hydrophobic Grid Membrane Filter Method

First Action 1983
Final Action 1985

A. Principle

Hydrophobic grid membrane filter (HGMF) uses membrane filter imprinted with hydrophobic material in grid pattern. Hydrophobic lines act as barriers to spread of colonies, thereby dividing membrane filter surface into sep. compartments of equal and known size. Number of squares occupied by colonies is enumerated and converted to most probable number value of organisms by using formula given below.

B. Apparatus, Culture Media and Reagents

(a) *Hydrophobic grid membrane filter (HGMF).*—Membrane filter has pore size of 0.45 μm and is imprinted with nontoxic hydrophobic material in grid pattern. ISO-GRID (available from QA Laboratories Ltd, 135 The West Mall, Toronto, Ontario, Canada M9C 1C2) or equiv. meets these specifications.

(b) *Filtration units for HGMF.*—Equipped with 5 μm mesh prefilter to remove food particles during filtration. One unit is required for each sample. ISO-GRID (available from QA Laboratories Ltd.) or equiv. meets these specifications.

(c) *Pipets.*—1.0 mL serological with 0.1 mL graduations; 1.1 mL or 2.2 mL milk pipets are satisfactory. 5.0 mL serological with 0.1 mL graduations.

(d) *Blender.*—Waring Blendor, or equiv., multispeed model, with low-speed operation at 10 000–12 000 rpm, and 250 mL glass or metal blender jars with covers. One jar is required for each sample.

(e) *Vac. pump.*—Water aspirator vac. source is satisfactory.

(f) *Manifold or vac. flask.*

(g) *Filter paper.*—Whatman No. 1 or No. 4, or equiv.

(h) *Peptone/Tween 80 diluent.*—Dissolve 1.0 g peptone (Difco 0118) and 10.0 g Tween 80 in 1 L H_2O. Dispense enough vol. into diln bottles to give 90 ± 1 mL after autoclaving 15 min at 121°.

Table 978.23 Most Probable Numbers per 100 mL of Sample, Planting 5 Portions in Each of 3 Dilutions in Geometric Series

Number of Positive Tubes				Number of Positive Tubes				Number of Positive Tubes				Number of Positive Tubes				Number of Positive Tubes				Number of Positive Tubes			
10 mL	1 mL	0.1 mL	MPN	10 mL	1 mL	0.1 mL	MPN	10 mL	1 mL	0.1 mL	MPN	10 mL	1 mL	0.1 mL	MPN	10 mL	1 mL	0.1 mL	MPN	10 mL	1 mL	0.1 mL	MPN
0	0	0		1	0	0	2.0	2	0	0	4.5	3	0	0	7.8	4	0	0	13	5	0	0	23
0	0	1	1.8	1	0	1	4.0	2	0	1	6.8	3	0	1	11	4	0	1	17	5	0	1	31
0	0	2	3.6	1	0	2	6.0	2	0	2	9.1	3	0	2	13	4	0	2	21	5	0	2	43
0	0	3	5.4	1	0	3	8.0	2	0	3	12	3	0	3	16	4	0	3	25	5	0	3	58
0	0	4	7.2	1	0	4	10	2	0	4	14	3	0	4	20	4	0	4	30	5	0	4	76
0	0	5	9.0	1	0	5	12	2	0	5	16	3	0	5	23	4	0	5	36	5	0	5	95
0	1	0	1.8	1	1	0	4.0	2	1	0	6.8	3	1	0	11	4	1	0	17	5	1	0	33
0	1	1	3.6	1	1	1	6.1	2	1	1	9.2	3	1	1	14	4	1	1	21	5	1	1	46
0	1	2	5.5	1	1	2	8.1	2	1	2	12	3	1	2	17	4	1	2	26	5	1	2	64
0	1	3	7.3	1	1	3	10	2	1	3	14	3	1	3	20	4	1	3	31	5	1	3	84
0	1	4	9.1	1	1	4	12	2	1	4	17	3	1	4	23	4	1	4	36	5	1	4	110
0	1	5	11	1	1	5	14	2	1	5	19	3	1	5	27	4	1	5	42	5	1	5	130
0	2	0	3.7	1	2	0	6.1	2	2	0	9.3	3	2	0	14	4	2	0	22	5	2	0	49
0	2	1	5.5	1	2	1	8.2	2	2	1	12	3	2	1	17	4	2	1	26	5	2	1	70
0	2	2	7.4	1	2	2	10	2	2	2	14	3	2	2	20	4	2	2	32	5	2	2	95
0	2	3	9.2	1	2	3	12	2	2	3	17	3	2	3	24	4	2	3	38	5	2	3	120
0	2	4	11	1	2	4	15	2	2	4	19	3	2	4	27	4	2	4	44	5	2	4	150
0	2	5	13	1	2	5	17	2	2	5	22	3	2	5	31	4	2	5	50	5	2	5	180
0	3	0	5.6	1	3	0	8.3	2	3	0	12	3	3	0	17	4	3	0	27	5	3	0	79
0	3	1	7.4	1	3	1	10	2	3	1	14	3	3	1	21	4	3	1	33	5	3	1	110
0	3	2	9.3	1	3	2	13	2	3	2	17	3	3	2	24	4	3	2	39	5	3	2	140
0	3	3	11	1	3	3	15	2	3	3	20	3	3	3	28	4	3	3	45	5	3	3	180
0	3	4	13	1	3	4	17	2	3	4	22	3	3	4	31	4	3	4	52	5	3	4	210
0	3	5	15	1	3	5	19	2	3	5	25	3	3	5	35	4	3	5	59	5	3	5	250
0	4	0	7.5	1	4	0	11	2	4	0	15	3	4	0	21	4	4	0	34	5	4	0	130
0	4	1	9.4	1	4	1	13	2	4	1	17	3	4	1	24	4	4	1	40	5	4	1	170
0	4	2	11	1	4	2	15	2	4	2	20	3	4	2	28	4	4	2	47	5	4	2	220
0	4	3	13	1	4	3	17	2	4	3	23	3	4	3	32	4	4	3	54	5	4	3	280
0	4	4	15	1	4	4	19	2	4	4	25	3	4	4	36	4	4	4	62	5	4	4	350
0	4	5	17	1	4	5	22	2	4	5	28	3	4	5	40	4	4	5	69	5	4	5	430
0	5	0	9.4	1	5	0	13	2	5	0	17	3	5	0	25	4	5	0	41	5	5	0	240
0	5	1	11	1	5	1	15	2	5	1	20	3	5	1	29	4	5	1	48	5	5	1	350
0	5	2	13	1	5	2	17	2	5	2	23	3	5	2	32	4	5	2	56	5	5	2	540
0	5	3	15	1	5	3	19	2	5	3	26	3	5	3	37	4	5	3	64	5	5	3	920
0	5	4	17	1	5	4	22	2	5	4	29	3	5	4	41	4	5	4	72	5	5	4	1,600
0	5	5	19	1	5	5	24	2	5	5	32	3	5	5	45	4	5	5	81				

(**i**) *M-FC agar.*—10.0 g tryptose, 5.0 g proteose peptone No. 3, 3.0 g yeast ext, 5.0 g NaCl, 12.5 g lactose, 1.5 g bile salts No. 3, 0.1 g aniline blue, and 15.0 g agar dild to 1 L with H_2O (mFC Agar, Difco 0677, is satisfactory). Heat to boiling. Temper to 50–55°. Adjust pH to 7.4 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use.

(**j**) *Tryptone bile agar (TBA).*—20.0 g tryptone, 1.5 g bile salts No. 3, and 15.0 g agar dild to 1 L with H_2O (Tryptone bile agar (Oxoid CM 595) is satisfactory). Heat to boiling. Autoclave 15 min at 121°. Temper to 50–55°. Adjust pH to 7.2 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use.

(**k**) *Tryptic soy-magnesium sulfate agar (TSAM).*—15.0 g tryptone, 5.0 g phytone (or soytone), 5.0 g NaCl, 1.5 g $MgSO_4.7H_2O$, and 15.0 g agar dild to 1 L with H_2O. Heat to boiling. Autoclave 15 min at 121°. Temper to 50–55°. Adjust pH to 7.3 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use.

(**l**) *Indole reagent.*—(*1*) *Soln A:* Dissolve 2.5 g *p*-dimethylamino benzaldehyde and 10 mL HCl in 90 mL alcohol. (*2*) *Soln B:* Dissolve 2.0 g potassium persulfate in 200 mL H_2O. Mix equal vols of Soln A and Soln B just before use.

(**m**) *Tris buffer.*—1.0M, pH 7.6. Dissolve 121.1 g tris(hydroxymethylamino)methane and dil. to 1 L with H_2O. Adjust pH to 7.6 with 1N HCl.

(**n**) *Trypsin stock soln.*—Dil. 10 g trypsin to 100 mL with tris buffer. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material, then filter-sterilize using 0.45 μm membrane filter.

C. Sample Preparation

(**a**) *Nut meat pieces.*—Aseptically weigh 50 g sample into sterile jar. Add 50 mL peptone/Tween 80 diluent (**h**) and shake vigorously (50 times thru 30 cm arc). Let stand 3–5 min and shake just before doing filtrations.

(**b**) *Cheese.*—Aseptically weigh 10 g sample into sterile blender jar. Add 90 mL diluent (**h**) and blend 2 min at low speed (10 000–12 000 rpm). Aseptically combine, in 16 × 150 mm tube, 3.5 mL of this 1:10 homogenate and 3.5 mL trypsin soln (**n**). Incubate 20–30 min at 35 ± 0.5° in H_2O bath. Vortex to remix suspension just before doing filtrations.

(**c**) *Other foods needing digestion.*—Aseptically weigh 10 g sample into sterile blender jar. Add 90 mL diluent (**h**) and blend 2 min at low speed (10 000–12 000 rpm). Aseptically combine in 16 × 150 mm tube 5.0 mL of this 1:10 homogenate and 1.0 mL trypsin soln (**n**). Incubate 20–30 min at 35 ± 0.5° in H_2O bath. Vortex to remix suspension just before doing filtrations.

(**d**) *Other foods.*—Aseptically weigh 10 g sample into sterile blender jar. Add 90 mL diluent (**h**) and blend 2 min at low speed (10 000–12 000 rpm).

D. Analysis

(*See* Figs. **986.32A** and **986.32B**). Turn on vac. source. Place sterile filtration unit on manifold or vac. flask. Open clamp A. Rotate back funnel portion C. Aseptically place sterile HGMF on surface of base D. Rotate funnel forward. Clamp shut by sliding jaws L of stainless steel clamp over entire length of flanges B extending from both sides of funnel C and base D, and rotating moving arm K into horizontal (locked) position.

Aseptically add ca 15–20 mL sterile H_2O to funnel. Pipet required volume (see below) of sample suspension into funnel. Apply free end of vac. tubing E to suction hole F to draw liq. thru prefilter mesh G. Aseptically add addnl 10–15 mL H_2O to funnel and draw thru mesh as before. Close clamp A to direct vac. to base of filtration unit and draw liq. thru HGMF.

Food	Filtering Diln	Filtering Vol., mL	Multiplication Factor
Nut meat pieces	10^0	0.5	2
Cheese	10^{-1}	2.0[a]	10
Other foods needing digestion	10^{-1}	1.2[a]	10
Other foods	10^{-1}	1.0	10

[a] Filtering vol. of enzyme-digested suspension.

Open clamp A. Rotate moving arm K of stainless steel clamp into unlocked (ca 45° angle) position and slide jaws L off of flanges B. Rotate back funnel C. Aseptically remove HGMF and place on surface of pre-dried agar plate (see below). Avoid trapping air bubbles between filter and agar.

(**a**) *Total coliform count.*—Place HGMF on surface of pre-dried M-FC agar (**i**). Incubate 24 ± 2 h at 35°. Count all squares contg one or more blue colonies. Include any shade of blue. Score each square as either pos. (blue) or neg. Convert pos. square count to MPN with the formula

$$\text{MPN} = [N \log_e (N/(N - x))]$$

where N = total number of squares and x = number of pos. squares. Multiply by reciprocal of diln factor and report as MPN of total coliform bacteria/g.

(**b**) *Fecal coliform count.*—Place HGMF on surface of pre-dried TSAM (**k**). Incubate 4–5 h at 25° for dry foods and 4–5 h at 35° for all other foods. Transfer HGMF to surface of pre-dried M-FC agar (**i**) and incubate 24 ± 2 h at 44.5 ± 0.5° in closed container. Proceed as in (**a**), and report as MPN of fecal coliform bacteria/g.

(**c**) *E. coli count.*—Place HGMF on surface of pre-dried TSAM (**k**). Incubate 4–5 h at 25° for dry foods and 4–5 h at 35° for all other foods. Transfer HGMF to surface of pre-dried TBA (**j**) and incubate 24 ± 2 h at 44.5° ± 0.5° in closed container. Prepare indole reagent (**l**) by combining equal vol. of Soln A and Soln B. Place 9 cm filter paper disk in petri dish lid and flood with 1–2 mL indole reagent (**l**). Transfer HGMF to filter paper, ensuring that no air bubbles are trapped between HGMF and paper. Let stand 10–15 min, then transfer HGMF back to surface of TBA. Count all squares contg one or more pink (indole pos.) colonies. Score each square as either pos. (pink) or neg. Convert pos. square count to MPN with formula above. Multiply by reciprocal of diln factor and report as MPN of *E. coli* (biotype I)/g.

Refs.: JAOAC **66,** 897(1983); **67,** 812(1984).

ESCHERICHIA COLI

988.19 *Escherichia coli* in Chilled or Frozen Foods

Fluorogenic Assay for Glucuronidase

First Action 1988

(Applicable only to chilled or frozen foods, except chilled or frozen shellfish)

A. Principle

Lauryl sulfate tryptose broth with added 4-methyl-umbelliferyl-β-D-glucuronide (MUG) is used as medium in 3-tube MPN method. Tubes are incubated 24 ± 2 h at 35°. Fluorescent-pos. tubes are streaked onto eosin methylene blue agar (Levine) plates, which are incubated 24 ± 2 h at 35°. Typical colonies are picked and confirmed as *E. coli.*

B. Media and Reagents

See introductory par. to **966.23A**.

(**a**) *Plate count agar.*—See **940.36A(g)**.

(**b**) *Eosin methylene blue agar (Levine).*—See **940.36A(d)**.

(c) *Tryptophane broth.*—Dissolve by heating, with stirring, 10.0 g tryptone or trypticase in 1 L H_2O. Dispense in 10 mL portions into test tubes and autoclave 15 min at 121°. Final pH, 6.9 ± 0.2.

(d) *Buffered glucose broth (MR-VP medium).*—For Me red-Voges Proskauer (MR-VP) tests. Dissolve 7.0 g proteose peptone, 5.0 g glucose, and 5.0 g K_2HPO_4 in ca 800 mL H_2O with gentle heat and occasional stirring. Filter, cool to 20°, and dil. to 1 L. Dispense 10 mL portions into test tubes and autoclave 12–15 min at 121°. Max. exposure to heat should be ≤30 min. Final pH, 6.9 ± 0.2. BBL, Division of Bioquest, or Difco dehydrated medium may be used.

(e) *Koser's citrate broth.*—See **940.36A(e)**.

(f) *Butterfield's buffered phosphate diluent.*—See **966.23A(m)**.

(g) *Lauryl sulfate tryptose broth with MUG.*—Prep. lauryl sulfate tryptose broth, **966.23A(b)**, and add 50 mg 4-methylumbelliferyl-β-D-glucuronide (MUG). Dissolve with gentle heat, if necessary. Dispense 10 mL portions into 20 × 150 mm test tubes. Autoclave 15 min at 121°. Final pH, 6.8 ± 0.1. Difco dehydrated medium, or equiv., may be used.

(h) *Peptone dilution water.*—Dissolve 1.0 g peptone in 1 L H_2O. Prep. diln blanks with this soln, dispensing enough to allow for losses during autoclaving. Adjust pH to 7.0 ± 0.1. Autoclave 15 min at 121°.

(i) *Lactose broth.*—Dissolve on H_2O bath, with stirring, 3.0 g beef ext and 5.0 g polypeptone or peptone in 1 L H_2O. Add 5.0 g lactose. Dispense 450 mL portions into 750 mL flasks and autoclave 15 min at 121°. Max. exposure to heat should be ≤30 min. Final pH, 6.7 ± 0.2.

C. Preparation of Sample

Prep. all decimal dilns with 90 mL sterile diluent, peptone diln H_2O (h) or **966.23A(m)**(2), plus 10 mL previous diln unless otherwise specified. Shake all dilns 25 times in 30 cm arc. Pipets must accurately deliver required vol. Do not use to deliver <10% of their total vol. For example, to deliver 1 mL, do not use pipet >10 mL; to deliver 0.1 mL, do not use pipet >1 mL.

Frozen or chilled foods.—Use balance with capacity of ≥2 kg and sensitivity of 0.1 g to aseptically weigh 50 g unthawed (if frozen) sample into sterile high-speed blender jar. Add 450 mL diluent, (i) or **966.23A(m)**(2), and blend 2 min at 10 000–12 000 rpm. (If necessary to temper frozen sample to remove 50 g portion, hold ≤18 h at 2–5°.) Not >15 min should elapse from time sample is blended until all dilns are made in appropriate media.

If entire sample consists of <50 g, weigh portion equiv. to $^1/_2$ sample and add vol. of sterile diluent required to make 1:10 diln. Total vol. in blender jar must completely cover blades.

D. Determination

Notes: (1) Test tubes used in MPN method should be checked under UV light to be sure glass does not fluoresce. (2) To avoid false-pos. fluoresence, longwave UV light used in method should not exceed 6 watts (Blak-Ray, Model UVL-56 (available from UVP, Inc.), or equiv.).

Seed 3-tube most probable number (MPN) series into lauryl sulfate tryptose broth contg MUG (g), using 1 mL inocula of 1:10, 1:100, and 1:1000 dilns, with triplicate tubes at each diln. Incubate 24 ± 2 h at 35° and examine for fluorescence of medium when tube is held under longwave UV light (366 nm).

Streak fluorescent-pos. tubes on eosin methylene blue agar plates (b), and incubate plates 24 ± 2 h at 35°.

Pick 2 or more well isolated typical colonies from eosin methylene blue agar plates and transfer to agar slants prepd from agar medium (a). Incubate 18–24 h at 35°. If typical colonies are not present, pick 2 or more colonies most likely to be *E. coli*. Pick ≥2 from every plate.

Confirm *E. coli* as specified in **966.24**.

Ref.: JAOAC **71,** 589(1988).

982.36 Invasiveness by *Escherichia coli* of Mammalian Cells

Microbiological Method

First Action 1982
Final Action 1987

A. Principle

Invasiveness is detected by intracellular growth on monolayer of HeLa cells on slides. To minimize extracellular bacterial multiplication, host-pathogen interaction is resolved into 2 phases, infective and intracellular, using appropriate substrates and the following protocols: growth of monolayer in chamber slides, using controlled inoculum and period of incubation; detn of optimal pre-infection growth conditions for pathogen; washing pathogen to remove toxic end products; infection of host cell under controlled conditions of number and multiplicity of infection, and medium and length of incubation; subsequent removal of unattached bacteria; use of post-infection medium to permit only intracellular bacterial growth for limited period.

B. Culture Media

(a) *Minimal essential medium (MEM).*—Eagle-type with Earle's salts. Dissolve 126.4 mg L-arginine.HCl, 24 mg L-cystine, 292 mg L-glutamine, 41.9 mg L-histidine.HCl.H_2O, 52.5 mg L-isoleucine, 52.4 mg L-leucine, 73.1 mg L-lysine.HCl, 14.9 mg L-methionine, 33.0 mg L-phenylalanine, 47.6 mg L-threonine, 10.2 mg L-tryptophan, 36.2 mg L-tryosine, 46.8 mg L-valine, 1 mg D-calcium pantothenate, 1 mg choline chloride, 1 mg folic acid, 2 mg isoinositol, 1 mg pyridoxal HCl, 1 mg nicotinamide, 0.1 mg riboflavin, 1 mg thiamine.HCl, 1 g glucose, 265 mg $CaCl_2.2H_2O$, 400 mg KCl, 200 mg $MgSO_4.7H_2O$, 6.8 g NaCl, 2.2 g $NaHCO_3$, 140 mg $NaH_2PO_4.H_2O$, and 10 mg phenol red in 1 L H_2O. Sterilize by filtration. Final pH should be 7.2 ± 0.2. Check sterility of all culture fluids before use. Store at 4–8°.

(b) *Fetal bovine serum (FBS).*—Sterile, virus-screened, myco-plasma-free, obtained aseptically during slaughter (Flow Laboratories, Inc., 7655 Old Springhouse Rd, McLean, VA 22102). Store at 4–8°.

(c) *Antibiotic concentrate (AC).*—Dissolve 500 000 international units (IU) penicillin G and 500 mg streptomycin (Flow Laboratories, Inc.) in 100 mL H_2O and sterilize by filtration. Store at −10°.

(d) *MEM-FBS-AC medium.*—Routine medium for cultivation of HeLa mammalian cells. Mix 90 mL MEM (a), 10 mL FBS (b), and 1 mL AC (c). Store at 4–8°.

(e) *MEM-FBS medium.*—Medium for cultivation of HeLa cells before infection. Mix 90 mL MEM (a) and 10 mL FBS (b). Store at 4–8°.

(f) *Earle's salts.*—Prep. without phenol red as follows: Dissolve 6.8 g NaCl, 400 mg KCl, 265 mg $CaCl_2$, 200 mg $MgSO_4.7H_2O$, 140 mg $NaH_2PO_4.H_2O$, 1.0 g glucose, and 2.2 g $NaHCO_3$ in 1 L H_2O. Sterilize by filtration. Final pH should be 7.2 ± 0.2.

(g) *Veal infusion broth.*—Dissolve 500 g veal (infusion) and 10 g proteose peptone in 1 L H_2O with gentle heating. Dispense 5 mL portions into 13 × 100 mm screw-cap tubes. Autoclave 15 min at 121°. Final pH should be 7.3 ± 0.2.

(h) *Brain-heart infusion (BHI).*—Dissolve 12.5 g BHI

(powder) in 1 L Earle's salts (**f**). Sterilize by filtration. Final pH should be 7.2 ± 0.2.

(**i**) *Bile salts No. 3.*—Dissolve 5 g bile salts No. 3 formulation in 1 L Earle's salts (**f**). Sterilize by filtration.

(**j**) *Heat-inactivated HFBS.*—Heat FBS (**b**) 2 h at 55 ± 1°. Store at 4–8°.

(**k**) *HFBS-BHI-BS medium.*—Mix 20 mL heat-inactivated FBS (**j**), 10 mL BHI (**h**), 10 mL bile salts No. 3 (**i**), and 60 mL Earle's salts (**f**). Store at 4–8°.

(**l**) *Veal infusion agar slant.*—For maintenance of cultures, dissolve 500 g veal (infusion), 10 g proteose peptone No. 3, 5 g NaCl, and 15 g agar in 1 L H_2O with gentle heating. Dispense 7 mL aliquots to 16 × 150 mm screw-cap tubes. Autoclave 15 min at 121°. Final pH should be 7.3 ± 0.2.

(**m**) *Dulbecco's phosphate-buffered saline (PBS).*—Dissolve 8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, 200 mg KH_2PO_4, 100 mg $CaCl_2$, and 100 mg $MgCl_2.6H_2O$ in 1 L H_2O. Sterilize by filtration. Final pH 7.2 ± 0.2.

(**n**) *Calcium- and magnesium-free Dulbecco's PBS.*—Dissolve 8.0 g NaCl, 200 mg KCl, 1.15 g Na_2HPO_4, and 200 mg KH_2PO_4 in 1 L H_2O. Sterilize by filtration. Final pH 7.2 ± 0.2.

(**o**) *Calcium magnesium, phenol red-free Hanks' PBS.*—Dissolve 8.0 g NaCl, 400 mg KCl, 90 mg $Na_2HPO_4.7H_2O$, 60 mg KH_2PO_4, 1.0 g glucose, and 350 mg $NaHCO_3$ in 1 L H_2O. Sterilize by filtration. Final pH 7.2 ± 0.2.

(**p**) *Trypsin stock soln.*—2.5%. Suspend 2.5 g 1:250 trypsin (Difco Laboratories) in 100 mL Ca- and Mg-free Hanks' PBS (**o**) and let particles settle. Sterilize by filtration. Dil. 10 mL stock soln with 90 mL sterile Ca- and Mg-free Dulbecco's PBS (**n**) to prep. 0.25% trypsin. Store at −10°.

(**q**) *Gentamicin stock soln.*—Dissolve 50 mg gentamicin (Schering Corp., 2000 Galloping Hill Rd, Kenilworth, NJ 07033) in 100 mL Dulbecco's PBS (**m**) to give soln contg 500 μg/mL. Dil 1 + 9 with Dulbecco's PBS to soln contg 50 μg/mL. Store at 4–8°.

(**r**) *Lysozyme soln.*—Weigh 0.3 g lysozyme, 3× crystalline, salt-free, ca 12 000 Shugar units/mg (Calbiochem Corp.), into 100 mL Dulbecco's PBS and stir to dissolve. Store at 4–8° not >2 weeks.

(**s**) *Intracellular growth phase medium.*—Mix 80 mL MEM-FBS medium (**e**), 10 mL gentamicin soln (50 μg/mL) (**q**), and 10 mL lysozyme soln (**r**). Prep. immediately before use.

C. Diagnostic Reagents

(**a**) *May-Grunwald stain.*—Weigh 2.5 g stain (EM Science) into 50 mL absolute MeOH, dissolve by grinding, and dil. to 1 L with MeOH. Stir 16 h at 37°. Hold stain 1 month at 22° (room temp.). Filter for use.

(**b**) *Giemsa stain.*—Dissolve 1 g stain (EM Science, No. GX0080) in 66 mL glycerol by heating 1.5–2.0 h at 55–60°. Add 66 mL absolute MeOH. Store stain 2 weeks in tightly stoppered bottle at 22°. Dil. stock soln (1 + 9) before use.

(**c**) *Decolorizing and dehydrating reagents.*—Acetone; acetone-xylene (50 + 50) and (33 + 67); xylene.

(**d**) *Mounting medium.*—Dil. mounting medium with xylene to give easily dispensed colloidal suspension; 20 mL Permount® (Fisher Scientific Co.) dild with 5 mL xylene is satisfactory.

(**e**) *Human cervical epithelial cell culture.*—ATCC HeLa culture. Other cultures, including Henle 407 human intestine and human laryngeal carcinoma gave comparable data; however, HeLa cell culture was more suitable with regard to culture characteristics.

D. Apparatus

(**a**) *Water baths.*—Maintained at 35 ± 1° and 55 ± 1°.

(**b**) *Microscopes.*—Standard 900 × magnification; inverted stage, 100× magnification (Preiser Scientific, 94 Oliver St, St Albans, WV 25177), or equiv.; microscope illuminator.

(**c**) *Carbon dioxide incubator.*—95% air-5% CO_2-moisture-satd atmosphere, maintained at 36 ± 1° (Lab-Line Instruments, Inc. Melrose Park, IL 60160, or equiv.).

(**d**) *Tissue culture chamber slides.*—Clean microscope slides mounted with partitions on plastic gasket to facilitate multiple testing. Lab-Tek units contg 4 chambers are satisfactory (Nunc, Inc., 200 N. Aurora Rd, Naperville, IL 60566), or equiv.

(**e**) *Culture containers.*—Sterile 3 fluid oz (85 mL) glass prescription bottles or plastic tissue culture flasks (Costar, 205 Broadway, Cambridge, MA 02139, or equiv.).

(**f**) *Glass cover slips.*—1 × 2 in. (2.5 × 5.1 cm).

(**g**) *Cell-counting chamber.*—Spencer Bright Line, Fuchs-Rosenthal (Preiser Scientific), or equiv.

(**h**) *Refrigerated centrifuge with adapter.*—To accommodate 13 × 100 mm tubes and covered centrf. cups to prevent aerosolization of pathogens.

(**i**) *Membrane filters.*—0.45 μm pore diam. (Millipore Corp., or equiv.).

E. Preparation of HeLa Cell Culture

Using std cell culture technics, grow HeLa strain on inner surface of 3 oz glass or plastic container, using 5 mL MEM-FBS-AC medium, (**d**), for 7 days at 36° in CO_2 incubator. Replace with fresh culture medium on fourth day to prevent accumulation of toxic metabolites. In prepg cells in monolayer for transfer to chamber slides, wash once with 5 mL Dulbecco's PBS (**m**) prewarmed at 36°. Add 5 mL prewarmed (36°) 0.25% trypsin and hold at room temp. 2 min. Aseptically remove ca 4.5 mL trypsin. Incubate flask at 36° with occasional agitation. After monolayer has detached and cells are fairly uniformly distributed in residual trypsin, add 25 mL prewarmed (36°) MEM-FBS medium, (**e**). Est. cell density, using counting chamber. Add MEM-FBS medium, if necessary, to dil. suspension to density of 1 × 10^5 cells/mL. With occasional agitation, rapidly transfer 1 mL aliquots to chambers of slide. Incubate 20–24 h at 36° in CO_2 incubator. Aseptically remove spent medium before infection. Wash each monolayer once with 1 mL prewarmed (36°) Earle's salts, (**f**), and 1 mL prewarmed (36°) uninoculated infection medium, (**k**) (see below).

F. Preparation of Bacteria

Inoculate, with needle, 5 mL veal infusion broth, (**g**), using growth from veal infusion agar slant (**l**) incubated at 22°. Incubate presumptive *E. coli* broth cultures 18–24 h at 36°. Centrf. suspension 20 min at 1200 × g at 18°. Resuspend cells in equal vol. of Earle's salts, (**f**). Recentrifuge 20 min at 1200 × g. Resuspend cells in 5 mL Earle's salts. Dil. latter suspension with prewarmed (36°) HFBS-BHI-BS medium, (**k**), to final density of 5 × 10^7 cells/mL. Add 0.2 mL of each suspension to prepd chamber (above). Use 0.2 mL HFBS-BHI-BS for uninoculated neg. control.

G. Infection Stage

Incubate chambers 2.5 h at 36° in CO_2 incubator. Time factor is critical; shorter period results in min. number of infected host cells and longer period may result in cytotoxic effect arising from medium and possibly bacterial metabolites.

H. Intracellular Growth Stage

Remove infection medium from chamber with Pasteur pipet. To prevent contamination, use sep. pipet for each chamber. Wash each chamber twice with 1 mL aliquots of prewarmed (36°) Earle's salts. Subsequently wash with 1 mL aliquot of prewarmed intracellular growth phase medium (**s**) prepd immediately before use. Add 0.8 mL prewarmed intracellular growth phase medium to each chamber. Incubate 5 h at 36° in

CO_2 incubator. Control of extracellular growth is critical at this stage; sensitivity of culture to gentamicin and other antibiotics should be examined by std procedures before pathogenicity testing. Problem is critical in meats and dairy products where antibiotics may have been used in therapy or in feeds.

I. Staining

Remove fluid contents of chambers. Wash monolayer 3 times with 1 mL Dulbecco's PBS (**n**). Add 1 mL absolute MeOH fixative per chamber. Hold at room temp. 5 min. Remove MeOH and side walls of chamber slide. Insert single-edge razor blade between gasket and slide, and gently pry gasket from slide. If necessary, cautiously remove remnants of gasket from slide with razor blade. Do *not* let specimen dry while slide is prepd for staining. Immerse slides in May-Grunwald stain (**a**) 10 min. Withdraw slides, remove excess stain, and immerse in Giemsa stain (**b**) 20 min. Withdraw slides, remove excess stain, and immerse in H_2O 10–20 s. Briefly rinse twice in acetone. Briefly immerse slides in following sequence of solvs; acetone-xylene (50 + 50), acetone-xylene (33 + 67), and xylene. Evenly distribute 4 drops of mounting medium, (**d**) to slide. Place large cover slip on prepn. Remove excess mounting medium and xylene by gently blotting. Gently apply pressure to remove air bubbles from prepn.

J. Detection and Criteria of Invasiveness

Examine specimens with 900× magnification. Criterion for intracellular location of bacteria is parfocality of cytoplasmic ground substance and bacteria. If invasive, *E. coli* occurs within cytoplasm. Frequently, they may be located along nuclear membrane. In addition, they may be elongated. Finally, bacteria may occur within a membrane (phagolysosome) individually or in groups, indicative of intracellular growth. Examine, at random, 10 fields contg 15–25 HeLa cells. Count bacteria in each cell. Criterion for infection is ≥5 bacteria per cell. Criterion for invasiveness of bacterial culture is ≥1.0% infected HeLa cells.

HeLa cell results with *E. coli* strains must be confirmed by Sereny keratoconjunctivitis test.

Refs.: *Acta Microbiol. Acad. Sci. Hung.* **2,** 292(1955); **4,** 367(1957). *J. Hyg. Epidemiol. Microbiol. Immunol.* **3,** 292(1959). JAOAC **65,** 602(1982).

984.34 Detection of *Escherichia coli* Producing Heat-Labile Enterotoxin

DNA Colony Hybridization Method

First Action 1984
Final Action 1987

(*Caution:* This procedure uses radioactive and mutagenic compounds. Personnel must receive adequate training and monitoring and have proper facilities available for handling these substances.)

A. Method Performance

Results	Percent	95% Confidence Range (approx.)
Correct	96.9	95–99
False positive	2.1	1–5
False negative	4.6	1–11

Of 13 laboratories, 8 (62%) correctly identified all unknown samples (25/25); 11 laboratories (85%) identified ≥96% of the samples.

B. Principle

Isolated and purified genes (DNA) that code for determinants of bacterial virulence can be used to detect pathogenic strains. Specific fragments of DNA are isolated by cleaving plasmid DNA with appropriate restriction endonucleases and sepg resulting pieces by gel electrophoresis. Purified fragments are radioactively labeled in vitro. Bacterial cultures to be tested are spotted on nitrocellulose filters on agar medium and incubated until colonies are visible. Cells are lysed in situ, DNA is fixed to filter, and radioactive virulence gene DNA fragments are added. Colonies which contain same gene as radioactive DNA will bind this DNA and become radioactive. These colonies are detected by autoradiography.

C. Reagents

(Prep. all media according to manufacturer's instructions.)

(**a**) *10X M9 Salts.*—Dissolve 10 g NH_4Cl, 60 g Na_2HPO_4, 30 g KH_2PO_4, and 5 g NaCl in final vol. of 1 L H_2O. Dispense into 100 mL aliquots and autoclave 15 min at 121°.

(**b**) *Amplification medium.*—Sterilize all components sep. Aseptically combine 100 mL 10X M9 salts (**a**), 835 mL H_2O, 10 mL 0.1M $MgSO_4$, 10 mL 0.01M $CaCl_2$, 25 mL 20% (w/v) casamino acids, 20 mL 20% (w/v) glucose, and 0.2 mL thiamine (10 mg/mL).

(**c**) *TE Buffer.*—Combine 10 mL 1.0M Tris-HCl (tris-hydroxymethyl aminomethane HCl) and 2 mL 0.5M Na_2EDTA. Adjust to pH 8.0 with 10N NaOH. Add H_2O to final vol. of 1 L.

(**d**) *TES Buffer.*—Combine 30 mL 1.0M Tris, 10 mL 0.5M Na_2EDTA, and 10 mL 5.0M NaCl. Add H_2O to final vol. of 1 L.

(**e**) *CsCl saturated isopropanol.*—Add ca 50 mL TE buffer (**c**) to ca 350 mL isopropanol. Add solid CsCl (reagent or optical grade) until bottom layer is satd.

(**f**) *Triton lytic mix.*—Add 0.1 mL Triton X-100, 5 mL 1.0M Tris, pH 8.0, and 12.5 mL 0.5M Na_2EDTA, pH 8.0, to H_2O (final vol. 100 mL).

(**g**) *10X TBE electrophoresis buffer.*—Dissolve 108 g Tris, 9.3 g Na_2EDTA, and 55 g boric acid in ca 800 mL H_2O. Adjust pH to 8.2 with concd HCl and bring final vol. to 1 L with H_2O.

(**h**) *10X HindIII reaction buffer.*—Combine 50 mL 1.0M Tris, pH 8.0, 10 mL 1.0M $MgCl_2$, 10 mL 5.0M NaCl, and 10 mL 100 mM dithiothreitol in final vol. of 1 L H_2O.

(**i**) *Stop soln.*—Combine 1.0 mL 10% (w/v) sodium dodecyl sulfate, 10 mg bromophenol blue, 2 mL 0.5M Na_2EDTA, pH 8.0,5 g glycerol in a final vol. of 10 mL of H_2O.

(**j**) *10X nick translation buffer.*—Combine 500 μL 1.0M Tris, pH 7.8, 50 μL 1.0M $MgCl_2$, 7 μL 2-mercaptoethanol, and 500 μL nuclease-free bovine serum albumin (1 mg/mL). (Commercially available nick translation kits contain similar reagents. Follow supplier's instructions.)

(**k**) *Hybridization mixture.*—Combine 22 mL distd formamide, 12.5 mL 20X SSC (**l**), 0.5 mL 10% (w/v) sodium dodecyl sulfate, 5.0 mL 10X Denhardt's soln (**m**), 0.1 mL 0.5M Na_2EDTA, pH 8.0, and 9.9 mL H_2O.

(**l**) *20X Standard saline citrate soln (SSC).*—Add 175.4 g NaCl and 88.2 g Na citrate to final vol. of 1 L H_2O. 5X and 2X SSC may be prepd by dilg 20X SSC with H_2O.

(**m**) *10X Denhardt's soln.*—Combine 2.0 g Ficoll (400,000 mol. wt), 2.0 g polyvinyl pyrrolidone (360,000 mol. wt), and 2.0 g nuclease-free bovine serum albumin in 1 L H_2O. Store 5 mL aliquots at −20°.

(**n**) *Calf thymus DNA.*—Dissolve 1 g purified calf thymus DNA in 100 mL H_2O by stirring for several hours. Sonicate until av. mol. wt is 300,000–500,000 which may be detd by

electrophoresis with appropriate stds. Store in 1 mL portions at −20°.

(**o**) *Brain heart infusion broth.*—Prep. and sterilize according to supplier's instructions.

(**p**) *DE 52 column chromatography medium.*—Prep. according to manufacturer's instructions in loading buffer, (**q**).

(**q**) *DE 52 loading buffer.*—Combine 30 mL 5.0M NaCl, 0.2 mL 0.5M Na_2EDTA, and 10 mL 1.0M Tris (pH 8.0) in final vol. of 1 L H_2O.

(**r**) *DE 52 eluting buffer.*—Combine 200 mL 5.0M NaCl, 0.2 mL 0.5M Na_2EDTA, and 10 mL 1.0M Tris (pH 8.0) in final vol. of 1 L H_2O.

(**s**) *Sephadex G-50 column chromatography medium.*—Prep. according to manufacturer's instructions in TE buffer, (**c**).

D. Apparatus and Materials

(**a**) *Preparative ultracentrifuge and fixed angle rotor.*—100,000 × g and 13 mL tubes.

(**b**) *Shaker.*—In 37 ± 1° H_2O bath with clips for holding 1 L erlenmeyers.

(**c**) *Longwave ultraviolet lamp.*—302 nm transilluminator preferred. Camera for photographing gels is useful.

(**d**) *Refrigerated superspeed centrifuge and fixed angle rotor.*—37,000 × g and −20°, capable of holding 50 mL tubes and adapters for 15 or 30 mL tubes.

(**e**) *Siliconized glass tubes.*—15 or 30 mL capable of withstanding 10,000 × g.

(**f**) *Spectrophotometer or colorimeter and sample holder.*—Measure bacterial cell growth at 550 or 600 nm.

(**g**) *Escherichia coli strain C600(pEWD299)(ATCC 37218).*—Contains cloned heat-labile enterotoxin gene. Pos. and neg. strains such as *E. coli* H10407 (ATCC 35401) and plasmid pBR313 (ATCC 37018) are needed as controls during hybridization.

(**h**) *Alpha-^{32}P deoxycytosine triphosphate.*—dCTP, 2000–3000 Ci/mmole, aq. stabilized (ICN Biomedicals, Inc., ICN Plaza, 3300 Hyland Ave, Costa Mesa, CA 92626; New England Nuclear, 549 Albany St, Boston, MA 02118; Amersham Corp., Div. of Amersham International, 2636 S. Clearbrook Dr, Arlington Heights, IL 60005-4692).

(**i**) *Ultralow temperature freezer.*—Capable of −70° is preferred; however, −20° (not frost-free) may be substituted.

(**j**) *Vacuum desiccator.*—Large enough to contain 15 or 30 mL tubes.

(**k**) *Polycarbonate tubes.*—50 mL.

(**l**) *Variable volume micropipettors and tips.*—To cover range of 1–1000 μL.

(**m**) *Electrophoresis apparatus.*—Horizontal and vertical units with bed dimensions ca 12 × 12 cm and appropriate power supplies (to 125 mA; 200 V).

(**n**) *Incubator.*—H_2O bath or dry heating block capable of maintaining 37 ± 1°.

(**o**) *Plastic conical centrifuge tubes.*—500 and 1500 μL sizes able to withstand 15,000 × g with appropriate racks.

(**p**) *Centrifuge.*—For spinning tubes (**o**) at greater than 10,000 × g.

(**q**) *Dialysis tubing.*—$^1/_4$ in. diam., 10–12,000 molecular weight cut-off. Boil 3 min before use.

(**r**) *Glass wool.*—Boiled or siliconized.

(**s**) *Disposable plastic syringes.*—1 mL.

(**t**) *Vacuum side arm flask.*—250 mL for degassing.

(**u**) *Cooling block or refrigerated H_2O bath.*—15 ± 1°.

(**v**) *Plastic column.*—Disposable, ca 4 × 0.9 cm.

(**w**) *Scintillation counter.*—Or Geiger-Mueller counter if calibrated in cpm.

(**x**) *Nitrocellulose filters.*—0.45 μm pore size, 82 mm diam.

(**y**) *Absorbent paper filters.*—82 mm diam.; similar in characteristics to Whatman No. 1.

(**z**) *Petri dishes.*—100 × 15 or 20 mm, plastic.

(**aa**) *Vacuum oven.*—Maintain 80 ± 3°.

(**bb**) *X-ray film.*—8 × 10 in. is convenient size.

(**cc**) *X-ray film holder cassette.*—With intensifying screens (Kodak regular or Dupont Cronex lightening plus).

E. Isolation of Plasmid DNA

Inoculate 25 mL brain heart infusion broth (**o**) contg 10 μg ampicillin (filter-sterilized)/mL with frozen stock of strain C600 (pEWD299). Incubate overnight at 37° with shaking. Read *A* at 550 nm, using 25-fold diln. Inoculate 1.5 L amplification medium (**b**) to A_{550} = 0.02. [*Note:* This procedure can be scaled up to 10 L.] Shake or aerate well at 37°. When A_{550} = 0.4, add solid chloramphenicol to 100 μg/mL. Reduce shaking to 75 rpm or aeration to 2 Lpm. Incubate overnight. Harvest cells by centrifugation at 4°, resuspend pellets in TES buffer (**d**), and centrf. again. Resuspend cells in 8 mL 25% sucrose (w/v, nuclease-free) in TE buffer (**c**) in 50 mL polycarbonate centrf. tube. Add 1 mL 1% lysozyme (egg white, grade 1), mix gently, and let sit on ice 5 min. Add 13 mL Triton lytic mix (**f**), stir briefly to mix, and incubate on ice 30 min. Centrf. 30 min at 27,000 × g. Decant supernate thru gauze. If pellet is very soft, centrf. again at 37,000 × g for 30 min and combine this supernate with first one. Measure vol. of supernate (to 0.1 mL) and add 0.97 g solid CsCl for each mL. Add soln to ultracentrf. tubes and layer on surface 0.1 mL ethidium bromide, 10 mg/mL, for each mL supernate (before addn of CsCl) on liq. surface. [*Caution:* Ethidium bromide is mutagenic. Handle with care.] Fill tubes with light mineral oil, balance to 50 mg, and cap or seal. Centrf. 40 h in fixed angle rotor at 100,000 × g or 18 h in vertical rotor at 180,000 × g at room temp. (23°).

Observe ultracentrifuge tube in subdued room light, without fluorescent lights. Locate lower, orange band with longwave UV light and remove band with needle and syringe. Place band into polystyrene or siliconized glass tube. Ext and discard ethidium bromide with isopropanol satd with TE buffer and CsCl (**e**). Repeat until pink color is gone and then ext twice more. Measure remaining sample vol. and add 3 vols H_2O and 25 μg yeast transfer RNA (2.5 mg/mL, stored at −20°). After addition of water, add one-ninth total vol. of 3.0M Na acetate-10mM $MgCl_2$. Add 2.5 vols −20° alcohol and hold at −20° 1 h. Centrf. 10 min. at 9,000 × g at 0°. Discard supernate and let pellets drain until alcohol odor is gone. Tubes may be dried 15 min in vac. desiccator but do not over-dry. Gently resuspend pellet in 1 mL TE buffer (**c**). Est. DNA concn by electrophoresis against known stds. If A_{258} is measured, DNA concn will be over-estd because of presence of RNA. [For pure DNA, A_{258} = 1.0 corresponds to 50 μg/mL and ratio $A_{258}/A_{280} \simeq 1.8$]. Store DNA in plastic or siliconized glass tubes at 4°.

Enterotoxin Gene DNA Isolation

F. Enzyme Titration

Titr. restriction endonuclease against plasmid (pEWD299), using estd DNA concn to det. correct amt of enzyme. Usually, one unit of enzyme will digest about 1 μg DNA. However, this can vary by several fold, depending on plasmid, enzyme, or impurities. Generally, it is best to follow methods suggested by supplier.

If *Hind*III is used to cleave pEWD299, an 850 base-pair fragment will be generated which contains nucleotide sequence for entire B subunit and about one-third of the A subunit of

the heat-labile enterotoxin. Dispense ca 1 μg DNA into four 500 μL conical plastic tubes. Add 2.5 μL 10X – *Hind*III reaction buffer (**h**). Add 0, 2, 5, or 25 units of enzyme to each tube. Add 2.5 μL bovine serum albumin (1 mg/mL, nuclease-free). Add H_2O to bring vol. to 25 μL. Incubate 1 h at 37°. Add 5 μL stop soln (**i**) and electrophorese 25 μL of each mixture for 3 h at 100 V in 0.7% agarose in 1X TBE dild from (**g**). As control, run 30–50 ng linear bacteriophage lambda DNA. Stain gel with ethidium bromide (2 μg/mL) until lambda DNA band is visible under longwave UV light. If record is desired, rinse gel briefly with H_2O, and photograph with 302 nm transilluminator and camera with Wratten No. 23A or 9 filter.

G. Preparative Digest

Scale up titrn digest using lowest amt of enzyme that achieves complete digestion. After 1 h of incubation at 37°, add one-tenth vol. of stop soln (**i**). Prep. 10% polyacrylamide gel. For 50 mL gel, combine 32.5 mL H_2O, 5.0 mL 10X TBE (**g**), 12.5 mL 40% acrylamide (w/v; caution: acrylamide monomer is a neurotoxin). Degas 15–30 min in sidearm flask under vac. Add 0.5 mL freshly prepd 10% (w/v) ammonium persulfate soln. Add 50 μL TEMED (*N,N,N′,N′*-tetramethylethylenediamine) but mix gently so as not to aerate degassed soln. Pour vertical gel which should harden in 10–20 min. Layer digest on gel and electrophorese for 3 h at 100 V in 1X TBE (diluted from **g**). Stain with ethidium bromide (2 μg/mL) until bands are visible with longwave UV. Slice 850 base-pair band (nearest bottom) from gel and place into dialysis tubing with 1–2 mL 1X TBE. Place bag in horizontal electrophoresis unit and cover with 1X TBE. Electroelute band from gel at 50 V for 16 h. Reverse polarity of electrodes and turn on power for 15 s at 150 V. Remove buffer contg DNA from dialysis bag with plastic pipet. Add one-tenth vol. of stop soln (**i**). Repeat electrophoresis and electroelution as described above.

H. DE52 Chromatography

Prep. DE52 according to manufacturer's instructions, using loading buffer (0.15M NaCl, 1mM Na_2EDTA, pH 8.0, 0.01M Tris, pH 8.0). Construct 0.3 to 0.4 mL DE52 column in 1 mL plastic syringe plugged with boiled or siliconized glass wool. Wash column with 2 mL loading buffer. Apply 1–2 mL DNA to top of column bed. Wash column with 3–4 mL loading buffer. Elute DNA with 10 column vols of eluting buffer (1.0M NaCl, 1mM Na_2EDTA, pH 8.0, 10mM Tris, pH 8.0) in 0.3 mL aliquots. Collect 0.3 mL fractions in 500 μL plastic tubes. Most of DNA should be in first 2 or 3 fractions. Spot 2 μL of each fraction onto 1% agarose with 2 μg/mL ethidium bromide and illuminate with UV light. Fractions contg DNA will fluoresce; pool these fractions and alcohol-ppt by measuring total vol. and adding 10 μL transfer-RNA (2.5 mg/mL) and one-ninth soln vol. of 3.0M NaOAC-10mM $MgCl_2$. Add 2.5 vols of −20° alcohol and hold at −20° ≥1 h. Centrf. at 10,000 × g for 10 min. Discard supernate and gently rinse pellet (which may not be visible) with 0.5 mL −20° alcohol. Drain well until alcohol odor is gone but do not dry completely. Gently resuspend DNA pellet in 200–300 μL TE buffer (**c**).

I. In Vitro DNA Labeling

Kits are available commercially for nick translation reaction. Following procedure uses 50 ng DNA rather than 1 μg often suggested by suppliers. To 500 μL conical plastic centrf. tube, add 50 ng DNA (as prepd above in max. vol. of 5 μL). Add 3 μL 10X reaction buffer. Add 1.5 μL of soln 333 μM in each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, and thymidine triphosphate. Add 16 μL alpha-^{32}P deoxycytosine triphosphate (dCTP) (**h**). Add H_2O to final vol. of 26 μL. Add 2 μL DNase I (100 ng/mL, dild immediately before use). Incubate 10 min at 15°. Add 2 μL DNA polymerase I (1 IU/μL). Incubate 1 h at 15°. Add 2 μL 0.5M Na_2EDTA, pH 8.0. Prep. 2 mL column of Sephadex G-50 (prepd according to manufacturer's instructions) using TE buffer (**c**). Load reaction mix onto column and elute by adding 100 μL portions of TE buffer (**c**). Collect twenty 2-drop fractions into 500 μL tubes. Spot 2 μL of each fraction onto 2 × 2 cm paper squares (e.g., Whatman 3MM), dry, add scintillation fluid (e.g., 5 g 2,5-diphenyloxazole/L toluene), and count. Geiger-counter may suffice to assay fractions. Labeled DNA is eluted from column usually between fractions 6 and 12. Unincorporated dCTP elutes as larger peak, starting between fractions 12 and 15. Pool fractions from earliest peak and count 2 μL as previously described. Using 3000 Ci/mmol of $dCT^{32}P$, specific activities of 2–8 × 10^8 cpm/μg usually result.

J. Colony Hybridization Filter Preparation

When received, transfer sample cultures to 5 mL rich broth and incubate at 37° for 18–24 h. Aseptically add 2 mL culture to 0.5 mL sterile 50% (v/v) glycerol. Store at −70° if possible. [*Note:* Frost-free freezers will decrease culture viability. If cultures must be stored at −20°, use non-frost-free unit. This caveat holds for all frozen material in this procedure.]

Boil nitrocellulose filters (0.45 μm, 82 mm diam.) for 2–3 min in ca 2 L H_2O. While still wet, flatten filters (to minimize wrinkles) between paper filters (such as Whatman No. 1 or Schleicher and Schuell No. 597), using forceps to avoid touching filters. Loosely wrap filters in Al foil and sterilize at 121°, 15 lb, for 10–20 min on liq. (slow exhaust) cycle.

Store at room temp. Aseptically inoculate ca 5 mL rich broth with portion of frozen bacterial culture. [It is not necessary to completely thaw culture and it may be re-used several times.] Incubate cultures 18–24 h at 37°. Aseptically, place sterile nitrocellulose filter on dry MacConkey agar plate. Ensure that no bubbles are trapped under filter and that it wets completely. Discard filters that do not lie flat. Label filters, using soft lead pencil or by perforating filter in distinctive pattern with needle. This may be more easily done after baking at 80° (see below). Filters marked with 5 mm square grid are useful for arranging cultures in orderly array. Inoculate filters with sterile microbiological needle, using 1:100-fold diln, in sterile normal saline, of overnight culture. Always inoculate each filter with known pos. and neg. control cultures. Record location of each culture; 30–50 cultures should fit on each filter. It is vital that filters and the resulting autoradiogram can be oriented unambiguously. Make duplicate filters, since procedure may have to be repeated. Incubate filters 18–24 h at 37°. Mark cultures which have failed to grow, or a false-neg. result may be reported.

Lyse colonies by transferring filters for 10 min onto paper filters (in 100 × 15 mm plastic petri plates) wetted with 1.5 mL 0.5M NaOH. Ensure that no bubbles are trapped under filters. Transfer nitrocellulose filters for ≥1 min each, to series of 3 paper filters each wetted with 1.5 mL 1.0M ammonium acetate-0.02N NaOH. Shift nitrocellulose filter to fourth ammonium acetate-NaOH filter for 10 min. Keep filters horizontal during transfers so that lysed colonies will not run together. Air dry nitrocellulose filter on absorbent paper ≥30 min. Bake in vac. oven 2 h at 80°. Cool filters to room temp. and label with H_2O-proof ink or pencil. Store between paper filters under vac.

K. Colony Hybridization

Freshly prep. 50 mL hybridization mixt. (**k**). Boil 0.5 mL sonicated calf thymus DNA (**n**) 10 min and add to hybridiza-

tion mixt. (**k**). Pre-incubate each nitrocellulose filter 3 h at 37° in 100 × 15 mm plastic petri dish contg 5 mL hybridization mixt. with boiled calf thymus DNA. After 3 h, alkali-denature radioactive toxin gene DNA. Det. vol. of DNA required to contain 1×10^6 cpm. Correct for 14.2-day half-life of ^{32}P. Add 1×10^6 cpm DNA to 500 μL plastic conical tube and bring total vol. to 300 μL with H_2O. Add 6 μL 10N NaOH and mix briefly with pipet tip. After 10 min, neutze with 6 μL 10N HOAc. Boil 0.5 mL sonicated calf thymus DNA for 10 min and add 50 mL hybridization mixt. (**k**). Place nitrocellulose filter into 5.0 mL fresh hybridization mixt. and 1×10^6 cpm alkali denatured and neutzd probe DNA. Incubate 18–24 h at 37°.

Rinse filters 5–10 s in 10–15 mL 5X SSC (dild from soln l)-0.1% (w/v) sodium dodecyl sulfate (SDS). Place filter into clean petri dish and cover with 10–15 mL 5X SSC-0.1% SDS and incubate 1 h at 70°. Place filter in fresh 5X SSC-0.1% SDS and incubate addnl 1 h. Rinse filter 5–10 s in 2X SSC (dild from soln l). Air-dry 15–30 min. Mount filter with small pieces of tape onto paper and cover with plastic sheet (such as document holder).

L. Autoradiography

In dark room, place film on plastic-covered filters in cassette film holder with intensifying screens. Enclose film holder in plastic bag and expose film preferably at −70° but at least −20°. Exposure length is dictated by amt of radioactive DNA bound to filter. If increase of 2–3 cps is observed when Geiger-Mueller counter is held over filter, it is likely that pos. reaction will be visible after 1 d exposure. After exposure, let cassette reach room temp. Develop following manufacturer's instructions. If spots are too faint for analysis, expose new film for longer period.

M. Interpretation of Results

DNA of cells having gene for heat-labile enterotoxin of *Escherichia coli* bind radioactive toxin gene DNA. Film will be exposed and dark spots will appear after development. Since colony size and hybridization efficiency can vary, this test is best used qual. and not quant. If there are dark areas on film where no colonies should be, unhybridized radioactive DNA has probably not been completely washed away. Rewash filter twice in 5X SSC-0.1% SDS at 70° for 1 h. Let dry and expose film. After film development, make pos. or neg. detn of each unknown culture by comparing intensity of spot with pos. and neg. control cultures. Neg. control should show no darkening of film or, at most, very faint darkening. Pos. control should show distinct darkening of film clearly discernible above background.

N. Troubleshooting

If autoradiograms are unsatisfactory, a number of factors might be responsible. False-neg. results could be due to spontaneous loss of virulence determinants, insufficient growth of colonies on filters, failure to bake filters to fix DNA, or insufficient radioactivity during hybridization. False-pos. results may result from insufficient filter washing after hybridization, failure to add Denhardt's soln or sonicated calf thymus DNA, or use of probe DNA fragment which was not purified adequately. Possible remedies include use of new bacterial cultures, prepg new filters with lysed colonies, reviewing procedures and reagent composition, rewashing filters, or checking darkroom methods.

Ref.: JAOAC **67,** 801(1984).

986.34 Enterotoxigenic *Escherichia coli*

DNA Colony Hybridization Method Using Synthetic Oligodeoxyribonucleotides and Paper Filters

First Action 1986
Final Action 1987

(*Caution:* This procedure uses radioactive compd. Personnel must receive adequate training and monitoring and have proper facilities available for handling this substance.)

A. Principle

Chemically synthesized pieces of DNA (oligodeoxyribonucleotides) that code for regions of genes detg bacterial virulence can be used to identify pathogenic strains of bacteria. These oligomers are radioactively labeled in vitro and hybridized with colonies of bacterial cells that have been lysed and fixed to paper filters. Colonies contg same region of a gene will bind labeled DNA and become radioactive. Such colonies can be detected by autoradiography.

B. Reagents

(Prep. all media according to manufacturer's instructions and use analytical grade materials whenever possible. *Note:* DNA often adheres to unsiliconized glass. When working with solns contg DNA, use siliconized glassware or disposable plasticware unless otherwise specified.)

(**a**) *Lysis mixture A.*—Combine 50 mL 10N NaOH, (**s**), 300 mL 5.0M NaCl, (**u**), and 650 mL H_2O.

(**b**) *Lysis mixture B.*—Combine 50 mL 2.0M Tris, pH 7.0, (**v**), 400 mL 5.0M NaCl, (**u**), and 550 mL H_2O.

(**c**) *Hybridization mixture.*—Combine in plastic tube or beaker: 28.9 mL H_2O, 15.0 mL 20X SSC, (**d**), 5.0 mL 50X Denhardt's soln, (**e**), and 0.1 mL 0.5M EDTA soln, pH 8.0, (**f**). Final vol. is 49 mL. Use immediately.

(**d**) *20X std saline citrate soln (SSC).*—Dissolve 175.4 g NaCl and 88.2 g Na citrate in final vol. of 1 L H_2O.

(**e**) *50X Denhardt's soln.*—Dissolve 2.0 g Ficoll (av. molecular wt 400 000), 2.0 g polyvinyl pyrrolidone (av. molecular wt 360 000), and 2.0 g bovine serum albumin in 200 mL H_2O. Store at −20° in 5.0 mL aliquots.

(**f**) *0.5M Disodium ethylenediamine tetraacetate soln, pH 8.0.*—Dissolve 186.12 g Na_2EDTA in 800–900 mL H_2O. Adjust to pH 8.0 with 10N NaOH, (**s**). Dil. to 1 L with H_2O.

(**g**) *Sonicated calf thymus DNA.*—Dissolve 1 g purified calf thymus DNA in 100 mL H_2O by stirring 3–4 h. Sonicate until av. molecular wt is 300 000–500 000, which may be detd by electrophoresis with appropriate stds such as 123-base ladder (Bethesda Research Laboratories (BRL), Div. Life Technologies, Inc., 8717 Grovemont Circle, Gaithersburg, MD 20877). Store in 1 mL portions in 13 × 100 mm screw-cap tubes. Glass may be used in this instance only.

(**h**) *6X SSC soln.*—Combine 300 mL 20X SSC, (**d**), with 700 mL H_2O.

(**i**) *2X SSC soln.*—Combine 100 mL 20X SSC, (**d**), with 900 mL H_2O.

(**j**) *Synthetic DNA stock soln.*—Approx. 150–350 μg/mL. (A_{260} = 5–10 units.) Soln of 22-base, single stranded DNA molecules [STH (human) and STP (porcine) oligodeoxyribonucleotide probes for enterotoxin genes] will have concn ca 20–50μM. Store at −20°.

(**k**) *Synthetic DNA working soln.*—Dil. stock soln, (**j**), in H_2O to 10μM. Store at −20°.

(**l**) *2.0M Tris soln, pH 7.6.*—Dissolve 242.28 g Tris in ca 800 mL H_2O. Adjust to pH 7.6 with concd HCl. Dil. to 1 L with H_2O.

(**m**) *1.0M $MgCl_2$ soln.*—Dissolve 9.52 g $MgCl_2$ in final vol. of 100 mL H_2O.

(**n**) *0.5M Dithiothreitol soln.*—Weigh 0.77 g dithiothreitol and combine with H_2O to final vol. of 10.0 mL. Store at 4°.

(**o**) *10mM Spermidine soln.*—Dissolve 14.5 mg spermidine in final vol. of 10.0 mL H_2O. Store at −20°.

(**p**) *10X Kinase buffer.*—Combine 2.5 mL 2.0M Tris, pH 7.6, (**l**), 1.0 mL 1.0M $MgCl_2$, (**m**), 1.0 mL 0.5M dithiothreitol, (**n**), 1.0 mL 10mM spermidine, (**o**), 20 μL 0.5M EDTA, (**f**), and 4.5 mL H_2O. Store at 4°.

(**q**) *(γ-^{32}P) ATP.*—Aq. soln of adenosine triphosphate, specific activity 3000–7000 Ci/mmole. ("Crude" prepn from ICN Biomedicals, Inc., ICN Plaza, 3300 Hyland Ave, Costa Mesa, CA 92626, or equiv.). Store at −70° if possible.

(**r**) *Bacteriophage T4 polynucleotide kinase.*—20 units/μL (BRL or equiv.).

(**s**) *10N NaOH soln.*—Dissolve 400 g NaOH in final vol. of 1 L H_2O.

(**t**) *2.0M Tris soln, pH 8.0.*—Follow instructions for (**l**) but adjust pH to 8.0.

(**u**) *5.0M NaCl soln.*—Dissolve 292.2 g NaCl in final vol. of 1 L H_2O.

(**v**) *2.0M Tris soln, pH 7.0.*—Follow instructions for (**l**) but adjust pH to 7.0.

(**w**) *Glycerol freezing soln.*—Combine 50.0 mL glycerol and 50.0 mL H_2O. Dispense 0.5 mL aliquots into 1 dram vials. Sterilize by autoclaving 15 min at 121°.

(**x**) *NACS PREPAC column loading buffer.*—Dissolve 19.3 g ammonium acetate in final vol. of 1 L H_2O.

(**y**) *NACS PREPAC column eluting buffer.*—Dissolve 308.4 g ammonium acetate in final vol. of 1 L H_2O.

(**z**) *Brain heart infusion or trypticase soy broth and agar.*—For microbial growth.

(**aa**) *Scintillation fluid.*—Dissolve 5.0 g 2,5-diphenyloxazole in 1 L toluene.

(**bb**) *ST probe soln.*—Combine equal vols of STH and STP working soln, (**k**).

(**cc**) *Phosphoramidite soln.*—0.5 g (Applied Biosystems, Inc., 850 Lincoln Centre Dr, Foster City, CA 94404; American Bionetics, Inc., 21377 Cabot Blvd, Hayward, CA 94545, or equiv.), reagent grade (≥95%), made up to 0.1M using anhyd. CH_3CN, (**nn**), and glass syringe transfer procedures with protection from atm. H_2O. Vortex mix until dissolved.

(**dd**) *Thiophenol soln.*—Mix 80 mL *p*-dioxane (≤0.01% H_2O), 80 mL triethylamine (99$^+$%), and 40 mL thiophenol (99$^+$%) ("Gold Label," Aldrich Chemical Co., or equiv.).

(**ee**) *1H-Tetrazole soln.*—Add 300 mL anhyd. CH_3CN, (**nn**), to 10 g resublimed tetrazole, (**oo**), with protection from atm. H_2O, and sonicate until dissolved. Warm (30–40°), if necessary.

(**ff**) *Ammonium hydroxide soln.*—28–30% NH_3, as supplied.

(**gg**) *Acetic anhydride soln.*—Combine 160 mL tetrahydrofuran (≤0.01% H_2O), 20 mL 2,6-lutidine ("Spectro Grade," Eastman Kodak Co., or equiv), and 20 mL acetic anhydride (99$^+$%).

(**hh**) *4-Dimethylaminopyridine soln.*—Dissolve 13 g recrystd 4-dimethylaminopyridine, (**pp**), in 200 mL tetrahydrofuran (≤0.01% H_2O).

(**ii**) *Trichloroacetic acid soln.*—Weigh 125 g trichloroacetic acid (Aldrich Chemical Co., Inc., No. 25,139, or equiv., 99$^+$%) in beaker with min. exposure to atm. moisture and transfer to storage container using 4 L CH_2Cl_2 (≤0.006% H_2O).

(**jj**) *Iodine soln.*—Combine 320 mL tetrahydrofuran, 80 mL 2,6-lutidine, and 10.2 g I crystals. Sonicate until dissolved. Add 8.0 mL H_2O, dropwise, with stirring.

(**kk**) *Dimethoxytrityl (DMT) assay soln.*—Dissolve 19 g *p*-toluenesulfonic acid monohydrate in 1 L LC grade CH_3CN (0.1M).

(**ll**) *Triethylammonium acetate (TEAA) buffer.*—With const stirring, add 28 mL triethylamine, (**qq**), to 1.8 L H_2O followed by 10 mL glacial acetic acid. Titr. slowly with more acid to pH 7.0 and then vac. filter thru type HA 0.45 μm filter (Millipore Corp. or equiv.).

(**mm**) *Detritylation soln.*—Add 3 mL glacial acetic acid to 97 mL H_2O.

(**nn**) *Anhydrous acetonitrile.*—Store 1 L LC grade CH_3CN (≤0.007% H_2O, Burdick & Jackson Laboratories, Inc., or equiv.) over type 4A molecular sieves ≥24 h.

(**oo**) *Resublimed 1H-tetrazole.*—Sublime 20 g 1*H*-tetrazole (99$^+$%, Aldrich "Gold Label" or equiv.) in std sublimation app. at ≤0.25 torr and 130–140°. (Yields ca 15 g sublimate.)

(**pp**) *Recrystallized 4-dimethylaminopyridine.*—Dissolve 200 g 4-dimethylaminopyridine in ca 1 L hot (50–60°) tetrahydrofuran contg 20 g decolorizing charcoal. Filter while still hot thru glass fiber paper (Grade 934-AH, "Reeve Angel," Whatman, Inc., or equiv).

(**qq**) *Triethylamine.*—99$^+$% (Aldrich "Gold Label" or equiv. LC grade).

C. Apparatus and Materials

(**a**) *Labware.*—100 × 15 mm glass petri plates; plastic beakers and tubes to contain up to 100 mL; 100 × 15 or 20 mm plastic petri plates; plastic conical tubes to contain up to 500 μL; plastic pipets to cover range 1–10 mL; variable vol. micropipettors and tips to cover range 1–1000 μL.

(**b**) *Incubators.*—(*1*) Capable of maintaining 37 ± 1°; (*2*) capable of maintaining 40 ± 1°; (*3*) capable of maintaining 50 ± 1°; (*4*) H_2O bath or dry block capable of maintaining 37 ± 1°.

(**c**) *UV spectrophotometer.*—To measure DNA concn at 260 nm. (1 A_{260} unit is 50 μg/mL for double stranded DNA and 33 μg/mL for single stranded DNA.)

(**d**) *Ultralow temperature freezer.*—Capable of maintaining −70° is preferred, but freezer (not frost-free) at −20° may be substituted.

(**e**) *Freezer.*—Capable of maintaining −20° (not frost-free).

(**f**) *Cellulose filters.*—No. 541 (Whatman), 82–85 mm diam.

(**g**) *Absorbent filters.*—Whatman No. 1 or similar, ca 85 mm diam.

(**h**) *NACS PREPAC column.*—DNA binding resin (BRL or equiv.).

(**i**) *Scintillation counter.*—Or Geiger-Mueller counter if calibrated in cpm.

(**j**) *X-ray film and developing chemicals.*—8 × 10 in. is convenient size. Kodak XAR X-ray film or equiv.

(**k**) *Darkroom.*—Facilities for X-ray film development with appropriate safelight.

(**l**) *X-ray film holder cassette.*—With intensifying screens (Kodak regular, Eastman Kodak Co.; Dupont Cronex Lightening Plus, E.I. Dupont de Nemours & Co.; or equiv.).

(**m**) *Centrifuge.*—Capable of spinning 500 μL conical plastic tubes (Eppendorf Model 5412, Brinkmann Instruments, Inc., or equiv.).

(**n**) *Vacuum desiccator.*—Needed only if prepd colony hybridization filters must be stored 1 week.

(**o**) *DNA synthesizer.*—Manual or automated synthesis system (i.e., Applied Biosystems synthesizer Model 380A; other synthesis systems providing equiv. results are also acceptable).

(**p**) *Synthesis ("reaction") columns.*—1 μmol long chain alkylamine-functionalized controlled pore glass, either prepacked or handpacked (Applied Biosystems or equiv.).

(**q**) *Fraction collector.*—To collect fractions from automated synthesis system. Should have auxilliary signal input.

(**r**) *Liquid chromatographic system.*—App. with gradient elution capability, UV detection at 254 or 260 nm, and μBondapak® C_{18}, 7.8 mm × 30 cm column (Waters Associates, Inc., or equiv.).

(**s**) *Rotary vacuum centrifuge.*—To conc. LC-purified oligodeoxyribonucleotides (SpeedVac concentrator/dryer, Savant Instruments, Inc., 110-103 Bi-County Blvd, Farmingdale, NY 11735, or equiv.).

(**t**) *Glass syringes.*—Capacity up to 10 mL for transfer of anhyd. CH_3CN with protection from atm. moisture.

(**u**) *Type HV, 0.45 μm filters.*—To remove LC column particulates (Millipore or equiv.).

D. Colony Hybridization Filter Preparation

Transfer candidate cultures to 5 mL brain heart infusion or trypticase soy broth and incubate 18–24 h at 37°. If culture must be stored before analysis can be performed, aseptically add 2.0 mL culture to 0.5 mL freezing soln, (**w**). Store at −70° if possible. (Note: Frost-free freezers will decrease culture viability and may result in loss of virulence determinants. If cultures must be stored at −20°, use non-frost-free unit. This precaution holds for all frozen material in this procedure.)

Aseptically inoculate 5 mL rich broth with portion of frozen bacterial culture. Sterile cotton swabs are well suited for this purpose. Always include known pos. and neg. control cultures on every fiber (see below). (If culture is not thawed, it may be reused innumerable times.) Incubate culture 18–24 h at 37°. At same time, aseptically prepare 100 × 15 mm petri plates contg either brain heart infusion or trypticase soy agar and dry inverted 18–24 h at 37°. After inoculating cultures in orderly array and ensuring that resulting colonies will not ultimately merge while growing, inoculate agar plates with test cultures, using sterile microbiological needle, toothpick, cotton swab, or replicator; 9–10 mm is convenient distance between cultures. Record location of each culture; it is vital that culture patterns and resulting autoradiogram(s) can be oriented unambiguously. Prep. multiple plates and concomitant filters because hybridization procedure may have to be repeated and number of steps to be repeated is thereby lessened. Incubate plates inverted 18–24 h at 37°. Mark cultures failing to grow; otherwise, false-neg. results may be reported.

Label Whatman No. 541 cellulose filters, (**f**), 82–85 mm diam., using soft lead pencil, and also mark filter so it can be oriented unambiguously after replication. (*Note:* Other manufacturers make filters with physical properties equiv. to Whatman No. 541. However, DNA binding abilities of such filters are not always suitable for use in DNA hybridization.) Apply filter so that side with pencil markings faces colony array on agar surface of plate contg colonies. Wetting initial edge of filter paper and rolling to opposite edge usually eliminates formation of air pockets. If air bubbles are entrapped between filter and agar plate, remove by applying gentle pressure with glass spreader. This maneuver also ensures more efficient attachment of cultures to filter paper, but care must be taken to avoid spreading colonies because of excessive pressure. Filters may be peeled from plate immediately, but more definitive reactions are usually obtained if filter remains situated 1–2 h. (*Note:* Colony array on filter is now mirror image of array originally applied to agar plate.)

Lyse colonies replicated onto filters by transferring filters with colony side up onto absorbent cellulose filters, ca 85 mm diam. (such as Whatman No. 1 or Schleicher & Schuell No. 597) contained in glass 100 × 15 mm petri plates and previously wetted with 1.5–2.0 mL lysis mixt. A, (**a**). Be sure that no air is entrapped between filters. Heat filters in glass plate for 3–5 min in steam. Transfer steamed filters to glass petri plates contg absorbent cellulose filters previously wetted with 1.5–2.0 mL lysis mixt. B, (**b**). Again, be sure that no air pockets result. Maintain filters in horizontal position when transferring so that lysed colonies (DNA) will not become confluent. Let filters become completely neutralized by remaining situated 5–10 min.

If filters are not to be used immediately, air-dry on absorbent paper at room temp. and store under vac. between filter papers. Such filters have been kept ca 1 year without noticeable change in results.

E. Oligodeoxyribonucleotide Synthesis

(*Note* 1: A number of companies will custom-synthesize oligodeoxyribonucleotides. Also, several oligodeoxyribonucleotide synthesis systems are com. available, both automated and manual. Results are generally satisfactory if manufacturer's instructions are followed. This method uses one of com. available, automated synthesizers and procedure described below is meant to serve only as example.)

(*Note* 2: All solns for prepn and isolation of synthetic oligodeoxyribonucleotides should be prepd in deionized H_2O passed thru 0.2 μm filter ("Versacap Filter Unit," Gelman Sciences, Inc., or equiv.).)

According to manufacturer's instructions, use Applied Biosystems, "fast" cycle but with following modifications of step times: trichloroacetic acid to column detritylation step, 75 s (retained in fraction collector); CH_3CN to column post-detritylation step, 50 s (also retained and pooled with above in fraction collector); CH_3CN to column, pre-coupling step, 120 s; coupling step, 180 s; capping step, 120 s. Synthesis is ended with dimethoxytrityl (DMT) group retained at 5′ terminus. Automated cleavage from support is achieved with concd NH_4OH at room temp. for 1 h. Dil. delivered NH_4OH soln with 1 mL concd NH_4OH, heat 10 h at 60° in 3.7 mL vial with Teflon-lined screw cap (Supelco, or equiv.). Let cool to room temp. Add 50 μL triethylamine, (**qq**). Evap. NH_3 with N stream to ca 2 mL.

F. Quantitation of Coupling Yield

To det. isolated product yield (see below) and ensure satisfactory coupling at each addn, theoretical yields of product must be calcd. Dil. each collected fraction (from detritylation and post-detritylation steps above) to 5 mL with DMT-assay soln (**kk**). Mix each fraction well and read *A* at 530 nm. Use assay soln (**kk**) as reference std. Compare *A* with that of previous fraction to det. coupling efficiency of each step (generally 97–99%). To det. overall theoretical yield, multiply all individual step-yields.

G. Oligodeoxyribonucleotide Purification and Isolation

To det. chromatgc properties of prepn, perform anal. run. Set detector for 0.1 AUFS. Inject 10 μL soln evapd to 2.0 mL. In ambient temp. column, start 20–30% gradient (at 1%/min) of CH_3CN in triethylammonium acetate buffer, (**ll**). Continue at 30% CH_3CN after 10 min. Generally, major DMT-product elutes at 10 ± 3 min. After elution time is detd, repeat chromatgy on preparative scale (inject 100 μL crude soln, 1.0 AUFS). Collect center position of major peak.

H. Oligodeoxyribonucleotide Processing

Before synthetic oligonucleotide can be used as substrate for polynucleotide kinase, LC solvs and DMT group must be removed. Conc. collected LC fraction using N ca 10–20 min to remove most CH_3CN. Conc. sample to dryness using concentrator/dryer, (**s**). Add 1 mL 3% (v/v) acetic acid to remove DMT protecting group. Vortex-mix to dissolve. After 5–10 min at room temp., freeze in crushed dry-ice and conc. using

concentrator/dryer, (**s**). Dissolve residue in 1 mL H_2O. Add 1 mL anal. grade ethyl acetate to ext org. impurities and vortex-mix thoroly. Let org. layer sep. from aq. layer contg DNA and possible LC column particulates (centrf. if necessary). Remove org. layer with Pasteur pipet and discard. If insoluble LC column particulates are present, syringe-filter DNA soln thru type HV, 0.45 μm filter (**u**). Let DNA soln gravity-filter and collect residual soln by rapidly depressing syringe plunger. Remove 50 μL aliquot from 1 mL filtered DNA soln for *A* measurement. Conc. both remaining sample and *A* aliquot to dryness. Dissolve aliquot in 1 mL H_2O and measure *A* at 260 nm. Since $^1/_{20}$ of sample has been removed, multiply reading by 19 to obtain *A* units in total purified sample. Discard *A* aliquot. Multiply *A* in total purified sample by 10 (because only 10% of total synthesis reaction was purified) to obtain *A* units of entire isolable product. Compare this yield with calcd value (1 μmole × theoretical yield [see above] × molar *A* of oligonucleotide synthesized × 10^{-3}) to det. yield of isolable product. Molar *A* is calcd by adding number of purines (dA plus dG) times 14 000 plus number of pyrimidines (dC plus T) times 7000. These factors are molar extinction coefficients and 10^{-3} is used to convert molar *A* to μmoles/mL which is a millimolar concn.

I. End-Labeling of Synthetic DNA

Synthetic oligodeoxyribonucleotides are rehydrated to ca 5–10 A_{260} units (ca 150–350 μg/mL) to serve as stock soln (**j**). One A_{260} unit corresponds to ca 33 μg/mL single-stranded DNA. Molecular wt of 22-base, single-stranded DNA molecule is ca 7260. Prep. 10μM working soln for each DNA probe (10 pmoles/μL, 72.6 μg/mL). If desired, STH and STP synthetic DNA probes can be combined into single soln, 5μM in each probe, (**bb**).

Mix 5 μL DNA probe soln, (**bb**), 2.5 μL 10X kinase buffer, (**p**), 15 μL H_2O, 1.5 μL (γ-^{32}P) ATP, (**q**), and 1 μL T4 kinase, (**r**), in 500 μL plastic conical centrf. tube, (**a**), on ice. Add kinase, (**r**), last and return enzyme immediately to −20° because it is quite heat-labile. Centrf., (**m**), 2–3 s to adequately mix reagents. Incubate at 37° in H_2O bath or dry block heater, (**b**), 1 h. Add 2 μL 0.5M EDTA, (**f**), to terminate reaction. Add 1.6 μL 4.0M ammonium acetate soln, (**y**), to bring ammonium acetate concn to 0.25M before applying sample to NACS PREPAC column.

Unincorporated ^{32}P is removed by binding DNA to NACS PREPAC column, (**h**). Equilibrate column with 0.25M ammonium acetate, (**x**), 2 h. Load reaction mixt. onto column and wash, using gravity or very gentle pressure, with ca 4 mL loading buffer, (**x**), to remove free ATP. Elute bound DNA with 200 μL aliquots of eluting buffer, (**y**). Do not force liq. thru column rapidly. Collect three 200 μL fractions in 500 μL plastic tubes, (**a**). Spot 2 μL of each fraction onto ca 2 × 2 cm paper (e.g., Whatman 3MM), dry, add ca 5 mL scintillation fluid, (**aa**), and assay radioactivity by scintillation counting. Geiger-Mueller counter, (**i**), may suffice if properly calibrated and used. Most labeled DNA is eluted from column in fractions 1 and 2. Pool fractions and count triplicate 2 μL portions as described above. Est. total vol. of prepn by carefully drawing into plastic 1 mL pipet. Calc. total amt of radioactivity recovered in prepn. Usually, 1–2 × 10^8 cpm is obtained if specific activity of ATP, (**q**), is 3000–7000 Ci/mmol. Store at −20°.

J. Colony Hybridization

Freshly prep. 50 mL hybridization mixt., (**c**). Boil 1.0 mL sonicated calf thymus DNA, (**g**), 5 min in H_2O bath and add to hybridization mixt., (**c**). Dispense 10 mL sonicated calf thymus DNA-hybridization mixt. into 100 × 15 or 20 mm *plastic* petri dish and insert cellulose filter contg lysed colony array. To use std amt of probe for each hybridization, det. vol. of probe DNA soln required to contain 1 × 10^6 cpm after correcting for 14.2 day half-life of ^{32}P. Add 1 × 10^6 cpm probe DNA to soln contg filter. Mix briefly and incubate plate overnight at 40°.

Wash hybridized filters free of ^{32}P-labeled DNA not specifically bound to DNA from colonies on filter by removing filter from hybridization mixt. and rinsing 5–10 s in plastic petri dish contg 10 mL 6X SSC, (**h**). Drain and recover filter with 6X SSC. Incubate 1 h at 50°. Again, drain plate, recover with 6X SSC, and incubate 1 h at 50°. Finally, rinse filter 5–10 s at room temp. in 2X SSC, (**i**). Air-dry on absorbent paper at room temp. to prevent curling. Mount filter to 8 × 10 in. stiff paper (e.g., Whatman 3MM) using small pieces of tape. Cover with plastic or glassine sheet (such as document or neg. holder) to prevent contamination of intensifying screens in X-ray film holders.

K. Autoradiography

Exposure time is dictated by amt of radioactive DNA bound to filter. If increase above background exceeds 10 cps when Geiger-Mueller counter is held over filter, it is likely that pos. reaction will be visible after 4 h exposure at room temp. However, if increase of 2–3 cps is observed, enclose loaded film cassette in sealed plastic bag and expose film overnight, preferably at −70° or at least −20°. If −70° is not available, cassette can be sandwiched between slabs of dry ice to reduce exposure time.

In darkroom, place X-ray film onto plastic-covered filter in cassette film holder with intensifying screens. Expose film for appropriate length of time as detd above. After exposure, let cassette equilibrate at room temp. (to prevent moisture accumulation) before removing plastic bag. Develop X-ray film by following manufacturer's instructions. If spots are too faint or too intense for analysis, expose new film for appropriate length of time.

L. Reporting of Results

Lysed colonies of *E. coli* strains contg DNA coding for heat-stable enterotoxins will bind radioactively labeled oligonucleotide probe for ST. These radioactive lysed colonies will expose X-ray film, and dark spots will be evident after development. Det. if each unknown culture is pos. or neg. by comparing spot intensity to that of pos. and neg. culture controls. However, many factors can influence quality of these results: size of colonies, amt of cellular debris, amt of DNA per lysed colony, hybridization and washing temps, hybridization time, specific activity of probe, and length of autoradiogram exposure. Well documented pos. and neg. controls must be present on every filter to ensure that the procedure has been performed correctly and that compensation for nonspecific binding of labeled probe DNA (neg. colonies that may be seen as faint spots) has been made.

If neg. control cultures exhibit faint spots, and pos. culture spots are intense, re-wash filter(s) in 6X-SSC, (**h**), at 52–55° twice for 1 h each time. Dry filters and re-expose autoradiogram. Take care because thermal stability of oligonucleotide hybrids is much less than that of longer DNA molecules.

M. Troubleshooting

Unsatisfactory autoradiograms can result from several factors, some of which have been listed in the previous section. False-neg. results can be due to spontaneous loss of plasmids, especially when strains are cultivated excessively under nonselective laboratory conditions (i.e., re-isolation or further subculture). Also, hybridization and/or washes at excessively high temps can result in decreased DNA probe binding which

in turn can lead to neg. observation. Occasionally, very large colonies do not become affixed to filters and cellular material is lost from hybridization filters. False-pos. results can be observed if either hybridization or washing temp. is too low. Nonspecific DNA probe binding will occur. Autoradiogram exposures of excessive time can result in overemphasis of limited, nonspecific binding of probe to neg. cultures; this may be falsely reported as pos. results. Other possible sources of error and their remedies have been discussed (**984.34N**; JAOAC **67**, 801(1984)).

Finally, it is essential to note that resulting autoradiogram spot arrays are mirror images of plate inoculation patterns. This is not the case with **984.34**. Results are accurately read if autoradiograms are reversed (left to right) before interpretation. Films must be marked so that they can be unambiguously oriented with recorded location of each test culture.

Ref.: JAOAC **69,** 531, 151A(1986).

984.35 *Escherichia coli* Enterotoxins

Mouse Adrenal Cell and Suckling Mouse Assays

First Action 1984
Final Action 1987

A. Principle

When exposed to cholera toxin or heat-labile enterotoxin of *Escherichia coli,* mouse adrenal cell line, designated Y1, responds by change in morphology from flat to round. Response is mediated by adenyl cyclase and is irreversible. Intragastric administration of heat-stable enterotoxin of *E. coli* to suckling mouse causes fluid accumulation in intestinal lumen. This measurable response is mediated by guanyl cyclase.

B. Media and Reagents

(**a**) *Casamino acids-yeast extract (CAYE) broth.—Soln a:* Casamino acids, 20 g; yeast ext, 6 g; NaCl, 2.5 g; K_2HPO_4, 8.71 g; adjust to pH 8.5 with 0.1N NaOH, and to final vol. of 1 L. *Soln b:* $MgSO_4$, 50 g; $MnCl_2$, 5 g; $FeCl_2$, 5 g; dissolve in min. amt of 0.01N H_2SO_4, and adjust to final vol. of 1 L with H_2O.

Add 1 mL soln b to soln a before sterilizing; autoclave 15 min at 121°C after dispensing.

(**b**) *Trypticase soy-yeast extract (TSYE) broth.*—Com. trypticase soy broth rehydrated as directed with 0.6% yeast ext added.

(**c**) *Tissue culture media.—(1) Growth medium:* Ham's F-10 with glutamine and $NaHCO_3$ (Flow Laboratories), 100 mL; newborn calf serum, 10 mL; antibiotic conc. (5000 IU penicillin G, and 5000 μg streptomycin/mL), 1 mL. *(2) Maintenance medium:* Same as *(1)* except serum level is 1%.

(**d**) *Dulbecco's PBS, pH 7.5.*—NaCl, 8 g; KCl, 200 mg; $Na_2HPO_4.7H_2O$, 2.16 g; KH_2PO_4, 200 mg; make up to 1 L with H_2O and autoclave 15 min at 121°.

(**e**) *Trypsin.*—0.25% in Dulbecco's PBS.

(**f**) *Cholera enterotoxin.*—1 mg/mL when reconstituted as directed (Schwartz/Mann).

(**g**) *Mice.*—Outbred white Swiss mice, 3–5 days old.

(**h**) *Evans blue.*—2% soln.

C. Equipment and Materials

(**a**) *Serological pipets.*—1 and 5 mL, small tip.

(**b**) *Pipets.*—25 μL.

(**c**) *Swinnex filters.*—25 mm, 0.45 μm membrane.

(**d**) *Disposable syringes.*—5 mL, accommodating Swinnex filters.

(**e**) *Tissue culture flasks.*—Plastic, 75 sq. cm.

(**f**) *Vertical laminar flow hood.*—Biological containment, equipped with HEPA filters.

(**g**) *Incubator.*—CO_2, set at 35° and 5% CO_2.

(**h**) *Microtiter tissue culture plates.*—96 wells, with lids, sterile.

(**i**) *Syringe.*—1 mL, disposable.

(**j**) *Animal feeding needle.*—24 gage, 1 in., straight.

(**k**) *Needle.*—27 gage. Not needed if *per os* procedure is followed.

Labile Toxin (LT)

D. Day 1

(**a**) Inoculate control cultures and cultures to be assayed into TSYE broth in 16 × 125 mm screw-cap tubes. Incubate in shaker incubator overnight at 37°. Both known enterotoxin-pos. and enterotoxin-neg. *E. coli* cultures should be used as controls, in addition to cholera toxin-pos. control.

(**b**) Remove growth medium from confluent layer of Y1 cells in 75 sq. cm flask. (One flask will provide enough cells for 2 microtiter assay plates.) Wash cell layer with PBS. Remove PBS wash and add 5 mL trypsin. After 1 min exposure, remove 4.5 mL trypsin and place flask in 35° incubator. Observe at 5 min intervals for cell detachment. When cell sheet has detached, add 5 mL growth medium and pipet repeatedly to break up cell clumps.

(**c**) Add cells from Day 1 (**b**) to 35 mL growth medium (total vol. is 40 mL) in small beaker. Stir this suspension while pipeting 0.2 mL into each well of two 96-well microtiter plates, using macroliter pipet. Cover finished plates and incubate ca 48 h at 35° in CO_2 incubator.

E. Day 2

(**d**) Add 2 drops of previously prepd starter culture, Day 1 (**a**), to 10 mL CAYE broth in 50 mL erlenmeyer and incubate 24 h at 37° in shaker incubator at 250 rpm.

F. Day 3

(**e**) Centrf. 24 h culture from Day 2 (**d**). (Twenty min at 2500 rpm will clarify most cultures of *E. coli.*). Filter supernate thru 0.45 μm membrane in Swinnex syringe-end filter holder.

(**f**) Divide filtrate into 2 portions. Heat one portion 30 min at 80°; leave other portion unheated. Both heated and unheated portions are assayed. Heated portion serves as neg. control. Store both at 4°.

(**g**) Prep. cholera toxin soln of 1 ng CT/mL in PBS. Soln is used as pos. control for cell reactivity. Note: CT is unstable at this concn, even at 4°; prep. daily from stock soln.

(**h**) Remove microtiter plates prepd in Day 1 (**c**) and replace growth medium with maintenance medium, 0.2 mL/well.

(**i**) Add 0.025 mL assay and control solns to one or more wells (4/test substance recommended) of microtiter plate, using microtiter syringe. Incubate microtiter plates 30 min at 35° in CO_2 incubator.

Replace maintenance medium, 0.2 mL/well. Incubate microtiter plates overnight at 35° in CO_2 incubator.

G. Day 4

(**j**) Examine microtiter plates for degree of rounding, starting with controls. Score rounding as follows:

0 = no rounding
1 = ca 25% rounding
2 = ca 50% rounding
3 = ca 75% rounding
4 = 100% rounding

Score ≥2 is reported as pos. for LT. Score <2 is recorded as neg. Neg. controls should show <10% rounding.

Stable Toxin (ST)

H. Day 1

(**a**) Inoculate starter culture. Procedure is identical to Day 1 (**a**), and need not be repeated when both assays (LT and ST) are done concurrently.

I. Day 2

(**b**) Inoculate CAYE assay culture. Procedure is identical to Day 2 (**d**), and need not be repeated when both assays (LT and ST) are done concurrently.

J. Day 3

(**c**) Prep. cultures for assay. Procedure is same as Day 3 (**e**) and (**f**), and need not be repeated if both assays (LT and ST) are done concurrently. Heated portion only is used in ST assay (suckling mouse). Material for ST assay may be stored at 4° for several days without noticeable loss of activity.

K. Day 4

(**d**) Add 2 drops of sterile Evans blue to 1 mL filtrate to be assayed.

(**e**) Inject suckling mice precutaneously with 0.1 mL filtrate into milk-filled stomach. Use tuberculin syringe and 27 gage needle. Inject min. of 4 mice for each filtrate. Discard all injections in which blue filtrate is not confined to stomach (immediate visual inspection) *or*

(**f**) Inject *per os* 0.1 mL filtrate into stomach of each mouse, using tuberculin syringe equipped with 24 gage feeding needle. This procedure may be used instead of precutaneous injection described in Day 4 (**e**). Either method works and preference is based on analyst's familiarity. Both methods yield equiv. results.

(**g**) Hold mice 3 h at *room temp*. Sacrifice mice by CO_2 inhalation. Open each abdomen and remove intestinal tract with exception of stomach and liver. Pool intestines treated with same filtrate in tared weighing vessel. Pool remainder of carcasses in another tared weighing vessel. Weigh both vessels on balance accurate to 0.01 g. Compute ratio of intestine wt/carcass wt.

L. Interpretation

Report ratio ≥0.083 as pos. for ST. Report ratio ≤0.074 as neg. for ST. Ratio 0.075–0.082 calls for re-examination of filtrate involved.

Ref.: JAOAC **67,** 946(1984).

STAPHYLOCOCCUS

980.37 *Staphylococcus aureus* in Foods

Microbiological Method

Final Action 1984
Repealed First Action 1987

(Applicable to detection and enumeration of small numbers of *S. aureus* in raw food ingredients and non-processed foods expected to contain large population of competing species.)

Inoculate 3 tubes of trypticase soy broth with 10% NaCl, **966.23A(f)**, at each test diln with 1 mL aliquots of decimal dilns of sample. Max. diln of sample must be high enough to yield neg. end point. Incubate 48 hr at 35–37°.

Using 3 mm loop, transfer 1 loopful from each growth-pos. tube to dried Baird-Parker medium plates, **966.23A(e)**(*3*). Streak so as to obtain isolated colonies. Incubate 45–48 hr at 35–37°.

From each plate showing growth, pick ≥1 colony suspected to be *S. aureus*, **966.23A(e)**(*4*). Transfer colonies to tubes contg 0.2 mL brain heart infusion (BHI) broth, **967.25A(r)**, and emulsify thoroly. Withdraw 1 loopful of resulting culture suspension and transfer to agar slant contg any suitable maintenance medium, e.g., trypticase soy agar: Suspend 40 g powder in 1 L H_2O. Let stand 5 min and mix thoroly. Heat gently with occasional agitation and boil ca 1 min or until soln is complete. Autoclave 15 min at 121°. Incubate BHI culture suspensions and slants 18–24 hr at 35–37°. Retain slant cultures at room temp. for ancillary or repeat tests, in case coagulase test results are questionable.

To BHI cultures add 0.5 mL reconstituted coagulase plasma with EDTA, **966.23A(i)**, and mix thoroly. Incubate at 35–37° and examine periodically over 6 hr interval for clot formation. Any degree of clot formation is considered pos. reaction. Small or poorly organized clots may be observed by gently tipping tube so that liq. portion of reaction mixt. approaches lip of tube; clots will protrude above liq. surface. Coagulase-pos. cultures are considered to be *S. aureus*. Test pos. and neg. controls simultaneously with cultures of unknown coagulase reactivity. Recheck doubtful coagulase test results on BHI cultures which have been incubated at 35–37° for >18 but ≤48 hr.

Report most probable number (MPN) of *S. aureus*/g from tables of MPN values, Table **966.24**.

987.09 *Staphylococcus aureus* in Foods

Most Probable Number Method for Isolation and Enumeration

First Action 1987

(Applicable to detection and enumeration of small numbers of *S. aureus* in food ingredients and food expected to contain large population of competing species)

A. Apparatus

(**a**) *Pipets.*—1.0 mL with 0.1 mL graduations; 5.0 mL and 10.0 mL with 0.5 and 1.0 mL graduations.

(**b**) *Blender.*—Waring Blendor, or equiv., 2-speed model, with high-speed operation at 16 000–18 000 rpm, and 1 L glass or metal blender jars with covers. One jar is required for each analytical unit.

(**c**) *Mixer.*—Vortex Genie, or equiv.

(**d**) *Water bath.*—Maintained at 35–37°.

(**e**) *Incubator.*—Maintained at 35°.

B. Media and Reagents

(**a**) *Trypticase (tryptic) soy broth with 10% sodium chloride and 1% sodium pyruvate.*—Add 95 g NaCl to 1 L soln of 17.0 g trypticase or tryptose (pancreatic digest of casein), 3.0 g phytone (papaic digest of soya meal), 5.0 g NaCl, 2.5 g K_2HPO_4, 2.5 g dextrose (dehydrated trypticase or tryptic soy broth is satisfactory), and 10 g sodium pyruvate. Adjust to pH 7.3. Heat gently if necessary. Dispense 10 mL into 16 × 150 mm tubes. Autoclave 15 min at 121°. Final pH, 7.3 ± 0.2. Store ≤1 month at 4 ± 1°.

(**b**) *Physiological salt soln.*—Dissolve 8.5 g NaCl in 1 L H_2O. Autoclave 15 min at 121° and cool to room temp.

(**c**) *Baird-Parker medium (egg tellurite glycine pyruvate agar, ETGPA).*—(*1*) *Basal medium.*—Suspend 10.0 g tryptone, 5.0 g beef ext, 1.0 g yeast ext, 10.0 g Na pyruvate, 12.0 g glycine, 5.0 g $LiCl.6H_2O$, and 20.0 g agar in 950 mL H_2O. Heat to bp with frequent agitation to dissolve ingredients completely.

Dispense 95 mL portions into screw-cap bottles. Autoclave 15 min at 121°. Final pH, 7.0 ± 0.2 at 25°. Store ≤1 month at 4 ± 1°.

(*2*) *Enrichment.*—Bacto EY tellurite enrichment (Difco Laboratories) or prep. as follows: Soak fresh eggs ca 1 min in diln of satd $HgCl_2$ soln (1 + 1000). Aseptically crack eggs and sep. yolks from whites. Blend yolk and physiological saline soln, (**b**), (3 + 7, v/v) in high-speed blender ca 5 s. To 50 mL egg yolk emulsion add 10 mL filter-sterilized 1% K tellurite soln. Mix and store at 4 ± 1°.

(*3*) *Complete medium.*—Add 5 mL warmed enrichment to 95 mL molten basal medium cooled to 45–50°. Mix well, avoiding bubbles, and pour 15–18 mL into sterile 100 × 15 mm petri dishes. Store plates at room temp. (≤25°) for ≤5 days before use. Medium should be densely opaque; do not use nonopaque plates. Dry plates before use by 1 of following methods: (*a*) in convection oven or incubator 30 min at 50° with lids removed and agar surface downward; (*b*) in forced-draft oven or incubator 2 h at 50° with lids on and agar surface upward; (*c*) in incubator 4 h at 35° with lids on and agar surface upward; or (*d*) on laboratory bench 16–18 h at room temp. with lids on and agar surface upward.

(**d**) *Brain-heart infusion (BHI) broth.*—Dissolve infusion from 200 g calf brain and from 250 g beef heart, 10.0 g proteose peptone or gelysate, 5.0 g NaCl, 2.5 g $Na_2HPO_4.12H_2O$, and 2.0 g glucose in 1 L H_2O, heating gently if necessary. Dispense 5 mL portions into 16 × 150 mm test tubes and autoclave 15 min at 121°. Final pH, 7.4 ± 0.2.

(**e**) *Desiccated coagulase plasma (rabbit) with EDTA.*—Reconstitute according to manufacturer's directions. If not available, reconstitute *desiccated coagulase plasma (rabbit)* and add Na_2H_2EDTA to final concn of 0.1% in reconstituted plasma.

(**f**) *Butterfield's buffered phosphate diluent.*—(*1*) *Stock soln.*—Dissolve 34.0 g KH_2PO_4 in 500 mL H_2O, adjust to pH 7.2 with ca 175 mL 1N NaOH, and dil. to 1 L. Store in refrigerator. (*2*) *Diluent.*—Dil. 1.25 mL stock soln to 1 L with H_2O. Prep. diln blanks with this soln, dispensing enough to allow for losses during autoclaving. Autoclave 15 min at 121°.

C. Preparation of Food Homogenate

Aseptically weigh 50 g unthawed food sample into sterile blender jar. Add 450 mL phosphate-buffered diln H_2O and homogenize 2 min at high speed (16 000–18 000 rpm). Use this 1:10 diln to prep. serial dilns from 10^{-2} to 10^{-6} by transferring 10 mL of 1:10 diln to 90 mL diln blank, mixing well with vigorous shaking, and continuing until 10^{-6} is reached.

D. Most Probable Number Technique

Inoculate 3 tubes of trypticase soy broth with 10% NaCl and 1% sodium pyruvate, (**a**), at each test diln with 1 mL aliquots of decimal dilns of sample. Max. diln of sample must be high enough to yield neg. end point. Incubate 48 h at 35°.

Using 3 mm loop, transfer 1 loopful from each growth-pos. tube to dried Baird-Parker medium plates, (**c**)(*3*). Vortex-mix tubes before streaking if growth is visible only on bottom or sides of tubes. Streak so as to obtain isolated colonies. Incubate 48 h at 35–37°.

E. Interpretation

Colonies of *S. aureus* are typically circular, smooth, convex, moist, 2–3 mm in diam. on uncrowded plates, gray-black to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone (ppt), and frequently with outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle. Occasional non-lipolytic strains may be encountered which have same appearance, except that surrounding opaque and clear zones are absent. Colonies isolated from frozen or desiccated foods which have been stored for extended periods are frequently less black than typical colonies and may have rough appearance and dry texture.

F. Confirmation Technique

For each plate showing growth, pick ≥1 colony suspected to be *S. aureus*. With sterile needle transfer colonies to tubes contg 0.2 mL BHI broth, (**d**), and to agar slants contg any suitable maintenance medium, e.g., trypticase soy agar, std plate count agar, etc. Incubate BHI culture suspensions and slants 18–24 h at 35°. Retain slant cultures at room temp. for ancillary or repeat tests, in case coagulase test results are questionable.

To BHI cultures add 0.5 mL reconstituted coagulase plasma with EDTA, (**e**), and mix thoroly. Incubate at 35–37° and examine periodically over 6 h interval for clot formation. Any degree of clot formation is considered pos. reaction. Small or poorly organized clots may be observed by gently tipping tube so that liq. portion of reaction mixt. approaches lip of tube; clots will protrude above liq. surface. Coagulase-pos. cultures are considered to be *S. aureus*. Test pos. and neg. controls simultaneously with cultures of unknown coagulase reactivity. Recheck doubtful coagulase test results on BHI cultures which have been incubated at 35–37° for >18 but ≤48 h.

Report most probable number (MPN) of *S. aureus*/g from tables of MPN values, Table **966.24**.

Ref.: JAOAC **70,** 35(1987).

975.55 *Staphylococcus aureus* in Foods

Surface Plating Method for Isolation and Enumeration

First Action 1975
Final Action 1976

(Applicable for general purpose use in testing foods expected to contain ≥10 cells of *S. aureus*/g. For small numbers, *see* **987.09**.)

A. Apparatus

Sterile, bent glass streaking rods.—Hockey stick or hoe-shape, with fire-polished ends, 3–4 mm diam., 15–20 cm long, with angled spreading surface 45–55 mm long.

B. Determination

At each diln plated, aseptically transfer 1 mL of sample suspension, **987.09C**, to triplicate plates of Baird-Parker medium, **987.09B(c)**(*3*), and equitably distribute the 1 mL inoculum over the triplicate plates (e.g., 0.4 mL–0.3 mL–0.3 mL). Spread inoculum over surface of agar using sterile, bent glass streaking rods. Avoid extreme edges of plate. Retain plates in upright position until inoculum is absorbed by medium (ca 10 min on properly dried plates). If inoculum is not readily absorbed, plates may be placed in incubator in upright position ca 1 hr before inverting. Invert plates and incubate 45–48 hr at 35–37°. Select plates contg 20–200 colonies, unless only plates at lower dilns (>200 colonies) have colonies with typical appearance of *S. aureus*, **987.09E**. If several types of colonies are observed which appear to be *S. aureus*, count number of colonies of each type and record counts sep. When plates at lowest diln plated contain <20 colonies, these may be used. If plates contg >200 colonies have colonies with typical appearance of *S. aureus* and typical colonies do not appear at higher dilns, use these plates for enumeration of *S. aureus*, but do not count non-typical colonies. Select ≥1 colony of each type counted and test for coagulase production, **987.09F**. Add number of colonies on triplicate plates represented by colonies giving pos. coagulase test and multiply by sample diln

factor. Report this number as number of *S. aureus*/g of food tested.

Ref.: JAOAC **58,** 1154(1975).

976.31 Staphylococcal Enterotoxin in Foods

Microslide Gel Double Diffusion Test

First Action 1976
Final Action 1977

(Detects 0.1–0.01 μg enterotoxin/mL and is applicable to detection of enterotoxin in culture fluids and concd food exts)

A. Principle

Pptn line occurs when serological type of enterotoxin diffuses thru gel and reacts with its specific antibody. Coalescence with ref. pptn line which results from serological reactivity of enterotoxin serotype and specific antibody confirms identity.

B. Apparatus

(a) *Debubblers.*—Fine glass rods. Prep. by pulling glass tubing very fine, as in making capillary pipets. Break into ca 6 cm lengths and seal ends in flame.

(b) *Electrical tape.*—Insulating tape, 0.25 × 19.1 mm (Temflex 1700, 3M Co., Electrical Products Div., Bldg 225-4N-05, 3M Center, St Paul, MN 55144-1000, or equiv.)

(c) *Microscope slides.*—Plain glass, pre-cleaned, 7.62 × 2.54 cm (3 × 1″), 0.96–1.06 mm thick.

(d) *Pasteur pipets.*—Prep. by drawing out ca 7 mm od glass tubing or use disposable 30 or 40 μL pipets (Kensington Scientific Corp., 1399 64th St, Emeryville, CA 94608, or equiv.).

(e) *Petri dishes.*—20 × 150 mm and 15 × 100 mm.

(f) *Plastic templates.*—*See* Fig. **976.31A**. (Available from Toxin Technology, 845 E. Johnson St, Madison, WI 53703.)

(g) *Silicone lubricant.*—High vac. grease (Dow Corning Corp., or equiv.).

(h) *Staining jars.*—Coplin or Wheaton jars.

(i) *Sterile bent glass spreaders.*—Bend glass rods like hockey sticks and fire polish.

(j) *Water-saturated synthetic sponge strips.*—Approx. 1.5 × 1.5 × 6.5 cm H_2O-satd absorbent cotton is also satisfactory.

C. Media and Reagents

(a) *Agar soln for coating slides.*—0.2%. Add 2 g bacteriological grade agar to 1 L boiling H_2O and heat until agar dissolves. Pour 20–30 mL portions agar into 180 mL (6 oz) prescription bottles or equiv. containers and store at room temp. Remelt when needed for coating slides.

(b) *Brain-heart infusion (BHI) agar.*—0.7% (w/v). Adjust BHI broth to pH 5.3; add bacteriological grade agar to prep. 0.7% concn and dissolve by boiling gently. Distribute in 25 mL portions into 25 × 200 mm test tubes, and autoclave 10 min at 121°. Immediately before use, aseptically empty tubes of sterile medium into 15 × 100 mm petri dishes.

(c) *Enterotoxin antisera.*—Dil. lyophilized sera (Toxin Technology) with normal physiological saline according to specific instructions of supplier. Store liq. stocks (highly concd) and working dilns of antisera at 4°; for long term storage, freeze-drying or freezing is recommended.

(d) *Enterotoxin references.*—Rehydrate lyophilized enterotoxin prepns, (c), according to specific instructions of supplier.

(e) *Gel diffusion agar.*—Add 1.2% purified agar (Noble special agar, Difco Laboratories) to boiling fluid base (0.85% NaCl–0.80% Na barbital with final concn of 1:10,000 merthiolate (Eli Lilly and Co., Pharmaceutical Div., Lilly Corporate Center, Indianapolis, IN 46285, or equiv.) adjusted to pH 7.4). Filter hot agar thru 2 layers of anal. grade paper and store in 15–25 mL portions in screw-cap bottles.

(f) *Staining soln.*—0.1% Thiazine Red R stain (Fluka Chemical Corp., or equiv.) in 1% HOAc.

(g) *Sterile distilled water.*—Dispense 5 mL distd H_2O into tubes and autoclave 15 min at 121°. Normal physiological saline may be substituted for H_2O.

(h) *Turbidity std.*—1% $BaCl_2$–1% H_2SO_4 (1 + 99) (No. 1 of McFarland nephelometer scale).

D. Preparation of Sample

Select ≥4 isolated staphylococcal colonies from enumeration and recovery media, and streak nutrient media agar slants, or equiv. Incubate slants 18–24 hr at 35–37°. Add loopful of growth from agar slants to 5.0 mL sterile distd H_2O or saline and prep. aq. suspension of organisms from each slant which is equiv. to turbidity of No. 1 tube of McFarland nephelometer scale (ca 3 × 10^8 organisms/mL). Inoculate surface of semi-solid BHI agar with 4 drops aq. suspension of organisms de-

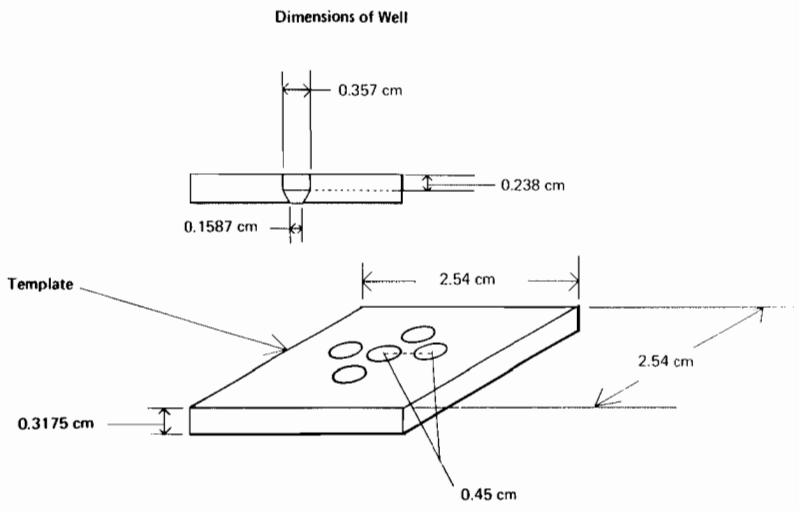
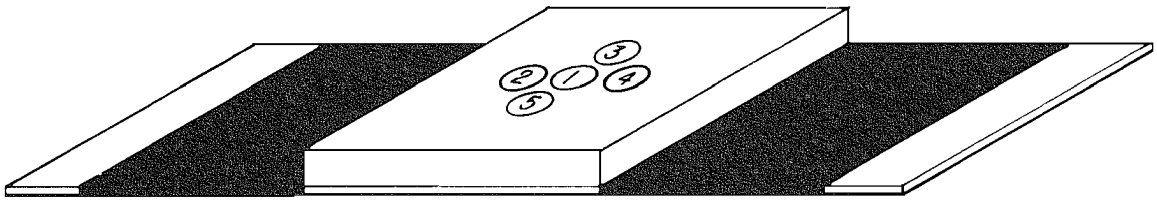


FIG. 976.31A—Plastic template schematic for microslide assembly



(1) Bivalent

1. Combination Antisera (e.g., Anti A and B)
2. Prepn under test
3. Ref. enterotoxin (e.g., Type A)
4. Prepn under test
5. Ref. enterotoxin (e.g., Type B)

(2) Monovalent

1. Antiserum (e.g., Anti A)
2. Dilns of prepn under test
3. Ref. enterotoxin (e.g., Type A)
4. Dilns of prepn under test
5. Dilns of prepn under test

FIG. 976.31B—Arrangement of antisera and homologous reference enterotoxins (*1*) when assaying preparation(s) under test for presence of 2 staphylococcal enterotoxins simultaneously (bivalent detection system) or (*2*) when assaying dilutions of preparation under test with apparent enterotoxin excess (monovalent detection system)

livered from sterile 1.0 mL pipet. Spread drops of aq. culture suspension over entire surface of semisolid agar with sterile glass rod and incubate plates upright 48 hr at 35–37°. Transfer contents of petri dish to 50 mL centrf. tube with aid of wood applicator stick and centrf. 10 min at 32,800 *g* to remove agar and organisms. Examine culture fluid for presence of serologically identifiable enterotoxins.

E. Preparation of Slides

Wrap double layer of elec. tape around pre-cleaned microscope slide, leaving 2.0 cm space in center, as follows: Start piece of tape ca 9.5–10 cm long ca 0.5 cm from edge of bottom surface of slide and wrap tightly around slide twice. Wipe area between tapes with cheesecloth soaked with alcohol, and dry with dry cheesecloth. Coat upper surface area between tapes with 0.2% bacteriological grade agar as follows: Melt 0.2% agar, and maintain at ≥55° in screw-cap bottle. Hold slide over beaker on hot plate adjusted to 65–85° and pour or brush 0.2% agar over slide between 2 pieces of tape. Let excess agar drain off, wipe bottom surface of slide, and collect agar in beaker for reuse. Place slide on tray and dry in dust-free atm. (e.g., incubator). If slides are not clean, agar will not coat slides uniformly.

F. Preparation of Slide Assemblies

Prep. plastic templates according to specifications in Fig. **976.31A**. Spread *thin* film of silicone grease on side of template that will be placed next to agar (i.e., side with smaller holes). Place ca 0.4 mL melted and cooled (55–60°) 1.2% gel diffusion agar between tapes. Immediately lay silicone-coated template on melted agar and edges of bordering tapes. Place 1 edge of template on 1 piece of tape, and bring opposite edge to rest gently on other piece. Sat. strips of synthetic sponge (ca 1.5 × 1.5 × 6.5 cm) with H_2O, and place 2 strips on periphery of each 20 × 150 mm petri dish. Place slide in prepd petri dish (2–4 slide assemblies/dish) soon after agar hardens, and label slide.

G. Slide Gel Diffusion Test

To prep. record of assay, draw hole pattern of template on record sheet and indicate number (same as that used for slide) and contents of each well. Place suitable diln of antiserum or sera in central well, homologous ref. enterotoxin in peripheral well(s), and material under examination in well adjacent to that contg ref. enterotoxin. See Fig. **976.31B**(*1*) for reagent arrangement for simultaneous detection of 2 enterotoxin types (bivalent detection system). Prep. control slide with only ref. toxin and antienterotoxin serum to det. proper reactivity of reagents. Fill wells to convexity with reagents, using Pasteur or disposable 30 or 40 μL pipet. Partially fill capillary pipet with soln and remove excess liq. by touching pipet to edge of sample tube. Slowly lower pipet into well until it touches agar surface, and fill to convexity. Remove trapped air bubbles from *all* wells by probing with debubbler, (**a**), against dark background. Let slides incubate 48–72 hr at room temp. in covered petri dishes contg moist sponge strips (24 hr slide incubation at 35° is generally sufficient for testing of culture fluids). Carefully remove template by sliding it to 1 side. If necessary, clean slide by dipping in H_2O and wiping bottom of slide. Enhance lines of pptn by immersing slide in staining soln, (**f**), 5–10 min. To preserve slide as permanent record, rinse any reactant liq. remaining on slide by dipping in H_2O and then immerse slide in each of following baths 10 min: staining soln, 1% HOAc, 1% HOAc, and 1% HOAc contg 1% glycerol. Drain excess fluid from slide and dry in 35° incubator. After prolonged storage, lines of pptn may not be visible until slide is immersed in H_2O.

H. Interpretation

Examine slide for lines of pptn by holding at oblique angle to light source against dark background. Coalescence of test sample lines of pptn with ref. line(s) of pptn indicates pos. reaction. Fig. **976.31C** shows microslide gel diffusion test as bivalent detection system: Antisera to enterotoxins A and B are in well 1; known ref. enterotoxins A and B are in wells 3 and 5, resp., to produce ref. lines of A and B; prepns under test are in wells 2 and 4. Interpret 4 reactions as follows: (1) No line development between test prepns—absence of enterotoxins A and B; (2) coalescence of test prepn line from well 4 with enterotoxin A ref. line (intersection of test prepn line with enterotoxin B ref. line)—absence of enterotoxins A and B in well 2, presence of enterotoxin A and absence of enterotoxin B in well 4; (3) presence of enterotoxin A and absence of enterotoxin B in both test prepns; and (4) absence of enterotoxins A and B in test prepn in well 2, presence of enterotoxins A and B in well 4. Operator can simplify assay by

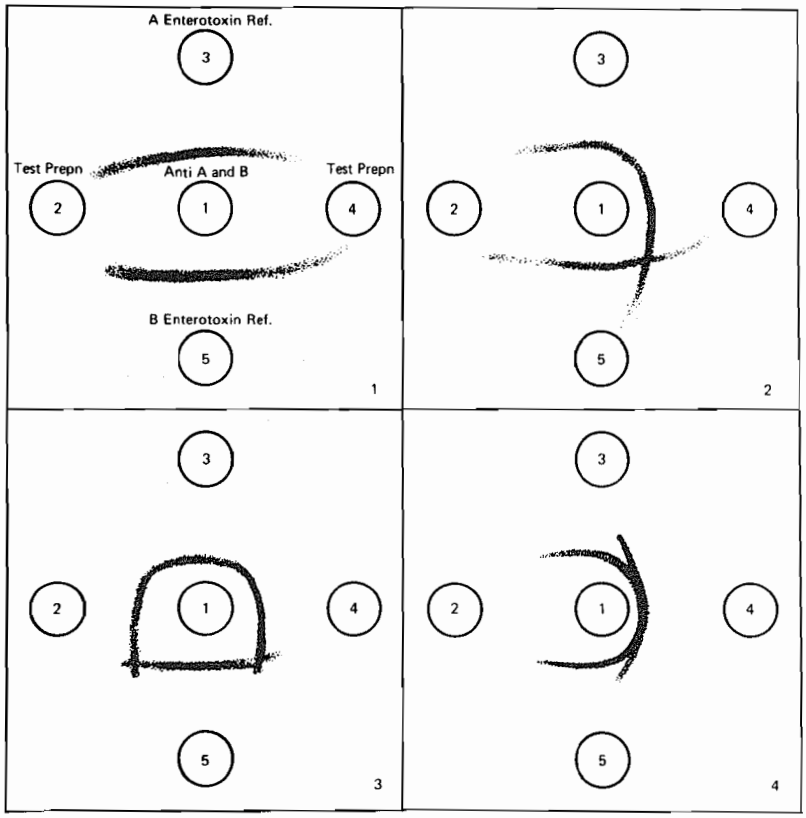
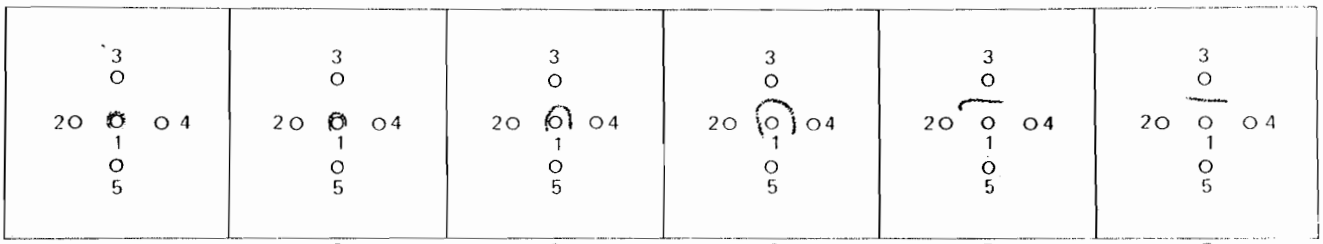


FIG. 976.31C—Examples of 4 possible reactions in bivalent detection system. *See* 976.31H for explanation of reactions

testing only 1 prepn for presence of 2 different enterotoxins on same set of slides.

If concn of enterotoxin in test material is excessive, formation of ref. line will be inhibited because of fast migration of toxin thru gel, thus localizing antibody in its well. Fig. **976.31D**(*A*) shows this inhibition of ref. line formation when 10 and 5 μg enterotoxin/mL, resp., are used. Figs. **976.31D**(*B*)–(*F*) show ppt patterns when successively less enterotoxin is used. If test prepn inhibits formation of ref. line as in Fig. **976.31D**(*A*), dil. test material, utilizing monovalent system shown in Fig. **976.31E**. Reactant arrangement for assaying dilns of prepn under test is shown in Fig. **976.31B**(*2*). Figure **976.31E** shows microslide gel diffusion test as monovalent system in which antiserum is placed in well 1; ref. enterotoxin in well 3; and dilns of test prepn in wells 2, 4, and 5. Do not make starting diln of culture fluid (test material) so high as to dil. beyond reactive concn of enterotoxin.

Occasionally, atypical ppt patterns form which may be difficult for inexperienced analysts to interpret. One of most common atypical reactions is formation of lines not related to toxin, but caused by other antigens in test material. Examples of such patterns are given in Fig. **976.31F**, which shows microslide



A	B	C	D	E	F
(1) Antiserum	(1) Antiserum	(1) Antiserum	(1) Antiserum	(1) Antiserum	(1) Antiserum
(2) 10 μg/ml Enterotoxin	(2) 4 μg/ml Enterotoxin	(2) 2 μg/ml Enterotoxin	(2) 0.5 μg/ml Enterotoxin	(2) 0.125 μg/ml Enterotoxin	
(3) Enterotoxin Ref.	(3) Enterotoxin Ref.	(3) Enterotoxin Ref.	(3) Enterotoxin Ref.	(3) Enterotoxin Ref.	(3) Enterotoxin Ref.
(4) 5 μg/ml Enterotoxin	(4) 3 μg/ml Enterotoxin	(4) 1 μg/ml Enterotoxin	(4) 0.25 μg/ml Enterotoxin	(4) 0.0625 μg/ml Enterotoxin	

FIG. 976.31D—Effect of amount of enterotoxin in test preparation on development of reference line of precipitation. *See* 976.31H for explanation of reactions

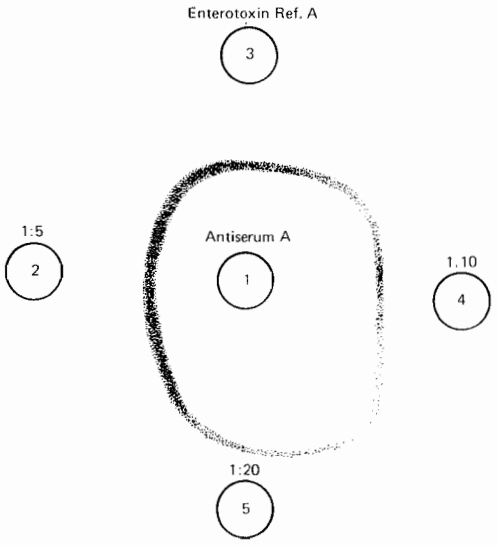


FIG. 976.31E—Appearance of microslide gel diffusion test as monovalent system

gel diffusion test as bivalent detection system. (*See* reactant arrangement in Fig. **976.31B**(*1*).) In ppt pattern **976.31F**(*1*), test prepn in well 4 produced atypical reaction indicated by nonspecific line of pptn (lines of nonidentity with enterotoxin refs A and B), which intersects enterotoxin ref. lines. In ppt pattern **976.31F**(*2*), both test prepns (wells 2 and 4) are neg. for enterotoxins A and B but produce nonspecific lines of pptn which intersect enterotoxin A and B ref. lines of pptn.

I. Slide and Template Recovery

To recover slides for reuse, clean without removing tape. Rinse slides with tap H_2O to remove agar gel, boil 3–5 min in tap H_2O contg mild detergent, rinse in tap H_2O and then in distd H_2O, immerse momentarily in alcohol, and wipe dry with cheesecloth. Wash templates with hot (not boiling) H_2O contg moderately strong detergent, using cheesecloth to remove silicone film. Rinse templates with tap H_2O, distd H_2O, and alcohol; dry with cheesecloth, and tap alcohol out of wells. In cleaning plastic templates, avoid exposure to excessive heat or plastic-dissolving solvs. Templates and especially wells must be dry before reuse.

Ref.: JAOAC **59,** 594(1976).

980.32 Staphylococcal Enterotoxin in Foods

Extraction and Separation Methods

First Action 1980
Final Action 1981

A. Apparatus

(**a**) *Centrifuge.*—High-speed, preferably refrigerated, with 285 mL stainless steel bottles, or equiv.

(**b**) *Dialysis sac.*—1.25 in. (32 mm) flat width tubing, av. pore diam. 4.8 μm. Cut piece of tubing long enough to accommodate vol. of food to be extd. Soak tubing in 2 changes of H_2O to remove glycerol coating. Tie 1 end with 2 knots close together. Test for leaks by filling sac with H_2O and squeezing, while untied end is held tightly with fingers. Empty sac and place in H_2O until ready for use.

(**c**) *Chromatographic columns.*—400 × 20 (id) mm, with stopcock (or use rubber tube attachment with finger clamp), packed with carboxymethyl cellulose (CMC), Whatman CM 22, 0.6 meq/g, or equiv. Pack as follows: Suspend 1 g CMC in 100 mL 0.005 *M* Na phosphate buffer, pH 5.7, in 250 mL beaker, and adjust to pH 5.7 with 0.005 *M* H_3PO_4. Stir intermittently 15 min, recheck pH, and adjust, if necessary. Pour suspension into tube containing plug of glass wool, and let settle. Withdraw liq. from column to within ca 25 mm of surface of settled CMC in column. Place loosely packed plug of glass wool on column. Pass 0.005 *M* Na phosphate buffer, pH 5.7, thru column until washing is clear and pH is 5.7 (150–200 mL). Leave enough buffer in column to cover glass wool to prevent column from drying out.

(**d**) *Reservoir.*—Attach ca 60 cm latex tubing to stem of separator of appropriate size and attach other end of tube to piece of glass tubing inserted thru No. 3 rubber stopper to fit chromatgc column. Suspend separator from ring stand above chromatgc tube.

B. Reagents

(**a**) *Polyethylene glycol (PEG) soln.*—30%. *See* **974.38B(f)**.

(**b**) *Sodium phosphate buffer solns.*—(*1*) *pH 5.7, 0.2M.*—Add 0.2 *M* NaH_2PO_4 (27.60 g $1H_2O$/L) to 0.2 *M* Na_2HPO_4 (53.61 g $7H_2O$/L) to pH 5.7. (*2*) *pH 5.7, 0.005M.*—Dil. 0.2 *M*, pH 5.7, buffer with H_2O (1 + 39). Adjust to pH 5.7 with 0.005 *M* H_3PO_4. (*3*) *pH 6.4, 0.2M.*—Add 0.2 *M* Na_2HPO_4 to 0.2 *M* NaH_2PO_4 to pH 6.4. (*4*) *pH 6.5, 0.05M Na phosphate-NaCl.*—Add NaCl (11.69 g/L) to pH 6.4, 0.2 *M* soln to give 0.2 *M* NaCl (pH is ca 6.3). Dil. with H_2O (1 + 3) and adjust to pH 6.5 with 0.05 *M* H_3PO_4 or 0.05 *M* Na_2HPO_4.

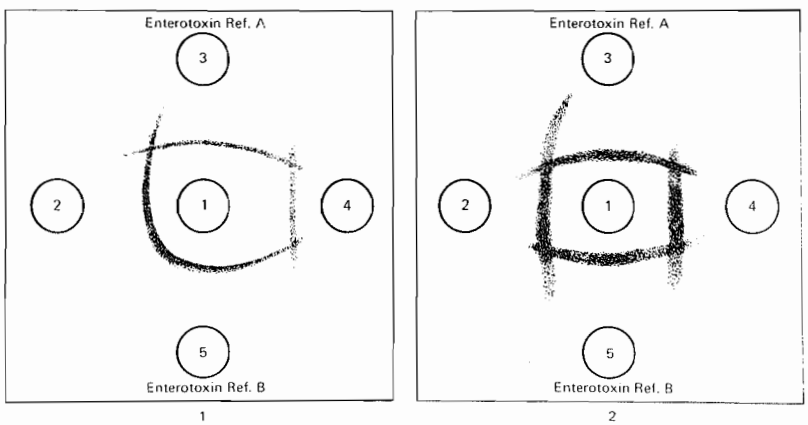


FIG. 976.31F—Precipitate patterns in microslide gel diffusion test demonstrating nonspecific (atypical) lines of precipitation

C. Extraction of Toxin

Homogenize 100 g sample in 500 mL (or 20 g with 100 mL) 0.2*M* NaCl 3 min in high-speed blender to very fine consistency. Adjust to pH 7.5 with 1*N* NaOH or HCl if food is highly buffered or with 0.1*N* if weakly buffered. Let stand 10–15 min, recheck pH, and readjust to pH 7.5, if necessary. Transfer homogenate to two 285 mL stainless steel bottles and centrf. 20–30 min at 27,300 × *g* at 5° in refrigerated centrf. (Lower speeds for longer times may be used.) If refrigerated centrf. is not available, centrf. at room temp., but chill supernate 1 h at 4° before filtering.

Decant supernate into beaker thru fine mesh screen (or other filtering material (e.g., miracloth) placed in funnel). Re-homogenize solids left in centrf. bottles with 125 mL (for 100 g sample; 25 mL for 20 g) 0.2*M* NaCl as above. Centrf., filter and combine filtrate with original supernate.

D. Purification of Toxin

Place combined exts in dialysis sac, immerse sac in 30% PEG soln and let conc. at 5° to ≤15 mL. Remove sac from soln and wash outside thoroly with tap H_2O. Soak sac in distd H_2O 1–2 min and let stand in 0.2*M* NaCl few min. Pour contents of sac into 50 mL beaker. Rinse inside of sac with 2–3 mL portions 0.2*M* NaCl by running fingers up and down outside of sac to remove material adhering to insides. Add rinsings to beaker. Repeat rinsings until clear, keeping vol. at min.

Quant. transfer ext to separator, add $^1/_4$–$^1/_2$ vol. $CHCl_3$, and shake vigorously 10 times thru arc of 90°. Centrf. 10 min at 32,800 × *g* at 5°. Return mixt. to separator. Slowly drain lower $CHCl_3$ layer and discard. Repeat extn at least once (twice with high protein foods). After final extn, measure vol. of aq. phase, and dil. with 40 vols pH 5.7, 0.005*M* Na phosphate buffer. Adjust pH to 5.7 with 0.005*M* H_3PO_4 or Na_2HPO_4. Place adjusted soln in separator large enough to accommodate vol. for percolation thru CMC column.

Place stopper (attached thru tubing to separator) loosely into top of chromatgc column and slowly fill tube nearly to top with dild ext from separator. Tighten stopper in tube and open stopcock of separator. Let liq. percolate thru column at 5° at 1–2 mL/min by adjusting flow rate with stopcock at bottom of tube. Stop flow when liq. reaches top of glass wool layer. (If liq. has passed, rehydrate column with 25 mL H_2O.)

Wash column with 100 mL pH 5.7 0.005*M* Na phosphate buffer at same flow rate, stopping flow when liq. level reaches top of glass wool. Discard wash.

Elute enterotoxin from CMC column with 200 mL pH 6.5 0.05*M* Na phosphate-NaCl buffer at rate of 1–2 mL/min at room temp. Force last of liq. from column by applying air pressure to top of tube.

E. Concentration of Toxin

Place eluate in dialysis sac. Place sac in 30% PEG at 5° and conc. to almost dryness. Remove sac and wash thoroly with tap H_2O. Soak sac in pH 6.5, 0.05*M* phosphate-NaCl buffer, and remove conc. from sac by rinsing with five 2–3 mL portions pH 6.5, 0.05*M* phosphate-NaCl buffer.

Transfer soln to separator, add $^1/_4$–$^1/_2$ vol. $CHCl_3$, and shake vigorously 10 times thru arc of 90°. Centrf. 10 min at 32,800 × *g* at 5°. Return mixt. to separator. Slowly drain lower $CHCl_3$ layer and discard.

Place ext in short dialysis sac (ca 16 cm). Place sac in 30% PEG and let stand until all liq. has been removed from inside of sac. Remove sac from soln and wash outside thoroly with tap H_2O. Place sac in distd H_2O 1–2 min. Remove contents of sac by rinsing inside with 1 mL portions distd H_2O until rinse is clear, keeping vol. to min. Place rinsings in 18 × 100 mm test tube or other container (e.g., 2–3 dram vial), and freeze-dry. Dissolve freeze-dried sample in as small vol. saline soln. **974.38B(e)** as possible (0.15–0.1 mL).

Det presence of enterotoxin as in **976.31**.

Ref.: JAOAC **63,** 1205(1980).

STERILITY (COMMERCIAL) OF FOODS (CANNED, LOW ACID)

972.44 Microbiological Method

First Action 1972
Final Action 1978

(Personnel with beards, mustaches, or sideburns below ear lobe should not perform sterility examination unless these are completely covered with sterile caps and masks. Wear clean laboratory coat for examination.)

A. Principle

"Low acid foods" means any food with finished equilibrium pH value >4.6. Method applies only to containers which show no distention of either end. Incubate containers ≥10 days at 21–35° before examination.

Com. sterility is defined as that condition achieved by application of heat which renders food free of viable forms of microorganisms having public health significance, as well as microorganisms not of health significance capable of reproducing in the food under normal non-refrigerated conditions of storage and distribution.

B. Media and Reagents

See also **966.23A**.

(a) *Tryptone broth.*—(Aerobic medium.) Dissolve 10.0 g tryptone or trypticase, 5.0 g glucose, 1.25 g K_2HPO_4, 1.0 g yeast ext, and 2.0 mL 2% alc. soln of bromocresol purple in 1 L H_2O with gentle heat, if necessary. Dispense 10 mL portions into 20 × 150 mm screw-cap test tubes and autoclave 20 min at 121°. Do not exhaust before using.

(b) *Modified PE-2 medium.*— (Anaerobic medium.) Dissolve 20.0 g peptone, 3.0 g yeast ext, and 2.0 mL 2% alc. soln of bromocresol purple in 1 L H_2O with gentle heat, if necessary. Dispense 19 mL portions into 20 × 150 mm screw-cap test tubes contg 8–10 *untreated* Alaska seed peas (Rogers Brothers Co., Seed Div., PO Box 2188, Idaho Falls, ID 83401, No. 423; or hardware store). Autoclave 30 min at 121°. If not freshly prepd, heat to 100° and cool to 55° before using.

(c) *Glucose starch agar.*— (Aerobic medium.) Dissolve 15.0 g proteose peptone No. 3, 2.0 g glucose, 10.0 g sol. starch, 5.0 g NaCl, 3.0 g Na_2HPO_4, 20.0 g gelatin, and 10.0 g agar in 1 L H_2O, heat to bp, and autoclave 15 min at 121° in erlenmeyer. Aseptically pour into sterile petri dishes and allow to solidify.

(d) *Nutrient agar.*—(Aerobic medium for spore production; Difco dehydrated, or equiv.) Dissolve 3.0 g beef ext, 5.0 g peptone, and 15.0 g agar in 1 L H_2O, heat to bp, and autoclave 30 min at 121°.

(e) *Detergent sanitizer soln.*—pHisoHex (3% hexachlorophene), or equiv.

C. Apparatus

(a) *Can opener.*—Bacti-Disc Cutter (Wilkens-Anderson Co., 4525 W Division St, Chicago, IL 60651, No. 10810-01), bacteriological can opener (Marmora Machine Co., 1956 N Latrobe Ave, Chicago, IL 60639), or equiv.

(b) *Caps.*—Disposable, operating room-type (Baxter Hos-

pital Supply Div., 1450 Waukegan Rd, McGaw Park, IL 60085, or equiv.).

(c) *Pipets.*—Straight wall, 200–250 mm long × 7 mm id, 9 mm od (Scientific Products, Inc., No. G6100–9, cut and fire polished, or equiv.).

D. Sampling

Conduct test in clean room. (If necessary, open room may be used but outside windows must be closed and direct drafts across work area must be eliminated.) If available, use laminar flow cabinet. Strip labels from cans, examine cans for external defects, and record descriptions. Wash cans with soap (or detergent sanitizer soln) and H_2O, and dry with clean paper towels. Wipe counter top with 100 ppm Cl soln (e.g., Clorox or dild NaOCl soln) immediately before placing washed and dried can on it. Place code end of can in down position and number cans in ink or with $CuSO_4$ marking soln to right of side seam.

Wash hands and face with soap, and rewash hands and face with detergent sanitizer soln. Completely cover hair with clean disposable operating room cap.

Hold noncoded end of can over large Meker burner, just above blue portion of flame. Heat this end of can until all condensation is evapd; then return can to table in former position. Clean handle and blade of special can opener, (a), with paper towel moistened with 70% alcohol, flame metal portion enough to destroy all microorganisms, and use it to make 4 cm (1.5″) diam. hole in noncoded, heated end of can. Immediately and without moving can, use straight-wall sterile glass pipet, (c), to transfer ca 2 g food to sep. tubes, 2 each of aerobic and 2 of anaerobic media (4 total). (No other transferring tool may be substituted.) Preloosen screw cap and hold it between little and ring fingers while transfer is being made. Flame lips of media tubes both before and after addn of food. When transferring food to anaerobic tubes, food must be inoculated into lower portion of medium. Tighten screw caps after inoculation, incubate tubes 72 hr at 35°, and observe daily. Record results for each tube sep.

Remove addnl ≥10 g food sample from each container with sterile pipet and place in sterile 25 × 200 mm screw-cap test tube. Use pipet-like spatula, if necessary, for this operation (thermophilic contamination unlikely). Number tube to correspond to can and refrigerate for later testing, if necessary.

E. Contamination Control

Use sterile loop or glass rod to streak plate of glucose starch agar, (c). On table, open plate of glucose starch agar for time equal to longest duration that any medium tube or plate is exposed. Incubate plates 72 hr at 35°, and observe daily.

F. Microscopic Examination

With pair of metal cutting shears, enlarge hole in can and record odor. Microscopically (oil immersion) examine heat-fixed thin smear of food, stained 10 sec with 1% gentian (or crystal) violet and washed in running tap H_2O, or, alternatively, examine wet mounts with phase contrast microscope. If food contains appreciable fat, xylol should be dripped across food smear while it is still hot from heat fixing. Compare stained smear with one made from normal product, if possible.

G. pH Determination

Det. pH with pH meter, using ref. buffer near normal pH of food. Record both ref. buffer pH and sample pH. Compare to normal can of food, if available.

H. Confirmation of Results

If there is any abnormal odor, abnormal appearance, abnormal pH, numbers of bacteria on microscopic examination, and/or growth in media from any can of food, subculture corresponding refrigerated tube as follows: Flame lip of tube and, with straightwall sterile glass pipet, (c), transfer ca 2 g food to 2 tubes each of aerobic and anaerobic media (4 total). Flame lips of media tubes both before and after addn of food. Tighten caps after inoculation, incubate tubes 72 hr at 55°, and observe daily. Record results for each tube sep.

Any organisms isolated from normal cans having obvious vac. which produce gas in anaerobic medium at 35° should immediately be suspected as being from laboratory contamination. Aseptically inoculate growing organism into another normal can, close hole with solder, and incubate 14 days at 35°. Any swelling of container indicates that organism was not in original sample. Record as laboratory contamination and review results of addnl cans to verify finding of contamination.

Growth in aerobic medium at 35° from normal cans indicates either non-com. sterility or laboratory contamination. Unless there is abnormal odor, abnormal appearance, abnormal pH, and/or numbers of bacteria on microscopic examination from product in original can, record results as laboratory contamination and review results of addnl cans to verify finding of contamination. Otherwise, observe subculture results at 55°. Growth at 35° and absence of growth at 55° confirm nonsterility of original container. Check growth under aerobic conditions on nutrient agar plates, (d), at 55° and confirm for spores after 72 hr. Confirmation indicates nonsterility due to flat sour spoilage. Record growth at 55° under anaerobic conditions with gas production as com. sterile. Growth is caused by dormant spores incapable of growth at normal temps of storage and distribution.

If only one of duplicate tubes is pos. after incubation and streaked glucose starch agar is also neg., record as laboratory contamination. Growth on air control plate of glucose starch agar also indicates potential laboratory contamination.

Ref.: JAOAC **55,** 613(1972).

984.36 Microleak Detection in Low-Acid Canned Food Containers

Helium Leak Test

First Action 1984
Final Action 1987

A. Principle

(This test does not detect bacterial contamination.)

He is inert gas with small MW that can be forced through micron size openings and be easily detected by gas chromatgc analysis. After can is pierced aseptically, and sample is taken for microbiological analysis, can is sealed with rubber disc and subjected to He at 45 psi for 30 min. Headspace sample is then taken and analyzed for He.

B. Apparatus

(a) *Gas chromatograph.*—Instrument capable of sepg He from N, O, H, and CO_2 as described, or equiv., with strip chart recorder, gas partitioner (Model 1200, Fisher Scientific) with dual thermal conductivity cells and dual in-line columns. Column 1: $6^1/_2$ ft × $^1/_8$ in. Al packed with 80–100 mesh Columpak™ PQ. Column 2: 11 ft × $^3/_{16}$ in. Al packed with 60–80 mesh Molecular Sieve 13×.

Operating conditions: column temperature 75°; attenuation 128; Ar carrier gas inlet pressure 40 psi, flow rate 26 mL/min thru gas partitioner; bridge current 125 mA; column mode 1 & 2; temperature mode, column; injector temp., off.

(b) *Puncturing press* (*Fig. **984.36***).—Made from drill press for electric hand drill with internal spring reversed to push head

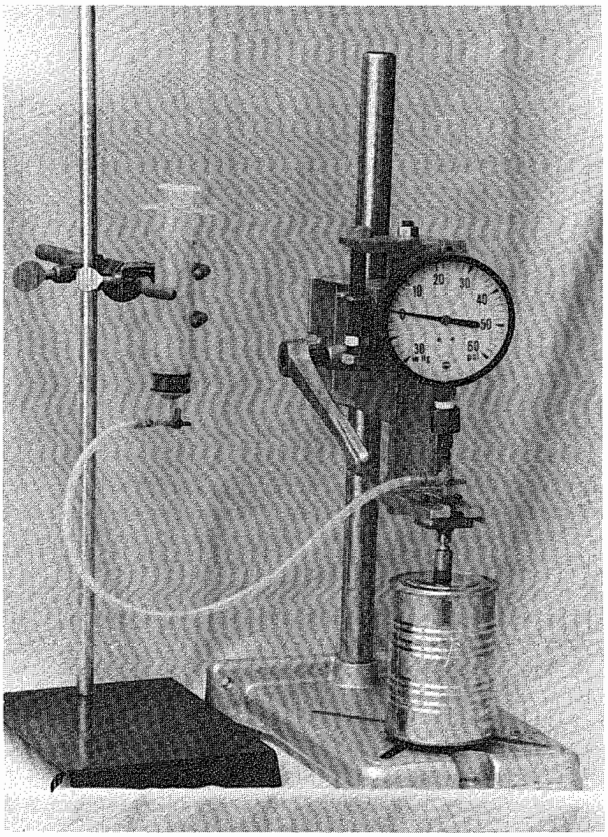


FIG. 984.36—Puncturing press

down. Metal valve, 3-way (stopcock No. 3161 Becton-Dickinson & Co., Stanley St, Rutherford, NJ 07070); vac. pressure gage, 30 in. Hg/0-60 psi, $2^1/_2$ in. face (Ametek, US Gauge Div., PO Box 152, Sellerville, PA 18960). Stainless steel piercer $1^1/_2$ in. × $^1/_2$ in. (machined in local machine shop) with No. 2 taper in piercer top, beveled $^1/_8$ in. × $^3/_{16}$ in. at bottom. $^1/_4$ in. × $^1/_2$ in. silicone rubber gasket around beveled $^1/_8$ in. piercer to maintain seal.

(c) *Helium exposure tank.*—ASME paint tank, 10 gal., tested to 100 psi, equipped with inlet and outlet microcontrol valves (Harrison Rubber and Supply Co., Court and Race Sts, Cincinnati, OH 45202).

(d) *Pressurized helium tank.*—With 2 stage regulator.

(e) *Timer and solenoid.*—To automate release of He from exposure tank.

(f) *Helium gas standards.*—Scott Specialty Gasses, 2330 Hamilton Blvd, South Plainfield, NJ 07090.

(g) *Cyanoacrylate glue.*—SuperGlue (3M, AC&S Div., 3M Center, St Paul, MN 55144-1000, or equiv.).

(h) *Can opener.*—Bacteriological, **972.44C(a)**.

(i) *Rubber discs.*—$2^3/_8$ in. × $^1/_8$ in. and 70 durometer (Netherland Rubber Co., 629 Burbank, Cincinnati, OH 45206).

C. Calibration Test Procedure

For gas chromatographs equipped with side port loop (0.5 mL), inject 5.0 mL calibrated He stds (suggested range of 5, 15, 25, 50, and 75% He). For instruments not equipped with side port loop, inject appropriate vol. of stds. Use same vol. for analysis of headspace gas samples. Plot percent He vs He peak ht at attenuation used. Depending on qual. of instrument, plot should approximate a straight line.

Check gage on can piercer against known pressure and vac. Test resealing procedure, **984.36E**, on control cans.

D. Helium Exposure Tank

Control introduction rate of He into exposure tank, and time cans are exposed to He pressure at 45 ± 2 psi. Timer, solenoid, and microvalves with vernier scales can facilitate procedure. Connect He source to exposure tank. Turn timer on to close outlet solenoid valve. Approx. 15–20 min are needed to reach 45 psi in tank. Make minor adjustments if necessary. Adjust timer to expose cans to He pressure at 45 psi for 30 min (30 min exposure period is in addition to time necessary to reach 45 psi.). Tank pressure should be reduced to 0 psi within 5–10 min.

E. Preparation of Can for Helium Test

If sample is to be taken for microbiological testing, proceed as in **972.44D**.

For nonsterile opening of can, use opener, **972.44C(a)** to cut $1^1/_2$ in. hole in can lid. Remove and discard portion of contents.

Push down any sharp metal projections around 1.5 in. hole. Wipe lid dry and lightly sand area where rubber disc will be inserted. Pool cyanoacrylate glue around surface covered by edge of rubber disc. Place disc over hole and smooth edges with fingers to remove air bubbles. Place wt (>500 g) on disc ≥1 h.

F. Collection and Analysis of Headspace Gas

Can piercing assembly is shown in Fig. **984.36**. Before piercing can, close gage valve and pull plunger on syringe to remove air from silicone tubing. Close syringe valve and expel air from syringe. Puncture can and open gage valve to read vac. or pressure. Turn gage valve and syringe valve to release gas into syringe. If gas sample is >5.0 mL, withdraw this amt (as shown in Fig. **984.36**) and inject into port of gas chromatograph. If gas sample is <5.0 mL, force collected gas back into can. Close syringe valve to retain gas in tubing and can. Use syringe to add 40 mL room air to can, and pump syringe twice to mix gas. Let syringe equilibrate to atm. pressure and record syringe vol. From this dil. gas, sample may be obtained for gas chromatograph. Percent He measured should be divided by diln factor to obtain correct percent He in headspace gas. Use following formula to det. diln factor:

$$\text{Diln factor} = (\text{equilibrated syringe vol.} - 40\text{ mL air} + \text{headspace vol.})/(\text{equilibrated syringe vol.} + \text{headspace vol.})$$

For example: (43−40+9)/(43+9) = 12/52 = 0.23 diln factor.

$$\%\text{ He in can} = \%\text{ He measured/diln factor}$$

For example: 5% He/0.23 = 22% He in can.

Headspace vol. may be measured by piercing control can that still has vac. Assume sample and control can same volume. Measure amt of vac. (in. Hg) and vol. air pulled in from syringe.

$$\text{Headspace vol.} = \text{measured vol. from syringe} \times 30\text{ in Hg/measured vac. in can (in. Hg)}$$

For example, if 6 mL air is pulled into can and vac. is 20 in. Hg, then,

$$\text{Headspace vol.} = 6\text{ mL} \times 30\text{ in Hg}/20\text{ in. Hg} = 9\text{ mL}$$

To perform addnl work on can, collected gas may be stored in capped syringe 2–3 h without appreciable change in its composition.

G. Interpretation of Results

Report can as leaker if, after exposure to pressurized He, can internal pressure is ≥8 psi or percentage He is ≥1%. Report can as nonleaker if, after exposure to pressurized He, can internal vac. is ≥5 in., or percentage He is <1%.

Ref.: JAOAC **67,** 942(1984).

972.45 Thermophilic Bacterial Spores in Sugars

Microbiological Method

First Action 1972
Final Action 1989

(Sugar, both beet and cane, may carry spores of all 3 groups of thermophilic bacteria that are important as spoilage agents in low-acid canned foods, i.e., flat sour bacteria (*Bacillus stearothermophilus*), thermophilic anaerobes not producing H_2S (*Clostridium thermosaccharolyticum*), and sulfide spoilage bacteria or thermophilic anaerobes producing H_2S (*C. nigrificans*). These bacteria are not of health significance, but excessive numbers may survive com. heat processes.)

A. Sampling

Take 225 g (0.5 lb) samples from 5 sep. bags or barrels of shipment or lot, place in clean containers, and seal.

Sample liq. sugar by drawing 5 sep. 200–250 mL (6–8 oz) portions during pumping transfer from tank trucks to storage tanks or at refinery during filling of tank trucks.

Number of samples will vary in relation to size of shipment or lot. If there is significant variability in lot, this fact will become evident, in majority of cases, thru individual tests on the 5 samples.

B. Preparation of Sample

(a) *Dry sugar.*—Place 20 g sample in sterile 150–250 mL erlenmeyer marked to indicate 100 mL. Add sterile H_2O to 100 mL mark. Bring rapidly to bp, and boil 5 min. Replace liq. evapd with sterile H_2O.

(b) *Liquid sugar.*—Add sample contg 20 g dry sugar, detd on basis of °Brix (e.g., 29.41 g 68° Brix (%) liq. sugar is equiv. to 20 g dry sugar), to sterile 250 mL flask and proceed as in **972.45D(a)**.

C. Culture Media

(a) *Glucose tryptone agar.*—For detection of flat sour bacteria. Use com. stdzd dehydrated medium (Bacto-Dextrose Tryptone Agar) preferably, or prep. as follows: Suspend 10.0 g tryptone, 5.0 g glucose, 15.0 g agar, and 0.04 g bromocresol purple in 1 L H_2O, and mix thoroly. Final pH, 6.7 ± 0.1. Autoclave 30 min at 121° and cool to 55°.

(b) *Liver broth.*—For detection of thermophilic anaerobes not producing H_2S (*C. thermosaccharolyticum*). Mix 500 g chopped beef liver with 1 L H_2O. Slowly boil mixt. 1 hr, adjust to ca pH 7.0, and boil addnl 10 min. Press boiled material thru cheesecloth and dil. liq. to 1 L. To broth, add 10.0 g peptone and 1.0 g K_2HPO_4, and adjust to pH 7.0. To test tube, add 1–2 cm previously boiled ground beef liver and 10–12 mL broth. Sterilize 20 min at 121°. Before using medium, unless freshly prepd, exhaust by subjecting to flowing steam ≥20 min, and, after inoculation, stratify with 5–6 cm layer of plain nutrient agar (common formula) that has been cooled to 50°.

(c) *Sulfite agar, modified.*—For detection of sulfide spoilage bacteria. Suspend 10.0 g tryptone, 1.0 g Na_2SO_3, and 20.0 g agar in 1 L H_2O, and mix thoroly. At time agar is added to tube, place clean iron strip or nail in each tube. No adjustment of reaction is necessary. Prep. medium and Na_2SO_3 soln, if used in place of solid Na_2SO_3, fresh weekly. Autoclave medium 20 min at 121° and cool to 55°.

D. Culture Technic

(a) *Flat sour spores.*—Into 5 sep. petri dishes, pipet 2 mL boiled sugar soln. Cover, and mix inoculum with glucose tryptone agar. Incubate plates 35–48 hr at 55° and, to prevent drying of agar, humidify incubator. Combined count from 5 plates represents number of spores in 2 g original sugar. Multiply this count by 5 to express results in terms of number of spores/10 g sugar.

Characteristic colonies are round, 1–5 mm in diam., with typical opaque central "spot," and, usually, surrounded by yellow halo in field of purple. This halo may be insignificant or missing with certain low acid-producing types or if plate is so thickly seeded that entire plate has yellow tinge. Typical subsurface colonies are compact and may approach "pin point" conditions.

If identity of subsurface colonies is doubtful, observe nature of surface colonies. If they show reasonable purity of formed flora, assume that subsurface colonies have been formed by similar bacterial groups. If plate is heavily seeded, counts may not be accurate and colony structure and size may be atypical. If plates are so heavily seeded that counting is impractical, dil. original soln and repeat procedure.

To det. if typical subsurface colonies are flat sour organisms, apply streak from colonies to agar plates to det. surface characteristics.

(b) *Thermophilic anaerobes not producing hydrogen sulfide.*—Divide 20 mL boiled sugar soln equally among 6 liver broth tubes and stratify liq. medium with plain nutrient agar. After agar has solidified, preheat to 55° and incubate 72 hr at that temp.

Thermophilic anaerobes not producing H_2S are identified by splitting of agar, presence of acid, and, occasionally, cheesy odor. Method is suitable as qual. test but provides only rough estn; results cannot be expressed as number of spores/unit wt sugar.

(c) *Sulfide spoilage bacteria.*—Divide 20 mL boiled sugar soln equally among 6 freshly exhausted tubes contg modified sulfite agar. Incubate 48 hr at 55°.

In sulfite agar, sulfide spoilage bacteria form characteristic blackened spherical areas. Due to solubility of H_2S and its fixation by Fe, no gas is noted. Some thermophilic anaerobes not producing H_2S generate relatively large amts of H, which splits agar and reduces sulfite, thereby causing general blackening of medium. This condition, however, is readily distinguishable from restricted blackened area mentioned above. Count blackened areas to obtain quant. results.

E. Reporting Results

Report flat sour and sulfide spoilage results as number of spores/10 g sugar. Report thermophilic anaerobes not producing H_2S as number of tubes pos. or neg. (+ or −).

Refs.: JAOAC **19,** 438(1936); **21,** 457(1938); **55,** 445(1972).

985.41 Sporeformers in Low-Acid Canned Foods

Gas Chromatographic Method

First Action 1985
Final Action 1989

A. Principle

Two org. compds produced by sporeforming organisms in low-acid canned foods, D-(−)-2,3-butanediol (BD) and butyric acid (BA), but not produced by nonsporeformers, are

measured by gas chromatgy. Identification of BD and BA is based on relative retention times (RRTs) to internal std, propionic acid. Identification of sporeforming organisms as cause of spoilage is based on ratio of peak hts for BD and butyric acid (in external std) and BA and butyric acid (in external std).

B. Apparatus

(a) *Gas chromatograph.*—Suitable for use with 2 heated flash vaporizer injectors contg glass sleeves; equipped with flame ionization detectors (FID); linked to data processor (if available) with printer/plotter (Sigma Series Instrument with console and printer/plotter, Perkin-Elmer, or equiv.).

(b) *Gas chromatographic columns.—(1)* 1.8 m (6 ft) × 2 mm id glass column packed with 15% SP 1220/1% H_2PO_4 on 100–120 mesh Chromosorb W(AW); *(2)* 1.8 m × 2 mm (id) glass column packed with 0.3% CW 20M/0.1% H_3PO_4 on 60–80 mesh Carbopack C (Supelco Inc.). Condition both columns, using recommended procedures: purge column at ambient temp. ≥30 min with carrier gas at 20 mL/min; then program from 50° to 150° at 2°/min and hold overnight. Cool column 1, attach to detector, and set column temp. at 118°. Inject twenty 10 μL portions of freshly boiled H_2O on column 2 at 150°. Then cool, attach to detector, and set column temp. at 125°.

(c) *Operating conditions.*—Select 2 methods from available chromatgc data systems software (if data processor is available) that provide relative retention time (RRT) = 1.00 for internal std. Column 1: injector 200°, detector 240°, column 118° for 12 min isothermal run; He carrier flow rate 24 mL/min; propionic acid elution time 2.70–3.30 min; electrometer range 10, attenuation 4; chart speed 5 mm/min; injection vol. 1 μL. For butyric acid, retention time = ca 6 min, RRT = 1.7, and peak ht = 60% FSD (10 cm). Column 2: injector 200°, detector 240°, column 125° for 25 min isothermal run; N carrier flow rate 15 mL/min; propionic acid elution time 2.25–2.45 min; electrometer range 10, attenuation 2; chart speed 10 mm/min; injection vol. 0.3 μL. For butyric acid, retention time = ca 6.5 min, RRT = 2.7, and peak ht = 60% FSD (10 cm). Theoretical plates for each column ≥1600.

(d) *Syringes.*—1 and 5 μL (Hamilton 7001-N and 1705-N, or equiv.).

(e) *Centrifuge.*—With adapters suitable to accept 5 mL minivials, 1.0 and 5.0 mL capacity, with silicone stopper and screw cap (95010 and 95050, Alltech Associates, Inc., 2051 Waukegan Rd, Deerfield, IL 66015), or equiv.

(f) *Disposable Pasteur pipets.*—7760-F 30 series (Thomas Scientific), or equiv.

C. Reagents

(a) *Water.*—Distd H_2O that elutes with no detectable peaks on 2 columns used for assay.

(b) *External std solns.—(1)* For column 1, WSFA-2 (Supelco Inc.,), or equiv. It must contain aq. soln of propionic, isobutyric, and butyric acids. Concn of butyric acid should be 0.1%. Concns of propionic and isobutyric acids should be sufficient to produce peak hts ranging from detectable to 100% scale deflection (SD).

(2) For column 2, dil. WSFA-2 with equal vol. H_2O or prep. equiv. soln. It must contain aq. soln of propionic and butyric acids. Concn of butyric acid should be 0.05%. Concn of propionic acid should be sufficient to produce peak ht ranging from detectable to 100% SD.

(c) *Internal std solns.—(1)* For column 1, dil. 5 μL reagent grade propionic acid to 5 mL with H_2O. For injection, take up 1 μL H_2O into 5 μL syringe, followed by 0.1 μL soln. Modify proportions of 2 components, if necessary, to give ca 50% FSD for propionic acid peak eluting in 2–3 min. *(2)* For column 2, dil. protion of internal std for column 1 with equal vol. H_2O. For injection, take up 0.3 μL H_2O into 1 μL syringe, followed by 0.02 μL of this diln. Modify proportions of 2 components, if necessary, to give ca 50% FSD for propionic acid peak eluting in 2–3 min.

D. Assay

Pierce each can aseptically by any microbiologically acceptable technic (see **972.44C(a)**, (c) and **972.44D**). Transfer portion of liq. can contents to 5 mL mini-vial, using sterile Pasteur disposable pipet, or equiv. and store at ca −20° overnight or longer. Warm 5 mL vials to room temp. and then centrf. 15 min at 1000 × *g* or until phases sep. Transfer portion of clear upper phase to 1 mL mini-vial, using sterile Pasteur disposable pipet, or equiv. Store this upper phase at −20° overnight or until day of assay.

Let 1 mL mini-vials of upper phase of centrfd can contents warm to room temp. Use syringe to remove (and discard) any particulate matter from bottom of cone; then mix sample, using syringe. For assays on either column, assay external std to ensure optimal instrumental conditions; propionic acid must be eluted in time range such that RRT = 1.000; theoretical plates should be ca ≥1600; and butyric acid peak should be ca 60% FSD. Then replace glass liner in injector and inject sample of H_2O, using sample syringe. Repeat H_2O injections, if necessary, until chromatogram has no ghost peaks. For assays on column 1, take up 1 μL portion of sample into 5 μL syringe, followed by 0.1 μL internal std soln 1 and inject into gas chromatograph. For assays on column 2, take up 0.3 μL portion of sample into 1 μL syringe followed by 0.02 μL internal std soln 2 and inject into gas chromatograph.

E. Interpretation

For each sample, examine chromatograms from both columns, together with chromatograms for external std assayed same day. On column 1, BD elutes as tailing peak with retention time between those for isobutyric and butyric acids; it will not elute on column 2 in 25 min run. Sample contg BA gives peak eluting in both sample chromatograms in retention time range for butyric acid. Measure peak ht for BD peak and divide by peak ht for butyric acid in external std 1 assayed on column 1. Measure BA peak ht from column 2 and divide by peak ht for butyric acid in external std 2 assayed on column 2. Peak ht ratio for BD ≥0.39 or peak ht ratio for BA ≥0.30 indicates sporeformers as cause of spoilage. Record cause of spoilage as from either sporeformer or nonsporeformer.

Ref.: JAOAC **68,** 626(1985).

CLOSTRIDIUM

977.26 *Clostridium botulinum* and Its Toxins in Foods

Microbiological Method

First Action 1977
Final Action 1979

(*Caution:* See safety notes on pipets.)

A. Principle

Mice injected intraperitoneally (IP) with food ext contg ≥1 min. lethal dose (MLD) of botulinum toxin die within 72 hr after exhibiting sequence of symptoms characteristic of botulinum intoxication. Homologous antitoxin will protect mice from symptoms while other antitoxins will not, thus detg serological type. Viable spores in food will grow in suitable culture medium and produce toxin, which is detected and typed.

B. Apparatus

(a) *Can opener.*—*See* **972.44C(a)**.

(b) *Anaerobic jars.*—GasPak (BBL) or Case-nitrogen replacement.

(c) *Petri dishes.*—100 mm diam. Dry prepd plates ca 24 hr at 35° before streaking.

(d) *Centrifuge.*—High-speed, refrigerated.

(e) *Syringes.*—1.0 or 3.0 mL with 25 gage $^5/_8$″ needles for inoculating mice.

C. Media and Reagents

(a) *Cooked meat broth.*—Use either liver or heart medium. (*1*) *Chopped liver broth.*—Grind 500 g fresh beef liver into 800 mL H_2O. Heat to bp and simmer 1 hr. Cool, adjust to pH 7.0, and boil 10 min. Filter thru cheesecloth, pressing out excess liq. To broth add 10 g peptone, 1 g K_2HPO_4, and 1 g sol. starch. Adjust to pH 7.0 and dil. to 1 L with H_2O. Filter thru coarse paper. (If desired, broth and liver may be stored sep. in freezer for future use.) To 18 or 20 × 150 mm test tubes, add liver to ht of 1–2 cm and 10–12 mL liq. Autoclave 20 min at 121°. (2) *Cooked meat medium.*—Use com. medium of following formula: beef heart 454 g, proteose peptone 20 g, dextrose 2 g, and NaCl 5 g. Suspend 12.5 g medium in 100 mL cold H_2O. Mix thoroly and let stand until particles are thoroly wetted (ca 15 min). (Alternatively, add 1.25 g solid medium into test tubes, add 10 mL cold H_2O, and mix thoroly to wet all particles.) Autoclave 15 min at 121°. Final pH, 7.2 ± 0.1.

(b) *Trypticase-peptone-glucose-yeast extract broth with trypsin (TPGYT).*—Dissolve 50 g trypticase, 5 g Bacto-peptone, 20 g yeast ext, 4 g dextrose, and 1 g Na thioglycollate in 1 L H_2O, and dispense 15 mL portions into 20× 150 mm culture tubes or 100 mL portions into 6 fl oz prescription bottles. Autoclave 10 min (tubes) or 15 min (bottles) at 121°. Final pH, 7.0 ± 0.1. Refrigerate, and discard if not used within 2 weeks. Immediately before use, steam or boil 10–15 min to remove O, cool quickly, and aseptically add 1.0 mL trypsin soln/15 mL broth.

Prep. trypsin soln by dissolving 1.5 g trypsin (Difco 1:250) in 100 mL H_2O. Sterilize by filtering thru 0.45 μm Millipore or equiv. filter, and refrigerate.

(c) *Liver-veal-egg yolk agar or anaerobic egg yolk agar.*—(*1*) *Liver-veal-egg yolk agar (LVEY).*—Wash 2 or 3 eggs with stiff brush, and drain. Soak eggs in 0.1% $HgCl_2$ soln 1 hr. Drain $HgCl_2$ soln and replace with 70% alcohol, soaking 30 min. Remove eggs, crack aseptically, and discard whites. Remove yolk with syringe, place in sterile container, and add equal vol. sterile 0.85% NaCl soln. Mix thoroly. To each 500 mL prepd sterile com. dehydrated liver veal agar at 50°, add 40 mL egg yolk-NaCl suspension. Mix thoroly and pour plates. Dry plates 2 days at room temp. or 24 hr at 35°. Discard contaminated plates, and store sterile plates in refrigerator. (*2*) *Anaerobic egg agar.*—Dissolve 5 g yeast ext, 5 g tryptone, 20 g proteose peptone, 5 g NaCl, and 20 g agar in 1 L H_2O. Adjust to pH 7.0, dispense 500 mL into 1 L flask, and autoclave 20 min at 121°. To 500 mL melted agar at 45–50°, add 40 mL egg yolk-NaCl suspension, prepd as in (*1*). Mix, and pour plates immediately. Dry and store sterile plates as in (*1*).

(d) *Gel-phosphate buffer.*—pH 6.2. Dissolve 2 g gelatin and 4 g Na_2HPO_4 in 1 L H_2O with gentle heat. Dispense into 100 mL milk diln bottle. Autoclave 20 min at 121°.

(e) *Clostridium botulinum antitoxin preparations.*—Types A thru F or polyvalent A–F. Available from Centers for Disease Control, CID, Office of Official Services, Atlanta, GA 30333.

D. Preparation of Sample

(a) *Preliminary examination.*—Keep samples refrigerated. Unopened canned foods, unless badly swollen and in danger of bursting, need not be refrigerated. Record code and condition of container. Clean and identify container.

(b) *Solid foods.*—Aseptically transfer portion, with little or no free liq., to sterile mortar. Add equal amt sterile gel-phosphate buffer, (**d**), and grind with sterile pestle. Alternatively, inoculate small pieces of sample with sterile forceps directly into enrichment broth.

(c) *Liquid foods.*—Inoculate with sterile pipets directly into enrichment broth.

(d) *Canned foods.*—Prep., disinfect with alc. I soln, and open cans as in **972.44D**. If can has swelled, position can so vertical side seam is away from operator. If can has buckled ends, chill before opening, and flame cautiously to avoid bursting can.

(e) *Visual examination.*—Note appearance, odor, and any evidence of decomposition. DO NOT TASTE PRODUCT under any circumstances.

(f) *Reserve sample.*—After culturing, aseptically remove portion to sterile sample jars for further tests which may be needed later.

E. Detection of Viable C. botulinum

(a) *Enrichment.*—Remove dissolved O from media before inoculation by steaming 10–15 min and cooling quickly without agitation. Inoculate 2 tubes of cooked meat broth, (**a**), with 1–2 g solid or 1–2 mL liq. food or ext/15 mL broth, introducing inoculum slowly beneath surface of broth. Incubate at 35°. Similarly inoculate 2 tubes of TPGYT broth, **977.26C(b)**, and incubate at 26°.

(b) *Examination.*—After 5 days, examine cultures for turbidity, gas production, digestion of meat particles, and odor. Also examine microscopically by wet mount under high power phase contrast or by bright field illumination of smear stained by Gram stain, crystal violet, or methylene blue. Observe morphology of organisms and note existence of typical clostridial cells, occurrence and relative extent of sporulation, and location of spores within cells.

(c) *Further treatment.*—Usually 5 day incubation produces active growth and highest concn of toxin, as well as peak sporulation. Retain culture in refrigerator for pure culture isolation. If there is no growth after 5 days, incubate addnl 10 days to detect possible delayed germination of *C. botulinum* spores before discarding culture as sterile.

F. Isolation of Pure Cultures

If good sporulation has occurred, *C. botulinum* is more readily isolated from mixed flora in enrichment culture or from original sample.

(a) *Pretreatment.*—Add equal vol. filter-sterilized absolute alcohol to 1–2 mL culture or sample in sterile screw-cap tube. Mix well and incubate at room temp. 1 hr. Alternatively, heat 1–2 mL enrichment culture 10–15 min at 80° to destroy vegetative cells. (Do not use heat treatment for nonproteolytic type *C. botulinum.*)

(b) *Plating.*—With inoculating loop, streak 1 or 2 loopfuls of alcohol or heat-treated cultures, dild if necessary, to either or both liver-veal-egg yolk agar or anaerobic egg yolk agar dried plates in manner to obtain isolated colonies. Incubate plates ca 48 hr at 35° under anaerobic conditions of Case anaerobic jar or Gas-Pak systems, or equivs.

(c) *Selection of colonies.*—Typical colonies are raised or flat, smooth or rough, and commonly show some spreading and have irregular edge. On egg yolk media, colonies usually

exhibit surface iridescence when examined by oblique light. This luster zone is referred to as "pearly layer." Zone usually extends beyond and follows irregular contour of colony. Besides pearly zone, colonies of types C, D, and E are ordinarily surrounded by wide (2–4 mm) zone of yellow ppt. Colonies of types A and B generally show smaller zone of pptn. Not all typical colonies will produce toxin. Some members of genus *Clostridium* have typical morphological characteristics but do not produce toxins.

(**d**) *Cultures.*—With sterile transfer loop, inoculate each of 10 selected colonies into tube of sterile medium: (*1*) TPGYT broth for *C. botulinum* Type E, incubating 5 days at 26°; and (*2*) cooked meat broth for other toxin types, incubating 5 days at 35°. Use cultures for confirmation as in (**e**) and for detection and identification of toxin as in **977.26G**.

(**e**) *Confirmation.*—Streak culture from (**d**) in duplicate on egg yolk agar plates, incubating 1 plate anaerobically and other plate aerobically at 35°. If colonies typical of *C. botulinum* are found on anaerobic plate and no growth is found on aerobic plate, culture may be pure. Failure to isolate *C. botulinum* from ≥1 of selected colonies may indicate that its population relative to mixed flora is low. Repeated serial transfers thru addnl enrichment steps, **977.26E(a)**, may increase numbers sufficiently to permit isolation. Store pure culture, (**d**), either under refrigeration, on glass beads, or lyophilized.

G. Detection of Toxin

(**a**) *Preparation of sample.*—Ext solid foods with equal vol. gel-phosphate buffer, **977.26C(d)**, macerating with sterile, prechilled mortar and pestle. Centrf. ext and liq. foods contg suspended solids under refrigeration. Rinse empty containers suspected of having held toxic foods with few mL gel-phosphate buffer. Use min. vol. to avoid diln of toxin.

(**b**) *Trypsin treatment.*—Toxins of nonproteolytic types, if present, may need trypsin activation to be detected. Do not use trypsin treatment with TPGYT culture which already contains trypsin. Further treatment may degrade any fully activated toxin present in culture.

Adjust portion of food supernate, (**a**), liq. food, or cooked meat culture, if necessary, to pH 6.2 with 1*N* NaOH or HCl. Prep. satd trypsin soln by dispersing 1 g trypsin (Difco 1:250) in 10 mL H_2O in clean culture tube. Mix 0.2 mL trypsin soln with 1.8 mL liq. to be tested. Incubate 1 hr at 37° with occasional gentle agitation.

(**c**) *Toxicity testing.*—Conduct each test in duplicate, *i.e.*, on trypsin treated and untreated materials. Dil. portions of untreated and treated food supernate, liq. food, or culture 1:2, 1:10, and 1:100, resp., with gel-phosphate buffer. Inject sep. pairs of mice, ca 15–20 g, IP with original and dild fluids, treated and untreated, using syringe, (**e**). Heat 1.5 mL original untreated fluid 10 min at 100° for control. Cool, and inject pair of mice each with 0.5 mL heated fluid. These mice should not die because botulinum toxin, if present, is inactivated by this heat treatment.

Observe mice periodically for 72 hr, recording symptoms and time of deaths. Typical symptoms of botulism usually begin within 24 hr with ruffling of fur, followed in sequence by labored breathing, weakness of limbs, and finally total paralysis with gasping for breath, followed by death due to respiratory failure. Death without symptoms of botulism is not sufficient evidence that injected material contained botulinum toxin. Deaths may occur from chems present in fluid or from trauma.

If after 72 hr, all but mice receiving heated prepn have died, repeat toxicity test, using higher dilns of fluids. It is necessary to have dilns that kill as well as dilns that do not kill to establish an end point or MLD (min. lethal dose) as est. of amt of toxin present. MLD is contained in highest diln killing both (or all) mice inoculated. Calc. MLD/mL.

H. Typing of Toxin

Dil. monovalent antitoxins to types A, B, E, and F in 0.85% NaCl soln to concn of 1 International Unit/0.5 mL. Prep. enough dild antitoxin to inject 0.5 mL into each of 2 mice for each diln of prepn to be tested.

Use toxic prepn which gave greatest number of MLD, either treated or untreated. If untreated, same prepn can be used as was used for toxicity testing; if trypsinized prepn was most lethal, prep. freshly trypsinized fluid since continued action of trypsin may destroy toxin. Prep. dilns to cover range of at least 10, 100, and 1000 MLD below previously detd end point of toxicity.

Inject several groups of mice IP, each mouse receiving 0.5 mL of 1 of dild antitoxins, 30–60 min before challenging them with IP injection of toxic prepns.

Inject pairs of mice protected by specific monovalent antitoxin injection IP with each diln of toxic prepn. Also inject pair of unprotected mice (no injection of antitoxin) with each toxic diln as control. (This protocol requires 30 mice: 3 pairs for each of the 4 monovalent antitoxins (A, B, E, and F), each pair to receive challenge of 1 of the 3 dilns of toxic prepn ($2 \times 3 \times 4 = 24$) plus 1 pair of unprotected mice for each diln of toxic material as control ($2 \times 3 = 6$).

Observe mice 72 hr for symptoms of botulism and record time of deaths. If results indicate that toxin was not neutzd, repeat test, using monovalent antitoxins to types C and D, plus polyvalent antitoxin pool of types A thru F.

I. Interpretation

Toxin in food means that product, if consumed without thoro heating, could cause botulism. Presence of toxin in food is required for botulism to occur. Viable *C. botulinum* but no toxin in food is not proof that food in question caused botulism. Ingested organisms may be found in alimentary tract, but are considered to be unable to multiply and produce toxin *in vivo*.

Presence of botulinum toxin and/or organisms in low-acid (pH >4.6) canned foods means that items were underprocessed or were contaminated thru post-processing leakage. Swollen cans are more likely than flat cans to contain botulinum toxin since organism produces gas during growth. Presence of toxin in flat can may imply that seams were loose enough to let gas escape. Toxin in canned foods is usually of type A or of proteolytic type B strain, since spores of proteolytics can be among more heat resistant bacterial spores. Spores of nonproteolytics, types B, E, and F, generally are of low heat resistance and would not normally survive even mild heat treatment.

Protection of mice from botulism and death with 1 of monovalent botulinum antitoxins confirms presence of botulinum toxin and dets serological type of toxin in sample.

If mice are not protected by 1 of monovalent antitoxins, there may be too much toxin in sample, there may be more than 1 kind of toxin present, or deaths may be due to some other cause. In such cases, retesting at higher dilns of test fluids is required and mixts of antitoxins must be used in place of monovalent antiserum. If mice are still not protected, some other toxic material, which is not heat labile, could be responsible if both heated and unheated fluids cause death. It is also possible that heat stable toxic substance could mask botulinum toxin.

Ref.: JAOAC **60,** 541(1977).

976.30 *Clostridium perfringens* in Foods

Microbiological Method

First Action 1976
Final Action 1979

(Applicable to examination of outbreak foods in which relatively small numbers of vegetative cells are expected to be present)

A. Apparatus

(a) *Pipets.*—1.0 mL serological with 0.1 mL graduations and 10.0 mL with 1.0 mL graduations.

(b) *Colony counter.*—Quebec, or equiv., dark field model.

(c) *High-speed blender.*—Waring Blendor, or equiv., multispeed model, with low-speed operation at 13,000 rpm, and 1 L glass or metal blender jars with covers. One jar is required for each sample.

(d) *Anaerobic jars.*—BBL GasPak jars equipped with GasPak H + CO_2 generator envelopes are recommended. Anaerojar (Pfizer Diagnostics, 1407 N Dayton St, Chicago, IL 60622) with replacement of air by purified N or N-CO_2 (9 + 1) is satisfactory.

(e) *Freezer, ultra-low temperature.*—REVCO Model ULT-107 (Revco Scientific, Inc., 275 Aiken Rd, Asheville, NC 28804) or equiv., capable of maintaining temp. of −68°.

(f) *Shipping container.*—Heavy duty styrofoam, including hermetically sealable metal canister (friction-fit paint can is satisfactory).

B. Reagents

(a) *Peptone dilution water.*—Dissolve 2.0 g peptone (Difco B118) in 2 L H_2O for each sample, and adjust to pH 7.0 ± 0.1. Dispense enough vol. in 175 mL (6 oz) bottles to give 90 ± 1 mL and in 750 mL erlenmeyers to give 450 ± 5 mL after autoclaving 15 min at 121°.

(b) *Nitrite test reagents.*—(*1*) *Reagent A.*—Dissolve 8 g sulfanilic acid in 1 L 5*N* HOAc (2 + 5). (*2*) *Reagent B.*—Dissolve 5 g α-naphthol in 1 L 5*N* HOAc.

(c) *Buffered glycerol-salt soln.*—Dissolve 4.2 g NaCl in 900 mL H_2O. Add 12.4 g anhyd. K_2HPO_4, 4.0 g anhyd. KH_2PO_4, and 100 mL glycerol. Mix well to dissolve, and adjust pH to 7.2. Autoclave 15 min at 121°. For double-strength glycerol soln (20%), use 200 mL glycerol and 800 mL H_2O.

C. Culture Media

(Sizes of culture media containers (test tubes, flasks, and petri dishes) are specified for each medium. All media except tryptose-sulfite-cycloserine (TSC) agar are incubated in air at 35°. Media not used ≤4 hr after prepn must be heated 10 min in boiling H_2O or flowing steam to expel O and cooled rapidly in tap H_2O without agitation just before use.)

(a) *Tryptose-sulfite-cycloserine agar.*—15.0 g tryptose, 20.0 g agar, 5.0 g soytone, 5.0 g yeast ext, 1.0 g Na metabisulfite, and 1.0 g ferric ammonium citrate (NF Brown Pearls) dild to 1 L with H_2O (SFP agar base, Difco 0811-01, is satisfactory). Adjust to pH 7.6 ± 0.1, dispense 250 mL portions into 500 mL flasks, and sterilize 15 min at 121°. Before plating, add 20.0 mL 0.5% filter-sterilized soln of D-cycloserine to each 250 mL sterile melted medium at 50°. To make egg yolk-contg plates, add 20 mL 50% egg yolk emulsion, (c), to 250 mL sterile medium contg D-cycloserine. Dispense 15 mL portions into 100 × 15 mm sterile petri dishes. Cover plates with towel and let dry overnight at room temp. before use.

(b) *D-Cycloserine soln.*—Dissolve 1 g D-cycloserine (Sigma Chemical Co. or Serva Feinbiochemicia, Heidelberg, West Germany) without heating in 200 mL 0.05*M* phosphate buffer (pH 8.0 ± 0.1) and sterilize by filtering thru 0.45 μm membrane filter.

(c) *Egg yolk emulsion.*—Wash fresh eggs with stiff brush and drain. Soak 1 hr in 70% alcohol. Aseptically remove yolk and mix with equal vol. sterile 0.85% NaCl soln. Store at 4°.

(d) *Buffered motility-nitrate medium.*—3.0 g beef ext, 5.0 g peptone, 5.0 g KNO_3, 2.5 g Na_2HPO_4, 3.0 g agar, 5.0 g galactose, and 5.0 g glycerol dild to 1 L with H_2O. Adjust to pH 7.3 ± 0.1, dispense 11 mL portions into 150 × 16 mm tubes, and sterilize 15 min at 121°.

(e) *Lactose-gelatin medium.*—15.0 g tryptose, 10.0 g yeast ext, 10.0 g lactose, 5.0 g Na_2HPO_4, 0.05 g phenol red, and 120.0 g gelatin dild to 1 L with H_2O. Adjust to pH 7.5 ± 0.1 before adding lactose and phenol red. Dispense 10 mL portions into 150 × 16 mm screw-cap tubes and sterilize 15 min at 121°.

(f) *Sporulation broth.*—15.0 g polypeptone, 3.0 g yeast ext, 3.0 g sol. starch, 0.1 g $MgSO_4$, 1.0 g Na thioglycollate, and 11.0 g Na_2HPO_4 dild to 1 L with H_2O. Adjust to pH 7.8 ± 0.1, dispense 15 mL portions into 150 × 20 mm screw-cap tubes, and sterilize 15 min at 121°.

(g) *Polypeptone-yeast extract (PY) medium.*—20.0 g polypeptone, 5.0 g yeast ext, and 5.0 g NaCl dild to 1 L with H_2O. Adjust to pH 6.9 ± 0.1, dispense 9 mL portions into 125 × 16 mm screw-cap tubes, and sterilize 15 min at 121°.

(h) *Fluid thioglycollate medium.*—(BBL No. 11260 or Difco No. 0256). Dispense 10 mL portions into 150 × 16 mm screw-cap tubes. Sterilize 15 min at 121°, and cool quickly. Final pH is 7.1 ± 0.1.

D. Preparation of Sample

(a) *For storage and shipping.*—Using aseptic technic, transfer 50 g sample to sterile container such as Whirl-Pak plastic bag and add 50 g sterile buffered glycerol-salt soln. Mix well by kneading bag or stirring with sterile pipet. Let soln penetrate solid foods 10 min before freezing. Treat liq. samples such as beef juice or gravy with double-strength (20% glycerol) soln to obtain final concn of 10% glycerol. Freeze samples as quickly as possible in ultra-low temp. freezer at −68° or, alternatively, by placing in sealable metal canister and storing with solid CO_2 in insulated shipping container. To ship samples, place in sealable metal canister and pack in well insulated styrofoam shipping carton with sufficient solid CO_2 to keep samples frozen during transit. Ship by most rapid means possible. Upon receipt, transfer samples to ultra-low temp. freezer at −68° or replenish solid CO_2 in shipping carton to maintain temp. at ca −56° until samples can be examined. Thaw samples and proceed as in (b) without delay.

(b) *For analysis.*—Using aseptic technic, weigh 50 g food sample into sterile blender jar. Add 450 mL peptone diln H_2O and homogenize 2 min at low speed (13,000 rpm). Use this 1:10 diln to prep. serial dilns from 10^{-2} to 10^{-6} by transferring 10 mL of 1:10 diln to 90 mL diln blank, mixing well with gentle shaking, and continuing until 10^{-6} diln is reached.

E. Plate Count Technic

Pour ca 5 mL TSC agar without egg yolk into each of ten 100 × 15 mm petri dishes and spread evenly by rapidly rotating dish. When agar has solidified, label plates and aseptically pipet 1 mL of each diln of homogenate in duplicate onto agar surface in center of dish. Pour addnl 15 mL TSC agar without egg yolk into dish and mix well with inoculum by gently rotating dish.

Alternatively, with sterile glass rod spreader, spread 0.1 mL diln over previously poured plates of TSC agar contg egg yolk emulsion. Let plates absorb inoculum 5–10 min; then overlay

with 10 mL TSC agar without egg yolk. (TSC agar contg egg yolk is preferred for foods which may also contain other sulfite-reducing *Clostridium* sp.)

When agar has solidified, place plates in upright position in anaerobic jar. Produce anaerobic conditions, and incubate jar 20 hr at 35° for TSC agar without egg yolk and 24 hr at 35° for TSC agar with egg yolk. After incubation, remove plates from jar and observe macroscopically for growth and black colony production. Select plates showing estd 20–200 black colonies. Using Quebec colony counter with piece of white tissue paper over counting area, count black colonies and calc. number of *Clostridium* sp./g food. *C. perfringens* colonies in medium contg egg yolk are black and usually surrounded by 2–4 mm zone of white ppt due to lecithinase activity. However, since a few strains are weak or neg. for lecithinase, count any black colonies suspected to be *C. perfringens* and confirm identity as in **976.30F**.

F. Confirmation Technic

Select 10 characteristic colonies from countable plates (20–200 colonies), inoculate each into tube of fluid thioglycollate medium, and incubate 18–24 hr at 35°. Make Gram-stained smear of fluid thioglycollate cultures and check for purity and presence of short, thick, Gram-pos. bacillus characteristic of *C. perfringens*. Streak contaminated cultures on TSC agar contg egg yolk and incubate plates anaerobically 24 hr at 35° to obtain pure cultures. Stab-inoculate buffered motility-nitrate and lactose gelatin media with 2 mm loopfuls of pure fluid thioglycollate culture or portion of isolated colony from TSC agar plate. Inoculate sporulation broth with 1 mL fluid thioglycollate culture and incubate 24 hr at 35°. Examine tubes of buffered motility-nitrate medium by transmitted light for type of growth along stab. Nonmotile organisms produce growth only in and along line of stab. Motile organisms produce diffuse growth out into medium away from stab.

Test buffered motility-nitrate medium for presence of nitrite by adding 0.5 mL Reagent A and 0.2 mL Reagent B. Orange which develops within 15 min indicates presence of nitrites. If no color develops, add few grains of powd Zn metal, and let stand 10 min. No color change after addn of Zn indicates that nitrates are completely reduced; change to orange indicates that organism is incapable of reducing nitrates.

Examine lactose-gelatin medium for gas and color change from red to yellow, indicating that lactose is fermented with production of acid. Chill tubes 1 hr at 5° and check for gelatin liquefaction. If medium solidifies, reincubate addnl 24 hr at 35° and repeat test for gelatin liquefaction. Make Gram-stained smear from sporulation broth and examine microscopically for spores. Report whether or not spores are produced. Store sporulated cultures at 4° if further testing of isolates is desired.

Nonmotile, Gram-pos. bacilli which produce black colonies in TSC agar, reduce nitrates to nitrites, produce acid and gas from lactose, and liquefy gelatin within 48 hr are provisionally identified as *C. perfringens*.

Organisms suspected to be *C. perfringens* that do not meet criteria stated above must be confirmed by further testing. Subculture into fluid thioglycollate medium isolates that do not liquefy gelatin or which are atypical in other respects. Incubate 24 hr at 35°, make Gram-stained smear, and check for purity. Inoculate 1 tube of PY medium, (**g**), contg 1% salicin and 1 tube contg 1% raffinose with 0.1 mL fluid thioglycollate culture. Incubate media 24 hr at 35° andj check PY-salicin for acid and gas. Transfer 1.0 mL culture to test tube and add 1–2 drops 0.04% phenol red. Yellow indicates acid is produced from salicin. (Salicin usually is not fermented by *C. perfringens* but is rapidly fermented with production of acid and gas by closely related species.) Reincubate media addnl 48 hr and test both media for production of acid. Acid is usually produced from raffinose by *C. perfringens* but not by closely related species. Acid is produced from salicin in PY medium by a few strains of *C. perfringens*.

Calc. number of *C. perfringens* in sample on basis of % colonies tested that are confirmed as *C. perfringens*. (*Example:* If av. plate count of 10^{-4} diln was 85, and 8 of 10 colonies tested were confirmed as *C. perfringens*, number of *C. perfringens*/g food is $85 \times (8/10) \times 10{,}000 = 680{,}000$.) (*Note:* Diln factor with plates contg egg yolk is 10-fold higher than diln plated.)

Refs.: JAOAC **59,** 606(1976); **61,** 785(1978).

974.38 *Clostridium perfringens* in Foods

alpha-Toxin Estimation Method

First Action 1974
Final Action 1979

(Applicable to examination of outbreak foods in which presence of large numbers of vegetative cells are suspected but which may no longer be viable)

A. Apparatus

(**a**) *Centrifuge.*—High-speed, preferably refrigerated, with 250 mL bottles.

(**b**) *Seitz filter.*—100–250 mL with sterilizing filter pads.

(**c**) *High-speed blender.*—Waring Blendor or Omni-Mixer homogenizer (DuPont Co., Sorvall Operations, Newtown, CT 06470), with blending vessels.

(**d**) *Vacuum flask.*—Sidearm 1 L erlenmeyer fitted with 1-hole rubber stopper to receive 200 mm glass tubing with 125 cm of 6 mm od (3 mm id) rubber tubing attached.

(**e**) *Tubing.*—Stainless steel thin wall (No. 9 surgical), 3 (od) × 180 mm (Tubesales, 175 Tubeway St, Forrest Park, GA 30051).

(**f**) *Dialysis tubing.*—1.21″ flat width (Fisher Scientific Co., No. 8667C).

B. Reagents

(**a**) *N-2-Hydroxyethyl piperazine-N′-2-ethane sulfonic acid (HEPES) buffer soln.*—Dissolve 6.0 g HEPES (Calbiochem Corp.) and 11.7 g NaCl in 500 mL H_2O. Adjust to pH 8.0 with 3*N* NaOH and store at 4°.

(**b**) *Lecithovitellin soln.*—Mix 1 egg yolk with 250 mL saline soln, (**e**), and clarify by centrfg 20 min at 14,000×*g* at 4°. Filter-sterilize supernate with Seitz filter and store at 4°.

(**c**) *Saline agar base.*—Add 15.0 g purified agar (Difco Laboratories) and 8.5 g NaCl to 1 L H_2O. Adjust to pH 7.0, heat to dissolve agar, dispense in 100 mL portions, and autoclave 15 min at 121°.

(**d**) *Washed red blood cells.*—Wash packed human red blood cells 3 times by mixing with 4 vols saline soln, (**e**). Centrf. 10 min at low speed (2500 rpm) to sediment cells. Remove supernate with vac. flask. Resuspend cells in addnl saline soln and repeat these steps twice. After final wash, mix cells with equal vol. saline soln. Use sterile precautions.

(**e**) *Sterile saline soln.*—Dissolve 8.5 g NaCl in 1 L H_2O. Adjust to pH 7.0, dispense 250 mL portions into Pyrex containers, and autoclave 15 min at 121°.

(**f**) *Polyethylene glycol soln.*—30%. Dissolve 120 g polyethylene glycol (Carbowax Compound 20M, Union Carbide Corp., PO Box 8361, S Charleston, WV 25303) in 400 mL H_2O.

(**g**) *Antiserum.—Clostridium perfringens* Type A diagnostic

serum (Coopers Animal Health Inc., PO Box 41967, Kansas City, MO 64141-6167).

C. Preparation of Hemolysin Plates

Melt 100 mL saline agar base, (**c**), cool to 50°, and add 11 mL washed red cells, (**d**). Mix thoroly and dispense 7 mL into 15 × 100 mm sterile plastic petri dishes. Dry plates overnight at room temp. and store at 4°. Just before use, cut test wells by applying vac. to sterile stainless steel tube, (**e**), and plunging tube into agar. Using template, space 9 test wells 3 cm apart and 2 cm from edge, and place 2 addnl wells 3 cm apart near center of plate.

D. Toxin Extraction

Homogenize 25 g food (do not include fat) in 100 mL HEPES buffer soln, (**a**), 1 min in high-speed blender. Centrf. homogenate 20 min at 14,000–20,000×*g* at 5°. Filter supernate thru Whatman No. 31 paper, or equiv., to remove fat (chill ext centrfd without refrigeration 1 hr at 4° before filtering). Discard solids. Rinse Seitz filter pad with 15 mL saline soln. Discard saline soln and filter-sterilize ext, rinsing filter pad with 10 mL saline soln.

E. Concentration

Soak 90 cm dialysis tubing 1 hr in H_2O. Tie one end and fill with saline soln. Check for leaks and rinse out twice with saline soln. Transfer sterile ext to dialysis sack and conc. to <10 mL by dialyzing 4–5 hr against 400 mL 30% polyethylene glycol, (**f**), at 4°. Rinse outside of sack with tap H_2O and collect concd ext in sterile tube.

F. Toxin Testing

Adjust vol. of concd ext to 10 ± 0.5 mL with saline soln. Set up 10 sterile 13 × 100 mm test tubes and add 0.5 mL saline soln to all tubes except first and last. Add 0.5 mL ext to first and second tubes. Mix ext and saline soln in second tube and transfer 0.5 mL to third tube, etc., to serially dil. ext from 0 to 1 + 255. Change pipet after 3 dilns to prevent excessive carry-over. Mix 0.25 mL ext, 0.25 mL saline soln, and 0.1 mL antiserum, (**g**), in last tube. Fill 1 peripheral well of duplicate hemolysin plates with each diln of ext, using fine-tipped Pasteur pipet. Fill 1 center well of each plate with ext-antiserum mixt. and the other with saline soln. Add 0.5 mL lecithovitellin soln, (**b**), to remainder of dild ext in each tube, including ext-antiserum mixt. Mix well, and incubate tubes and plates (in plastic bag) 24 hr at 35°.

G. alpha-Toxin Titer

After incubation, refrigerate plates 2 hr at 4°. Measure hemolytic zone (width from edge of well in mm). Last 3 dilns before end point should exhibit ca 1 mm reduction in width for each 2-fold diln. If not, repeat α-toxin test. Hemolytic zone 1 mm in width is end point of titrn.

Examine ext-lecithovitellin mixt. in tubes for lecithinase activity and record results. Max. reaction (++++) is white pellicle 4–5 mm thick over clear liq. Activity decreases with diln to (+) reaction (opaque soln with no pellicle). This diln is end point of lecithovitellin test. Hemolytic and lecithinase activities neutzd by antiserum are due to α-toxin.

H. Population Estimate

Compare titer of α-toxin present in ext with data in Table **974.38** to est. population of *C. perfringens*. Hemolysin (HI) plate titer is preferred for this because lecithovitellin (LV) test is less sensitive with some food exts.

Ref.: JAOAC **57,** 91(1974).

Table 974.38 Correlation Between Population Levels of *C. perfringens* and Amount alpha-Toxin Produced in Food[a]

α-Toxin Titer[b]		Estd *C. perfringens* Population/g
HI Plate	LV Test	× 10^6
Undild		1.2
1+1	Undild	2.5
1+3	1+1	6.5
1+7	1+3	9.5
1+15	1+7	25
1+31	1+15	55
1+63	1+31	80
1+127	1+127	150
1+255	1+255	210

[a] Based on viable counts obtained with 6 strains in chicken broth.
[b] Diln which produces 1 mm zone of hemolysis in HI plate or one + reaction in LV test.

BACILLUS

980.31 *Bacillus cereus* in Foods

Enumeration and Confirmation Microbiological Methods

First Action 1980
Final Action 1981

A. Apparatus

(**a**) *Pipets.*—1.0 mL with 0.1 mL graduations; also 5.0 mL and 10.0 mL with 1.0 mL graduations.

(**b**) *Colony counter.*—Quebec, or equiv., dark field model.

(**c**) *High-speed blender.*—Waring blender, or equiv. 2 speed model with high-speed operation at 18,000–21,000 rpm, and 1 L glass or metal blender jars with covers. One jar is required for each sample.

(**d**) *Anaerobic jar.*—BBL GasPak jar equipped with GasPak H + CO_2 generator envelopes, or equiv.

(**e**) *Vortex mixer.*—Vortex Genie, or equiv.

(**f**) *Sterile bent glass spreading rods.*—Hockey stick or hoe shape with fire polished ends; 3–4 mm diam. with 45–55 mm spreading surface.

(**g**) *Inoculating loops.*—One each, 26 gage nichrome wire with loop 2 mm id and one 24 gage nichrome wire loop 3 mm id.

(**h**) *Staining rack.*—Rack must be accessible from below for heating slides.

B. Media and Reagents

(**a**) *Mannitol-egg yolk-polymyxin (MYP) agar.*—1.0 g beef ext, 10.0 g peptone, 10.0 g D-mannitol, 10.0 g NaCl, 0.025 g phenol red (as soln), and 15.0 g agar dild to 900 mL with H_2O. Adjust to pH 7.2 ± 0.1, heat to dissolve, and dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Cool to 50° in H_2O bath and add 12.5 mL egg yolk emulsion, (**b**), and 2.5 mL polymyxin B soln, (**c**), to each 225 mL medium. Mix well and dispense 18 mL portions into 100 × 15 mm sterile petri dishes. Dry plates 24 h at room temp. before use.

(**b**) *Egg yolk emulsion.*—50%. Wash fresh eggs with stiff brush and drain. Soak 1 h in 70% alcohol. Aseptically remove yolk and mix (1 + 1) with sterile 0.85% NaCl soln. (Difco egg yolk enrichment 50% is satisfactory).

(**c**) *Polymyxin B soln.*—Dissolve 500,000 units sterile polymyxin B sulfate in 50 mL sterile H_2O.

(**d**) *Trypticase-soy-polymyxin broth.*—17.0 g trypticase, 3.0 g phytone peptone, 5.0 g NaCl, 2.5 g K_2HPO_4, and 2.5 g dextrose dild to 1 L with H_2O. (Rehydrated trypticase soy broth

is satisfactory.) Boil 2 min. Dispense 15 mL portions into 150 × 20 mm tubes and autoclave 15 min at 121°. Final pH 7.3 ± 0.1. Just prior to use, add 0.1 mL 0.15% polymyxin B soln to each tube of medium and mix well. To make polymyxin B soln, dissolve 500,000 units sterile polymyxin B sulfate in 33.3 mL sterile H_2O.

(**e**) *Phenol red-dextrose broth.*—10.0 g proteose peptone No. 3, 1.0 g beef ext, 5.0 g NaCl, 0.018 g phenol red (as soln), and 5.0 g dextrose dild to 1 L with H_2O. (Phenol red dextrose broth, Difco 0093, is satisfactory). Dispense 3 mL portions into 100 × 13 mm tubes and autoclave 10 min at 121°. Final pH 7.4 ± 0.1.

(**f**) *Nitrate broth.*—3.0 g beef extract, 5.0 g peptone, and 1.0 g KNO_3 dild to 1 L with H_2O. (Rehydrated nitrate broth is satisfactory.) Adjust to pH 7.0 ± 0.1 and dispense 5 mL portions into 125 × 16 mm tubes. Autoclave 15 min at 121°.

(**g**) *Nutrient agar slants and plates.*—3.0 g beef ext, 5.0 g peptone, and 15.0 g agar dild to 1 L with H_2O (dehydrated nutrient agar is satisfactory). Heat to dissolve, and dispense 6.5 mL portions into 125 × 16 mm screw-cap tubes. Autoclave 15 min at 121° and slant tubes until medium solidifies. Final pH 6.8 ± 0.1. For plates, dispense 100–500 mL portions in bottles or flasks and autoclave 15 min at 121°. Cool to 50° in H_2O bath and dispense 18–20 mL portions in 100 × 15 mm sterile petri dishes. Dry plates 24–48 h at room temp. before use.

(**h**) *Nutrient agar with* L-*tyrosine.*—Prep. nutrient agar as in (**g**) and dispense 100 mL portions into bottles. Autoclave 15 min at 121°. Cool to 45° in H_2O bath and add 0.5 g sterile L-tyrosine suspended in 10 mL H_2O to each 100 mL of medium. Mix thoroly by rotating or inverting bottle and aseptically dispense 3.5 mL portions of complete medium into sterile 100 × 13 mm tubes. Slant tubes and cool rapidly to prevent sepn of tyrosine. To prep. L-tyrosine suspension, add 0.5 g to 150 × 20 mm tube and suspend in 10 mL H_2O with Vortex mixer. Autoclave 15 min at 121°.

(**i**) *Nutrient broth with lysozyme.*—3.0 g beef ext and 5.0 g peptone dild to 1 L with H_2O. (Rehydrated nutrient broth, Difco 0003, is satisfactory.) Dispense 99 mL portions in bottles and autoclave 15 min at 121°. Final pH 6.8 ± 0.1. Mix 1.0 mL 0.1% lysozyme soln with 99 mL broth and aseptically dispense 2.5 mL complete medium into sterile 100 × 13 mm tubes. To make lysozyme soln, dissolve 0.1 g lysozyme in 65 mL sterile 0.01*N* HCl, boil for 20 min, and dil. to 100 mL with sterile 0.01*N* HCl. Alternatively, dissolve 0.1 g lysozyme hydrochloride in 100 mL H_2O and sterilize with 0.45 μm membrane filter.

(**j**) *Modified Voges-Proskauer (VP) medium.*—7.0 g proteose peptone, 5.0 g dextrose, and 5.0 g NaCl dild to 1 L with H_2O. Dispense 5 mL portions into 150 × 20 mm tubes. Autoclave 10 min at 121°. Final pH 6.5 ± 0.1.

(**k**) *Motility medium.*—10.0 g trypticase, 2.5 g yeast ext, 5.0 g dextrose, 2.5 g Na_2HPO_4, and 3.0 g agar dild to 1 L with H_2O. Heat to dissolve. Dispense 2 mL portions into 13 × 100 mm tubes, and autoclave 10 min at 121°. Final pH 7.4 ± 0.2. Alternatively, dispense 100 mL amts in 150 mL bottles and autoclave 15 min at 121°. Cool at 50° and aseptically dispense 2 mL into sterile 13 × 100 mm tubes. For best results, store at room temp. 2–4 days before use to prevent growth along side of medium.

(**l**) *Trypticase-soy-sheep blood (TSSB) agar.*—Dil. 15.0 g trypticase, 5.0 g phytone peptone, 5.0 g NaCl, and 15.0 g agar to 1 L with H_2O. Adjust pH to 7.0 ± 0.2. Heat to boiling to dissolve, and dispense 100–500 mL portions in bottles or flasks. Autoclave 15 min at 121° and cool to 48° in H_2O bath. Add 5 mL sterile defibrinated sheep blood per 100 mL medium. Mix well, and dispense 18–20 mL portions into 100 × 15 mm petri dishes. (Trypticase-soy or tryptic-soy agar plates contg 5% sheep blood are satisfactory.)

(**m**) *Butterfield's buffered phosphate diluent.*—(*1*) *Stock soln.*—Dissolve 34.0 g KH_2PO_4 in 500 mL H_2O, adjust to pH 7.2 with ca 175 mL 1*N* NaOH, and dil. to 1 L with H_2O. Store in refrigerator. (*2*) *Diluent.*—Dil. 1.25 mL stock soln to 1 L with H_2O. Prep. 90 ± 1 mL diln blanks with this soln and autoclave 15 min at 121°.

(**n**) *Nitrite test reagents.*—(*1*) *Reagent A.*—Dissolve 8 g sulfanilic acid in 1 L 5*N* HOAc (2 + 5). (*2*) *Reagent B.*—Dissolve 2.5 g α-naphthol in 1 L 5*N* HOAc.

(**o**) *Voges-Proskauer (VP) test reagents.*—(*1*) *Alpha-naphthol soln.*—5%. Dissolve 5.0 g α-naphthol in 100 mL absolute alcohol. (*2*) *Potassium hydroxide soln.*—40%. Dissolve 40 g KOH in H_2O and dil. to 100 mL. (*3*) *Creatine crystals.*

(**p**) *Basic fuchsin stain.*—Dissolve 0.5 g basic fuchsin in 20 mL alcohol and dil. to 100 mL with H_2O. Filter soln if necessary thru fine paper to remove excess dye particles. Store in tightly stoppered container. (TB Carbol-fuchsin ZN stain is satisfactory.)

C. Preparation of Food Homogenate

Using aseptic technic, weigh 50 g food sample into sterile blender jar. Add 450 mL phosphate buffered diln H_2O and homogenize 2 min at high speed (ca 20,000 rpm). Use this 1:10 diln to prep. serial dilns from 10^{-2} to 10^{-6} by transferring 10 mL of 1:10 diln to 90 mL diln blank, mixing well with vigorous shaking, and continuing until 10^{-6} is reached.

D. Plate Count Technic

Inoculate duplicate MYP agar plates with each diln of homogenate by spreading 0.1 mL evenly onto each plate with sterile glass rod spreader. Incubate plates 24 h at 30° and check for colonies surrounded by ppt zone indicating lecithinase is produced. *B. cereus* colonies usually are pink which becomes more intense after addnl incubation. If reactions are not clear, incubate plates for addnl 24 h before counting.

Select plates showing estd 15–150 eosin pink colonies surrounded by lecithinase zones. Mark bottom of plates into zones with black felt pen to facilitate counting and count colonies. This is the presumptive count of *B. cereus*/g of food. Pick 5 or more colonies from plates counted and transfer to nutrient agar slants for confirmation tests.

E. Most Probable Number Technic

(For foods containing $\leq 10^3$ *B. cereus*/g)

Inoculate 3-tube most probable number (MPN) series in trypticase-soy-polymyxin broth, (**d**), using 1 mL inocula of 1:10, 1:100, and 1:1000 dilns with triplicate tubes at each diln. Incubate 48 ± 2 h at 30° and examine tubes for dense growth typical of *B. cereus*. Streak pos. tubes on sep. MYP agar plates, (**a**), and incubate 24–48 h at 30°. Pick 1 or more eosin pink colonies surrounded by ppt zone due to lecithinase from each plate and transfer to nutrient agar slants for confirmation tests. Confirm as *B. cereus* and compute MPN of *B. cereus*/g using Table **966.24** on basis of number of tubes in which *B. cereus* was present.

F. Confirmation Technic

Pick ≥5 presumptive pos. colonies from MYP agar plates and transfer to nutrient agar slants. Incubate 24 h at 30°. Make Gram stained smears from slants and examine microscopically. *B. cereus* will appear as large Gram pos. bacilli in short to long chains; spores are ellipsoidal, central to subterminal, and do not swell sporangium.

Transfer 3 mm loopful culture from each slant to 100 × 13

mm tube contg 0.5 mL sterile phosphate buffered diln H_2O and suspend culture in diluent with Vortex mixer. Inoculate following media with suspended culture:

(**a**) *Phenol red dextrose broth,* (***e***).—Inoculate broth with 2 mm loopful culture and incubate anaerobically 24 h at 35° in Gas-Pak anaerobic jar. Shake tubes and check for growth. Change from red to yellow indicates acid was produced from dextrose anaerobically.

(**b**) *Nitrate broth,* (*f*).—Inoculate with 3 mm loopful culture and incubate 24 h at 35°. Test for presence of nitrite by adding 0.25 mL nitrite test reagent A and 0.25 mL reagent B. Orange which develops within 10 min indicates presence of nitrites.

(**c**) *Modified VP medium,* (*j*).—Inoculate with 3 mm loopful of culture and incubate 48 h at 35°. Transfer 1 mL culture to empty tube to test for acetylmethylcarbinol. Add 0.2 mL 40% KOH soln, 0.6 mL 5% alc. α-naphthol soln, and few crystals creatine. Let stand 1 h. Test is pos. if eosin pink develops.

(**d**) *Nutrient agar with* L-*tyrosine,* (***h***).—Inoculate entire surface of slant with 3 mm loopful of culture. Incubate 48 h at 35°. Check for clearing of medium near growth indicating tyrosine is decomposed. Check neg. tubes for growth and incubate addnl 72 h before discarding.

(**e**) *Nutrient broth with lysozyme,* (*i*).—Inoculate nutrient broth contg 0.001% lysozyme with 2 mm loopful of culture; also inoculate control tube of plain nutrient broth. Incubate 24 h at 35° and record growth as + or −. Incubate neg. tubes addnl 24 h before discarding.

(**f**) *MYP agar,* (***a***).—(Test may be omitted if reactions of all isolates on MYP agar plates were typical.) Inoculate premarked 4 sq. cm area of MYP agar plate by gently touching surface with 2 mm loopful of culture. Let inoculum be absorbed and incubate 24 h at 35°. Check for lecithinase production as indicated by zone of ppt surrounding growth. Mannitol fermentation is neg. if growth and surrounding medium are eosin pink.

Large Gram pos. bacilli which produce lecithinase and are neg. for mannitol fermentation on MYP agar, grow and produce acid from dextrose anaerobically, reduce nitrate to nitrite, produce acetylmethylcarbinol, decompose L-tyrosine, and grow in the presence of 0.001% lysozyme are provisionally identified as *B. cereus.* (These characteristics are shared by all members of *B. cereus* group. See Differentiation of Members of *Bacillus cereus* Group, **983.26**.)

Calc. number of *B. cereus* in sample on basis of % colonies tested that are confirmed as *B. cereus.* (*Example:* If av. plate count with 10^{-4} diln of sample was 65 and 4 of 5 colonies tested were confirmed as *B. cereus,* number of *B. cereus*/g food is $65 \times (4/5) \times 10{,}000 \times 10 = 5{,}200{,}000$.) (Diln factor is 10-fold higher than sample diln because only 0.1 mL was tested.)

Ref.: JAOAC **63,** 581(1980).

983.26 Differentiation of Members of *Bacillus cereus* Group

Microbiological Method

First Action 1983
Final Action 1984

(Typical strains of *B. cereus* isolated from foods by **980.31** can be differentiated from other members of *B. cereus* group including: (1) insect pathogen *B. thuringiensis,* (2) mammalian pathogen *B. anthracis,* and (3) rhizoid strains of *B. cereus* var. *mycoides.*)

A. Apparatus

(**a**) *Staining rack.*—Rack must be accessible from below for heating slides.

(**b**) *Inoculating loops.*—One each, 26 gage nichrome wire with loop 2 mm id and one 24 gage nichrome wire loop 3 mm id.

B. Media and Reagents

(**a**) *Mannitol-egg yolk-polymyxin (MYP) agar.*—1.0 g beef ext, 10.0 g peptone, 10.0 g D-mannitol, 10.0 g NaCl, 0.025 g phenol red (as soln), and 15.0 g agar dild to 900 mL with H_2O. Adjust to pH 7.2 ± 0.1, heat to dissolve, and dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Cool to 50° in H_2O bath and add 12.5 mL sterile 50% egg yolk emulsion (**b**) and 2.5 mL polymyxin B soln contg 10 000 units per mL (if available) to each 225 mL medium. (Addn of polymyxin B soln is optional when medium is to be used for testing reactions of pure cultures.) Mix well and dispense 18 mL portions into 100 × 15 mm sterile petri dishes. Dry plates 24 h at room temp. before use. (Dehydrated mannitol-egg yolk-polymyxin (MYP) agar contg 50% egg yolk enrichment is satisfactory.)

(**b**) *Egg yolk emulsion.*—50%. Wash fresh eggs with stiff brush and drain. Soak 1 h in 70% alcohol. Aseptically remove yolk and mix (1 + 1) with sterile 0.85% NaCl soln. (50% egg yolk enrichment is satisfactory.)

(**c**) *Nutrient agar slants and plates.*—3.0 g beef ext, 5.0 g peptone, and 15.0 g agar dild to 1 L with H_2O (dehydrated nutrient agar is satisfactory). Heat to dissolve, and dispense 6.5 mL portions into 125 × 16 mm screw-cap tubes. Autoclave 15 min at 121° and slant tubes until medium solidifies. Final pH 6.8 ± 0.2. For plates, dispense 100–500 mL portions in bottles or flasks and autoclave 15 min at 121°. Cool to 50° in H_2O bath and dispense 18–20 mL portions in 100 × 15 mm sterile petri dishes. Dry plates 24–48 h at room temp. before use.

(**d**) *Motility medium.*—10.0 g trypticase, 2.5 g yeast ext, 5.0 g dextrose, 2.5 g Na_2HPO_4, and 3.0 g agar dild to 1 L with H_2O. Heat to dissolve. Dispense 2 mL portions into 13 × 100 mm tubes, and autoclave 10 min at 121°. Final pH 7.4 ± 0.2. Alternatively, dispense 100 mL amts in 150 mL bottles and autoclave 15 min at 121°. Cool to 50° and aseptically dispense 2 mL into sterile 13 × 100 mm tubes. For best results, store at room temp. 2–4 days before use to prevent growth along side of medium.

(**e**) *Trypticase-soy-sheep blood (TSSB) agar.*—Dil. 15.0 g trypticase, 5.0 g phytone peptone, 5.0 g NaCl, and 15.0 g agar to 1 L with H_2O. Adjust pH to 7.0 ± 0.2. Heat to boiling to dissolve, and dispense 100–500 mL portions in bottles or flasks. Autoclave 15 min at 121° and cool to 48° in H_2O bath. Add 5 mL sterile defibrinated sheep blood per 100 mL medium. Mix well, dispense 18–20 mL portions into 100 × 15 mm petri dishes. (Trypticase-soy or tryptic-soy agar plates contg. 5% sheep blood are satisfactory.)

(**f**) *Basic fuchsin stain.*—Dissolve 0.5 g basic fuchsin in 20 mL alcohol and dil. to 100 mL with H_2O. Filter soln if necessary thru fine paper to remove excess dye particles. Store in tightly stoppered container. (TB Carbol-fuchsin ZN stain is satisfactory.)

(**g**) *Butterfield's buffered phosphate diluent.*—(*1*) *Stock soln.*—Dissolve 34.0 g KH_2PO_4 in 500 mL H_2O, adjust to pH 7.2 with ca 175 mL 1N NaOH, and dil. to 1 L with H_2O. Store in refrigerator. (*2*) *Diluent.*—Dilute 1.25 mL stock soln to 1 L with H_2O. Prep. 90 ± 1 mL diln blanks with this soln and autoclave 15 min at 121°. Dispense 0.5 mL portions sterile diluent into sterile 13 × 100 mm tubes for preparing suspension of cultures to be tested.

(**h**) *Methanol fixative.*—Dispense undild methanol in plastic squeeze bottle for use in fixing slides.

C. Differential Tests

(**a**) *Preparing test inoculum.*—Inoculate sep. nutrient agar slants with each culture to be tested. Incubate slants 18–24 h at 30° and transfer 3 mm loopful of culture from each slant to 100 × 13 mm tube contg 0.5 mL sterile phosphate buffered diluent. Suspend culture in diluent with vortex mixer. Alternatively, inoculate 5 mL trypticase-soy broth and incubate tubes 18 h at 30°. Mix culture well and use for performing differential tests. Latter procedure is preferred for rhizoid strains and other strains which do not disperse well in phosphate buffer.

(**b**) *Reaction on MYP agar.*—Mark bottom of MYP agar plate into 6–8 equal segments with black felt pen as indicated in Fig. **983.26** and label each section. Place plate in upright position on piece of white paper and inoculate one or more of the prelabeled sections by gently touching surface of agar with 2 mm loopful of culture. Let inoculum be absorbed and incubate plates in upright position 24–48 h at 30–35°. Check for lecithinase production as indicated by zone of ppt surrounding growth. Mannitol fermentation is neg. if growth and surrounding medium are eosin pink. These reactions should be observed with all organisms of *B. cereus* group except rare lecithinase-neg. variants.

(**c**) *Motility tests.*—Inoculate BC motility medium by stabbing down center with 3 mm loopful of culture. Incubate 18–20 h at 30° and examine for type of growth along stab. Motile strains produce diffuse growth into medium away from stab. Nonmotile strains except *B. cereus* var. *mycoides* grow only in and along stab. Strains of *B. cereus* var. *mycoides* often produce "fuzzy" growth in semisolid media resulting from cellular expansion but are not motile by means of flagella. Recheck doubtful results by alternative microscopic motility test as follows: Add 0.2 mL sterile H_2O to nutrient agar slant and inoculate with 3 mm loopful of culture. Incubate slant 6–8 h at 30°, and mix small loopful of liq. culture from base of slant with drop of sterile H_2O on microscope slide. Apply cover glass and examine immediately for signs of motility. *B. cereus* and *B. thuringiensis* cultures are usually actively motile by means of peritrichous flagella. *B. anthracis* and typically rhizoid strains of *B. cereus* var. *mycoides* are nonmotile.

(**d**) *Rhizoid growth.*—Inoculate predried nutrient agar plate by touching medium surface near center with 2 mm loopful of culture. Let inoculum be absorbed, and incubate plate in upright position 24–48 h at 30°. Check plate for rhizoid growth characterized by root or hairlike structures which may extend several cm from point of inoculation. Many *B. cereus* strains produce rough irregular colonies that should not be confused with rhizoid growth. This property is characteristic only of strains which are classified as *B. cereus* var. *mycoides*.

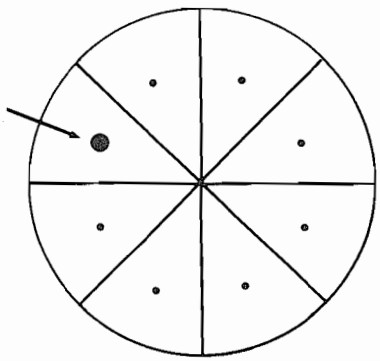


FIG. 983.26—Diagram of template for marking and inoculating *B. cereus* confirmatory plates. Each section is labeled and inoculated in the center, as indicated by arrow

(**e**) *Hemolytic activity.*—Mark bottom of trypticase-soy-sheep blood agar plate into 6–8 equal segments (see Fig. **983.26**) with black felt marking pen. Label each segment and inoculate one or more segments near center by gently touching agar surface with 2 mm loopful of culture. Let inoculum be absorbed, and incubate plates 24 h at 30–32°. Check plates for hemolytic activity as indicated by 2–4 mm zone of complete (beta) hemolysis surrounding growth. *B. cereus* is usually strongly hemolytic, whereas *B. thuringiensis* and *B. cereus* var. *mycoides* are often weakly hemolytic and produce complete hemolysis only underneath colonies. *B. anthracis* is usually nonhemolytic after 24 h of incubation. *Caution:* Nonmotile, nonhemolytic cultures could be *B. anthracis*. See precautions under *interpreting test results,* (**g**).

(**f**) *Detection of toxin crystals.*—Inoculate nutrient agar slant with loopful of culture. Incubate slant 24 h at 30° and hold at room temp. 2–3 days. Make smear on microscope slide with sterile H_2O. Air-dry and briefly heat-fix by passing slide slowly over burner flame; let cool, and place slide on staining rack. Flood slide with methanol, wait 30 s, and pour off methanol. Dry thoroughly by passing through burner flame. Return slide to staining rack, and flood completely with 0.5% aq. soln of basic fuchsin or TB Carbol-fuchsin ZN stain. Heat slide gently from below with micro burner until steam is seen. Wait 1–2 min and repeat this step. Let stand 30 s, pour off stain, and rinse slide thoroughly in 1 L clean tap H_2O. Dry slide without blotting and examine microscopically under oil immersion for presence of free spores and darkly stained tetragonal (diamond-shaped) toxin crystals. Free toxin crystals are usually abundant after 3 days but will not be detectable unless sporangia have lysed. Therefore, if free spores are not seen, leave cultures at room temp. for a few more days and repeat test. *B. thuringiensis* produces protein toxin crystals that usually can be detected by staining, but are not produced by other members of *B. cereus* group.

(**g**) *Interpreting test results.*—On basis of test results, identify as *B. cereus* those isolates which are actively motile, strongly hemolytic, and do not produce rhizoid growth or protein toxin crystals. Nonmotile strains of *B. cereus* may be encountered and a few are weakly hemolytic. These strains can be differentiated from *B. anthracis* by their resistance to penicillin and to gamma bacteriophage. *Caution:* Nonmotile, nonhemolytic strains could be *B. anthracis,* and should be handled with special care and submitted to pathology laboratory such as Centers for Disease Control for identification or destroyed by autoclaving. Noncrystalliferous variants of *B. thuringiensis* and nonrhizoid strains derived from *B. cereus* var. *mycoides* cannot be differentiated from *B. cereus* by tests described.

Ref.: JAOAC **65,** 1134(1982).

SALMONELLA

967.25 *Salmonella* in Foods

Preparation of Culture Media and Reagents

First Action 1967
Final Action 1970

(Applicable to the detection and identification of *Salmonella* from dried active yeast, dried whole egg, dried egg yolk, and dried egg white, edible casein, milk chocolate, nonfat dry milk and dry whole milk, and onion and garlic powders. Method described is minimal. Depending upon history of sample, addnl

types of examinations may be applied. Use *Edwards and Ewing's Identification of Enterobacteriaceae*, W. H. Ewing, Elsevier Science Publishing Co., Inc., New York, NY 10017, 4th ed., 1986, as guide for further study of isolated microorganisms. For food sampling plans and initial sample handling, refer to Chapter 1, *Bacteriological Analytical Manual*, 6th ed., 1984.)

A. Preparation

(Sizes of culture media containers (test tubes, flasks, and petri dishes) are specified in prepn of each medium. Different size containers may be used if they give identical results. All media containers must have covers, caps, or plugs which prevent contamination but maintain aerobic conditions unless otherwise directed.)

(**a**) *Lactose broth.*—*See* **940.36A(f)**. Dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Aseptically det. vol. and adjust, if necessary, to 225 mL. Final pH, 6.7 ± 0.2.

(**b**)(*1*) *Selenite cystine broth.*—Suspend 5.0 g tryptone or polypeptone, 4.0 g lactose, 10.0 g Na_2HPO_4, 4.0 g $NaHSeO_3$, and 0.01 g L-cystine in 1 L H_2O and mix thoroly. Heat with frequent agitation. Dispense 10 mL portions into sterile 16 × 150 mm test tubes. Heat 10 min in flowing steam. *Do not autoclave*. Final pH, 7.0 ± 0.2. Medium is not sterile. Use same day as prepd.

(*2*) *Selenite cystine broth* (*North and Bartram*).—Prep. as in (*1*), using 5.0 g polypeptone or 4.0 g tryptone, 4.0 g lactose, 4.0 g $NaHSeO_3$, 5.5 g Na_2HPO_4, 4.5 g KH_2PO_4, and 1 mL 1% L-cystine (10 mg) soln prepd by dissolving 1.0 g L-cystine in 15 mL 1*N* NaOH and dilg to 100 mL with sterile H_2O.

(**c**) *Tetrathionate broth* (*with iodine and brilliant green*).—Suspend 5.0 g polypeptone, 1.0 g bile salts, 10 g $CaCO_3$, and 30 g $Na_2S_2O_3.5H_2O$ in 1 L H_2O, mix thoroly, and heat to bp. (Ppt will not dissolve completely.) Cool to <45° and store at 5–8°. Prep. I-KI soln by dissolving 5 g KI in 5 mL sterile H_2O, adding 6 g resublimed I, dissolving, and dilg to 20 mL with sterile H_2O. Prep. brilliant green soln by dissolving 0.1 g dye in sterile H_2O and dilg to 100 mL. On day medium is used, add 20 mL I-KI soln and 10 mL brilliant green soln per 1 L basal broth. Resuspend ppt by gentle agitation and aseptically dispense 10 mL portions into 20 or 16 × 150 mm sterile test tubes. *Do not heat medium after addn of I-KI and dye solns*.

(**d**) *Xylose lysine desoxycholate agar* (*XLD*).—Suspend ingredients (*1*) or (*2*) (varies with mfgr of formula) in 1 L H_2O and mix thoroly. Heat with frequent agitation just until medium boils. Do not overheat. Cool in H_2O bath and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with covers partially removed; then close plates. Final pH, 7.4 ± 0.2. Do not autoclave.

(*1*) 3.5 g xylose, 5.0 g L-lysine, 7.5 g lactose, 7.5 g sucrose, 5.0 g NaCl, 3.0 g yeast ext, 0.08 g phenol red, 2.5 g Na desoxycholate, 6.8 g Na thiosulfate, 0.8 g ferric ammonium citrate, and 13.5 g agar.

(*2*) 3.75 g xylose, 5.0 g L-lysine, 7.5 g lactose, 7.5 g sucrose, 5.0 g NaCl, 3.0 g yeast ext, 0.08 g phenol red, 2.5 g Na desoxycholate, 6.8 g Na thiosulfate, 0.8 g ferric ammonium citrate, and 15 g agar.

(**e**) *Hektoen enteric agar* (*HE*).—Suspend ingredients (*1*) or (*2*) (varies with mfgr of formula) in 1 L H_2O and mix thoroly. Heat to boiling with frequent agitation and let boil few moments. Do not overheat. Cool in H_2O bath and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with covers partially removed; then close plates. Final pH, 7.6 ± 0.2. Do not autoclave.

(*1*) 12.0 g thiotone peptone, 3.0 g yeast ext, 9.0 g bile salts, 12.0 g lactose, 12.0 g sucrose, 2.0 g salicin, 5.0 g NaCl, 5.0 g Na thiosulfate, 1.5 g ferric ammonium citrate, 0.064 g bromthymol blue, 0.1 g acid fuchsin, and 13.5 g agar.

(*2*) 12.0 g proteose peptone, 3.0 g yeast ext, 9.0 g bile salts No. 3, 12.0 g lactose, 12.0 g sucrose, 2.0 g salicin, 5.0 g NaCl, 5.0 g Na thiosulfate, 1.5 g ferric ammonium citrate, 0.065 g thymol blue, 0.1 g acid fuchsin, and 14.0 g agar.

(**f**) *Bismuth sulfite (BS) agar* (*Wilson and Blair*).—Suspend 10 g polypeptone or peptone, 5.0 g beef ext, 5.0 g glucose, 4.0 g Na_2HPO_4, 0.3 g $FeSO_4$, 8.0 g $Bi_2(SO_3)_3$ indicator, 0.025 g brilliant green, and 20 g agar in 1 L H_2O, mix thoroly, and heat with occasional agitation. Boil ca 1 min to obtain uniform suspension. (Ppt will not dissolve.) Cool to 45–50°. Suspend ppt by gentle agitation and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 hr with covers partially removed; then close plates. Final pH, 7.6 ± 0.2. *Do not autoclave*. Prepare plates day before streaking and store in dark at room temp. Selectivity of plates decreases 48 hr after prepn.

(**g**) *Triple sugar iron agar* (*TSI agar*).—Suspend ingredients (*1*) or (*2*) in 1 L H_2O, mix thoroly, and heat with occasional agitation. Boil ca 1 min until ingredients dissolve. Fill 16 × 150 mm tubes $^1/_3$ full and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca 3 cm) and adequate slants (ca 5 cm) are formed on solidification.

(*1*) 20 g polypeptone, 5.0 g NaCl, 10 g lactose, 10 g sucrose, 1 g glucose, 0.2 g $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.2 g $Na_2S_2O_3$, 0.025 g phenol red, and 13 g agar. Final pH, 7.3 ± 0.2.

(*2*) 3.0 g beef ext, 3.0 g yeast ext, 15 g peptone, 5.0 g proteosepeptone, 1.0 g glucose, 10 g lactose, 10 g sucrose, 0.2 g $FeSO_4$, 5.0 g NaCl, 0.3 g $Na_2S_2O_3$, 0.024 g phenol red, and 12 g agar. Final pH, 7.4 ± 0.2.

(**h**) *Tryptophane broth.*—*See* **940.36A(h)**. Use 16 or 20 × 150 mm test tubes.

(**i**) *Buffered glucose broth* (*MR-VP medium*).—*See* **940.36A(b)**. Use 16 or 20 × 150 mm test tubes.

(**j**) *Simmons' citrate agar.*—Dissolve 2.0 g Na citrate, 5.0 g NaCl, 1.0 g K_2HPO_4, 1.0 g $NH_4H_2PO_4$, 0.2 g $MgSO_4$, 0.08 g bromothymol blue, and 15 g agar in 1 L H_2O, and heat gently with occasional agitation. Boil 1–2 min until ingredients dissolve. Final pH, 6.9 ± 0.2. Fill 13 × 100 or 16 × 150 mm test tubes $^1/_3$ full and cap or plug so that aerobic conditions are maintained during use. Autoclave 15 min at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca 2 or 3 cm, resp.) and adequate slants (ca 4 or 5 cm, resp.) are formed on solidification.

(**k**)(*1*) *Urea broth.*—Dissolve 20 g urea, 0.1 g yeast ext, 9.1 g KH_2PO_4, 9.5 g Na_2HPO_4, and 4.0 mL 0.25% phenol red (10 mg) soln in 1 L H_2O. *Do not heat*. Sterilize by filtration and aseptically dispense 1.5–3 mL portions into 13 × 100 mm sterile test tubes. Final pH, 6.8 ± 0.2.

(*2*) *Rapid urea broth.*—Prep. as in (*1*), using 0.091 and 0.095 g phosphate salts, resp.

(**l**) *Malonate broth.*—Dissolve 1.0 g yeast ext, 2.0 g $(NH_4)_2SO_4$, 0.6 g K_2HPO_4, 0.4 g KH_2PO_4, 2.0 g NaCl, 3.0 g Na malonate, 0.25 g glucose, and 0.025 g bromothymol blue in 1 L H_2O, heating if necessary until dissolved. Dispense 3 mL portions into 13 × 100 mm test tubes and autoclave 15 min at 121°. Final pH, 6.7 ± 0.2.

(**m**)(*1*) *Lysine iron agar* (*Edwards and Fife*).—Dissolve 5.0 g gelysate or peptone, 3.0 g yeast ext, 1.0 g glucose, 10 g L-lysine, 0.5 g ferric ammonium citrate, 0.04 g anhyd. $Na_2S_2O_3$,

0.02 g bromocresol purple, and 15 g agar in 1 L H_2O, heating until dissolved. Dispense 4 mL portions into 13 × 100 mm test tubes and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min at 121°. Before medium solidifies, place tubes in slanted position so that 4 cm butts and 2.5 cm slants are formed on solidification. Final pH, 6.7 ± 0.2.

(*2*) *Lysine decarboxylase broth* (*Falkow*).—Dissolve 5.0 g gelysate or peptone, 3.0 g yeast ext, 1.0 g glucose, 5.0 g L-lysine, and 0.02 g bromocresol purple in 1 L H_2O, heating until dissolved. Dispense 10 mL portions into 16 × 125 mm screw-cap test tubes. Autoclave, loosely capped, 15 min at 121°. Screw caps on tightly for storage and after inoculation. Final pH, 6.5–6.8.

(**n**) *Motility test medium* (*semisolid medium*).—Dissolve 3.0 g beef ext, 10 g peptone or gelysate, 5.0 g NaCl, and 4.0 g agar in 1 L H_2O and heat gently with occasional agitation. Boil 1–2 min to dissolve. If medium is to be stored, dispense 20 mL portions into screw-cap containers, replacing caps loosely. Autoclave 15 min at 121°. Cool to 45°. To store, screw caps on tightly and refrigerate at 5–8°. To use, remelt in boiling H_2O or flowing steam and cool to 45°. Aseptically dispense 20 mL portions into 15 × 100 mm petri dishes and let solidify with dish completely covered. Use plates same day as prepd. Final pH, 7.4 ± 0.2.

(**o**) *Potassium cyanide* (*KCN*) *broth*.—Dissolve 3.0 g proteose peptone No. 3 or polypeptone, 5.0 g NaCl, 0.225 g KH_2PO_4, and 5.64 g Na_2HPO_4 in 1 L H_2O. Autoclave 15 min at 121°. Cool and refrigerate at 5–8°. Final pH, 7.6 ± 0.2. Dissolve 0.5 g KCN in 100 mL cold (5–8°) sterile H_2O. Using sterile bulb pipet or sterile syringe (*do not pipet by mouth*), aseptically add 15 mL cold KCN soln per L cold, sterile basal broth. Mix thoroly with gentle agitation and aseptically dispense 1.0–1.5 mL portions into sterile 13 × 100 mm test tubes. Using aseptic technic, immediately stopper tubes with No. 2 corks impregnated with paraffin. Prep. corks by boiling in paraffin ca 5 min. Place corks in tubes so that paraffin does not flow into broth but forms good seal between rim of tube and cork. Medium stored at 5–8° is usually stable ca 2 weeks.

(**p**)(*1*) *Phenol red carbohydrate broth*.—Dissolve 10 g trypticase or proteose peptone No. 3, 5.0 g NaCl, 1.0 g beef ext (optional), and 7.2 mL 0.25% phenol red (18 mg) soln in 1 L H_2O and heat with gentle agitation until dissolved. Dissolve 5 g dulcitol, 10 g lactose, or 10 g sucrose (as specified in title of test) in this basal broth. Dispense 2.5 mL portions into 13 × 100 mm test tubes contg inverted 6 × 50 mm fermentation tubes. Autoclave 10 min at 118° (12 psi). Final pH, 7.4 ± 0.2. Alternatively, dissolve ingredients, omitting carbohydrate, in 800 mL H_2O with heat and occasional agitation. Dispense 2.0 mL portions into 13 × 100 mm test tubes contg inverted fermentation tubes. Autoclave 15 min at 118° and let cool. Dissolve carbohydrate in 200 mL H_2O and sterilize by passing soln thru bacteria-retaining filter. Aseptically add 0.5 mL sterile filtrate to each tube of sterilized broth after cooling to <45°. Shake gently to mix. Final pH, 7.4 ± 0.2.

(*2*) *Purple carbohydrate broth*.—Prep. as in (*1*), using as basal broth 10 g proteose peptone No. 3 or gelysate, 5.0 g NaCl, and 0.015 or 0.020 g bromocresol purple. Final pH, 6.8 ± 0.2.

(**q**) *MacConkey agar*.—Suspend 3.0 g proteose peptone or polypeptone, 17 g peptone or gelysate, 10 g lactose, 1.5 g bile salts No. 3 or bile salts mixt., 5.0 g NaCl, 3.0 mL 1% neutral red (30 mg) soln, 1 mL 0.1% crystal violet (1.0 mg) soln, and 13.5 g agar in 1 L H_2O and mix thoroly until homogeneous. Heat, with occasional agitation, and boil 1–2 min until ingredients dissolve. Autoclave 15 min at 121°. Cool to 45–50° and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ≥2 hr with plates covered. Do not use wet plates. Final pH, 7.1 ± 0.2.

(**r**) *Brain-heart infusion broth*.—Dissolve infusion from 200 g calf brain and from 250 g beef heart, 10.0 g proteose peptone or gelysate, 5.0 g NaCl, 2.5 g $Na_2HPO_4.12H_2O$, and 2.0 g glucose in 1 L H_2O, heating gently if necessary. Dispense 5 mL portions into 16 × 150 mm test tubes and autoclave 15 min at 121°. Final pH, 7.4 ± 0.2.

(**s**) *Trypticase soy-tryptose broth*.—Combine 15 g com. dehydrated trypticase soy broth medium (contg 17.0 g trypticase, 3.0 g phytone, 5.0 g NaCl, 2.5 g K_2HPO_4, and 2.5 g glucose), 13.5 g com. dehydrated tryptose broth medium (contg 20 g tryptose, 5 g NaCl, and 1.0 g glucose), 3 g yeast ext, and 1 L H_2O. Heat, if necessary, until dissolved. Dispense 5 mL portions into 16 × 150 mm test tubes and autoclave 15 min at 121°. Final pH, 7.2 ± 0.2.

(**t**) *Trypticase* (*tryptic*) *soy broth*.—Suspend 17.0 g trypticase or tryptose (pancreatic digest of casein), 3.0 g phytone (papaic digest of soya meal), 5.0 g NaCl, 2.5 g K_2HPO_4, and 2.5 g glucose in 1 L H_2O. Heat gently to dissolve completely. Dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Aseptically det. vol. and adjust, if necessary, to 225 mL. Final pH, 7.3 ± 0.2.

(**u**) *Lauryl sulfate tryptose broth*.—Prep. as **966.23A(b)**, but without inverted fermentation tubes, 10 × 75 mm.

(**v**) *Reconstituted nonfat dry milk with brilliant green dye* (*NFDM-BG*).—Suspend 100 g dehydrated NFDM in 1 L H_2O; mix by swirling until dissolved. Autoclave 15 min at 121°. Add brilliant green dye soln, **966.24(n)**, after blending sample/broth mixture as in **978.23A(e)**.

(**w**) *Brilliant green* (*BG*) *water*.—Prep. sterile H_2O as **966.24(m)** and add 2 mL 1% aq. brilliant green dye, **966.24(n)**, per L sterile H_2O and mix well.

B. Diagnostic Reagents

(**a**) *Kovacs reagent for indole test*.—Dissolve 5 g *p*-dimethylaminobenzaldehyde in 75 mL amyl alcohol and slowly add 25 mL HCl.

(**b**) *Voges-Proskauer* (*VP*) *test reagents*.—(*1*) *Alpha-naphthol soln*.—5%. Dissolve 5.0 g α-naphthol in 100 mL absolute alcohol.

(*2*) *Potassium hydroxide soln*.—40%. Dissolve 40 g KOH in H_2O and dil. to 100 mL.

(**c**) *Sodium hydroxide soln*.—1*N*. Dissolve 42.11 g 95% reagent NaOH in sterile H_2O and dil. to 1 L.

(**d**) *Hydrochloric acid soln*.—1*N*. Dil. 89 mL to 1 L with sterile H_2O.

(**e**) *Methyl red indicator*.—Dissolve 0.10 g Me red in 300 mL alcohol and dil. to 500 mL with H_2O.

(**f**) *Sterile physiological saline soln*.—*See* **940.36B(c)**.

(**g**) *Formalinized physiological saline soln*.—Add 6 mL HCHO soln (36–38%) to 1 L sterile saline soln, (**f**), mix, and store in tightly stoppered containers.

(**h**) *Salmonella polyvalent somatic* (*O*) *antiserum**.—("Serological Identification of the *Salmonella* Serotypes," No. 1229, Difco Laboratories, November 1977, p. 13, or equiv.) Contains agglutinins for at least somatic (O) antigens 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 19, 22, 23, 24, 25, 34, and Vi. They are agglutinins for somatic (O) groups: A, B, C_1, C_2, D, E_1, E_2, E_3, E_4, F, G_1, G_2, H, I, and Vi.

(**i**) *Salmonella individual somatic* (*O*) *antisera**.—(*See* ref.

* Conform to specifications issued by Diagnostic Products Evaluation Branch, Biological Products Div., Bureau of Laboratories, Centers for Disease Control, Atlanta, GA 30333.

in (**h**).) For at least each of the somatic (O) groups listed in (**h**).

(**j**) *Salmonella polyvalent flagellar (H) antiserum Poly a-z*.*—(*See* p. 12 of ref. in (**h**).) Contains agglutinins for at least the following flagellar (H) antigens: a, b, c, d, e, f, g, h, i, k, l, m, n, p, q, r, s, t, u, v, w, x, y, z, z_4, z_6, z_{10}, z_{13}, z_{15}, z_{23}, z_{24}, z_{28}, z_{29}, z_{32}, 1, 2, 5, 6, 7.

(**k**) *Salmonella "Spicer-Edwards" flagellar (H) antisera*.*—(From pp. 11 and 12 of ref. in (**h**).) Consists of 7 pooled or polyvalent antisera which react as in Table **967.25**.

(**l**) *pH Test paper.*—Min. range 6.0–7.6 with max. gradations of 0.4 pH unit per color change.

(**m**) *Sterile distilled water.*—Dispense 1 L H_2O into 2 L wide-mouth flask or wide-mouth jar; plug or cap loosely. Autoclave 20 min at 121°.

(**n**) *Brilliant green dye soln.*—1%. Dissolve 1 g in sterile H_2O and dil. to 100 mL. (Since some batches of dye are unusually toxic, test all batches of dye before use and use only those producing satisfactory results when tested with known pos. and neg. test organisms.)

(**o**) *Bromcresol purple soln.*—0.2%. Dissolve 0.2 g in sterile H_2O and dil. to 100 mL.

Refs.: JAOAC **50,** 753(1967); **51,** 870(1968); **52,** 455(1969); **56,** 1027(1973); **59,** 731(1976); **62,** 499(1979); **64,** 893(1981); **64,** 899(1981); **65,** 356(1982).

967.26 *Salmonella* in Foods

Detection

First Action 1967
Final Action 1974

A. Preparation of Sample

(**a**) *Dried whole egg, dried egg yolk, and dried egg white.*—Aseptically open sample container and aseptically weigh 25 g sample into sterile, empty, wide-mouth, screw-cap pt (500 mL) jar. Add ca 15 mL sterile lactose broth, **967.25A(a)**. Stir with sterile glass rod, sterile spoon, or sterile tongue depressor to smooth suspension. Add 3 addnl portions lactose broth, 10, 10, and 190 mL for total of 225 mL. Stir after each addn until sample is suspended without lumps. Cap jar securely and let stand at room temp. 60 min. Mix well by shaking, and det. pH with test paper, **967.25B(l)**. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1*N* NaOH or HCl, **967.25B(c)** or (**d**), capping jar securely and mixing well before detg final pH. Loosen jar cap ca $^1/_4$ turn and incubate 24 ± 2 hr at 35°.

(**b**) *Dry whole milk.*—Aseptically weigh 25 g sample into sterile, wide-mouth screw-cap 500 mL (1 pt) jar. Add 225 mL sterile H_2O and mix well. Cap jar securely and let stand 60 min at room temp. Mix well by swirling and det. pH with test paper, **967.25B(l)**. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1*N* NaOH or HCl, **967.25B(c)** or (**d**). Add 0.45 mL of 1% aq. brilliant green dye soln, **967.25B(n)**, and mix well. Loosen jar cap ca $^1/_4$ turn and incubate 24 ± 2 hr at 35°.

(**c**) *Dried active yeast.*—Aseptically weigh 25 g sample into sterile, empty, wide-mouth, screw-cap pt (500 mL) jar. Add 225 mL sterile trypticase (tryptic) soy broth, **967.25A(t)**, and let yeast form smooth suspension. Cap securely and let stand 60 min at room temp. Det. pH with test paper, **967.25B(l)**. Adjust pH, if necessary to 6.8 ± 0.2 with sterile 1*N* NaOH or HCl, **967.25B(c)** or (**d**), capping jar securely and mixing well before detg final pH. (If pH is adjusted before yeast is evenly suspended, final pH will be less than desired.) Incubate 24 ± 2 hr at 35°, with jar cap loosened $^1/_4$ turn.

(**d**) *Onion powder and garlic powder (First Action 1979; Final Action 1980).*—Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap 500 mL (1 pt) jar. Sample is preenriched in trypticase (tryptic) soy broth, **967.25A(t)**, with added K_2SO_3 (5 g/L) for final 0.5% K_2SO_3 concn. Autoclave 225 mL portions in 500 mL flasks for 15 min at 121°. Aseptically det. vol. and adjust, if necessary, to 225 mL. Add 225 mL sterile trypticase (trypic) soy broth, **967.25A(t)**, with K_2SO_3, to sample, and mix thoroly using sterile glass rod or spoon. Let stand 60 min and det. pH with test paper, **967.25B(l)**. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1*N* NaOH or 1*N* HCl, **967.25B(c)** or (**d**). Incubate 24 ± 2 hr at 35°, with jar cap loosened $^1/_4$ turn.

(**e**) *Milk chocolate and casein.*—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile reconstituted NFDM, **967.25A(v)**, to chocolate samples, and add 225 mL lactose broth, **967.25A(a)**, to casein samples. Blend each sample/broth mixt. 2 min at high speed and decant blended homogenate into sterile 500 mL jar. Cap jar securely and let stand 60 min at room temp. Mix well by shaking, and det. pH with test paper, **967.25B(l)**. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, **967.25B(c)** or (**d**), capping jar securely and mixing well before detg final pH. To chocolate-reconstituted NFDM samples, add 0.45 mL 1% aq. brilliant green dye, **967.25B(n)**, and mix well. Loosen jar caps $^1/_4$ turn and incubate jar 24 ± 2 h at 35°.

(**f**) *Instant nonfat dry milk* (NFDM) (*First Action 1984*).—Aseptically open sample container and aseptically weigh 25 g sample into sterile beaker (250 mL) or other appropriate container. Cover with sterile foil cover or sterile cap to prevent contamination. Using sterile glass or paper (made with tape to withstand autoclaving) funnel, pour 25 g analytical unit gently and slowly over surface of 225 mL brilliant green H_2O, **967.25A(w)**, contained in sterile 500 mL erlenmeyer or other appropriate container. Let container with sample-pre-enrichment broth stand undisturbed 60 ± 5 min. Incubate loosely capped container, without mixing or pH adjustment, for 24 ± 2 h at 35°.

Table 967.25 Spicer-Edwards *Salmonella* H Antisera and H Antigens with Which Each Antiserum Reacts

H Antigens	Spicer-Edwards *Salmonella* H Antisera			
	1	2	3	4
a	+	+	+	–
b	+	+	–	+
c	+	+	–	–
d	+	–	+	+
eh	+	–	+	–
G Complex[a]	+	–	–	+
i	+	–	–	–
k	–	+	+	+
r	–	+	–	+
y	–	+	–	–
z	–	–	+	+
z_4 Complex[b]	–	–	+	–
z_{10}	–	–	–	+
z_{29}	–	+	+	–

H Antigens	*Salmonella* H Antisera
enx, enz_{15}	EN complex
lv, lw, lz_{13}, lz_{28}	L complex
1, 2; 1, 5; 1, 6; 1, 7	1 complex

[a] The G complex component of Spicer-Edwards *Salmonella* H antisera 1 and 4 reacts with antigens f, g, m, p, q, s, t, and u.
[b] The z_4 complex component reacts with z_4, z_{23}, z_{24}, and z_{32}.
(From Difco Laboratories)

B. Isolation

(**a**) *Growth in selective broth.*—Gently shake incubated sample mixt., **967.26A**, and transfer 1 mL to 10 mL selenite cystine broth, **967.25A(b)**(*1*) or (*2*), and addnl 1 mL to 10 mL tetrathionate broth, **967.25A(c)**. Incubate 24 ± 2 h at 35°. (For

dried active yeast, substitute lauryl sulfate tryptose broth, **967.25A(u)**, for selenite cystine broth, **967.25A(b)**(*1*) or (*2*).

Vortex-mix, and streak 3 mm loopful of incubated selenite cystine broth on selective media plates of xylose lysine desoxycholate agar, **967.25A(d)**, Hektoen enteric agar, **967.25A(e)**, and BS agar, **967.25A(f)**. Repeat with 3 mm loopful of incubated tetrathionate broth. Incubate plates 24 ± 2 h at 35°.

(**b**) *Appearance of typical Salmonella colonies.*—(*1*) *On xylose lysine desoxycholate agar.*—Pink colonies with or without black centers. Many *Salmonella* may have large, glossy black centers or may appear as almost completely black colonies. Atypically, a few *Salmonella* cultures produce yellow colonies with or without black centers.

(*2*) *On Hektoen enteric agar.*—Blue-green to blue colonies with or without black centers. Many *Salmonella* cultures may have large glossy black centers or may appear as almost completely black colonies.

(*3*) *On bismuth sulfite agar.*—Brown, gray, or black, sometimes with metallic sheen. Surrounding medium is usually brown at first, turning black with increasing incubation time. Some strains produce green colonies with little or no darkening of surrounding medium.

Examine XLD and HE agar plates for typical or suspicious *Salmonella* colonies after 24 ± 2 h incubation at 35°. BS agar plates should be examined for typical or suspicious *Salmonella* colonies after 24 ± 2 h and 48 ± 2 h incubation at 35°.

C. Treatment of Typical or Suspicious Colonies

(**a**) *Inoculation of triple sugar iron (TSI) agar and lysine iron agar (LIA).*—Pick with needle 2 or more typical or suspicious colonies, if present, from each xylose lysine desoxycholate, Hektoen enteric, and BS agar plates having growth. Inoculate TSI agar slant, **967.25A(g)**, with portion of each colony by streaking slant and stabbing butt. After inoculating TSI agar with needle, do not obtain more inoculum from colony and do not heat needle, but inoculate LIA, **967.25A(m)**(*1*), as in **967.27C(a)**. Store picked selective plates at 5–8° or at room temp. (ca 26°).

(**b**) *Presumptive reactions.*—Incubate TSI and LIA slants at 35° for 24 ± 2 h and 48 ± 2 h, resp. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive H_2S production. *Salmonella* cultures typically have alk. (red) slant and acid (yellow) butt, with or without H_2S (blackening of agar) in TSI agar. In LIA, *Salmonella* cultures typically have alk. (purple) reaction in butt. Consider only a distinct yellow coloration in butt of tube as an acidic (neg.) reaction. Do not eliminate cultures that produce discoloration in butt solely on this basis. Most *Salmonella* cultures produce H_2S in LIA. Retain all presumptive pos. *Salmonella* cultures on TSI (alk. slant and acid butt) agar for biochem. and serological tests whether or not corresponding LIA reaction is pos. (alk. butt) or neg. (acid butt). Do not exclude a TSI culture that appears to be non-*Salmonella* if the reaction in LIA is typical (alk. butt) for *Salmonella*. Treat these cultures as presumptive pos. and submit them to further examination. LIA is useful in detection of *S. arizonae* and atypical *Salmonella* strains that utilize lactose and/or sucrose. Discard only apparent non-*Salmonella* TSI agar cultures (acid slant and acid butt) if corresponding LIA reactions are not typical (acid butt) for *Salmonella*. Test retained presumptive pos. TSI agar cultures as directed in **967.26C(c)** to det. if they are *Salmonella* sp., **967.27D(e)**(*1*), or *S. arizonae* organisms, **967.27D(e)**(*2*).

If TSI slants fail to give typical *Salmonella* reactions, pick addnl suspicious colonies from selective medium plate not giving presumptive pos. culture and inoculate TSI and LIA slants as in (**a**).

(**c**) *Selection for identification.*—Apply biochem. and serological identification tests to 3 presumptive pos. TSI agar cultures picked from selective agar plates streaked from selenite cystine broth and to 3 presumptive pos. TSI agar cultures picked from selective agar plates streaked from tetrathionate broth.

If 3 presumptive pos. TSI agar cultures are not isolated from 1 set of selective agar plates, test other presumptive pos. TSI agar cultures, if isolated, by biochem. and serological tests. A min. of 6 TSI cultures are examined for each 25 g sample tested.

Refs.: JAOAC **50,** 753(1967); **51,** 870(1968); **52,** 455(1969); **56,** 1027(1973); **59,** 731(1976); **61,** 401(1978); **62,** 499(1979); **64,** 893(1981); **64,** 899(1981); **65,** 356(1982); **67,** 807(1984); **69,** 277(1986).

967.27 *Salmonella* in Foods

Identification

First Action 1967
Final Action 1968

A. Cultures

Pure cultures on TSI agar are required for inoculation of biochem. test media.

(**a**) *Pure cultures.*—Proceed to **967.27B**.

(**b**) *Mixed cultures.*—Streak any culture that appears to be mixed on MacConkey agar, **967.25A(q)**, or xylose lysine desoxycholate agar, **967.25A(d)**, or Hektoen enteric agar, **967.25A(e)**. Incubate 24 ± 2 h at 35°.

(**c**) *Appearance of Salmonella colonies.*—(*1*) *On MacConkey agar.*—Typical colonies appear transparent and colorless, sometimes with dark centers. *Salmonella* will clear areas of pptd bile caused by other organisms sometimes present in medium.

(*2*) *On xylose lysine desoxycholate agar.*—See **967.26B(b)**(*1*).

(*3*) *On Hektoen enteric agar.*—See **967.26B(b)**(*2*).

Pick with needle ≥2 typical or suspicious colonies and inoculate TSI slants by streaking the slant and stabbing the butt as in **967.26C(a)**. Retest purified cultures as in **967.26C(b)**, and proceed with identification.

As alternative to conventional tube system for *Salmonella,* any one of the 4 commercial biochem. systems (API, Enterotube, Minitek, or Micro-ID) may be used for presumptive generic identification of foodborne *Salmonella. See* **978.24** and **989.12**.

B. Subcultures

(**a**) *Urease test.*—Subculture small amt of growth from presumptive pos. TSI agar culture to urea broth, **967.25A(k)**(*1*), and incubate 24 ± 2 hr at 35° or inoculate rapid urea broth, **967.25A(k)**(*2*) with two 3 mm loopfuls of growth from each presumptive-pos. TSI agar slant culture, and incubate 2 hr in H_2O bath at 37 ± 0.5°. Discard all cultures that give pos. test (purple-red color). Salmonellae are urease neg. (no change in orange color of medium).

(**b**) *Serological flagellar (H) screening test.*—To reduce number of presumptive pos. TSI agar cultures carried thru identification tests, perform serological flagellar (H) screening test by transferring one 3 mm loopful of each urease-neg. TSI agar culture to either:

(*1*) Brain-heart infusion broth, **967.25A(r)**, (for test on same day) and incubate at 35° until visible growth occurs (ca 4–6 hr); or

(*2*) Trypticase soy-tryptose broth, **967.25A(s)**, (for test on following day) and incubate 24 ± 2 hr at 35°.

To 5 mL of each of the 6 broth cultures add ca 2.5 mL formalinized physiological saline soln, **967.25B(g)**. Select 2 formalinized broth cultures and test with *Salmonella* flagellar (H) antisera, **967.25B(j)** or **(k)**, as in **967.28C** or **D**.

If selected formalinized broth cultures are pos., perform addnl tests on these cultures, beginning with **967.27C**, except step **967.27C(d)** may be omitted.

If both formalinized broth cultures are neg., perform serological test on the 4 additional broth cultures (**967.27B(b)**(*1*) or (*2*)) to obtain, if possible, 2 pos. cultures for addnl testing, **967.27C**.

If all urease-neg. TSI cultures from sample are *Salmonella* serological flagellar (H) test neg., then perform addnl tests, beginning with **967.27C**, on these cultures.

C. Testing Urease-Negative Cultures

Using needle, transfer portion of presumptive pos. TSI agar culture to lysine iron agar medium and small amt of growth from the TSI agar culture to each of other media:

(a) *Lysine iron agar*, **967.25A(m)**(*1*).—Stab butt twice and then streak slant. Replace tube cap *loosely* and incubate 48 ± 2 hr at 35°. Examine at least every 24 hr. Most salmonellae give purple color of alk. reaction thruout medium (final color is slightly darker than original purple color of medium). If H_2S is produced, butt of medium is blackened. Neg. test is purple or red slant and yellow butt. If LIA test, **967.26C(a)**, was satisfactory, it need not be repeated. Use lysine decarboxylase broth for final detn of lysine decarboxylase if culture gives doubtful LIA reaction.

If liq. medium is preferred, inoculate tube of lysine decarboxylase broth, **967.25A(m)**(*2*). Close tube cap *tightly* after inoculation and incubate 48 ± 2 hr at 35°. Examine at least every 24 hr. Salmonellae give purple color of alk. reaction thruout broth (final color is slightly darker than original purple color of medium). Sometimes tubes which have yellow color after 8–12 hr of incubation change to purple later. Neg. test is permanent yellow color thruout broth. If medium appears to be discolored (neither purple nor yellow) add few drops of 0.2% bromcresol purple dye **967.25B(o)**, and re-read tube reactions.

(LIA is incubated loosely capped so that aerobic conditions are maintained, while lysine decarboxylase broth is incubated tightly closed to exclude air.)

(b) *Phenol red dulcitol broth*, **967.25A(p)**(*1*).—Incubate 48 ± 2 hr at 35°. Examine at least every 24 hr. Most salmonellae give pos. test indicated by gas formation (displacement of liq. in inverted tube) and/or acid reaction (yellow). Neg. test is alk. reaction (red) and no gas formation.

(Purple broth base with dulcitol, **967.25A(p)**(*2*), may be substituted. Pos. test is acid reaction (yellow) and gas. Neg. test is alk. reaction (purple).)

(c) *Tryptophane broth*, **967.25A(h)**.—Incubate 24 ± 2 hr at 35° and test as follows:

(*1*) Transfer 3 mm loopful, excluding all solid particles, to KCN broth, **967.25A(o)**. Heat rim of tube to form good seal when restoppered. Incubate 48 ± 2 hr at 35°. Salmonellae do not grow in this broth as shown by lack of turbidity (neg. test).

(*2*) Transfer 3 mm loopful to malonate broth, **967.25A(l)**, and incubate 48 ± 2 hr at 35°. Salmonellae give neg. test as shown by green color (unchanged). Pos. test (alk. reaction) is shown by blue color.

(*3*) Transfer 5 mL to empty test tube and add 0.2–0.3 mL Kovacs reagent, **967.25B(a)**. Pos. test for indole is shown by deep red color in reagent on surface of broth. Most salmonellae are indole-neg.

(d) *Brain-heart infusion broth, **967.25A(r)**, or trypticase soy-tryptose broth, **967.25A(s)**.*—Incubate brain-heart infusion broth until visible growth occurs (ca 4–6 hr) or incubate trypticase soy-tryptose broth 24 ± 2 hr at 35°. To 5 mL broth culture add ca 2.5 mL formalized physiological saline soln, **967.25B(g)**. Refrigerate formalized broth at 5–8° if test is to be performed on another day. Perform *Salmonella* serological flagellar (H) test, **967.28C**, or "Spicer-Edwards" flagellar (H) test tube test, **967.28D**, using formalized broth culture as flagellar (H) antigen to be tested.

(e) *Tests indicating absence of Salmonella.*—Discard, as not *Salmonella*, cultures that show either:

(*1*) Pos. indole test (red) and neg. *Salmonella* serological flagellar (H) test.

(*2*) Pos. KCN broth test (growth) and neg. lysine decarboxylase test (yellow).

(f) *Testing of TSI agar cultures.*—Use *Salmonella* serological somatic (O) test, **967.28A**.

(g) *Classification.*—Classify as *Salmonella* sp. cultures that have all characteristics shown in Table **967.27A**. If 1 TSI culture from 25 g sample is classified as *Salmonella* sp., further testing of other TSI cultures from same 25 g sample is unnecessary.

(h) *Special cases.*—Cultures that contain demonstrable *Salmonella* antigens as shown by pos. *Salmonella* serological somatic (O) test and pos. flagellar (H) test but do not have biochem. characteristics of salmonellae should be purified as in **967.27A(b)** and retested, beginning with **967.27B**.

D. Additional Biochemical Tests

Perform addnl tests on cultures that do not give identical test results as in Table **967.27A** and do not classify as *Salmonella* sp. Transfer 1 loopful of culture from each unclassified TSI agar slant to each of following media:

(a) *Phenol red lactose broth*, **967.25A(p)**(*1*).—Incubate 48 ± 2 hr at 35°. Examine inoculated broth at least every 24 hr. Pos. test is shown by gas formation (displacement of liq. in inverted tube) and acid reaction (yellow). Most salmonellae give neg. test shown by alk. reaction (red) and no gas formation.

Discard, as not *Salmonella*, cultures that give pos. phenol red lactose broth test, except: (*1*) Cultures described in **967.26C(b)**, and (*2*) cultures that also give pos. malonate broth test. Cultures that are phenol red lactose broth pos. or neg. and malonate broth pos. are tested further to det. if they are *S. arizonae*, **967.27D(e)**(*2*).

(Purple lactose broth, **967.25A(p)**(*2*), may be substituted. Pos. test is acid reaction (yellow) and gas. Neg. test is alk. reaction (purple) and no gas formation.)

(b) *Phenol red sucrose broth*, **967.25A(p)**(*1*).—Incubate and read as in **(a)** above. Discard, as not *Salmonella*, cultures that give pos. test, except cultures described in **967.26C(b)**. (Purple sucrose broth may be substituted and read as in **(a)** above.)

Table 967.27A Characteristics of *Salmonella*

Test or Substrate	Results[a]
Urease, **967.27B(a)**	Negative (orange-red)
Lysine decarboxylase, **967.27C(a)**	Positive (alk.; purple thruout medium)
Phenol red dulcitol broth, **967.27C(b)**	Positive (yellow and/or gas)[b]
KCN broth, **967.27C(c)**(*1*)	Negative (no growth)
Malonate broth, **967.27C(c)**(*2*)	Negative (unchanged green)[c]
Indole test, **967.27C(c)**(*3*)	Negative (no red color)
Polyvalent flagellar test, **967.27B(b)**, **967.27C(d)**	Positive (visible agglutination)
Polyvalent somatic test, **967.27C(f)**	Positive (visible agglutination)

[a] +, ≥90% pos. in 1–2 days; −, ≥90% neg. in 1–2 days.
[b] Majority of *S. arizonae* cultures are neg.
[c] Majority of *S. arizonae* cultures are pos.

Table 967.27B Biochemical and Serological Reactions of *Salmonella*

Test or Substrate	Positive	Negative	*Salmonella* reaction[a]
Glucose (TSI), **967.26C(b)**	yellow butt	red butt	+
H_2S (TSI), **967.26C(b)**	blackening	no blackening	+
Urease, **967.27B(a)**	purple-red	no color change	–
Lysine decarboxylase broth, **967.27C(a)**	purple	yellow	+
Phenol red dulcitol broth, **967.27C(b)**	yellow and/or gas	no gas; no color change	+[b]
KCN broth, **967.27C(c)**(*1*)	turbidity	no turbidity	–
Malonate broth, **967.27C(c)**(*2*)	blue	no color change	–[c]
Indole test, **967.27C(c)**(*3*)	violet at surface	yellow at surface	–
Polyvalent flagellar test, **967.27B(b)**, **967.27C(d)**	agglutination	no agglutination	+
Polyvalent somatic test, **967.27C(f)**	agglutination	no agglutination	+
Phenol red lactose broth, **967.27D(a)**	yellow and/or gas	no gas; no color change	–[c]
Phenyl red sucrose broth, **967.27D(b)**	yellow and/or gas	no gas; no color change	–
Voges-Proskauer test, **967.27D(c)**(*1*)	pink to red	no color change	–
Methyl red test, **967.27D(c)**(*2*)	diffuse red	diffuse yellow	+
Simmons' citrate, **967.27D(d)**	growth; blue	no growth; no color change	v

[a] +, ≥90% pos. in 1–2 days; –, ≥90% neg. in 1–2 days; v, variable.
[b] Majority of *S. arizonae* cultures are neg.
[c] Majority of *S. arizonae* cultures are pos.

(**c**) *Buffered glucose broth* (*MR-VP medium*, **967.25A(i)**).—Incubate 48 ± 2 hr at 35°.

(*1*) Perform Voges-Proskauer (VP) test at room temp. by transferring 1 mL 48-hr culture to test tube and adding 0.6 mL α-naphthol soln, **967.25B(b)**(*1*), and 0.2 mL 40% KOH soln, **967.25B(b)**(*2*). Shake after each addn. To intensify and speed reaction, add few creatine crystals to test medium. Read results 4 hr after adding reagents. Pos. VP test is development of eosin pink color. Salmonellae give neg. test.

(*2*) Incubate remainder of MR-VP medium addnl 48 ± 2 hr at 35°. Perform Me red test by transferring 5 mL culture to test tube and adding 5–6 drops Me red soln, **967.25B(e)**, and read results immediately. Salmonellae give pos. test (red). Neg. test is indicated by yellow color.

(**d**) *Simmons' citrate agar*, **967.25A(j)**.—Inoculate by streaking slant and stabbing butt. Incubate 96 ± 2 hr at 35°. Salmonellae usually give pos. test shown by growth and color change from green to blue (alk.). Color change usually appears first on slant and then spreads thru medium. Neg. test is indicated by no or very little growth and no change in color of medium.

(**e**) *Classification*.—Classify cultures according to results listed in Table **967.27B**. If 1 TSI culture from 25 g sample is classified as *Salmonella* sp., further testing of other TSI cultures from same 25 g sample is unnecessary.

(*1*) *Salmonella* sp.—Cultures that have reaction patterns of Table **967.27B**.

(*2*) *Salmonella arizonae*.—Cultures that have reaction pattern of Table **967.27B**, except footnote reactions [b] and [c].

(*3*) *Non-Salmonella* sp.—Discard, as not *Salmonella*, cultures that give results listed in any 1 subdivision of Table **967.27C**.

E. Summary of Classification of Non-Salmonella Cultures

Classify, by performing addnl tests described in *Edwards and Ewing's Identification of Enterobacteriaceae*, any culture that is not clearly identified as *Salmonella* sp. or *S. arizonae* by classification schemes in Tables **967.27A** and **B** or not eliminated from these groups by test reactions listed in Table **967.27C**.

If neither of 2 TSI cultures carried thru biochem. tests, **967.27C** and **D** and Tables **967.27A–C**, confirms as *Salmonella*, perform biochem. tests, beginning with **967.27C**, on remaining urease-neg. TSI cultures from same 25 g sample.

Refs.: JAOAC **50,** 753(1967); **51,** 870(1968); **52,** 455(1969); **56,** 1027(1973); **59,** 731(1976); **62,** 499(1979); **64,** 893(1981); **64,** 899(1981); **65,** 356(1982).

Table 967.27C Criteria for Discarding Non-*Salmonella* Cultures

Test(s) or Substrate(s)	Results
(**a**) Urease test, **967.27B(a)**	Positive (purple-red)
(**b**) Indole test, **967.27C(c)**(*3*)	Positive (red/or violet at surface)
Polyvalent flagellar test, **967.27B(b)**, **967.27C(d)**, or Spicer-Edwards flagellar (H) test, **967.28D**	Negative (no agglutination)
(**c**) Lysine decarboxylase test, **967.27C(a)**	Negative (yellow)
KCN broth, **967.27C(c)**(*1*)	Positive (growth)
(**d**) Phenol red lactose broth[a], **967.27D(a)**	Positive (yellow and/or gas)[b]
(**e**) Phenol red sucrose broth, **967.27D(b)**	Positive (yellow and/or gas)[b]
(**f**) KCN broth, **967.27C(c)**(*1*)	Positive (growth)
Voges-Proskauer test, **967.27D(c)**(*1*)	Positive (red)
Methyl red test, **967.27D(c)**(*2*)	Negative (yellow)

[a] Malonate broth positive cultures are tested further to det. if they are *Salmonella arizonae*, **967.27D(e)**(*2*)
[b] Do not discard pos. broth cultures if corresponding LIA cultures give typical *Salmonella* reactions; test further to det. if they are *Salmonella* sp. *See* **967.26C(a)**

978.24 *Salmonella* sp. in Foods

Biochemical Identification Kit Method

Final Action

(Use of com. biochem. kit as alternative to conventional biochem.testing in **967.27B–E** is based upon demonstration in analyst's laboratory of adequate correlation between biochem. kit intended for use and conventional biochem. tests in **967.27B–E**. Com. biochem. kits should not be used as a substitute for serological tests as described in **967.27B–E**, **967.28**.)

A. Kits

(**a**) *API 20E*.—Available from Analytab Products Inc., 200 Express St, Plainview, NY 11803. Kit is series of 20 plastic microtubes contg biochem. test substrate affixed to plastic strip for conducting following 22 tests: urease; oxidase; tryptophan deaminase; *o*-nitrophenyl-β-D-galactosidase (ONPG); lysine and ornithine decarboxylase; arginine dihydrolase; gelatinase; citrate utilization; H_2S production; indole production; acetoin production (Voges-Proskauer or VP test); nitrate reduction; and fermentation of amygdalin, arabinose, glucose, inositol, mannitol, melibiose, rhamnose, sorbitol, and sucrose. Required reagents include Kovacs reagent, **967.25B(a)**; 10% $FeCl_3$ soln (for phenylalanine deaminase test); VP test reagents (5% α-naphthol soln and 40% KOH soln), **967.25B(b)**; nitrate re-

duction reagents (solns of sulfanilic acid and *N,N*-dimethyl-α-naphthylamine); sterile mineral oil; oxidase test reagents (1% *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine.2HCl soln and 0.2% ascorbic acid soln); sterile H_2O; and 1.5% H_2O_2.

(**b**) *Enterotube II*®.—Available from Roche Diagnostics Systems, Div. of Hoffmann-La Roche, Inc., One Sunset Ave, Montclair, NJ 07042-5188, order No. 43128. Consists of self-contained sterile compartmental plastic tube contg 12 different conventional media and enclosed inoculating needle for conducting following 15 tests: lysine and ornithine decarboxylase; phenylalanine deaminase; urease; Voges-Proskauer (VP); citrate utilization; H_2S production; indole production; and utilization of dulcitol, lactose, adonitol, arabinose, sorbitol, and glucose (acid and gas). Kovacs reagent, **967.25B(a)** (for indole test) and VP reagents, **967.25B(b)**, are also required.

(**c**) *Enterobacteriaceae II Set.*—(Formerly Minitek system.) Available from BBL Microbiological Systems, No. 25147. Consists of system for differentiation of microorganisms by observation of their effect upon chem. substrates impregnated into paper disks for conducting following 25 tests: urease; *o*-nitrophenyl-β-D-galactosidase (ONPG); phenylalanine deaminase; lysine and ornithine decarboxylase; arginine dihydrolase; nitrate reduction; citrate utilization; H_2S production; indole production; malonate utilization; Voges-Proskauer (VP) test; and fermentation of adonitol, arabinose, dulcitol, esculin, glucose, inositol, lactose, mannitol, raffinose, rhamnose, salicin, sorbitol, and sucrose. In addition to inoculum broth, required reagents include Kovacs reagent, **967.25B(a)**, 10% $FeCl_3$ soln (for phenylalanine deaminase test); VP test reagents (5% α-naphthol soln and 40% KOH soln), **967.25B(b)**; nitrate reduction reagents (solns of sulfanilic acid and *N,N*-dimethyl-α-naphthylamine); and sterile mineral oil. Required apparatus includes Minitek pipetter, disposable pipet tips, color comparator cards, disk dispenser, plastic multiwell plates, humidor with sponges for incubation of disks in plates after adding inoculum broth contg test culture, and paper disks impregnated with individual substrates for performing biochem. tests.

Systems (**a**)–(**c**) are also available from Fisher Scientific Co. Systems (**b**) and (**c**) are also available from Scientific Products, Inc., and VWR Scientific, Inc.

B. Isolation

Prep. samples and isolate presumptive cultures by **967.26**.

C. Identification

Assemble supplies and prep. reagents required for utilizing kit. Inoculate each unit according to directions supplied by manufacturer, incubating for time and temp. specified. Add reagents, observe, and record results. For presumptive identification, classify cultures according to flow charts and tables supplied by manufacturer as *Salmonella* or non-*Salmonella* sp.

For confirmation of cultures presumptively identified as *Salmonella* sp., perform *Salmonella* serological somatic (O) test, **967.28A**, and *Salmonella* serological flagellar (H) test, **967.28C**, or "Spicer-Edwards" flagellar (H) test, **967.28D**, and classify cultures according to following guidelines:

(**a**) Cultures classified as presumptive *Salmonella* sp. with com. biochem. kits are confirmed as *Salmonella* sp. when culture demonstrates pos. *Salmonella* somatic (O) test and pos. *Salmonella* (H) test.

(**b**) Cultures classified as presumptive non-*Salmonella* sp. with com. biochem. kits are discarded as non-*Salmonella* sp. when the cultures conform to manufacturer's criteria for classifying cultures as non-*Salmonella* sp.

(**c**) Cultures which do not conform to (**a**) or (**b**) should be classified according to addnl tests specified in **967.27B–E**, **967.28** or addnl tests specified in *Edwards and Ewing's Identification of Enterobacteriaceae*, or sent to ref. typing laboratory for definitive serotyping and identification.

Refs.: JAOAC **61,** 1043(1978); **64,** 408(1981).

989.12 *Salmonella* sp., *Escherichia coli*, and Other *Enterobacteriaceae* in Foods

Biochemical Identification Kit Method

First Action 1989

Use of com. biochem. kit as alternative to conventional biochem. testing in **966.24(a)–(f)** (*E. coli*) and **967.27** (*Salmonella* sp.) is based on demonstration in analyst's laboratory of adequate correlation between biochem. kit intended for use and conventional biochem. tests in **966.24(a)–(f)** and **967.27**. Com. biochem. kit should not be used as substitute for serological tests for *Salmonella* as described in **967.27**, **967.28**. Com. biochem. kit can be used for presumptive identification of other *Enterobacteriaceae* isolated from foods.

A. Principle

Method uses kit in which inoculum contains preformed enzymes at levels detectable in 4 h by means of sensitive indicator system. Kit contains filter paper discs impregnated with reagents which detect presence of specific enzymes and/or metabolic products produced by certain microorganisms. These reagents include substrate to be acted on by bacterial enzyme, and detection system which reacts with metabolic end product to yield readily identifiable color change. Precise quantities of substrate and/or detection reagents are supplied to each disc so that chem. incompatible materials are sepd until tray is inoculated. Tests included are Voges-Proskauer (VP), nitrate reductase, phenylalanine deaminase, H_2S, indole, ornithine decarboxylase, lysine decarboxylase, malonate utilization, urease, esculin hydrolysis, β-galactosidase, and arabinose, adonitol, inositol, and sorbitol fermentations.

B. Method Performance

Results	Percent Agreement	95% Confidence Range (Approx.)
Salmonella sp.	98.8	97.2–100
Escherichia coli	97.7	94.6–100
Other enterics[2]	84.6	81.2–88.0

[1] Agreement with conventional biochem. tests (AOAC methods).
[2] *Enterobacteriaceae* correctly identified to genera other than *Salmonella* and *E. coli*

C. Apparatus, Culture Media, and Reagents

Use distd or deionized H_2O.

(**a**) *Plate count agar (standard methods agar) slants.*—5.0 g tryptone, 2.5 g yeast ext, 1.0 g dextrose, and 15.0 g agar. Suspend ingredients in 1 L H_2O. Heat to boiling to dissolve medium completely. Dispense 8–10 mL portions into 16 × 150 mm tests tubes. Autoclave 15 min at 121°. Before medium solidifies, place tubes in slanted position so that adequate slants are formed.

(**b**) *Physiological saline.*—Dissolve 8.5 g NaCl in 1 L H_2O. Final pH *must be* 6.0 ± 0.5. Do not use saline prepns contg preservatives such as Na azide or other bacterial growth inhibitors. Saline does not need to be sterile but should be freshly prepd.

(**c**) *20% KOH soln.*—Slowly add 20 g KOH pellets to 60 mL H_2O. Dissolve by stirring. Add sufficient H_2O to prep. 100 mL soln. Keep KOH soln in tightly closed container when not in use. *Caution:* Caustic reagent. Handle with care.

(**d**) *Test tubes.*—16 × 100 mm or larger. One test tube is required for each isolate to be identified.

(**e**) *Pipets.*—1 mL and 5 mL serological, with cotton plug.

(**f**) *Pathotec cytochrome oxidase test.*—No. 34191 (Organon Teknika Corp., 100 Akzo Ave, Durham, NC 27704); or equiv.

(**g**) *MICRO-ID identification kit and manual.*—No. 34146 (Organon Teknika Corp.).

(**h**) *Support rack.*—To hold test kit units (Organon Teknika Corp., No. 34147).

D. Preparation of Inocula

(1) Select isolated colony from agar medium. Transfer colony to plate count agar slant. Incubate 18–24 h at 35°. *Note:* Cultures older than 30 h may give false neg. results.

(2) Perform cytochrome oxidase test on portion of growth from slant. Cytochrome oxidase-neg. rods should be further tested.

(3) Pipet ca 3.5 mL physiological saline (**b**) into 16 × 100 mm test tube for each isolate to be identified. Transfer growth from each slant into tube of saline until density of suspension of organisms is equiv. to McFarland No. 2.0. *Note:* Sterile test tubes are not required.

E. General Instructions

Components and procedures of test kit have been stdzd for use in MICRO-ID identification system. Components or procedures other then those supplied by Organon Teknika Corp. may yield unsatisfactory results, and should be pretested.

F. Inoculation and Reading of Unit

(1) Open sealed, moisture-proof, foil package and remove test unit. Do not remove clear plastic tape that covers test wells.

(2) Record sample no. and other required information on area provided on right side of cover.

(3) Open cover and let unit lie flat on laboratory bench.

(4) Pipet ca 0.2 mL of organism suspension into each inoculation well at top of unit.

(5) Close cover and stand tray upright in support rack. (Make sure that organism suspension is in contact with all substrate discs. DO NOT moisten detection discs.)

(6) Incubate 4 h at 35–37°. DO NOT use CO_2 incubator.

(7) After 4 h incubation, place each unit flat on bench, open lid, and add 2 drops (ca 0.1 mL) of 20% KOH soln (**c**) to inoculation well of VP test ONLY. Do not add KOH to any other inoculation well. Close lid and hold tray upright. Be certain that KOH flows down into VP test soln.

(8) Rotate unit clockwise ca 90° so upper discs in first 5 wells become wet. Hold tray upright and tap gently on bench to dislodge any suspension trapped under upper disc. Be certain that each upper disc in reaction chambers 1–5 is moistened by this procedure.

(9) Read all reactions immediately, except VP test, as pos. or neg. according to color changes listed below. Let color develop in VP well for ca 10 min, and then read. Read color of upper disc for first 5 tests; read color of organism suspension for remaining 10 tests. Record result for each biochem. test on encoding forms supplied with system.

Test	Positive Reaction	Negative Reaction
Voges-Proskauer	pink to red	light yellow
Nitrate reductase	red	colorless to light pink
Phenylalanine deaminase	green[1]	light yellow
H_2S	brown to black[2]	white
Indole	pink to red	light yellow to orange
Ornithine decarboxylase	purple to red-purple	amber to yellow
Lysine decarboxylase	purple to red-purple	amber to yellow
Malonate utilization	green to blue	yellow
Urease	orange to red-purple	yellow
Esculin hydrolysis	brown to black	no color change or beige
β-Galactosidase	light yellow to yellow	colorless
Fermentations:		
Arabinose	yellow to amber	red-purple to purple
Adonitol	yellow to amber	red-purple to purple
Inositol	yellow to amber	red-purple to purple
Sorbitol	yellow to amber	red-purple to purple

[1] In phenylalanine deaminase test, any green color in organism suspension also indicates pos. reaction.

[2] Pos. H_2S reaction might vary from thin, dark line at bottom of detection disc to entire disc turning black. It is often advisable to read this disc before it has been wetted.

(10) Use MICRO-ID identification manual (**g**) to det. 5 digit octal no. for each isolate, and record identification of isolate.

G. Confirmation (*Salmonella* sp. Only)

For confirmation of cultures presumptively identified as *Salmonella* sp., see **978.24C**.

Ref.: JAOAC **71**, 968(1988).

967.28 *Salmonella* in Foods

Serological Tests

First Action 1967
Final Action 1968

(Follow manufacturer's instructions for reconstitution, mixing, diln, and operation of *Salmonella* antisera. Dil. and pretest all *Salmonella* serological antisera with known test cultures to ensure reliability of results with unknown cultures. *Caution:* Handle viable cultures carefully to prevent contaminating environment.)

A. Polyvalent Somatic (O) Slide or Plate Test

Using wax pencil, mark off 2 sections ca 1 × 2 cm on inside of glass or plastic petri dish. Place $^1/_2$ of 3 mm loopful of culture from 24- or 48-hr TSI agar slant on dish in upper part of each marked section. Add 1 drop saline soln, **967.25B(f)**, to lower part of one section only. Add 1 drop *Salmonella* polyvalent somatic (O) antiserum, **967.25B(h)**, to other section only. With clean, sterile transfer loop or needle, emulsify culture in saline soln for one section and repeat for other section contg antiserum. Tilt mixt. in both sections back and forth 1 min and observe against dark background. Any degree of agglutination is pos. reaction.

Classify polyvalent somatic (O) test as:

(**a**) *Positive.*—Agglutination in culture-saline-serum mixt. and no agglutination in culture-saline mixt.

(**b**) *Negative.*—No agglutination in culture-saline-serum mixt. (Polyvalent somatic antisera do not contain agglutinins for antigens of some salmonellae isolated from foods. Neg. somatic reactions occur with *Salmonella* serotypes whose corresponding agglutinins are not contained in the antisera, i.e., *S. cerro,*

group K(18); *S. minnesota,* group L(21); *S. alachua,* group O(35).)

(**c**) *Non-specific.*—Both mixts agglutinate. Requires addnl testing as in *Edwards and Ewing's Identification of Enterobacteriaceae.*

B. Determination of Somatic Grouping (Optional)

Perform serological somatic (O) test on culture as in **967.28A**, using individual group somatic (O) antiserum (including Vi), **967.25B(i)**, instead of *Salmonella* polyvalent somatic (O) antiserum. Repeat test, using each group somatic antiserum or until culture reacts with specific group antiserum.

Suspend cultures pos. with Vi antiserum by emulsifying growth from slant surface in 1 mL physiological saline soln, **967.25B(f)**, to make heavy suspension. Heat in boiling H_2O 20–30 min and let cool. Retest heated suspension, using somatic group D, C_1, and Vi antisera. Vi-pos. cultures which react with somatic group D antiserum are probably *S. typhi,* and Vi-pos. cultures which react with somatic group C_1 antiserum are probably *S. paratyphi C.* For these cultures to be classified as *Salmonella* sp., they must have characteristics of salmonellae as in Table **967.27A** or **B**. Heated Vi-pos. cultures which do not react with any individual somatic serum but continue to react with Vi antiserum probably belong to *Citrobacter* and are not *Salmonella.* Confirm conclusion by biochem. tests listed in Table **967.27A**.

Cultures that give pos. somatic (O) test with any individual somatic (O) antiserum are recorded as pos. for that somatic (O) group; cultures that do not react with any individual somatic (O) antiserum are recorded as neg. for individual group somatic (O) test.

C. Polyvalent Flagellar (H) Test Tube Test

Place 0.5 mL appropriately dild *Salmonella* polyvalent flagellar (H) antiserum, **967.25B(j)**, in 10 × 75 or 13 × 100 mm serological test tube and add 0.5 mL antigen to be tested: formalinized brain-heart infusion broth, **967.27B(b)**(*1*), or formalinized trypticase soy-tryptose broth, **967.27B(b)**(*2*) or **967.27C(d)**. If formalinized culture contains granular particles, pellicles, or sediment, also prep. saline control by mixing 0.5 mL formalinized saline soln, **967.25B(g)**, with 0.5 mL formalinized trypticase soy-tryptose or brain-heart infusion broth culture in same size serological test tube. Incubate mixts 1 hr in H_2O bath at 48–50°. Observe preliminary results at 15 min intervals and read final results at 1 hr.

Classify polyvalent flagellar (H) test as:

(**a**) *Positive.*—Agglutination in culture-formalinized saline-serum mixt. and no agglutination in culture-formalinized saline mixt.

(**b**) *Negative.*—No agglutination in culture-formalinized saline-serum mixt. (Polyvalent flagellar antiserum does not contain agglutinins for antigens of some salmonellae isolated from foods. Neg. flagellar reactions occur with *Salmonella* serotypes whose corresponding agglutinins are not contained in the antisera (i.e., *S. simsbury,* z_{27}; *S. chittagong,* z_{35})).

(**c**) *Non-specific.*—Both mixts agglutinate. Requires addnl testing as in *Edwards and Ewing's Identification of Enterobacteriaceae.*

Cultures that give typical biochem. results as salmonellae but do not agglutinate in *Salmonella* flagellar (H) antisera must be tested to det. if enough flagellar (H) antigens are present. Test motility of culture as follows:

Inoculate petri dish contg motility test medium, **967.25A(n)**, with 3 mm loopful TSI culture by stabbing medium once, 10 mm from edge of plate to depth of 2–3 mm. (Do not stab to bottom of plate with inoculum.) Do not inoculate any other portion of plate. Incubate 24 hr at 35°. When organism has migrated 40 mm or more toward other side of plate, it is sufficiently motile to retest.

Transfer 3 mm loopful of growth which migrated farthest from inoculation point into tube of trypticase soy-tryptose broth, **967.27C(d)**. Incubate and retest this culture by adding $^1/_2$ vol. formalinized physiological saline soln, **967.25B(g)**, and repeat *Salmonella* serological flagellar (H) test, **967.28C** or **D**.

Incubate cultures that are not motile after first 24 hr incubation for addnl 24 hr at 35°. If still neg., incubate 5 days at 25° before classifying as nonmotile (flagellar (H) antigen not detected).

Cultures that are non-motile or cultures that are *Salmonella* serological flagellar (H) test-neg., when retested, are classified according to results of other tests in *Edwards and Ewing's Identification of Enterobacteriaceae.*

D. "Spicer-Edwards" Flagellar (H) Test Tube Test

(Alternative to polyvalent flagellar (H) test tube test, **967.28C**, to det. presence or absence of flagellar (H) antigens)

Test each culture, using each of the 7 "Spicer-Edwards" flagellar (H) antisera, **967.25B(k)**. Perform test as in **967.28C**, using 1 of the 7 "Spicer-Edwards" (H) antisera for each test instead of *Salmonella* polyvalent flagellar (H) antiserum. Since there are 7 "Spicer-Edwards" antisera, each culture must be tested 7 times.

Pos. agglutination indicates presence of flagellar (H) antigen. Identify by comparing pattern of agglutination reactions obtained with agglutinins known to be present in each of the 7 "Spicer-Edwards" (H) antisera. Results of these reactions are shown in Table **967.25**.

If culture produces pos. agglutination when tested with each of the 4 "Spicer-Edwards" antisera 1, 2, 3, and 4 (4 plus pattern), then results indicate presence of non-specific antigen other than *Salmonella* antigen or presence of more than single *Salmonella* H antigen which cannot be identified with this antisera until antigens are sepd.

Refs.: JAOAC **50,** 753(1967); **51,** 870(1968); **52,** 455(1969); **56,** 1027(1973); **59,** 731(1976); **62,** 499(1979); **64,** 893(1981); **64,** 899(1981); **65,** 356(1982).

975.54 *Salmonella* in Foods

Fluorescent Antibody (FA) Screening Method

First Action 1975
Final Action 1977

A. Precautions

Method is screening test for presence of *Salmonella*; it is not confirmatory test, since conjugate will react with some other members of *Enterobacteriaceae.*

Enrichment broths from samples pos. by FA method must be streaked on selective media as in **967.26B** and typical or suspicious colonies identified as in **967.26C**, **967.27**, **967.28**.

Method must be followed rigorously since errors in prepn of sample, smears, conjugate, and other reagents can lead to invalid results. Microscopic observation of stained smears must be performed with critically aligned and properly functioning equipment.

Visual estimation of degree of fluorescence of stained cells is somewhat subjective and should be conducted by analyst with prior training or experience in both FA methodology and in cultural technic for detection of *Salmonella.*

If sample prepn does not normally include pre-enrichment step (as with meat, poultry, and certain environmental samples), 4 hr post-enrichment incubation period may not be suf-

ficient for development of number of *Salmonella* cells required for detection by FA method. Therefore, include pre-enrichment step or extend post-enrichment incubation time. In some cases when pre-enrichment step is not used, sample is not adequately dild and carryover of debris into post-enrichment broth may interfere with observation of FA stained cells.

B. Apparatus

(**a**) *Multiwell coated slides.*—Clean thin (1.0–1.2 mm) slides thoroly with detergent and rinse with distd H_2O and alcohol. Apply double row of 4 sep. drops of glycerol (8 drops total) to each of series of slides and spray with fluorocarbon coating material (Fluoroglide, Ace Scientific Co., Inc., 1420 E Linden Ave, Linden, NJ 07036). After few min, rinse off each slide individually under tap and then with distd H_2O, and stand on end in rack to dry. (Prepd slides are available from Cell-Line Associates, PO Box 35, Newfield, NJ 08344 and Clinical Sciences, Inc., 30 Troy Rd, Whippany, NJ 07981.)

(**b**) *Fluorescence microscope.*—With exciter filter with wavelength transmission of 330–500 nm and barrier filter with wavelength reception >400 nm.

C. Reagents

(**a**) *Phosphate-buffered saline (PBS) soln.*—pH 7.5; 0.01*M*; 0.85% NaCl. Dissolve 12.0 g anhyd. Na_2HPO_4, 2.2 g $NaH_2PO_4.H_2O$, and 85.0 g NaCl in H_2O and dil. to 1 L. Dil. 100 mL this soln to 1 L with H_2O. Adjust pH to 7.5 with 0.1*N* HCl or 0.1*N* NaOH, if necessary.

(**b**) *Carbonate buffer.*—pH 9.0. Mix 4.4 mL 0.5*M* Na_2CO_3 (5.3 g in 100 mL H_2O) with 100 mL 0.5*M* $NaHCO_3$ (4.2 g in 100 mL H_2O). pH should be 9.0; if not, adjust by addn of 0.5*M* Na_2CO_3.

(**c**) *Glycerol saline soln.*—pH 9.0. Mix 9 mL glycerol with 1 mL carbonate buffer, (**b**). pH decreases on storage; prep. weekly.

(**d**) *Salmonella polyvalent fluorescent antibody conjugate.*—Fluorescein isothiocyanate-labeled *Salmonella* OH globulin, polyvalent, contg antibodies for all antigens within *Salmonella* O groups A–S, and meeting specifications of Centers for Disease Control, Atlanta, GA 30333 (1975). (Available from Difco Laboratories (FA Salmonella Poly); Clinical Sciences, Inc., 30 Troy Rd, Whippany, NJ 07981). Before use, titer each lot to det. appropriate routine test diln (RTD). Use pure cultures of *Salmonella* representative of several somatic groups. Prep. 5 dilns (1:2, 1:4, 1:8, 1:16, and 1:32) of conjugate in PBS soln, (**a**). Stain duplicate smears from cultures with each diln and det. intensity of fluorescence. RTD is that diln one less than highest diln giving 4+ fluorescence with representative *Salmonella* cultures. Store stock (undild) conjugate of known titer frozen, and dil. when needed. Dild conjugate can be stored at 4° for few weeks as long as control cultures remain pos.

D. Determination

(**a**) *Pre-enrichment.*—Pre-enrich product in noninhibitory broth to initiate growth of salmonellae. Methods used vary with product as in (*1*)–(*9*). In all cases loosen jar caps $^1/_4$ turn and incubate 24 ± 2 hr at 35°. Except where selenite cystine and tetrathionate broths, **967.25A(b)**(*1*) or (*2*) and (**c**), resp., have already been used ((*2*)(b) and (*5*)), transfer 1 mL incubated mixts to selenite cystine broth and tetrathionate broth for selective enrichment as in **967.26B(a)**. Where these broths have already been used ((*2*)(b) and (*5*)), proceed directly to post-enrichment, (**b**).

(*1*) *Dried yeast (inactive).*—Weigh 25 g into sterile, wide-mouth, screw-cap, 500 mL (pt) jar, add 225 mL sterile trypticase (tryptic) soy broth, **967.25A(t)**, and mix well to form smooth suspension. Cap jar securely and let stand 60 min at room temp. If pH is <6.6, adjust to 6.8 ± 0.2 with 1*N* NaOH.

(*2*) *Meats, animal substances, glandular products, and fish meal.*—(*a*) *Heated, processed, and dried products.*—Weigh 25 g into sterile blending jar, add 225 mL sterile lactose broth, **940.36A(f)**, and blend 2 min at 8000 rpm. If product is powd., ground, or comminuted, blending may be omitted. Transfer aseptically to sterile, wide-mouth, screw-cap, 500 mL (pt) jar and adjust pH to 6.8 ± 0.2 with 1*N* NaOH. If product contains large amt of fat, add 2.2 mL of steamed (15 min) Tergitol Anionic 7 (Na heptadecyl sulfate, Union Carbide Corp.).

(*b*) *Raw and highly contaminated products.*— Weigh duplicate 25 g samples into sep. sterile blending jars. Add 225 mL of selenite cystine broth to one jar and 225 mL of tetrathionate broth to other, and blend 2 min. Transfer aseptically to sterile, wide-mouth, screw cap, 500 mL (pt) jars.

(*c*) *Raw frog legs.*—Aseptically place 2 legs into single sterile, wide-mouth, screw cap, 500 mL (pt) jar contg 225 mL sterile lactose broth, **940.36A(f)**.

(*3*) *Dry whole milk.*—Weigh 25 g into sterile, wide-mouth, screw cap, 500 mL (pt) jar, add 225 mL sterile distd H_2O, and mix well. Adjust pH to 6.8 ± 0.2 with 1*N* NaOH, if necessary. Add 0.45 mL 1% aq. brilliant green soln and mix well.

(*4*) *Dried whole eggs, yolks, and whites; pasteurized liquid and frozen eggs; prepared powdered mixes (cake, cookie, donut, biscuit, and bread); and infant formula.*—If product is frozen, thaw rapidly at ≤45° for ≤15 min or overnight at 5–10°. Weigh 25 g into sterile, wide-mouth, screw cap jar. Add 225 mL lactose broth, little at time with mixing, cap jar, and let stand at room temp. 60 min. Mix well and adjust to pH 6.8 ± 0.2 with 1*N* NaOH or HCl.

(*5*) *Nonpasteurized frozen egg products.*—Thaw as in (*4*). Weigh duplicate 25 g samples into sep. sterile, wide-mouth, screw cap, 500 mL (pt) jars. Add 225 mL selenite cystine broth to one jar and 225 mL tetrathionate broth to other, and mix well. Adjust pH to 6.8 ± 0.2 with 1*N* NaOH.

(*6*) *Egg-containing foods (noodles, egg rolls, etc.)*—Proceed as in (*2*)(*a*).

(*7*) *Coconut.*—Proceed as in (*2*)(*a*), using Tergitol Anionic 7, but omitting blending.

(*8*) *Candy and candy coatings.*—Weigh 25 g into sterile blending jar. Add 225 mL sterile reconstituted nonfat dry milk, **967.25A(v)**, but without brilliant green dye, and blend 2 min. Adjust pH to 6.8 ± 0.2 with 1*N* NaOH, if necessary. Add 0.45 mL 1% aq. brilliant green soln and mix well.

(*9*) *Nonfat dry milk.*—Examine as in **967.26A(f)**.

(**b**) *Post-enrichment.*—Transfer 1 mL of incubated selenite cystine enrichment broth to 10 mL of sterile selenite cystine broth as post-enrichment. (Other vols may be used if 1:10 diln ratio is maintained.) Take aliquot from upper third of selective enrichment cultures to minimize product carryover. Similarly, transfer 1 mL of incubated tetrathionate enrichment broth to 10 mL of sterile selenite cystine broth. Incubate 4 hr in 35° H_2O bath.

(**c**) *Staining.*—Transfer ca 0.0075 mL of each post-enrichment medium with sterile 2 mm loop into sep. wells of multiwell coated slide, and dry thoroly in air at room temp. Fix by immersion in bath of alcohol-$CHCl_3$-formalin (60 + 30 + 10) 3 min. Rinse 2 or 3 times in alcohol, and air dry at room temp. Change alcohol periodically to prevent cell carryover (250 mL alcohol will rinse 5–10 slides). Slides may also be fixed and rinsed by flooding. Apply solns to one end of slide and allow to flow into wells.

Cover dried smears with titered *Salmonella* polyvalent FA conjugate and let stain in moist chamber 15–30 min. *FA conjugate must not dry on smear.* (Covered plastic petri dish contg piece of filter paper moistened with H_2O is excellent staining chamber.) Drain excess conjugate by standing slide on edge

few sec. (Avoid mixing conjugate from one well on slide to another.) Immediately rinse slides in PBS soln, **975.54C(a)**. Then soak slides 10 min in fresh PBS soln and rinse briefly with H_2O. Air-dry smears again at room temp. and then mount by placing drop of glycerol saline soln, (**c**), directly onto each smear and covering with No. 1 glass cover slip. Add enough glycerol saline soln to smear to ensure adequate, but not excessive, coverage of all wells after cover slips have been placed. Do not trap air bubbles under cover slip.

(**d**) *Examination.*—Examine smears with fluorescent microscope. Scan entire smear using 40–50× oil immersion objective to locate fluorescent cells. When found, change objective to 100× oil immersion lens for definitive detn of cell morphology and fluorescence. Objectives with iris diaphragm for adjusting numerical aperture are helpful for control of contrast between cells and background. Estimate degree of fluorescence of cells on scale of neg. to 4+ as follows:

4+ = Max. fluorescence; brilliant yellow-green; clearcut cell outline; sharply defined cell center.
3+ = Less brilliant yellow-green fluorescence; clearcut cell outline; sharply defined cell center.
2+ = Definite but dim fluorescence; cell outline less well defined.
1+ = Very subdued fluorescence; cell outline indistinguishable from cell center in most instances.
− = Negligible or complete lack of fluorescence.

Typical pos. smears for salmonellae exhibit ≥2 short to medium rod-shaped cells per field, using 100× objective. Cells should be distributed thruout entire smear. Intensity of fluorescence should be in range of 3+ to 4+. Occasionally cells are observed with proper morphology and cell distribution, but fluorescence is rated 2+. Sometimes 3+ to 4+ fluorescence is observed, but distribution is poor and not all fields contain cells, due to improper processing of slides. Score both cases pos. and subject to confirmatory tests.

Each time samples are tested, carry culture of known *Salmonella* strain thru all cultural, staining, and observation steps as control.

Report: (1) morphological characteristics of fluorescent cells; (2) number of typical cells per field under 100× oil immersion objective; and (3) degree of fluorescence of cells (1+ to 4+).

Ref.: JAOAC **58,** 828(1975).

985.42 *Salmonella* in Foods

Hydrophobic Grid Membrane Filter Screening Method

First Action 1985
Final Action 1986

(Applicable to detection of *Salmonella* from chocolate, raw poultry meat, pepper, cheese powders, powdered egg, and nonfat dry milk)

A. Principle

Hydrophobic grid membrane filter (HGMF) uses membrane filter imprinted with hydrophobic material in grid pattern. Hydrophobic lines act as barriers to spread of colonies, thereby dividing membrane filter surface into sep. compartments of equal and known size.

B. Apparatus, Culture Media, and Reagents

(**a**) *Hydrophobic grid membrane filter (HGMF)*—Membrane filter has pore size of 0.45 μm and is imprinted with nontoxic hydrophobic material in grid pattern. ISO-GRID (available from QA Laboratories Ltd, 135 The West Mall, Toronto, Ontario, Canada, M9C 1C2) or equiv. meets these specifications.

(**b**) *Filtration units for HGMF.*—Equipped with 5 μm mesh prefilter to remove food particles during filtration. One unit is required for each sample. ISO-GRID (available from QA Laboratories Ltd) or equiv. meets these specifications.

(**c**) *Pipets.*—1.0 mL serological with 0.1 mL graduations; 1.1 mL or 2.2 mL milk pipets are satisfactory.

(**d**) *Blender.*—Waring, or equiv. with high-speed operation at 20,000 rpm, and 500 mL glass or metal blender jars with covers. One jar is required for each sample.

(**e**) *Vacuum pump.*—H_2O aspirator vac. source is satisfactory.

(**f**) *Manifold or vacuum flask.*

(**g**) *Peptone diluent.*—Dissolve 1.0 g peptone (Difco 0118) in 1 L H_2O. Dispense enough vol. into diln bottles to give 99 ± 1 mL after autoclaving 15 min at 121°.

(**h**) *Lactose broth.*—*See* **940.36A(f)**. Dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Aseptically det. vol. and adjust if necessary to 225 mL. Final pH 6.7 ± 0.2.

(**i**) *Trypticase (tryptic) soy broth.*—Suspend 17.0 g trypticase or tryptose (pancreatic digest of casein), 3.0 g phytone (papaic digest of soya meal), 5.0 g NaCl, 2.5 g K_2HPO_4, and 2.5 g glucose in 1 L H_2O. Heat gently to dissolve completely. Dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Aseptically det. vol. and adjust if necessary to 225 mL. Final pH 7.3 ± 0.2.

(**j**) *Reconstituted nonfat dry milk with brilliant green dye (NFDM-BG).*—Suspend 100 g dehydrated NFDM in 1 L H_2O; mix by swirling until dissolved. Autoclave 15 min at 121°. Add brilliant green dye soln after blending sample/broth mixt. as described below.

(**k**) *Tetrathionate broth (with iodine and brilliant green).*—Suspend 5.0 g polypeptone, 1.0 g bile salts, 10 g $CaCO_3$, and 30 g $Na_2S_2O_3.5H_2O$ in 1 L H_2O, mix thoroly, and heat to bp. (Ppt will not dissolve completely.) Cool to <45° and store at 5–8°. Prep. I-KI soln by dissolving 5 g KI in 5 mL sterile H_2O, adding 6 g resublimed I, dissolving, and dilg to 20 mL with sterile H_2O. Prep. brilliant green soln by dissolving 0.1 g dye in sterile H_2O and dilg to 100 mL. On day medium is used, add 20 mL I-KI soln and 10 mL brilliant green soln per 1 L basal broth. Resuspend ppt by gentle agitation and aseptically dispense 10 mL portions in 16 × 150 mm sterile tubes. Do not heat medium after addn of I-KI and dye solns. Temper to 25–35° before use.

(**l**) *Selective lysine agar (SLA).*—Suspend 5.0 g proteose peptone No. 3, 3.0 g yeast ext, 10.0 g L-lysine.HCl, 3.5 g glucose, 1.5 g bile salts No. 3, 0.001 g crystal violet (1.0 mL of 0.1% (w/v) aq. soln), 0.03 g bromocresol purple, 0.3 g sulfapyridine, and 15.0 g agar in 1 L H_2O and heat to bp with stirring to dissolve completely. Autoclave 15 min at 121°. Cool to 45–50°. Dispense 20 mL vol. in 15 × 100 mm petri dishes. Final pH, 6.8 ± 0.1.

(**m**) *Hektoen enteric agar (HE).*—Suspend ingredients (*1*) or (*2*) (varies with manufacturer of formula) in 1 L H_2O and mix thoroly. Heat to boiling with frequent agitation and let boil few moments. Do not overheat. Cool in H_2O bath and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with covers partially removed; then close plates. Final pH, 7.6 ± 0.2. Do not autoclave.

(*1*) 12.0 g thiotone peptone, 3.0 g yeast ext, 9.0 g bile salts, 12.0 g lactose, 12.0 g sucrose, 2.0 g salicin, 5.0 g NaCl, 5.0 g Na thiosulfate, 1.5 g ferric ammonium citrate, 0.064 g bromothymol blue, 0.1 g acid fuchsin, and 13.5 g agar.

(*2*) 12.0 g proteose peptone, 3.0 g yeast ext, 9.0 g bile salts No. 3, 12.0 g lactose, 12.0 g sucrose, 2.0 g salicin, 5.0 g

NaCl, 5.0 g Na thiosulfate, 1.5 g ferric ammonium citrate, 0.065 g thymol blue, 0.1 g acid fuchsin, and 14.0 g agar.

(**n**) *Triple sugar iron agar (TSI agar).*—Suspend ingredients (*1*) or (*2*) in 1 L H_2O, mix thoroly, and heat with occasional agitation. Boil ca 1 min until ingredients dissolve. Fill 16 × 150 mm tubes $^1/_3$ full and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca 3 cm) and adequate slants (ca 5 cm) are formed on solidification.

(*1*) 20 g polypeptone, 5.0 g NaCl, 10 g lactose, 10 g sucrose, 1 g glucose, 0.2 g $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.2 g $Na_2S_2O_3$, 0.025 g phenol red, and 13 g agar. Final pH, 7.3 ± 0.2.

(*2*) 3.0 g beef ext, 3.0 g yeast ext, 15 g peptone, 5.0 g proteose peptone, 1.0 g glucose, 10 g lactose, 10 g sucrose, 0.2 g $FeSO_4$, 5.0 g NaCl, 0.3 g $Na_2S_2O_3$, 0.024 g phenol red, and 12 g agar. Final pH, 7.4 ± 0.2.

(**o**) *Lysine iron agar (LIA) (Edwards and Fife).*—Dissolve 5.0 g gelysate or peptone, 3.0 g yeast ext, 1.0 g glucose, 10 g L-lysine, 0.5 g ferric ammonium citrate, 0.04 g anhyd. $Na_2S_2O_3$, 0.02 g bromocresol purple, and 15 g agar in 1 L H_2O, heating until dissolved. Dispense 4 mL portions into 13 × 100 mm test tubes and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min at 121°. Before medium solidifies, place tubes in slanted position so that 4 cm butts and 2.5 cm slants are formed on solidification. Final pH 6.7 ± 0.2.

(**p**) *MacConkey agar (MAC).*—Suspend 3.0 g proteose peptone or polypeptone, 17 g peptone or gelysate, 10 g lactose, 1.5 g bile salts No. 3 or bile salts mixt., 5.0 g NaCl, 3.0 mL 1% neutral red (30 mg) soln, 1 mL 0.1% crystal violet (1.0 mg) soln, and 13.5 g agar in 1 L H_2O and mix thoroly until homogeneous. Heat, with occasional agitation, and boil 1–2 min until ingredients dissolve. Autoclave 15 min at 121°. Cool to 45–50° and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with plates covered. Do not use wet plates. Final pH 7.1 ± 0.2.

(**q**) *Sodium hydroxide soln.*—1N. Dissolve 42.11 g 95% reagent NaOH in sterile H_2O and dil. to 1 L.

(**r**) *Hydrochloric acid soln.*—1N. Dil. 89 mL to 1 L with sterile H_2O.

(**s**) *pH Test paper.*—Min. range 6.0–7.6 with max. gradations of 0.4 pH unit per color change.

(**t**) *Sterile distilled water.*—Dispense 1 L H_2O into 2 L wide-mouth flask or wide-mouth jar; plug or cap loosely. Autoclave 20 min at 121°.

(**u**) *Brilliant green dye soln.*—1%. Dissolve 1 g in sterile H_2O and dil. to 100 mL. (Since some batches of dye are unusually toxic, test all batches of dye before use, and use only those producing satisfactory results when tested with known pos. and neg. test organisms.)

(**v**) *Brilliant green dye water.*—Prep. sterile H_2O, (**t**), and add 2 mL of 1% aq. brilliant green dye, (**u**), per L sterile H_2O and mix well.

C. Preparation of Sample

(**a**) *Powdered egg.*—Aseptically open sample container and aseptically weigh 25 g sample into sterile, empty, wide-mouth, screw-cap pt (500 mL) jar. Add ca 15 mL sterile lactose broth. Stir with sterile glass rod, sterile spoon, or sterile tongue depressor to smooth suspension. Add 3 addnl portions lactose broth, 10, 10, and 190 mL for total of 225 mL. Stir after each addn until sample is suspended without lumps. Cap jar securely and let stand at room temp. 60 min. Mix well by shaking, and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Loosen jar cap ca $^1/_4$ turn and incubate 18–24 h at 35°.

(**b**) *Chocolate.*—Aseptically weigh 25 g sample into sterile blender jar. Add 255 mL sterile reconstituted NFDM-BG. Blend 2 min at high speed and decant blended homogenate into sterile 500 mL jar. Cap jar securely and let stand 60 min at room temp. Mix well by shaking, and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Add 0.45 mL 1% aq. brilliant green dye and mix well. Loosen jar cap $^1/_4$ turn and incubate 18–24 h at 35°.

(**c**) *Raw meat.*—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth and blend 2 min at high speed. Cap jar securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Aseptically transfer sample to sterile 500 mL wide-mouth screw-cap jar. Loosen jar cap $^1/_4$ turn and incubate 18–24 h at 35°.

(**d**) *Cheese powder.*—Aseptically weigh 25 g sample into sterile 500 mL wide-mouth screw-cap jar. Add 225 mL sterile lactose broth and mix well. Cap jar securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Loosen jar cap $^1/_4$ turn and incubate 18–24 h at 35°.

(**e**) *Pepper.*—Aseptically weigh 25 g sample into sterile 500 mL wide-mouth screw-cap jar. Add 225 mL sterile trypticase soy broth and mix well. Cap jar securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Loosen jar cap $^1/_4$ turn and incubate 18–24 h at 35°.

(**f**) *Powdered milk.*—Use sterile funnel to aseptically add 25 g sample slowly and gently to 225 mL sterile brilliant green dye water in 500 mL wide-mouth screw-cap jar. Do not mix. Allow to soak undisturbed 60 min at room temp. Do not mix or adjust pH. Loosen jar cap $^1/_4$ turn and incubate 18–24 h at 35°.

D. Isolation

(**a**) *Selective enrichment.*—Gently shake incubated sample mixt. and transfer 0.1 mL to 10 mL tempered (25–35°) tetrathionate broth. Mix inoculated broth on vortex mixer or by hand to disperse inoculum. Incubate in H_2O bath 6–8 h at 35 ± 0.5°.

(**b**) *Filtration and selective isolation.*—Mix incubated tetrathionate broth by hand or vortex-mixer to resuspend. For raw meats, prep. 10^{-2} diln by transferring 1.0 mL into 99 mL sterile peptone diluent. Mix by shaking. For all other products, use undild tetrathionate.

(*See* Figs **986.32A** and **986.32B**). Turn on vac. source. Place sterile filtration unit on manifold or vac. flask. Open clamp A. Rotate back funnel portion C. Aseptically place sterile HGMF on surface of base D. Rotate funnel forward. Clamp shut by sliding jaws L of stainless steel clamp over entire length of flanges B extending from both sides of funnel C and base D, and rotating moving arm K into horizontal (locked) position.

Aseptically add ca 15–20 mL sterile H_2O to funnel. Pipet 1.0 mL of required tetrathionate diln into funnel. Apply free end of vac. tubing E to suction hole F to draw liq. thru prefilter mesh G. Aseptically add addnl 10–15 mL H_2O to funnel and draw thru mesh as before. Close clamp A to direct vac. to base of filtration unit and draw liq. thru HGMF.

Open clamp A. Rotate moving arm K of stainless steel clamp into unlocked (ca 45° angle) position and slide jaws L off of flanges B. Rotate back funnel C. Aseptically remove HGMF and place on surface of pre-dried SLA. Avoid trapping air bubbles between filter and agar. For nonfat dry milk samples, insert second sterile HGMF into same filtration unit, repeat filtering procedure and place second HGMF on surface of pre-dried HE. Incubate SLA 24 ± 2 h at 43 ± 0.5°, and HE 24 ± 2 h at 35°. If HGMFs do not have typical or suspicious colonies or do not contain growth, record as neg. test result.

(c) *Appearance of typical Salmonella colonies.*—(*1*) *On SLA.*—Blue-green, blue, or purple colonies (lysine-pos. reaction). Typically, *Salmonella* produces relatively flat colonies which are neither watery nor mucoid. Lysine-neg. colonies are typically yellow or yellow-green. However, this can be masked if large no. of lysine-pos. colonies are present on HGMF.

(*2*) *On HE.*—Black, or green with black centers. Some *Salmonella* will produce yellow colonies with black centers or green colonies with no blackening. H_2S reaction can be partially suppressed if very heavy growth is present on HGMF.

E. Treatment of Typical or Suspicious Colonies

(a) *Inoculation of TSI, LIA, and MAC or HE.*—(*1*) *Raw meats.*—Select 5 typical or suspicious colonies from each HGMF.

(*2*) *All other products.*—Select 3 typical or suspicious colonies from each HGMF.

Using sep. sterile, completely cooled needle for each colony, pick each selected colony and inoculate TSI slant with portion of colony by stabbing butt and streaking slant. Without heating needle or obtaining more inoculum, inoculate LIA with portion of colony by stabbing butt in 2 places and streaking slant. Without heating needle or obtaining more inoculum, streak remainder of inoculum to MAC or HE. Incubate TSI, LIA, and MAC or HE 24 ± 2 h at 35°. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive H_2S production.

(b) *Presumptive positive reactions.*—*Salmonella* cultures typically have alk. (red) slant and acid (yellow) butt, with or without H_2S (blackening of agar) in TSI agar. In LIA, *Salmonella* cultures typically have alk. (purple) reaction in butt. Consider only distinct yellow coloration in butt of LIA tube as acidic (neg.) reaction. Do not eliminate cultures that produce discoloration in butt solely on this basis. Most *Salmonella* cultures produce H_2S in LIA. Retain all presumptive pos. *Salmonella* cultures on TSI agar (alk. slant and acid butt) for biochem. and serological tests whether or not corresponding LIA reaction is pos. (alk butt) or neg. (acid butt). Do not exclude TSI culture that appears to be non-*Salmonella* if reaction in LIA is typical (alk. butt) for *Salmonella*. Treat these cultures as presumptive pos. and submit them to further examination. LIA is useful in detection of *S arizonae* and atypical *Salmonella* strains that utilize lactose and/or sucrose. Discard only apparent non-*Salmonella* TSI agar cultures (acid slant and acid butt) if corresponding LIA reactions are not typical (acid butt) for *Salmonella*.

F. Purification and Identification

(a) *Appearance of Salmonella colonies.*—(*1*) *On MAC.*—Typical colonies appear transparent and colorless, sometimes with dark centers. *Salmonella* will clear areas of pptd bile caused by other organisms sometimes present in medium.

(*2*) *On HE.*—Blue-green to blue colonies with or without black centers. Many *Salmonella* cultures may have large glossy black centers or may appear as almost completely black colonies.

(b) *Purification of mixed cultures.*—Examine MAC or HE. If pure, proceed with identification. If mixed culture, pick with needle ≥2 well isolated typical or suspicious colonies and inoculate TSI, LIA, and MAC or HE as described above. Incubate and examine for presumptive pos. reactions.

(c) *Identification.*—Carry out biochem. and serological identification procedures on 3 presumptive pos. TSI cultures from each HGMF as described in **967.27B–E**, **967.28**. As alternative to conventional tube system for *Salmonella*, any one of 4 commercial biochem. systems (API, Enterotube, Minitek, or Micro-ID) may be used for presumptive generic identification of foodborne *Salmonella*. *See* **978.24**.

Ref.: JAOAC **68,** 555(1985).

986.35 *Salmonella* in Foods

Colorimetric Monoclonal Enzyme Immunoassay Screening Method

First Action 1986
Final Action 1988

Method is screening procedure for presence of *Salmonella* in all foods; it is not a confirmatory test because monoclonal antibodies used in test may cross-react with small percentage of non-*Salmonella*.

Enrichment broths and M-broth from samples pos. by enzyme immunoassay (EIA) method must be streaked on selective media as in **967.26B** and typical or suspicious colonies must be identified as in **967.26C**, **967.27**, **967.28**.

Detn of pos. result is objective and must be performed using filter photometer having 405–420 nm filter. Pos. result is valid only when neg. and pos. controls possess acceptable optical density readings.

A. Principle

Detection of *Salmonella* antigens is based on solid phase immunoassay and uses mag. force to transfer solid phase from one reaction mixt. to another. Monoclonal antibodies to *Salmonella* antigen are bound to surface of beads made of ferrous metal. Beads are placed in sample to be assayed. If *Salmonella* antigens are present in sample, they will attach to specific antibody on beads. Beads are washed and then released into reaction mixt. contg peroxidase-conjugated anti-*Salmonella* immunoglobulins. Conjugate will bind to *Salmonella* antigens if they are attached to antibody molecules on surface of beads. Beads are washed to remove unbound conjugate and then placed in substrate soln. Appearance of color indicates presence of *Salmonella* antigen in sample. Fig. **986.35A** shows schematic representation.

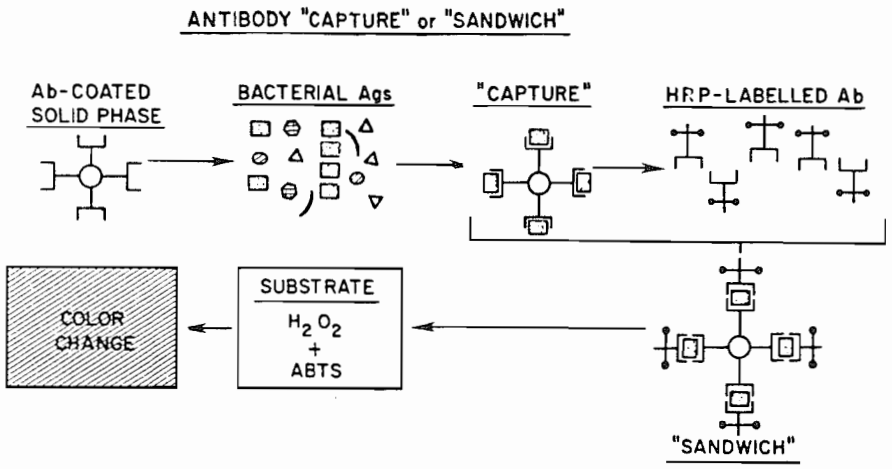
B. Method Performance

For all foods:

Results	Percent	95% Confidence Range (Approx.)
Agreement[1]	96.9	95.7–98.1
False neg. (BAM)[2]	1.5	0.4–2.5
False neg. (EIA)[3]	3.4	1.8–5.0

[1] This rate reflects no. of samples read identically between AOAC/BAM (Bacteriological Analytical Manual (1984) 6th ed., AOAC, Arlington, VA) culture method and EIA.
[2] This rate reflects no. of samples found to be pos. by EIA but detd to be neg. by AOAC/BAM culture method.
[3] This rate reflects no. of samples found to be pos. by AOAC/BAM culture method but detd to be neg. by EIA.

Of 21 laboratories, 10 (48%) had complete agreement between culture method and EIA (153/153); 17 laboratories (81%)



Ab = antibody
Ag = antigen
HRP = horseradish peroxidase
H_2O_2 = hydrogen peroxide
ABTS = 2,2′-azino-di[3-ethylbenzthiazoline-sulfonate(6)]

FIG. 986.35A—Antibody "capture" or "sandwich"

showed agreement on ≥96% of samples; 20 (95%) showed agreement on ≥93% of samples.

C. Reagents

Items (**a**)–(**m**) are available as *Salmonella* Bio-EnzaBead Screen Kit (Organon Teknika Corp., 100 Akzo Ave, Durham, NC 27704).

(**a**) *Antibody-coated beads.*—Monoclonal antibodies to *Salmonella*, 2 vials (48 beads/vial). Store bead vials tightly capped at 2–8°. Beads are stable 14 days after opening.

(**b**) *Control antigens.*—Pos. control (heat-treated *S. javiana*) which reacts with antibodies to *Salmonella*, 1 vial; neg. control which is nonreactive with antibodies to *Salmonella*, 1 vial. Reconstituted control antigens are stable 28 days when stored at 2–8°.

(**c**) *Conjugate diluent.*—1 vial (24 mL/vial). Contains 1% bovine serum in phosphate-buffered saline contg 0.05% Tween 20 and 0.01% thimerosal as preservative.

(**d**) *Reagent water.*—1 bottle (125 mL/bottle). Store at room temp. or warm to room temp. before use.

(**e**) *Phosphate-buffered saline.*—PBS, pH = 7.5 ± 0.2; 1 bottle (125 mL/bottle). Contains 1.2 g Na_2HPO_4, 0.22 g $NaH_2PO_4.H_2O$, and 8.5 g NaCl/L H_2O.

(**f**) *Peroxidase-conjugated antibodies to Salmonella.*—1 vial (lyophilized). When reconstituted, conjugate is stable 28 days when stored at 2–8°.

(**g**) *Wash solution (50×).*—1 vial (2.5 mL). Contains 2.5% surfactant.

(**h**) *ABTS substrate.*—2 vials (lyophilized). After reconstitution, each vial contains 0.03% 2,2′-azino-di(3-ethyl-benzthiazoline-sulfonate). Reconstituted substrate is stable 14 days when stored tightly capped at 2–8°. Let reconstituted substrate warm to room temp. before dispensing.

(**i**) *Substrate diluent for ABTS.*—2 vials (30 mL/vial). Contains H_2O_2.

(**j**) *"Stop" soln.*—1 vial (5 mL/vial). Contains 1.25% NaF. *Caution:* Avoid contact with skin. If contact occurs, wash area with H_2O.

(**k**) *Microtitration plates.*—Plate ($3^5/_{16}$ × 5 in.) possessing 96 wells, each having capability of holding >0.3 mL fluid. These must be designed in 8 × 12 format which will fit into mag. transfer device. Spaces between wells should be hollowed out, and not filled in with plastic coming to top of well. Available as "Accessory Package" (Organon Teknika Corp.), or equiv. may be used. *Note:* Not all microtitrn plates meet these criteria.

(**l**) *Package insert.*

(**m**) *Data record sheets.*

(**n**) *M-broth.*—5.0 g yeast ext, 12.5 g tryptone, 2.0 g D-mannose, 5.0 g Na citrate, 5.0 g NaCl, 5.0 g K_2HPO_4, 0.14 g $MnCl_2$, 0.8 g $MgSO_4$, 0.04 g $FeSO_4$, 0.75 g Tween 80. Suspend ingredients in 1 L H_2O, and heat to boiling for 1–2 min. Dispense 10 mL portions into 16 × 125 mm screw-cap test tubes. Cap tubes loosely and autoclave 15 min at 121°. Tighten caps securely for storage. Final pH should be 7.0 ± 0.2.

(**o**) *Diagnostic reagents.*—Necessary for cultural confirmation of presumptive pos. EIA tests; *see* **967.25B**.

D. Apparatus

Items (**a**)–(**e**) are available from Organon Teknika Corp.

(**a**) *Magnetic transfer device.*—Mag. app. which houses microtitrn plates and is used to transfer metal beads from one reagent to another as well as to wash metal beads.

(**b**) *Incubator.*—37° with 100 rpm agitator.

(**c**) *Enzyme immunoassay reader.*—Photometer with 405–420 nm screening filter which will read thru microtitrn plates. Must be able to be set to zero while reading thru unreactive substrate well (blank). Reader should be equipped with printer so that records of analysis can be kept. Semiautomated Organon Teknika 30 or equiv. meets these specifications.

(**d**) *Bead dispenser.*—Either single bead dispenser or 96-well bead dispenser, or suitable alternative. Places beads into wells of microtitrn plate.

(**e**) *Micropipet.*—Capable of delivering accurate amts in range 50–300 μL. Micropipets capable of delivering these vols to multiple wells simultaneously (multichannel) or individually (single channel) are needed.

(**f**) *Centrifuge.*—Having min. capacity to spin centrf. tubes (≤20 mm diam.) at 1500 × *g* in swinging bucket rotor or 3000

× *g* in fixed angle rotor for 20 min. IEC Centra 7 or Centra 8 tabletop centrf. with IEC 216 horizontal rotor (available from International Equipment Co., or equiv., meets these criteria.

(**g**) *Boiling water bath.*—Able to attain and maintain 100°. Must be able to hold centrf. tubes upright. Microwave or autoclave set at 100° is acceptable alternative, as are generators of flowing stream. *Caution:* H_2O baths which do not maintain boiling conditions are unacceptable.

(**h**) *Vortex mixer.*—Capable of vigorous agitation of centrf. tube, such that pellet at bottom of conical tube can be resuspended. S/P mixer (available from Scientific Products, Inc.) or equiv. meets these criteria.

E. General Instructions

Include pos. control antigen and duplicate neg. controls with each group of test samples. All controls must function properly for test to be valid. One addnl well per group of test samples should be left empty initially. This well, filled with substrate, will be used to "blank" assay reader. See sample data record sheet (Fig. **986.35B**).

Use data record sheets to identify location of each test sample.

Do not use mag. transfer device without top and bottom plate in position. Always insert plates in device with notched side facing operator.

Do not reuse wells of a plate or the beads.

Use sep. pipets for each sample and kit reagent to avoid cross-contamination. Take care not to contaminate substrate with conjugate.

The components and procedures of this test kit have been standardized for use in Bio-EnzaBead procedure. Use of components or procedures other than those supplied by Organon Teknika Corp. may yield unsatisfactory results.

F. Preparation of Sample

(**a**) *Pre-enrichment.*—Pre-enrich product in non-inhibitory broth to initiate growth of salmonellae. Methods used may vary with product, and should be performed as indicated in **967.26A**, or in Bacteriological Analytical Manual, 6th ed., AOAC, Arlington, VA, Chap. 7, section C, with the following exception:

Raw or highly contaminated products.—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth. Blend 2 min at high speed (ca 20 000 rpm). Cap jars securely and let stand at room temp. 60 min. Mix well by shaking and det. pH with test paper. Adjust pH to 6.8 ± 0.2, if necessary, using sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Aseptically transfer contents of each jar to sterile wide-mouth, screw-cap 500 mL jar. Loosen jar caps $^1/_4$ turn and incubate 24 ± 2 h at 35°.

(**b**) *Selective enrichment.*—Transfer 1 mL incubated pre-enrichment mixts to selenite cystine broth and 1 mL into tetrathionate broth as in **967.26B(a)**. (For dried active yeast, substitute lauryl sulfate tryptose broth **967.25A(u)** for selenite cystine broth.) Incubate 18–24 h at 35°.

(**c**) *Post-enrichment.*—Remove selective broths from incubation and mix by hand or by vortex mixer. Remove 0.5 mL from tetrathionate tube and transfer to 10 mL tube of sterile M-broth which has been warmed to 35°. Also remove 0.5 mL from selenite cystine tube and transfer to same tube of M-broth and vortex-mix well. Incubate M-broth tube 6 h at 35°. Return tetrathionate and selenite cystine tubes to 35° incubator for addnl 6 h.

(**d**) *Centrifugation and preparation of sample for EIA analysis.*—Remove M-broth from incubation and mix tube by hand or vortex mixer. Pipet 10 mL into centrf. tube (≤20 mm diam.) and label tube. Refrigerate (2–8°) remaining M-broth and tetrathionate and selenite cystine tubes from (**c**) above for cultural confirmation of any enzyme immunoassay pos. samples. These broths may be refrigerated, if necessary, for ≤18 h at 2–8°. Centrf. M-broth at *min.* speed of 1500 × *g* (swinging bucket rotor) or 3000 × *g* (fixed angle rotor) for 20 min. Suction off supernate from tube, using trap flask filled with disinfectant. Resuspend pellet with 1 mL PBS. Vortex-mix tube to mix well. Heat resuspended pellet in boiling H_2O bath or

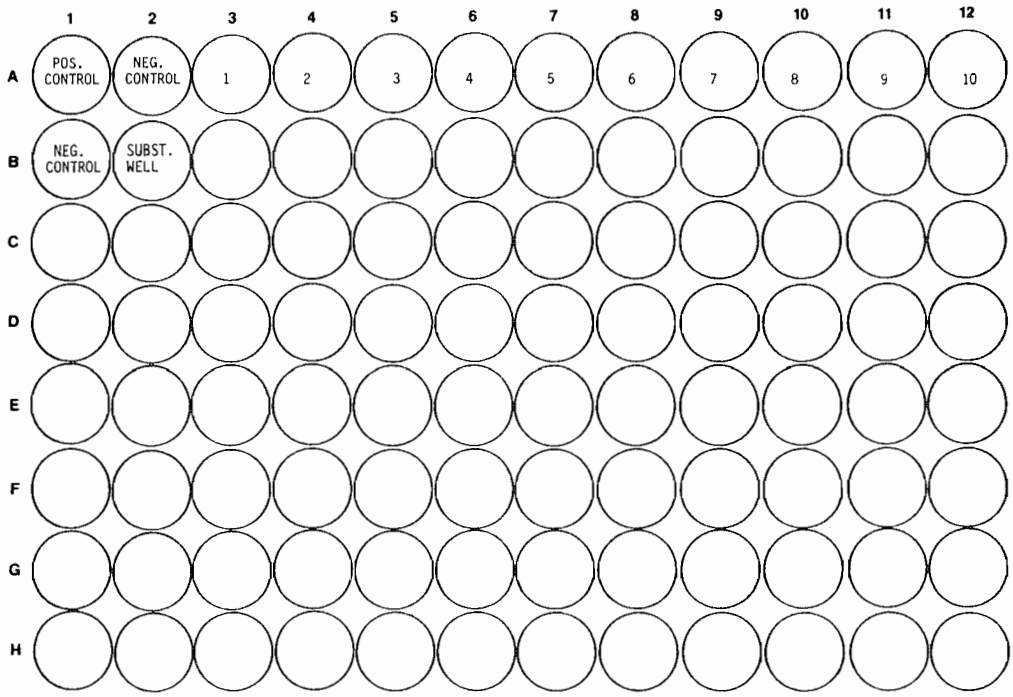


FIG. 986.35B—Data record sheet for identifying location of test samples

in flowing steam 20 min. Cool heated exts to 25–37° prior to analysis by EIA. *Note:* Heated exts which are not cooled to this temp. can destroy monoclonal antibodies on metal beads.

G. Enzyme Immunoassay

(*1*) Reconstitute control antigens by adding 2.5 mL PBS to each vial. Swirl gently to dissolve. Following record sheet, add 0.2 mL aliquot of neg. and pos. control antigens and 0.2 mL aliquot of test samples into designated wells of 96-well plate. *Note:* Use 2 neg. controls and one pos. control for each group of samples. Label plate "antigen."

(*2*) Using bead dispenser or forceps, place antibody-coated beads in wells of one of empty plates according to record sheet. If using forceps, remove beads from vials by rolling beads into cap or onto gauze pad. Do *not* put bead in substrate blanking well.

(*3*) To start assay, simultaneously transfer beads to "antigen" plate by using mag. transfer device as follows:

(a) Invert 96-well plate and insert it with notched end facing operator into top slot until it snaps into position. Magnet should be in UP position.

(b) Slide plate contg beads (with notched end facing operator) into lower slot until it snaps into position, centering it under inverted top plate.

(c) To remove beads from bottom plate, lower magnet to full DOWN position (all beads should now be in inverted plate). Without disturbing top plate, remove lower plate (save for later step) and slide plate contg samples under inverted plate.

(d) Raise magnet to allow all beads to drop into wells of "antigen" plate.

(*4*) Remove bottom plate contg samples and beads. Incubate plate with agitation (10–100 rpm) for 20 min at 37°. During incubation period, proceed to steps *5* and *6*.

(*5*) If entire plate is being used, prep. 1× wash soln by adding 1.5 mL 50× wash soln to 75 mL reagent water in clean glass or plastic screw-cap bottle. Mix by inverting bottle several times. Add 0.3 mL 1× wash soln into appropriate wells of 96-well plate previously saved. Label plate "wash 1." Similarly, fill second plate with 1× wash soln and label "wash 2." If entire plate is not being used, calc. amt of wash soln required by multiplying number of tests by 0.6 and prep. amt of 1× wash soln required based on 0.5 mL 50× wash soln to 25 mL reagent water.

(*6*) Prep. conjugate soln by adding 24 mL 1× conjugate diluent to lyophilized material in vial. Mix gently by inverting bottle several times. Date vial. Add 0.2 mL conjugate into appropriate wells of sep. plate. Label plate "conjugate."

(*7*) Following 20 min incubation (above), wash beads as follows:

(a) Assure that 96-well plate (from step *3*a) is inverted in top slot of mag. transfer device and that magnet is in UP position.

(b) Slide "antigen" plate contg beads under top plate and lower magnet to DOWN position (all beads should now be in inverted plate).

(c) Remove bottom "antigen" plate without disturbing top plate and place it in container for proper disposal.

(d) Slide "wash 1" under top plate and wash beads 12 times by raising and lowering magnet to extreme UP and full DOWN positions (count UP and DOWN as 1 wash).

(e) With magnet in DOWN position (beads in top inverted plate), remove wash plate without disturbing top plate and save for step *11*c. *Note:* Proceed immediately with next step. Do not let beads dry in top plate.

(*8*) Slide "conjugate" plate under top plate and raise magnet to let beads drop into wells.

(*9*) Remove bottom plate contg conjugate and beads and incubate with agitation (10–100 rpm) for 20 min at 37°.

(*10*) While beads are incubating, reconstitute ABTS substrate by adding contents of substrate diluent vial to lyophilized substrate. Mix gently by inverting bottle several times. Date substrate. Add 0.2 mL room temp. substrate into each appropriate well of unused plate. Also, put 0.2 mL into extra well which will be used to "blank" EIA reader.

(*11*) Following conjugate incubation period, wash beads as follows:

(a) Assure that 96-well plate (from step *3*a) is still inverted in top slot of mag. transfer device and that magnet is in UP position.

(b) Slide "conjugate" plate contg beads under top plate and lower magnet to DOWN position (all beads should now be in inverted plate).

(c) Remove bottom plate and slide "wash 1" under top plate. Raise magnet to extreme UP position, allowing beads to fall into "wash 1."

(d) Remove top plate and *replace with unused top plate*. Lower magnet to remove beads from "wash 1." Wash beads twice in "wash 1" by raising and lowering magnet to its extreme UP and full DOWN positions (count UP and DOWN as 1 wash).

(e) With magnet in DOWN position (beads in top plate), remove "wash 1" and insert "wash 2."

(f) Wash beads 9 times by raising and lowering magnet to extreme UP and full DOWN positions.

(g) With magnet in UP position (beads in "wash 2"), *replace top inverted plate with unused plate*. *Note:* This change must be made to avoid contamination of substrate with conjugate.

(h) Lower magnet and remove bottom "wash 2" without disturbing top plate.

(*12*) Immediately slide "substrate" plate under top plate and raise magnet to let all beads drop into the wells.

(*13*) Remove bottom plate contg substrate and beads and incubate uncovered at room temp. (20–25°) for 10 min. *Do not agitate plate*.

(*14*) After 10 min, add 0.025 mL (25 μL) "stop" soln to each well including substrate blanking well. Gently swirl beads in plate to disperse colored reaction product forming at surface of beads. Remove beads (be sure that 96-well plate is inverted in top slot of mag. transfer device) by placing "substrate" plate in mag. transfer device and lowering magnet.

(*15*) Remove "substrate" plate and release beads into used plate by sliding plate into mag. transfer device and raising magnet. Mix contents of substrate blanking well with pipet tip.

(*16*) Read results on EIA reader.

(*17*) Sterilize all used plates, tubes, etc., prior to disposal. Tightly close and return unused reagents to 2–8° storage.

H. Reading

Insert 405 nm filter and bring reader to zero (blank reader) on well contg only substrate and "stop" soln. Then read each individual control and sample well. Average optical density readings of the 2 neg. control wells. For test to be valid, pos. control should read ≥0.200 and av. of neg. controls should read ≤0.120. Record optical density (OD) of each well on data sheet. Samples reading ≥0.200 should be considered pos. Samples reading <0.200 should be considered neg.

I. Confirmation of Positive EIA Samples

Pos. EIA reading indicates that *Salmonella* may be present. However, since antibodies may cross-react with a few other organisms, cultural confirmations should be performed by streaking HE, XLD, and BS plates from tetrathionate broth,

selenite cystine broth, and M-broth tubes as described in **967.26B**, and typical or suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

Ref.: JAOAC **69,** 786(1986).

987.11 *Salmonella* in Low-Moisture Foods

Colorimetric Monoclonal Enzyme Immunoassay Screening Method

First Action 1987

Method is screening procedure for presence of *Salmonella* in low-moisture foods.

Enrichment broths and M-broths from samples pos. by enzyme immunoassay (EIA) method must be streaked on selective media as in **967.26B** and typical or suspicious colonies should be identified as in **967.26C**, **967.27, 967.28**.

Detn of pos. result is objective and must be performed using filter photometer having 405 nm filter. Pos. result is valid only when neg. and pos. controls exhibit acceptable optical density readings.

A. Principle

See **986.35A**.

B. Method Performance

Results	Percent	95% Confidence Range (Approx.)
Agreement[1]	97.0	95.8–98.2
False neg. rate[2]	3.3	1.7–4.9

[1] This rate reflects no. of samples read identically between 302AOAC/BAM (Bacteriological Analytical Manual (1984) 6th ed., AOAC, Arlington, VA) culture method and EIA.

[2] This rate reflects no. of samples found to be pos. by AOAC/BAM culture method but detd to be neg. by EIA.

Of 15 laboratories, 7 (47%) had complete agreement between culture method and EIA (156/156); 14 laboratories (93%) showed agreement on ≥97% of samples.

C. Reagents

See **986.35C**.

D. Apparatus

See **986.35D**.

E. General Instructions

See **986.35E**.

F. Preparation of Sample

(**a**) *Pre-enrichment.*—Pre-enrich low-moisture food product in non-inhibitory broth to initiate growth of salmonellae. Methods used may vary with product, and should be performed as indicated in **967.26A**, or in Bacteriological Analytical Manual (1984) 6th ed., AOAC, Arlington, VA, Chap. 7, section C, except incubation time is 18–24 h.

(**b**) *Selective enrichment.*—Transfer 1 mL incubated pre-enrichment mixt. to selenite cystine broth and 1 mL to tetrathionate broth as in **967.26B(a)**. Pre-warm both broths to 35° before inoculation. (For dried active yeast, substitute lauryl sulfate tryptose broth, **967.25A(u)**, for selenite cystine broth.) Incubate 6–8 h in 35° *water bath.*

(**c**) *Post-enrichment.*—Remove selective broths from incubation and mix by hand or by vortex mixer. Remove 1.0 mL from tetrathionate tube and transfer to 10 mL tube of sterile M-broth. Also remove 1.0 mL from selenite cystine tube and transfer to another 10 mL tube of sterile M-broth. Incubate both M-broths and remaining tetrathionate and selenite cystine broths for 14–18 h at 35°.

(**d**) *Preparation of sample for EIA analysis.*—Remove the 2 tubes of M-broth (M-broth-Tet and M-broth-SC) from incubation and mix tubes by hand or by vortex mixer. Remove 0.5 mL from M-broth-Tet tube and transfer to glass screw-cap test tube. Also remove 0.5 mL from M-broth-SC tube and transfer to same screw-cap test tube. Refrigerate (2–8°) remaining M-broths and tetrathionate and selenite cystine broths from (**c**) for cultural confirmation of EIA-pos. samples. Heat combined M-broths in boiling H_2O bath or in flowing steam 20 min. Cool heated exts to 25–37° prior to analysis by EIA. *Note:* Heated exts which are not cooled to this temp. can destroy monoclonal antibodies on metal beads.

G. Enzyme Immunoassay

See **986.35G**.

H. Reading

See **986.35H**.

I. Confirmation of Positive EIA Samples

Pos. EIA reading indicates that *Salmonella* may be present. However, since antibodies may cross-react with a few other organisms, culture confirmations should be performed by streaking HE, XLD, and BS plates from tetrathionate broth, selenite cystine broth, and the associated M-broth tubes as described in **967.26B**, and typical or suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

Ref.: JAOAC **70,** 530(1987).

989.14 Salmonella in Foods

Colorimetric Polyclonal Enzyme Immunoassay Screening Method

First Action 1989

Method is screening procedure for presence of *Salmonella* in all foods; it is not a confirmatory test because polyclonal antibodies used in test may cross-react with small percentage of non-*Salmonella*.

Enrichment broths and M-broths from samples pos. by enzyme immunoassay (EIA) method must be streaked on selective media as in **967.26B** and typical or suspicious colonies must be identified as in **967.26C**, **967.27**, **967.28**.

Detn of pos. result may be performed *(1)* visually by aid of color comparator card where pos. result is valid when neg. and pos. controls match those described on card or *(2)* instrumentally using filter photometer having 414 nm filter where pos. result is valid only when neg. and pos. controls possess acceptable optical density readings.

A. Principle

Detection of *Salmonella* antigens is based on enzyme immunoassay using highly purified antibodies prepd from antigens unique to *Salmonella*. Polyclonal antibodies to *Salmonella* antigen are adsorbed onto internal surface of 96-well microtiter tray. Sample to be assayed is placed into well of tray. If *Salmonella* antigens are present in sample, they will attach to specific antibody adsorbed on well. All other material in samples is washed away. Conjugate is added and will bind to *Salmonella* antigens if they are attached to adsorbed antibody on surface of well. Wells are washed to remove unbound conjugate, and enzyme substrate is added. Dark blue-green color indicates presence of *Salmonella* antigen in sample.

B. Method Performance

For all foods:

Results	Percent	95% Confidence Range (Approx.)
Agreement[1]	96.8	95.4–98.2
False neg. (BAM)[2]	1.6	0.5–2.7
False neg. (EIA)[3]	1.4	0.4–2.4

[1]This rate reflects no. of samples read identically between AOAC/BAM (Bacteriological Analytical Manual, 1984, 6th ed., AOAC, Arlington, VA) culture method and EIA.
[2]This rate reflects no. of samples found to be pos. by EIA but neg. by AOAC/BAM culture method.
[3]This rate reflects no. of samples found to be pos. by AOAC/BAM culture method but neg. by EIA.

Of 14 laboratories, 3 had complete agreement between culture method and EIA method. Excluding 1 food group, turkey, 13 of the 14 laboratories had perfect agreement between BAM/AOAC and EIA methods. Laboratory that did not have perfect agreement had difference in each of pepper, nonfat dry milk, and chocolate food groups.

C. Reagents

Items (**a**)–(**m**) are available as TECRA *Salmonella* Visual Immunoassay (Bioenterprises Pty Ltd, 28 Barcoo St, Roseville, NSW 2069, Australia). Substitutions must be pretested for equivalency.

(**a**) *Antibody adsorbed strips.*—Removawell® (Dynatech Laboratories, Inc.) strips. Polyclonal antobodies to *Salmonella,* 96 wells. Store wells at 2–8° when not in use.

(**b**) *Tray.*—Sufficient to secure individual wells or strips.

(**c**) *Control antigens.*—Pos. control (lyophilized). Purified *Salmonella* antigen, which reacts with antibodies to *Salmonella,* 1 vial. Neg. control (lyophilized lactose), which is nonreactive with antibodies to *Salmonella,* 1 vial. Reconstituted control antigens are stable 28 days when stored at 2–8°.

(**d**) *Controls diluent.*—1 vial (5 mL/vial). Contains 0.006 g Tris [tris(hydroxymethyl)aminomethane], 0.044 g NaCl, 0.0025 g Tween 20 (polyoxyethylene 20 sorbitan monolaurate), and 0.005 g thimerosal in H_2O.

(**e**) *Conjugate.*—1 vial (lyophilized). Contains 147 ng anti-*Salmonella* antibodies (from sheep) conjugated to horseradish peroxidase, 0.00686 g $Na_2B_4O_7$, 0.12 g Dextran T10, 0.06 g hydrolyzed gelatin, 0.0024 g $CaCl_2$, and 120 ng thimerosal. Reconstituted conjugate is stable 28 days when stored at 2–8°.

(**f**) *Conjugate diluent.*—1 vial (22 mL/vial). Contains 0.42 g $Na_2B_4O_7$, 0.193 g NaCl, 0.22 g hydrolyzed gelatin, and 0.0022 g thimerosal in H_2O.

(**g**) *Substrate.*—1 vial (lyophilized). Contains 0.011 g 2,2′-azino-di(3-ethylbenzthiazoline sulfonate) and 0.123 g NaH_2PO_4. Reconstituted substrate is stable 28 days when stored at 2–8°.

(**h**) *Substrate diluent.*—1 vial (22 mL/vial). Contains 0.116 g citric acid, 0.0011 g H_2O_2, and 0.0185 g NaOH in H_2O.

(**i**) *"Stop" soln.*—1 vial (6 mL/vial). Vial contains 0.15 g NaF in H_2O. *Caution:* Avoid contact with skin. If contact occurs, wash area with H_2O.

(**j**) *Wash soln concentrate.*—1 vial (25 mL/vial). Contains 1.45 g Tris, 7.03 g NaCl, 0.5 g Tween 20, and 0.0025 g thimerosal in H_2O.

(**k**) *Package insert.*

(**l**) *Data record sheet.*

(**m**) *Color comparator card.*—For visual interpretation of pos. and neg. tests.

(**n**) *M-broth.*—5.0 g yeast ext, 12.5 g tryptone, 2.0 g D-mannose, 5.0 g Na citrate, 5.0 g NaCl, 5.0 g K_2HPO_4, 0.14 g $MnCl_2$, 0.8 g $MgSO_4$, 0.04 g $FeSO_4$, 0.75 g Tween 80. Suspend ingredients in 1 L H_2O and heat to boiling for 1–2 min. Dispense 10 mL portions into 16 × 125 mm screw-cap test tubes. Cap tubes loosely and autoclave 15 min at 121°. Tighten caps securely for storage. Final pH should be 7.0 ± 0.2.

(**o**) *Diagnostic reagents.*—Necessary for culture confirmation of presumptive pos. EIA tests; see **967.25B**.

D. Apparatus

(**a**) *Incubator.*—35–37°.

(**b**) *Multipipets.*—Capable of delivering accurate amts in ranges 50–250 μL and 5–50 μL.

(**c**) *Water bath.*—Capable of maintaining 100°. Autoclave set at 100° is acceptable alternative, as are generators of flowing steam.

(**d**) *Plastic squeeze bottle.*—500 mL, for dispensing wash soln. Automatic washer may be used.

(**e**) *Plastic film wrap or sealable plastic container.*—To cover wells during incubation.

(**f**) *Enzyme immunoassay reader.*—Optional. Photometer with 414 ± 10 nm screening filter which will read thru microtiter plates.

E. General Instructions

Components of kit must be refrigerated when not in use. Kit is intended for 1-time use only; do not reuse wells containing sample, reagents, or wash solution.

Include duplicate pos. and neg. control antigens with each group of test samples. All controls must function properly for test to be valid.

Use data record sheet to identify location of each test sample.

Use sep. pipets for each sample and kit reagent to avoid cross-contamination. If plastic troughs are used to dispense conjugate and substrate, ensure that they are always kept separate.

Components in kit are intended for use as integral unit. Do not mix components of different batch numbers.

F. Preparation of Sample

(**a**) *Pre-enrichment.*—Pre-enrich product in noninhibitory broth to initiate growth of salmonellae. Methods used may vary with product and should be performed as indicated in **967.26A**, or in Bacteriological Analytical Manual, 6th ed., AOAC, Arlington, VA, Chap. 7, section C, with following exception:

Raw or highly contaminated products.—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth. Blend 2 min at high speed (ca 20 000 rpm). Cap jars securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH to 6.8 ± 0.2, if necessary, using sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Aseptically transfer contents of each jar to sterile wide-mouth, screw-cap 500 mL jar. Loosen jar caps 1/4 turn and incubate 24 ± 2 h at 35°.

(**b**) *Selective enrichment.*—Transfer 1 mL incubated pre-enrichment mixts to selenite cystine broth and 1 mL to tetrathionate broth as in **967.26B(a)**. For all foods other than raw or highly contaminated products, incubate 6–8 h at 35°. Selective enrichments of raw or highly contaminated product must be incubated 16–20 h at 35°.

(**c**) *Post-enrichment.*—Remove selective broths from incubation and mix by hand or by vortex mixer. Remove 1 mL from tetrathionate tube and transfer to 10 mL tube of sterile M-broth which has been warmed to 35°. Also remove 1 mL from selenite cystine tube and transfer to sep. tube of M-broth. For all foods other than raw or highly contaminated products, incubate M-broth tubes 14–18 h and return selective enrich-

ment broth tubes to 35° incubator and incubate for addnl 16–18 h. For raw or highly contaminated products, incubate M-broth tubes 6 h at 35° and return selective enrichment broth tubes to 35° and incubate for addnl 6 h at 35°.

(**d**) *Preparation of sample for EIA analysis.*—Remove M-broth tubes from incubation and mix tubes by hand or vortex mixer. Combine 1.0 mL from each M-broth tube in clean screw-cap tube and heat in boiling H_2O bath or in flowing steam 15 min. Refrigerate (2–8°) remaining M-broth and tetrathionate and selenite cystine tubes from (**c**) for culture confirmation of any EIA pos. samples. Cool heated M-broths to 25–37° prior to analysis by EIA.

G. Enzyme Immunoassay

(1) Following reagents must be prepd prior to commencing assay:

(a) Prep. working strength wash soln by dilg contents of 1 vial of wash soln conc. to 1 L with distd or deionized H_2O into plastic reagent bottle. Plastic squeeze bottle is ideal for washing trays manually.

(b) Prep. reconstituted neg. control by transferring 2 mL controls diluent to vial of lyophilized neg. control antigen; mix thoroly. Similarly prep. reconstituted pos. control by transferring 2 mL controls diluent to vial of lyophilized pos. control antigen; mix thoroly.

(c) Prep. reconstituted conjugate by adding 5 mL conjugate diluent to vial of lyophilized conjugate. Let conjugate rehydrate at room temp., mix, and then pour contents of vial into conjugate diluent vial. Finally, gently mix reconstituted conjugate.

(d) Prep. reconstituted substrate by adding vial of substrate diluent to lyophilized substrate. Be sure substrate has dissolved and mixt. is room temp. prior to use. Reconstituted substrate will appear pale green.

(e) Use stop soln as received. No reconstitution is required.

(2) Secure desired no. of test (Removawell) strips in tray, allowing 1 well per food sample plus 4 wells for controls. PRESS WELLS FIRMLY INTO PLACE. Remove sealing film from top of wells to be used. Transfer 0.2 mL of each heated M-broth sample to single well. Transfer 0.2 mL aliquots of reconstituted neg. control into 2 wells and 0.2 mL aliquots of reconstituted pos. control into 2 wells. Record sample position on sample record sheet provided. *Note:* Be sure numbered tag at end of each test strip has been removed.

(3) Cover tray and incubate 30 min at 35–37° in std laboratory incubator. Tray must be covered to prevent evapn. Plastic film or sealed plastic container may be used.

(4) After incubation, wash plate by hand using plastic squeeze bottle contg working strength wash soln or use automatic washer charged with working strength wash soln as follows:

(a) Quickly invert tray, emptying its contents into container.

(b) Remove any residual liquid by FIRMLY tapping tray face-down on paper towel several times.

(c) Completely fill each well with working strength wash soln.

(d) Repeat (a)–(c) **2** more times.

(5) Empty tray according to *4*(a) and (b); then add 0.2 mL reconstituted conjugate to each well. Cover tray and incubate 30 min at 35–37°.

(6) Empty contents of tray and wash it thoroly **4** times according to *4*(a)–(c); then empty tray according to *4*(a) and (b).

(7) Add 0.2 mL reconstituted substrate to each well. Incubate at room temp. (20–25°) until pos. control has reached color equiv. to pos. control on color comparator card or to $A \geq 1.0$. Because color development tends to conc. around edges of wells, it is important to tap sides of plate *gently* to mix contents prior to reading result. In this way, accurate readings will be obtained.

(8) Add 0.02 mL stop soln to each well. Incubation time should be ca 10–20 min. If >25 min has elapsed and A of 1.0 has not been attained, test is invalid.

H. Reading

Results of tests can be detd *(1)* visually or *(2)* with microtiter tray reader.

(1) Place tray on white background, and then compare individual test wells with color comparator. Pos. control should give strong blue-green color indicating that all reagents are functional. If pos. control is lighter than "Positive Control" on color comparator card, test is invalid; refer to "Troubleshooting Guide" in package insert (**k**). If neg. control is darker than "Negative" on color comparator card, it is probable that tray was inadequately washed, and assay must be repeated. Duplicate control antigens should appear equiv. by eye.

(2) A max. of blue-green end product occurs at 414 nm; therefore, tray can be read at 414 ± 10 nm. For single and dual wavelength readers, set reader to zero (blank) on air. For dual wavelength readers, set second ref. wavelength at 490 ± 10 nm. A >0.3 indicates pos. result. A >0.25 for neg. control indicates insufficient washing of tray. Pos. control should give $A \geq 1.0$.

I. Confirmation of Positive EIA Samples

Pos. EIA reading indicates that *Salmonella* may be present. However, since antibodies may cross-react with a few other organisms, culture confirmations must be performed by streaking HE, XLD, and BS plates from tetrathionate broth, selenite cystine broth, and M-broth tubes as described in **967.26B**, and typical or suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

Ref.: JAOAC **71,** 973(1988).

989.15 *Salmonella* in Foods

Fluorogenic Monoclonal Enzyme Immunoassay Screening Method

First Action 1989

Method is screening procedure for presence of *Salmonella* in all foods; it is not a confirmatory test because monoclonal antibodies used in test may cross-react with small percentage of non-*Salmonella*.

Enrichment broths and M-broths from samples pos. by enzyme immunoassay (EIA) method must be streaked on selective media as in **967.26B** and typical or suspicious colonies must be identified as in **967.26C**, **967.27**, **967.28**.

A. Principle

Detection of *Salmonella* antigens is based on enzyme immunoassay which measures *Salmonella* antigen in foods and feeds. Monoclonal antibodies to *Salmonella* antigen are coated on internal surface of plastic microtiter strip wells, and sample to be assayed is added to strip well. If *Salmonella* antigens are present in sample, they will be bound to antibody adsorbed onto surface of well. All other material in sample is washed away.

Salmonella antibody conjugated to alk. phosphatase is added and will bind to *Salmonella* antigens if they are attached to adsorbed antibody on surface of well. This forms antibody-antigen-antibody complex. Unbound conjugate is removed by washing and fluorescent substrate is added. Samples with fluorescent value greater than or equal to recommended cutoff value are considered pos. for *Salmonella* antigens.

B. Method Performance

For all foods:

Results	Percent	95% Confidence Range (Approx.)
Agreement[1]	98.5	97.9–99.1
False neg. (BAM)[2]	1.1	0.8–1.4
False neg. (EIA)[3]	0.8	0.0–2.7

[1] This rate reflects no. of samples read identically between AOAC/BAM (Bacteriological Analytical Manual (1984) 6th ed., AOAC, Arlington, VA) culture method and EIA.
[2] This rate reflects no. of samples found to be pos. by EIA but neg. by AOAC/BAM culture method.
[3] This rate reflects no. of samples found to be pos. by AOAC/BAM culture method but neg. by EIA.

Of 11 laboratories submitting usable data, 6 (55%) had complete agreement between culture method and EIA; 10 laboratories (91%) showed agreement on ≥96% of samples; all laboratories showed agreement on ≥93% of samples.

C. Reagents

Items (**a**)–(**j**) are available as Q-TROL *Salmonella* Detection Kit (Dynatech Laboratories, Inc., 14340 Sullyfield Circle, Chantilly, VA 22021).

(**a**) *Antibody coated microtiter wells.*—Monoclonal antibody to *Salmonella,* eight 12-well strips. Stable 28 days, after opening, when stored at 2–8°.

(**b**) *Microtiter strip well holder.*—Sufficient for securing individual wells or strips.

(**c**) *Control antigens.*—Pos. control (lyophilized boiled suspension of *S. typhimurium*) purified *Salmonella* antigen, which reacts with antibodies to *Salmonella,* 1 vial; neg. control (lyophilized boiled suspension of *Proteus mirabilis*), which is nonreactive with antibodies to *Salmonella,* 1 vial. Reconstituted control antigens are stable 28 days when stored at 2–8°.

(**d**) *Tween 20.*—1 vial. 25% Tween 20 (polyoxyethylene (20) sorbitan monolaurate) in H_2O. After opening, soln is stable 28 days when stored at 2–8°.

(**e**) *Phosphate buffer-saline tablets.*—For prepn of PBS-Tween soln. Dissolve 1 tablet in 100 mL distd or deionized H_2O to prep. 0.01M phosphate-buffered 0.85% saline. Add 8 drops Tween 20. PBS-Tween soln is used to rehydrate pos. and neg. control antigens and for wash steps, and is stable 7 days when stored at 2–8°.

(**f**) *Enzyme conjugate.*—1 vial contg antibody of *Salmonella* conjugated to alk. phosphatase (lyophilized). Reconstituted conjugate is stable 28 days when stored at 2–8°.

(**g**) *Conjugate diluent.*—1 vial (10 mL/vial). Contains 0.05M tris buffer (pH 8), 0.02% NaN_3, 1 mM $MgCl_2$, and 1% bovine serum albumin.

(**h**) *Substrate tablets.*—0.13 mg 4-methylumbelliferyl phosphate (4-MUP) per tablet. Reconstituted substrate must be used within 2 h.

(**i**) *Substrate diluent.*—1 vial (31.5 mL/bottle). Aq. soln of 10% diethanolamine with 0.02% NaN_3 as preservative.

(**j**) *Stop soln.*—1 vial (5.5 mL/vial). Aq. soln of 2% Na_3PO_4.

(**k**) *M-broth.*—5.0 g yeast ext, 12.5 g tryptone, 2.0 g D-mannose, 5.0 g Na citrate, 5.0 g NaCl, 5.0 g K_2HPO_4, 0.14 g $MnCl_2$, 0.8 g $MgSO_4$, 0.04 g $FeSO_4$, 0.75 g Tween 80. Suspend ingredients in 1 L H_2O and heat to boiling for 1–2 min. Dispense 10 mL portions into 16 × 125 mm screw-cap test tubes. Cap tubes loosely and autoclave 15 min at 121°. Tighten caps securely for storage. Final pH should be 7.0 ± 0.2.

(**l**) *Diagnostic reagents.*—Necessary for cultural confirmation of presumptive pos. EIA tests; see **967.25B**.

D. Apparatus

(**a**) *Fluorometer.*—To measure relative fluorescence of contents of microtiter well (Micro FLUOR® Reader, Dynatech Laboratories, Inc.; or equiv.).

(**b**) *Microtiter strip well-washer/aspirator.*—With 12 channels to wash entire strip.

(**c**) *Pipets.*—Capable of delivering 50–200 µL.

(**d**) *Water bath.*—Capable of maintaining 100°. Autoclave set at 100° is acceptable alternative, as are generators of flowing steam.

E. General Instructions

Components of kit must be refrigerated when not in use. Kit is intended for 1-time use only; do not reuse wells contg sample, reagents, or wash soln.

Include 3 neg. and 1 pos. control antigens with each group of test samples. All controls must function properly for test to be valid.

Caution: Diluents for conjugate and substrate contain NaN_3 as preservative. Flush drains with H_2O if any solns contg NaN_3 are discarded in sink. Flushing will prevent formation of lead or copper azide in plumbing, which may explode upon percussion (such as hammering). 4-MUP diluent and stop soln are basic and may cause skin irritation. If contact with skin occurs, flush area with H_2O.

Use data record sheet to identify location of each test sample.

Use sep. pipets for each sample and kit reagent to avoid cross-contamination. If plastic troughs are used to dispense conjugate and substrate, ensure that they are always kept separate.

Components in kit are intended for use as integral unit. Do not mix components of different batch numbers.

F. Preparation of Sample

(**a**) *Pre-enrichment.*—Pre-enrich product in noninhibitory broth to initiate growth of salmonellae. Methods used may vary with product and should be performed as indicated in **967.26A**, or in Bacteriological Analytical Manual, 6th ed., AOAC, Arlington, VA, Chap. 7, sec. C, with following exception:

Raw or highly contaminated products.—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth. Blend 2 min at high speed (ca 20 000 rpm). Cap jars securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH to 6.8 ± 0.2, if necessary, using sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Aseptically transfer contents of each jar to sterile wide-mouth, screw-cap 500 mL jar. Loosen jar caps 1/4 turn and incubate 24 ± 2 h at 35°.

(**b**) *Selective enrichment.*—Transfer 1 mL incubated pre-enrichment mixts to selenite cystine broth and 1 mL into tetrathionate broth as in **967.26B(a)**. For all foods, incubate 6–8 h in 35° water bath.

(**c**) *Post-enrichment.*—Remove selective broths from incubation and mix by hand or by vortex mixer. Remove 1 mL from tetrathionate tube and transfer to 10 mL tube of sterile M-broth which has been warmed to 35°. Also remove 1 mL from selenite cystine tube and transfer to a separate tube of M-broth. For all foods, incubate M-broth tubes for 14–18 h and return selective enrichment broth tubes to 35° incubator and incubate for addnl 16–18 h.

(**d**) *Preparation of sample for EIA analysis.*—Remove M-broth tubes from incubation and mix tubes by hand or vortex mixer. Combine 0.5 mL from each M-broth tube in clean screw-cap tube and heat 20 min in boiling H_2O bath or flowing steam. Refrigerate (2–8°) remaining M-broth and tetrathionate and selenite cystine tubes from (**c**) for cultural confirmation of any

EIA pos. samples. Cool heated M-broths to 20–30° prior to analysis by EIA.

G. Enzyme Immunoassay

(1) Following reagents must be prepd prior to commencing assay:

(a) *PBS-Tween soln.*—For every two 12-well strips to be used, dissolve 1 PBS tablet in H_2O and prep. soln as in (**e**).

(b) *Reconstituted control antigens.*—Transfer 3 mL PBS-Tween soln to neg. control vial and mix contents thoroughly. Transfer 3 mL PBS-Tween soln to pos. control vial and mix contents thoroughly. These solns are reconstituted neg. and pos. controls, resp.

(c) *Reconstituted enzyme conjugate.*—Add 10 mL (1 vial) conjugate diluent to conjugate vial. Mix and let contents of vial rehydrate at room temp.

(d) *Stop soln.*—No reconstitution is required. Warm soln at 35° if crystals are present.

(2) Turn on power to reader and printer. Allow at least 2 h warm-up.

(3) Remove necessary number of microtiter strips from Al foil pouch, allowing 1 well per food sample plus 4 wells for controls. Secure strips in strip well holder. Transfer 100 μL neg. control antigen into each of wells A-1, A-2, and A-3. Transfer 100 μL pos. control antigen into well designated A-4. Transfer 100 μL each heated M-broth sample to single well. Record sample position on sample record sheet provided.

(4) Incubate tray for 60 min at 20–25°.

(5) After incubation, aspirate samples from wells and add 300 μL PBS-Tween soln to each well by use of washer/aspirator.

(a) Repeat this step 4 more times.

(b) Aspirate last wash. Invert tray and firmly tap it on absorbent paper several times to remove last traces of fluid.

(6) Add 100 μL reconstituted enzyme conjugate to bottom of each well and incubate 40 min at 20–25°.

(7) During this incubation period, prep. substrate by adding one 4-MUP substrate tablet to 5.2 mL substrate diluent. Dissolve 1 substrate tablet for every 2 microtiter strips to be used. Swirl soln occasionally to dissolve tablet(s).

(8) Repeat steps *5*(a) and (b).

(9) Add 200 μL 4-MUP substrate to bottom of each well. Incubate 20 min at 20–25°.

(10) Add 50 μL stop soln to each well.

H. Reading

Place tray in reader. Read relative fluorescent units (RFU) of each control and sample well. Calc. av. RFU of the 3 neg. control wells. Individual neg. control values should be ≥0.85 av. RFU and ≤1.15 av. RFU. If 1 value is outside this range, discard that value and recalc. mean. If 2 values are outside range, test is invalid and must be repeated. Multiply av. valid neg. controls by 2.3 to det. cutoff value. Any sample with value at or above cutoff value is considered reactive.

If av. of neg. control values exceeds 1600 RFU, cutoff will exceed dynamic range of reader and test is invalid. Poor washing and deterioration of substrate may result in high readings of neg. control.

I. Confirmation of Positive EIA Samples

Pos. EIA reading indicates that *Salmonella* may be present. However, since antibodies may cross-react with a few other organisms, culture confirmations should be performed by streaking HE, XLD, and BS plates from tetrathionate broth, selenite cystine broth, and M-broth tubes as described in **967.26B**, and typical or suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

Ref.: JAOAC **72,** 318(1989).

987.10 *Salmonella* in Foods

DNA Hybridization Screening Method

First Action 1987
Final Action 1989

Method is test procedure for presence of *Salmonella* in all foods. Because a certain percentage of false pos. reactions are expected, all pos. assays should be confirmed by standard culture methods. Enrichment broths and GN broths from samples pos. by DNA hybridization method should be streaked to selective media as in **967.26B** and typical or suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

A. Principle

Detection of *Salmonella* DNA in cultured food samples uses specific DNA probes. Following pre-enrichment, selective enrichment, and post-enrichment of test samples, bacteria are collected on membrane filters by vac. filtration. Bacteria are lysed, DNA is denatured, and resultant single-stranded DNA is fixed to membrane filters. Filters are then incubated in hybridization soln contg ^{32}P-labeled *Salmonella*-specific DNA molecules. If *Salmonella* target DNA is present in test sample, radiolabeled DNA probes will hybridize to target DNA sequences. Unbound probe is washed away and radioactivity on filters is measured. Radioactivity on filter above threshold value indicates presence of *Salmonella* in test sample.

B. Method Performance

For all foods:

Results	Percent	95% Confidence Range
Agreement[1]	95.6	94.0–97.2
False neg. (BAM)[2]	5.9	3.7–8.1
False neg. (DNAH)[3]	0.2	0.0–0.6

[1] Rate reflects no. of samples read identically between AOAC/BAM (Bacteriological Analytical Manual (1984) 6th ed., AOAC, Arlington, VA) culture method and DNA hybridization (DNAH) method.

[2] Rate reflects no. of samples found to be pos. by DNAH method but detected as neg. by AOAC/BAM culture method.

[3] Rate reflects no. of samples found to be pos. by AOAC/BAM culture method but detected as neg. by DNAH method.

Of 11 laboratories, 2 had complete agreement between culture and DNAH methods; 4 showed agreement on ≥97% of samples; 7 showed agreement on ≥95% of samples; 10 showed agreement on ≥93% of samples.

C. Reagents

Items (**a**)–(**e**) are available as GENE-TRAK® DNA Hybridization Test for Detection of *Salmonella* (GENE-TRAK Systems, Inc., 31 New York Ave, Framingham, MA 01701).

(**a**) *Filter cups.*—Filter cup assemblies contg 25 mm diam. membrane filters (112 assemblies/box). Sufficient for 96 tests plus controls.

(**b**) *Soln set.*—Contains *(1)* 1 bottle (240 mL) denaturation soln (0.2N NaOH and 0.6M NaCl). (*Caution:* Contains NaOH. If contact with skin occurs, wash skin thoroly with H_2O.) *(2)* 1 bottle (240 mL) neutralization soln (1.0M Tris, pH 7.0, and 0.6M NaCl). *(3)* 1 bottle (240 mL) fixation soln (95% EtOH). (*Caution:* Flammable, contains alcohol.) *(4)* 1 bottle (120 mL) pre-hybridization soln (0.9M NaCl, 0.09M Na citrate, 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinyl pyrrolidone, 1mM Na_2 ethylenediamine tetraacetate, and 0.5% Na dodecyl sulfate (SDS), pH 6.5–7.5). *(5)* 1 bottle (60 mL) hybridization soln (same as soln *4*, with 10% dextran sulfate). *(6)* 3 bottles (240 mL each) wash soln (0.03M NaCl, 0.003M Na citrate, and 0.5% SDS, pH 6.5–7.5).

Sufficient for 96 tests plus controls. All solns are stable 6 months from date of manufacture when stored at 15–24°.

(c) *Salmonella probe and controls set.*—Contains *(1)* 1 bottle (20 mL) pos. control soln (heat-killed *S. typhimurium*). *(2)* 1 bottle (60 mL) neg. control soln (heat-killed *Escherichia coli*). *(3)* 1 vial (0.75 mL, 75 μCi ^{32}P) ^{32}P-labeled *Salmonella* DNA probe soln.

Sufficient for 96 tests plus controls. Shipped frozen on dry ice; thaw at room temp. (15–24°) before use. Solns are stable 8 days from receipt when stored at 2–8°.

(d) *Instruction manual.*

(e) *Data sheets.*

(f) *Gram negative (GN) broth.*—20.0 g tryptose, 1.0 g dextrose, 2.0 g D-mannitol, 5.0 g Na citrate, 0.5 g Na desoxycholate, 4.0 g K_2HPO_4, 1.5 g KH_2PO_4, 5.0 g NaCl. Dissolve ingredients in 1 L H_2O. Dispense 10 mL portions into 16 × 125 mm test tubes (or equiv.). Cap tubes loosely and autoclave 15 min at 121°. Final pH should be 7.0 ± 0.2 at 25°.

(g) *Diagnostic reagents.*—Necessary for cultural confirmation of pos. DNA hybridization tests; see **967.25B**.

D. Apparatus

Items (a)–(e) are available from GENE-TRAK Systems, Inc.

(a) *Manifold kit.*—Vac. filtration manifold device consisting of base with vac. control valve and top to house filter cup assemblies.

(b) *Bottle holder.*—Plastic rack to hold soln bottles in water bath.

(c) *Shield.*—$^1/_2$ in. thick Lucite used to protect worker from beta particle emissions from ^{32}P decay when radiolabeled DNA probe soln is used.

(d) *Vacuum pump.*—Adjustable between 8 and 15 in. Hg. Connected to manifold base thru trap consisting of 1 or 2 L vac. filtration flask and requisite tubing.

(e) *Beta detector.*—Any instrument capable of measuring radioactive decay of ^{32}P on dry, 25 mm membrane filters with efficiency of 0.4–0.5, e.g., scintillation counter. Use of scintillation counter requires appropriate carrier vials and insert minivials to hold filters upright.

(f) *Heating water bath.*—Capable of maintaining 65 ± 1.0°. Able to accommodate bottle holder 12 × 6 × $4^1/_2$ in. and H_2O level of $4^1/_2$ in.

(g) *Micropipet.*—Capable of delivering accurate amts in range continually adjustable in 1 μL increments between 100 and 200 μL.

(h) *Vortex mixer.*—For mixing broth in culture tubes.

(i) *Polypropylene screw-cap centrifuge tubes.*—50 mL, conical bottom.

E. General Instructions

Test uses radioactive compd. Personnel must receive appropriate training in use of radioactive materials and have proper facilities available for use of this substance. Facility must possess current, appropriate radioactive materials license issued by U.S. Nuclear Regulatory Commission or other agency with regulatory control.

Disposal of radioactive waste must be in accordance with radioactive materials license of facility.

Treat all materials in contact with bacterial cultures or culture filtrates as biohazardous material and decontaminate by appropriate methods.

Do not touch membrane filters. Handle with forceps only.

Return pos. and neg. controls and DNA probe soln to 2–8° storage immediately after use.

Include 1 pos. control and 3 neg. controls with each group of test samples.

Components and procedures of this test kit have been standardized for use in GENE-TRAK assay. Use of components or procedures other than those supplied or recommended by GENE-TRAK Systems, Inc., may yield unsatisfactory results.

F. Sample Preparation

(a) *Pre-enrichment.*—Pre-enrich product in non-selective medium to initiate growth of salmonellae. Procedure will vary with product type and should be performed as indicated in **967.26A** or in Bacteriological Analytical Manual (1984) 6th ed., AOAC, Arlington, VA, Chap. 7, section C, with the following exception:

Raw meats and raw milk products: Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth. Blend 2 min at high speed (ca 20 000 rpm). Cap jar securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 using sterile 1N NaOH or HCl; cap jar securely and mix well before detg final pH. Aseptically transfer contents to sterile wide-mouth, screw-cap 500 mL jar. Loosen jar cap $^1/_4$ turn and incubate 24 ± 2 h at 35°.

(b) *Selective enrichment.*—Transfer 1 mL incubated pre-enrichment culture to tube contg 10 mL selenite cystine broth and 1 mL to tube contg 10 mL tetrathionate broth (pre-warmed to 35°) as in **967.26B(a)**. Incubate 6 h at 35° with the following exception:

Raw meats and raw milk products: Incubate selenite cystine and tetrathionate broth 18 ± 2 h at 35°.

(c) *Post-enrichment.*—Remove selective enrichment cultures from incubation and mix by hand or with vortex mixer. Transfer 1 mL tetrathionate culture to tube contg 10 mL GN broth (prewarmed to 35°). Transfer 1 mL selenite cystine culture to sep. tube contg 10 mL GN broth. Incubate GN broths 12–18 h at 35° with the exception of raw meats and raw milk products (see below). Return tetrathionate and selenite cystine tubes to 35° for incubation up to total of 24 ± 2 h.

Raw meats and raw milk products: Incubate GN broths 6 h at 35°. Return tetrathionate and selenite cystine tubes to 35° for incubation up to total of 24 ± 2 h.

G. Filtration

(1) Connect manifold base to vac. pump thru trap. Add disinfectant soln to filtration flask. Two manifolds may be connected in parallel to single vac. source. This configuration will accommodate up to 24 food samples in 1 run plus requisite pos. and triplicate neg. controls.

(2) Place manifold top(s) on manifold base(s). Fit manifold top(s) with filter cup assemblies; 1 pos. control filter cup, 3 neg. control filter cups, and up to 24 pre-numbered sample filter cups for each set of assays. Fit unused manifold positions with No. 3 rubber stoppers (provided).

(3) Remove sample GN broths from 35° incubation. Vortex-mix or otherwise mix each culture. For each sample, pipet 1 mL from each of the 2 GN broths (one derived from tetrathionate, one from selenite cystine) into single filter cup. Record sample numbers and filter numbers on data sheet.

(4) Mix pos. and neg. control solns. Pipet 2 mL pos. control soln into pos. control filter cup. Pipet 2 mL neg. control soln into each of the 3 neg. control filter cups.

Note: Pos. and neg. controls are shipped frozen on dry ice. Thaw at room temp. (15–24°) before first use. Store controls at 2–8° between uses.

(5) When all filter cups have been loaded with samples, open vac. control valve on manifold base and turn on vac. pump. Apply vac. (8–10 in. Hg) until all samples are filtered. Avoid vac. of excessive pressure or prolonged duration. Close valve on manifold base and turn off pump. Be sure vac. on manifold base is released before proceeding.

(6) Add (squirt in) ca 1.5–2.0 mL denaturation soln (*Soln 1*) into each filter cup, completely covering surface of filters. Wait 2 min, then apply vac. as before (see step *5*) until soln has filtered thru all of the cups. Turn off vac.

(7) Add (squirt in) ca 1.5–2.0 mL neutralization soln (*Soln 2*) into each filter cup. Wait 2 min, then apply vac. Turn off vac. after soln has filtered thru all of the cups.

(8) Add (squirt in) ca 1.5–2.0 mL fixation soln (*Soln 3*) into each filter cup. Wait 2 min, then apply vac. Turn off vac. after soln has filtered thru all of the cups.

(9) Snap off top part of each cup assembly. Be careful not to discard membrane filters. With forceps, remove membrane filters from cup assembly bases and place on sheet of absorbent paper to dry. All other parts of cup assembly should be treated as biohazardous waste and discarded. Manifold bases and tops should be treated with disinfectant soln (do not autoclave).

H. DNA Hybridization Assay

(1) Using forceps, place filters (up to 24 sample filters plus 1 pos. and 3 neg. control filters) into 50 mL polypropylene centrf. tube, (**i**).

(2) Mix bottle of pre-hybridization soln (*Soln 4*) that has been equilibrated to 65°. Pour *Soln 4* into conical tube contg filters to 25 mL mark. Store remaining *Soln 4* at room temp. With back-and-forth motion, gently shake tube until all filters are completely immersed in soln and none are stuck to sides of tube. Filters should stack in vertical array at bottom of tube. Incubate tube for 30 min in 65° H_2O bath.

(3) Carefully drain pre-hybridization soln from tube and discard soln. Use of funnel will prevent losing stack of filters into collection vessel. Immediately mix bottle of hybridization soln (*Soln 5*) that has been equilibrated to 65° and add 12 mL to conical tube (measure accurately with pipet). Return remaining *Soln 5* to room temp. storage.

(4) Working behind Lucite shield, remove plastic vial contg thawed ^{32}P-labeled *Salmonella* probe soln from Lucite container. Mix probe soln by tapping on lower portion of plastic vial. Using precision micropipet, add probe soln to conical tube according to following schedule:

Age of Probe, Days	Calendar Day	μL of Probe Soln
0[1]	Mon.	—
1	Tue.	127
2	Wed.	133
3	Thur.	140
4	Fri.	147
5	Sat.	154
6	Sun.	162
7	Mon.	170
8	Tue.	179

[1] Day 0 is indicated by date on vial. Day 0 is normal shipping day; Day 1 is normal day of receipt, but this may vary. User should always refer to age and calendar day for detn of probe soln vol. to use.

Dispose of pipet tip in container reserved for solid radioactive waste. Return remaining probe soln to 2–8° storage in its Lucite container.

(5) Tighten cap on conical tube. With back-and-forth motion, gently shake tube until all filters are completely immersed in soln and none are stuck to sides of tube. Again, filters should stack in vertical array at bottom of tube. Incubate tube for 2 h in 65° H_2O bath.

(6) Working behind Lucite shield, carefully drain soln from conical tube into container reserved for liq. radioactive waste, using funnel to protect against losing stack of filters into waste vessel. Drain off as much soln as possible. (Radiochemical concn of waste is ca 1.0 μCi ^{32}P/mL in molecular form of DNA.)

(7) Immediately add 25 mL wash soln (*Soln 6*) that has been equilibrated to 65° by pouring soln into conical tube to 25 mL mark. Return bottle of *Soln 6* to 65° H_2O bath. Gently shake tube for at least 10 s, or until all filters are free from sides of tube and are stacked in vertical array at bottom of tube. Incubate conical tube for 5 min in 65° H_2O bath.

(8) Remove tube from H_2O bath and gently shake for at least 10 s, then carefully drain soln into liq. radioactive waste container. Drain off as much soln as possible.

(9) Repeat steps *7* and *8* five addnl times for total of 6 washes. Store *Soln 6* at room temp. after use.

(10) Using forceps, remove filters from conical tube and place them on sheet of absorbent paper. Discard conical tube into solid radioactive waste container. Using forceps, sep. filters and let dry briefly (5–10 min).

(11) Turn on power to beta detector, (**e**), and let instrument warm up ca 10 min. Counting time switch should be set to 0.5 min position.

To count each filter, center filter, using forceps, on lower window of beta detector. Detector windows are delicate; be careful not to damage them with forceps. Close detection unit and press start switch. Radioactivity on filter will be counted for 30 s, and result will be displayed as counts per minute (cpm). Record result for each filter in cpm on data sheet. Filters should be saved until data analysis is complete. Afterwards, they should be discarded as solid radioactive waste.

Alternatively, filters can be counted using std scintillation counter. Appropriate carrier vials and insert minivials are required so that filters can be positioned upright. Count each filter for 30 s using settings capable of counting ^{32}P on dry filters (do *not* use scintillation cocktail) with efficiency of 0.4–0.5. Record data as cpm.

I. Data Analysis

(1) Det. av. of 3 neg. control filters. If this av. is >500 cpm, *all* filters must be re-washed (repeat steps *7* and *8* under *DNA Hybridization Assay*) and re-counted. Accept data as final after addnl wash, even if av. of 3 neg. control filters is still >500 cpm.

(2) CPM of pos. control filter should be at least 5 times av. of neg. control filters; otherwise assay is invalid.

(3) Add 500 cpm to av. of the 3 neg. control filters. This sum is CUTOFF value.

Neg. criterion: Test sample is considered to be neg. (nonreactive for presence of *Salmonella*) if its cpm is ≤CUTOFF value.

Pos. criterion: Test sample is considered to be pos. (reactive for presence of *Salmonella*) if its cpm is >CUTOFF value.

J. Confirmation of Positive DNA Hybridization Assays

Because a certain percentage of false pos. DNA hybridization assays can be expected (3–4%), all samples found pos. by DNA hybridization assay must be confirmed by culture methods. HE, XLD, and BS plates should be streaked from tetrathionate broth, selenite cystine broth, and GN broth tubes as described in **967.26B**, and typical and suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

Ref.: JAOAC **70,** 527(1987).

989.13 Motile *Salmonella* in Foods

Immunodiffusion Screening Method

First Action 1989

Method is screening procedure for presence of motile *Salmonella* in all foods. It is not a confirmatory test because polyvalent H (flagellar) antibodies used in test may cross-react with

small percentage of non-*Salmonella*. *Note:* Method does not detect nonmotile salmonellae.

If test is pos., enrichment broth from inoculation chamber of test unit must be streaked onto selective/differential agar media as in **967.26B**, or, if test is performed on raw or highly contaminated product, enrichment broth must be transferred to tetrathionate broth and incubated 18–24 h in 43 ± 0.5° H_2O bath, and then streaked onto selective/differential agar media. Typical or suspicious colonies must be identified as in **967.26C**, **967.27**, **967.28**.

A. Principle

Detection of *Salmonella* is based on presence and observation of *Salmonella* immobilized in motility medium by polyvalent H (flagellar) antibodies. Immobilization of motile *Salmonella* results in development of well defined band of cells (immuno-band). Fig. **989.13** shows small disposable plastic device (1–2 TEST unit) which has 2 chambers. Smaller inoculation chamber contains selective tetrathionate broth supplemented with brilliant green and L-serine. Enriched sample is inoculated into this chamber. Central motility chamber of unit contains peptone-based, nonselective motility medium. Motility chamber is sealed with gel-former plug. Tip of this plug forms void in motility medium for addn of flagellar antibody prepn. For shipping, opening between 2 chambers is sealed with polyethylene chamber plug, which is removed and discarded prior to addn of inoculum. *Salmonella* inoculated into tetrathionate-brilliant green-serine broth move from this medium into motility medium to react with flagellar antibodies.

B. Method Performance

For all foods:

Results	Percent	95% Confidence Range (Approx.)
Agreement[1]	96.1	94.5–97.7
False neg. (BAM/AOAC)[2]	1.7	0.5–3.0
False neg. (immunodiffusion)[3]	3.6	1.8–5.5

[1] Rate reflects no. of samples read identically between BAM/AOAC (Bacteriological Analytical Manual (1984) 6th ed. AOAC, Arlington, VA) culture method and immunodiffusion method.
[2] Rate reflects no. of samples found to be pos. by immunodiffusion method but detd as neg. by BAM/AOAC culture method.
[3] Rate reflects no. of samples found to be pos. by BAM/AOAC culture method but detd as neg. by immunodiffusion method.

Of 17 laboratories, 8 had complete agreement between culture and immunodiffusion methods; 11 showed agreement on ≥97% of samples; 14 showed agreement on ≥95%; 16 showed agreement on ≥93%.

C. Reagents

Items (**a**)–(**c**) are available as BioControl 1–2 TEST (BioControl Systems, Inc., 19805 North Creek Pkwy, Bothell, WA 98011). Store at refrigerator temp. (4–8°C; 39–46°F). Items are stable 3 months (90 days) from date of receipt.

(**a**) *1–2 TEST unit.*—See Fig. **989.13**.

(**b**) *Iodine-iodide soln.*—1 vial for 12 units.

(**c**) *Antibody.*—1 vial for 12 units.

(**d**) *Diagnostic reagents.*—Necessary for cultural confirmation of presumptive pos. 1–2 TESTS. See **967.25B**.

D. Preparation of Sample

Most foods require only pre-enrichment of product in noninhibitory broth to initiate growth of salmonellae before inoculation into unit. Exceptions are given below. Methods used for sample prepn may vary with product and should be performed as in **967.26A** and **975.54D**.

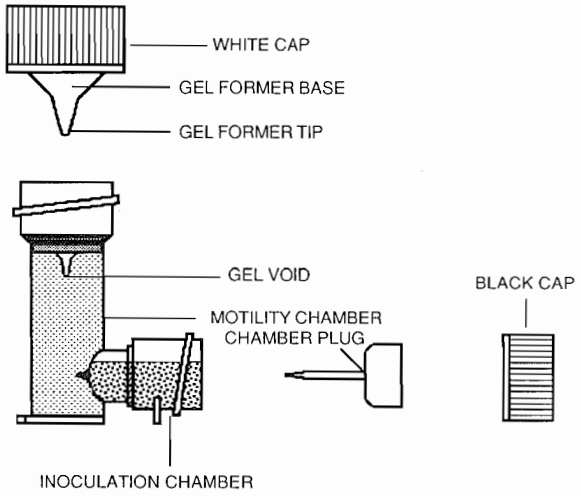


FIG. 989.13—Test unit for immunodiffusion screening method for motile *Salmonella*

Raw flesh foods or highly contaminated products.—No pre-enrichment is required. Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL tetrationate broth without brilliant green dye. Blend 2 min. Securely cap jars and let stand 60 min at room temp. Mix well by shaking. Add 2.25 mL 0.1% soln of brilliant green dye. Aseptically transfer contents of each jar to sterile 500 mL wide-mouth, screw-cap jar. Loosen jar caps $^1/_4$ turn and incubate 24 ± 2 h at 35°.

Flour-containing products (soy flour, wheat flour, dough, pasta, cake mix, and processed animal by-products).—Transfer 1 mL incubated pre-enrichment mixt. to tetrathionate broth as in **967.26B(a)**. Incubate 24 ± 2 h at 35°.

E. General Instructions

Components and procedures of test kit have been stdzd for use in 1–2 TEST procedures. Components or procedures other than those supplied by BioControl Systems, Inc., may yield unsatisfactory results, and should be pretested.

F. Immunodiffusion Detection

(**a**) *Test unit preparation.*—Each test unit has 2 chambers: inoculation chamber and motility chamber (Fig. **989.13**). Each step of prepn sequence can be performed on individual unit or multiple units as needed. Sample nos. can be recorded on lower portion of motility chamber but must NOT interfere with reading of results. Alternatively, sample nos. may be recorded on flat surface of white cap. When cap is replaced, it must be screwed on tightly.

(1) Position unit with black cap UP, and remove black cap. Add 1 drop of iodine-iodide soln to inoculation chamber, and replace black cap. Gently shake unit to mix and resuspend enrichment ingredients.

(2) Position unit with white cap UP, and remove white cap. Snip off tip of gel-former plug with scissors and discard tip. Cut should be made at point where tip meets base of plug. If tip of gel-former plug is not removed, antibody soln will be displaced from gel void when white cap is replaced.

(3) Add 1 drop of antibody prepn to gel void in motility chamber. Replace white cap. Antibody prepn should fill ca $^2/_3$ of gel void. This can be detd by observing blue antibody soln in gel void.

(4) Position unit with black cap UP, and remove black cap. Remove chamber plug from inoculation chamber with sterile forceps and discard plug. Do not replace black cap until unit is inoculated. If chamber plug is not removed, bacteria will be unable to move from inoculation chamber to motility chamber.

(**b**) *Inoculation.*—Prior to inoculation, be sure that enrichment broth contg sample is well mixed. Use pipet to transfer 0.1 mL enriched sample into inoculation chamber. Replace black cap.

(**c**) *Incubation.*—Place inoculated unit in incubator with white cap UP. Incubate unit in shipper/incubation tray at 35° for min. of 8 h.

(**d**) *Reading positive results.*—After 8 h incubation, unit may be inspected for pos. results: With white cap UP, hold unit next to strong light. Desktop fluorescent light is recommended for reading test results. Carefully observe motility chamber gel by rotating unit back and forth thru various angles in front of light source.

Pos. test is indicated by presence of white band that is U-shaped or meniscus-shaped. Band, which forms as motile *Salmonella* are immobilized by antibodies that have diffused into gel, is seen in upper half of motility chamber gel.

Pos. test indicates that sample contains *Salmonella*. Pos. test results should be confirmed by std culture methods outlined in *Confirmation of Positive Samples*.

Pos. unit can be stored up to 1 week at refrigerator temp. (4–8°).

(**e**) *Reading negative results.*—If no band is seen after initial 8 h incubation, reincubate units for min. of 6 h but not more than 12 h. After this incubation period, read units as described in (**d**), *Reading positive results*. Units that show no band after this second incubation indicate neg. test results. Neg. units that were incubated at least 14 h require no addnl incubation. Neg. test results indicate that sample does not contain levels of motile *Salmonella* detectable by immunodiffusion test.

Neg. units show uniform turbidity thruout motility chamber as result of movement of motile bacteria in gel. However, after initial 8 h incubation, movement of bacteria thru gel may not be complete.

G. Confirmation of Positive Samples

Presence of band of cells indicates that *Salmonella* may be present in sample. Perform cultural confirmation by using 3 mm loop to obtain inoculum from tetrathionate-brilliant green-serine broth in inoculation chamber and streaking HE, XLD, and BS plates.

For raw or highly contaminated products, transfer 0.1 mL of the tetrathionate-brilliant green-serine broth to test tube contg 10 mL tetrathionate-brilliant green broth. Incubate 18–24 h in 43 ± 0.5° H_2O bath, and then streak into HE, XLD, and BS plates. Identify typical or suspicious colonies from selective plates as in **967.26C**, **967.27**, **967.28**.

Ref.: JAOAC **72,** 303(1989).

VIBRIO

988.20 *Vibrio cholerae* in Oysters

Elevated Temperature Enrichment Method

First Action 1988

A. Principle

Recovery of *V. cholerae* is based on selection of typical colonies on isolation agar. Although *V. cholerae* grows well at 35°, many other species of competing bacteria also proliferate in enrichment broth when incubated at 35°. Some species of competing microflora mimic colonial appearance of *V. cholerae* on isolation medium. Subsequent selection of these mimicking colonies decreases probability of recovering any *V. cholerae* colonies that may be present, and increases labor and materials needed for analysis. Ability of almost all strains of *V. cholerae* to grow at 42° distinguishes them from many other bacterial species associated with oysters and results in higher confirmation rate of suspects as *V. cholerae*.

B. Culture Media and Reagents

(**a**) *AP broth.*—Suspend 10.0 g peptone and 10.0 g NaCl in 1 L H_2O and mix thoroly. Adjust pH so that value after sterilization is 8.5 ± 0.2. Dispense portions into 500 mL flasks so that final vol. after autoclaving 10 min at 121° is 225 mL.

(**b**) *TCBS agar.*—Suspend 5.0 g yeast extract, 10.0 g proteose peptone No. 3, 10.0 g Na citrate, 10.0 g $Na_2S_2O_3$, 8.0 g oxgall, 20.0 g sucrose, 10.0 g NaCl, 1.0 g ferric citrate, 0.04 g bromthymol blue, 0.04 g thymol blue, and 15 g agar in 1 L H_2O and mix thoroly. Heat with frequent agitation until medium just boils, 1–2 min. Cool in H_2O bath and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with covers partially removed; then close plates. Final pH, 8.6 ± 0.2. Do not autoclave. Do not use wet plates.

(**c**) *T_1N_1 agar.*—Suspend 10.0 g tryptone or typticase, 10.0 g NaCl, and 20.0 g agar in 1 L H_2O and mix thoroly. Heat with frequent agitation until medium boils. Dispense into 16 × 125 mm screw-cap tubes (if tubed medium is required). Autoclave 15 min at 121°. Slant tubes until cool or let medium cool to 50° and pour into 15 × 100 mm petri dishes. Let dry 2 h with plates covered. Do not use wet plates. Final pH, 7.2 ± 0.2.

(**d**) *Tryptone broth.*—Suspend 10.0 g tryptone or trypticase in 1 L H_2O and mix thoroly. Dispense 5 mL portions into 16 × 125 mm or 16 × 150 mm test tubes. Autoclave 15 min at 121°. Final pH, 6.9 ± 0.2.

(**e**) *Kligler iron agar (KIA).*—Suspend 3.0 g beef extract, 3.0 g yeast extract, 15.0 g peptone 5.0 g proteose peptone, 10.0 g lactose, 1.0 g dextrose, 0.2 g $FeSO_4$, 5.0 g NaCl, 0.3 g $Na_2S_2O_3$, 12.0 g agar, and 0.024 g phenol red in 1 L H_2O, mix thoroly, and heat with occasional agitation. Boil ca 1 min until ingredients dissolve. Fill 13 × 100 mm screw-cap tubes $^1/_3$ full and cap to maintain aerobic conditions during use. Autoclave 15 min at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca 3 cm) and adequate slants (ca 5 cm) are formed on solidification. Final pH, 7.4 ± 0.2.

(**f**) *Hugh-Leifson glucose broth (HLGB).*—Suspend 2.0 g peptone, 0.5 g yeast extract, 30.0 g NaCl, 10.0 g dextrose, 0.015 g bromcresol purple, and 3.0 g agar in 1 L H_2O, mix thoroly, and heat with agitation. Boil ca 1 min until ingredients are dissolved. Final pH, 7.4 ± 0.2. Fill 13 × 100 mm screw-cap test tubes $^1/_3$ full and cap. Autoclave 15 min at 121°. After inoculation cover with ca 1 mL sterile mineral oil to test for fermentation of dextrose.

(**g**) *Purple carbohydrate broth.*—Suspend 10.0 g proteose peptone No. 3, 1.0 g beef extract, 5.0 g NaCl, and 0.015 g bromcresol purple in 1 L H_2O and heat with gentle agitation until dissolved. Dissolve 10.0 g inositol or 10.0 g mannitol in basal broth. Dispense 2.5 mL portions into 13 × 100 mm test tubes. Autoclave 10 min at 121°. Final pH, 6.8 ± 0.2.

(**h**) *Decarboxylase test media (Moeller).*—Suspend 5.0 g peptone, 5.0 g beef extract, 0.5 g dextrose, 0.01 g bromcresol purple, 0.005 g cresol red, and 0.005 g pyridoxal in 1 L H_2O and heat with gentle agitation until dissolved. Dissolve 10.0 g L-lysine·2HCl, 10.0 g L-arginine·HCl, or 10.0 g L-ornithine·2HCl in basal broth. Use 1 portion of basal medium, without adding any amino acid, as control. Dispense 3–4 mL portions into 13 × 100 mm screw-cap tubes. Cap loosely and autoclave 10 min at 121°. Screw caps on tightly for storage. After inoculation cover with ca 1 mL sterile mineral oil. Final pH, 6.0 ± 0.2.

C. Diagnostic Reagents

(a) *Oxidase test soln.*—Dissolve 1.0 g *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine·2HCl in 100 mL H_2O. Store ≤7 days in dark glass bottle in refrigerator. Do not autoclave.

(b) *String test soln.*—Dissolve 0.5 g Na desoxycholate in 100 mL H_2O. Store tightly capped in refrigerator. Do not autoclave.

(c) *V. cholerae polyvalent (O) antiserum.*—Contains agglutinins for Inaba and Ogawa (O) antigens (Difco, or equiv.). Rehydrate with 5.0 mL sterile physiological saline soln (e). Store refrigerated.

(d) *V. cholerae individual somatic (O) antisera.*—For Inaba and Ogawa (O) groups (Difco, or equiv.). Rehydrate and store as described in (c).

(e) *Sterile physiological saline soln.*—Dissolve 8.5 g NaCl in 1 L H_2O and autoclave 15 min at 121°.

(f) *NaOH soln, 1N.*—Dissolve 42.11 g 95% reagent grade NaOH in sterile H_2O and dil. to 1 L.

(g) *HCl soln, 1N.*—Dil. 89 mL HCl to 1 L with sterile H_2O.

(h) *Sterile mineral oil.*—Autoclave 500 mL mineral oil in 1 L flask for 30 min at 121°.

(i) *Bromcresol purple soln, 0.2%.*—Dissolve 0.2 g bromcresol purple in sterile H_2O and dil. to 100 mL.

D. Apparatus

(a) *Incubator.*—Air, 35 ± 2°.

(b) *H_2O bath.*—Covered, 42 ± 0.2°.

(c) *High-speed blender.*—2 speed, with high-speed operation at 18 000–21 000 rpm, and 1 L glass or metal blender jars with covers. Use 1 jar for each test sample.

(d) *Sterile equipment.—(1)* Flasks or jars, 500 mL capacity. *(2)* Knives and spoons for opening and manipulating oysters. *(3)* Petri dishes. 15 × 100 mm. *(4)* Pipets, 1.0 and 10.0 mL with 0.1 mL graduations. *(5)* Inoculating needles and loops, ca 3 mm. *(6)* Culture tubes, 13 × 100 mm, 16 × 125 mm, and tube racks. *(7)* Wooden applicator sticks.

(e) *Balance.*—2000 ± 0.1 g capacity.

V. cholerae Recovery

E. Preparation of Test Sample

Aseptically remove oyster meats and liquor from ca 12 shell stock oysters or 12 shucked oysters from container. Aseptically weigh ca 200 g oyster meat and liquor into sterile empty blender jar. Blend at high speed 1 min. Aseptically weigh 25 g portions into 500 mL flasks contg 225 mL AP broth. Cover flask with sterile Al foil. Swirl mixt. 25 times clockwise and 25 times counterclockwise to suspend oyster homogenate. Incubate 6–8 h at 42 ± 0.2° in H_2O bath.

F. Isolation

Gently remove flasks from H_2O bath. Streak 3 mm loopful of surface or pellicle growth from incubated AP broth on TCBS agar plate. Incubate plates 18–24 h at 35°. Typical *V. cholerae* colonies on TCBS agar appear large, smooth, yellow, and slightly flattened with opaque centers and translucent peripheries. Colonies of *V. mimicus*, which is closely related to *V. cholerae*, appear as smooth, green, slightly flattened colonies.

G. Treatment of Typical or Suspicious Colonies

Inoculation of T_1N_1 agar.—Pick with needle 2–5 suspicious colonies from TCBS agar plate. Streak to T_1N_1 agar and incubate 18–24 h at 35°.

Initial screening reactions.—Scrape agar surface with sterile wooden applicator stick and touch to filter paper impregnated with oxidase reagent.

Oxidase test.—*V. cholerae* cultures are oxidase pos. and should produce dark purple spot within 1 min.

String test.—Emulsify oxidase pos. cultures in drop of 0.5% Na desoxycholate by stirring with same wooden applicator stick used previously. Within 1 min, *V cholerae* cultures form mucoid mass, which strings (string test) when stick is lifted 2–3 cm from slide. Treat oxidase and string test pos. cultures as presumptive *V. cholerae* and submit them to further examination.

Inoculation of Kligler iron agar (KIA) and tryptone broth.—Inoculate KIA slant with each suspect colony by streaking slant and stabbing butt with inoculating needle. After inoculating KIA with needle, do not obtain more inoculum from colony and do not heat needle, but directly inoculate tryptone broth. Incubate KIA and tryptone broth overnight at 35°. Cap tubes lightly to maintain aerobic conditions while incubating slants to prevent excessive H_2S production.

KIA.—*V. cholerae* cultures typically have alk. (red) slant and acid (yellow) butt, without H_2S (blackening of agar) or gas (cracking or lifting of agar). Do not eliminate KIA culture as *V. cholerae* solely on basis of acid slant.

Tryptone broth.—*V. cholerae* cultures typically produce growth in tryptone broth without added NaCl. Discard only apparent non-*V. cholerae* cultures. Test retained presumptive pos. KIA and tryptone cultures to det. if they are *V. cholerae*. Biochem. reactions characteristic of *V. cholerae* are summarized in Table **988.20**.

V. cholerae Identification

H. Identification Tests

Pure 18–24 h T_1N_1 agar cultures are required for inoculation of biochem. media. Select isolated colony and transfer with needle to each biochem. medium without obtaining more inoculum or heating needle.

Dextrose fermentation.—After inoculation, cover with ca 1 mL sterile mineral oil and incubate overnight at 35°. *V. cholerae* gives pos. test, shown by acid reaction (yellow). Discard all cultures that give neg. test.

Acid production from mannitol and inositol.—Incubate at 35° and read daily up to 4 days. Pos. tests are shown by acid production (yellow). *V. cholerae* gives pos. mannitol and neg. inositol test. Do not eliminate culture as *V. cholerae* solely on neg. mannitol test.

Decarboxylase broth.—After inoculation cover with ca 1 mL sterile mineral oil and incubate at 35°. Read daily up to 4 days. Pos. test is shown by purple alk. reaction thruout broth (final color is slightly darker than original purple of medium). Sometimes tubes that become yellow after 8–12 h incubation change to purple later. Neg. test is permanently yellow thruout broth and is seen with decarboxylase control tube without added

Table 988.20 Biochemical Reactions of *V. cholerae*

Test or substrate	Positive (+)	Negative (−)	*V. cholerae* reaction
H_2S (KIA)	blackening	no blackening	−
Gas (KIA)	lifting or cracking	no lifting or cracking	−
Tryptone	visible growth	no visible growth	+
HLGB	yellow	purple	+
Mannitol	yellow	purple	+
Inositol	yellow	purple	−
Decarboxylase broth:			
Lysine	purple	yellow	+
Arginine	purple	yellow	−
Ornithine	purple	yellow	+

amino acid. If medium appears to be discolored (neither purple nor yellow), add several drops of 0.2% bromcresol dye. *V. cholerae* gives pos. (purple) reaction in lysine and ornithine and neg. (yellow) reaction in arginine.

Serological Tests for V. cholerae

Reconstitute antisera with 5.0 mL sterile 0.85% saline and refrigerate. Pretest all antisera with known test cultures to ensure reliability of results with unknown cultures. *Caution:* Handle viable cultures carefully to prevent contaminating environment. Use pure 18–24 h T_1N_1 cultures for all serological tests. Perform serological test only on cultures that give biochem. reactions typical of *V. cholerae*.

I. Polyvalent Somatic O Group 1 Slide or Plate Test

Use wax pencil to mark off 2 sections ca 1 × 2 cm on inside of glass or plastic petri dish. Place 1 drop of 0.85% saline soln to one section and 1 drop of *V. cholerae* polyvalent somatic (O) antisera to other section. With sterile wooden applicator stick or inoculating loop or needle, emulsify culture in saline soln for one section and repeat for other section contg antiserum. Tilt mixt. in both sections back and forth 1 min and observe against dark background. Any degree of agglutination is pos. reaction.

Classify polyvalent somatic (O) group 1 test as:

Positive.—Agglutination in culture-saline-serum mixt.

Negative.—No agglutination in culture-saline-serum mixt.

Nonspecific.—Both mixts agglutinate.

J. Determination of Individual Somatic O Group 1 Serotypes

Test only somatic O group 1 pos. cultures in individual O group 1 antisera. Perform serological somatic O group 1 test on culture as above, by using Inaba and Ogawa antiserum instead of *V. cholerae* polyvalent somatic O group 1 antiserum.

Classify individual somatic O group 1 test as:

Inaba positive.—Agglutination in culture-saline-Inaba antiserum mixt. and no agglutination in culture-saline or in culture-saline-Ogawa antiserum mixt.

Ogawa positive.—Agglutination in culture-saline-Ogawa antiserum mixt. and no agglutination in culture-saline or in culture-saline-Inaba antiserum mixt.

Hikojima positive.—Agglutination in both culture-saline-Inaba antiserum mixt. and culture-saline-Ogawa antiserum mixt. but no agglutination in culture-saline mixt.

Negative.—No agglutination in culture-saline-Inaba antiserum mixt., culture-saline-Ogawa antiserum mixt., or in culture-saline mixt. This pattern indicates faulty individual somatic O group 1 antisera or presence of non-O group 1 antisera in polyvalent somatic O group 1 antiserum.

Nonspecific.—All mixts agglutinate.

Ref.: JAOAC **71,** 584(1988).

MISCELLANEOUS

975.56 Virus in Beef (Ground)

Microbiological Method

First Action 1975
Final Action 1989

A. Media and Reagents

(**a**) *Diethylaminoethyl (DEAE) dextran sulfate soln.*—Add 1 g DEAE dextran sulfate, 2 × 10^6 MW (Pharmacia Fine Chemicals, Inc., 800 Centennial Ave, Piscataway, NJ 08854), to H_2O, dil. to 100 mL, mix on mag. stirrer, and filter thru 0.22 μm filter.

(**b**) *Magnesium chloride soln.*—Add 50.75 g $MgCl_2.6H_2O$ to H_2O, dil. to 100 mL, mix on mag. stirrer, and filter thru 0.22 μm filter.

(**c**) *Neutral red soln.*—Add 1 g neutral red to 1 L H_2O, mix overnight on mag. stirrer, autoclave 15 min at 121°, and dispense into 100 mL bottles for storage at 10°.

(**d**) *Sodium bicarbonate soln.*—pH 8.0. Add 75 g $NaHCO_3$ to H_2O, dil. to 1 L, and filter thru 0.22 μm filter.

(**e**) *Tissue culture.*—Propagate Vero monkey kidney cell cultures (ATCC CCL 81) in 6 oz (45 sq cm) prescription bottles contg growth medium, (**f**). After cell sheets are confluent, ca 7 days, decant medium, add 10 mL 0.02% Na_4EDTA in phosphate buffered saline soln, **975.54C**(**a**), and shake. When cells resuspend, ca 20 min, pour suspension into centrf. tube, centrf. 15 min at 700 rpm in International PR-2, rotor 259, and decant supernate. Add 146 mL growth medium to cell pellet, mix, and distribute into 8 prescription bottles. Repeat propagation until enough cultures are prepd to perform analysis.

(**f**) *Growth medium.*—To Leibovitz medium (L-15) (Grand Island Biological Co., 3175 Staley Rd, Grand Island, NY 14072) add equal vol. Eagle's minimum essential medium (MEM) with Hank's salt (Grand Island Biological Co.). Add 10% fetal bovine serum (flow Laboratories). To final mixt. add 10 mL of 7.5% $NaHCO_3$ soln, (**d**). Medium will maintain cells 15 days without having to be changed.

(**g**) *Virus.*—Poliovirus 1, Chat, attenuated (ATCC VR-192). Passage in Vero cell cultures. Prep. virus pool and titer. Dil. pool to provide 10–50 plaque forming units (pfu)/g.

(**h**) *High antibiotic minimum essential medium (HAMEM).*—Prep. MEM with nonessential amino acids in Hank's salt soln contg in each L: 20 mL fetal bovine serum, 5.0 mL $MgCl_2$ soln, (**b**), 10 mL DEAE dextran sulfate soln, (**a**), 4.643 g K penicillin G, 5.0 g streptomycin sulfate, 0.25 g tetracycline.HCl, and 5.0 mg amphotericin B. Adjust to pH 8.5 with 1*N* NaOH for elution of virus and to prevent coagulation of sample slurry.

(**i**) *Agar medium.*—Mix 400 mL "2×" MEM (filtered thru 0.22 μm filter), 20 mL fetal bovine serum, 30 mL $NaHCO_3$ soln, (**d**), 15 mL neutral red soln, (**c**), 10 mL DEAE dextran sulfate soln, (**a**), 10 mL $MgCl_2$ soln, (**b**), 2 mL amphotericin B soln (10 μg/mL), 2 mL tetracycline.HCl soln (50 μg/mL), 5 mL K penicillin G soln (1435 units/mL), and 6 mL streptomycin sulfate soln (1 mg/mL).

(**j**) *Agar overlay medium.*—Add 9.5 g Oxoid Ion agar No. 2 or 12 g Difco purified agar to H_2O, dil. to 490 mL, mix on mag. stirrer, autoclave 15 min at 121°, and temper in 47° H_2O bath. Add tempered agar to 500 mL agar medium, (**i**), and temper in 43° H_2O bath. Add 10 mL canned sterile milk (Realfresh, Inc., PO Box 1551, Visalia, CA 93277) just before use.

B. Preparation of Sample

Place 100 g sample in plastic Whirl-Pac bag (Fisher Scientific Co.) and add 200 mL HAMEM, (**h**). Shake vigorously by hand, adjust pH of slurry to 8.5, and let stand 1 hr at room temp, shaking vigorously 1 min every 20 min. Readjust pH to 8.5 and pour thru funnel contg 5 g Pyrex glass wool pretreated with HAMEM. Let filter 1 hr (ca 180 mL filtrate is obtained) and compress glass wool and slurry with wooden tongue depressor to express remaining liq.

C. Assay

Inoculate 1 mL filtrate into each of 10 bottles of Vero cell monolayers, (**e**), rotating bottles to obtain even distribution of inoculum. Incubate 1 hr at 36°. Return bottles to room temp.

Dispense 18 mL agar overlay medium, (j), into each bottle against inside surface away from cell sheet. Cap bottles and turn so overlay gently floods cell surface. Let solidify at room temp. 30 min with bottles covered to exclude light. Turn bottles so that overlay side is up, and incubate in dark at 36°. Remove bottles daily from incubator, and count and mark plaques until no new plaques appear in 48 hr. Discard after 14 days.

Plaque forming units (pfu)/100 g sample = (Av. plaque count/bottle) × (total vol. filtrate/mL filtrate inoculated per bottle).

Ref.: JAOAC **58,** 576(1975).

985.43 Poliovirus 1 in Oysters

Microbiological Method

First Action 1985
Final Action 1989

A. Apparatus

(a) *Tissue culture flasks.*—Polystyrene, 150 sq. cm (Corning Glass Works, or equiv.).

(b) *Specimen containers.*—220 mL, polyethylene, disposable (Becton-Dickinson Labware, 2 Ridgewater Ln, Lincoln Park, NJ 07035, or equiv.).

(c) *Blender.*—Waring, or equiv.

(d) *Funnel.*—PF 100 polypropylene (No. 4252-0100, Nalge Co, 75 Panorama Creek Dr, PO Box 20365, Rochester, NY 14602, or equiv.).

(e) *Centrifuge bottle.*—250 mL, linear, polyethylene (Nalge No. 3121-0250, or equiv.).

(f) *Refrigerated centrifuge.*—Sorvall RC 5B (Ivan Sorvall, Inc., Norwalk, CT 06852, or equiv.).

(g) *Shaker.*—Wrist action (Burrell Corp., or equiv.).

B. Media

(Use double-distd H_2O for prepn of media and reagents.)

(a) *MEMH.*—Eagle's min. essential medium with Hanks' balanced salts (Gibco Laboratories, Life Technologies, Inc., 3175 Staley Rd, PO Box 68, Grand Island, NY 14072-0122, or equiv.).

(b) *Plaque assay agar.*—Add 12 g purified agar (Difco, or equiv.) to 500 mL H_2O autoclave 15 min at 121°, and temper fluid 30 min at 47°.

(c) *Plaque assay medium.*—Add 10 mL 50% $MgCl_2.6H_2O$, 10 mL 1% DEAE, 15 mL 7.5% $NaHCO_3$, 15 mL 0.1% neut. red, 30 mL 10% (w/v) nonfat dry milk autoclaved 10 min, and 1 mL (50 mg) gentamicin sulfate to 420 mL double strength MEMH. (All concns w/v with H_2O). Bring to 36° in H_2O bath and place in 36° incubator until use.

C. Reagents

(a) *Freon TF.*—(DuPont, Inc., or equiv.).

(b) *Antifoam.*—Antifoam C (Dow Corning, or equiv.)

(c) *Nonfat dry milk.*—Quality equiv. to Carnation Co., Los Angeles, CA, product.

(d) *Cat Floc.*—10% (w/v) (Calgon Corp., PO Box 1346, Ellwood, PA 15230, or equiv.).

(e) *DEAE.*—1% (w/v) diethylaminoethyl dextran, MW = 5×10^5 (Pharmacia Fine Chemicals, Inc., 800 Centennial Ave, Piscataway, NJ 08854 or equiv.).

D. Preparation of Cells

Buffalo African Green Monkey cell line (BGM).—(BGM cell line is available from several commercial sources.). Incubate cells at 36° in planting medium composed of equal vols of Leibovitz L-15 medium and MEMH supplemented with 10% fetal bovine serum plus 100 U penicillin G, 100 μg streptomycin sulfate, and 50 μg gentamicin/mL. Treat confluent cultures with 0.02% tetrasodium ethylenediamine tetraacetate in phosphate-buffered saline, split 1 to 10, and passage weekly in tissue culture flasks.

E. Preparation of Virus

Poliovirus 1.—Chat strain (ATTCC VR-192). Propagate in monolayers of BGM cells and harvest after observing 4 + cytopathic effect. Filter culture fluid contg virus thru 0.22 μm porosity membrane. Shake filtrate vigorously with equal vol. Freon TF, and centrf. 30 min at 4000 × *g* at 10°. Refilter supernate.

F. Sample Preparation

For each 100 g sample, select ca 10 std size oysters, shuck, and pour liquor and meat into specimen container. Adjust liquor and meat in container to 100 g.

Pour 100 g oyster sample into 1 L blender, and pipet 1 mL antifoam onto oysters. Rinse specimen container with 150 mL 37° H_2O, and pour rinse into blender. Pipet 2.5 mL 1N HCl into mixt. and blend contents 20 s at 18 500 rpm. Adjust homogenate, using 1 s blending mixes, to pH 4.8 ± 0.1 by adding 1 N HCl or 1 N NaOH as needed. Pour homogenate thru polypropylene funnel into 250 mL linear polyethylene centrif. bottle. Cap centrif. bottles tightly to prevent leakage during various assay procedures. Centrf. 10 min at 5000 × *g* without refrigeration in GSA rotor, using refrigerated centrif. Pour off supernate and discard.

G. Elution of Virus from Oyster Tissue

Add following cold reagents (5°) to pellet (ca 40 g): 50 mL MEMH without phenol red or $NaHCO_3$, 10 mL 10% w/v nonfat dry milk, 2 mL 50% $MgCl_2.6H_2O$, 50 mL Freon TF, and 3 mL 1N NaOH. Vigorously shake mixt. horizontally 5 min on wrist-action shaker, adjust pH to 9.1 ± 0.1, and centrif. mixt. 20 min at 5000 × *g* at 10°.

H. Concentration of Virus by Precipitation

Pipet supernate (ca 80 mL) into 250 mL centrf. bottle, taking care not to harvest Freon TF, which settles below supernate. Discard sediment and Freon TF. Add enough 37° H_2O to supernate to bring liq. level to shoulder of top of bottle. Form floc (15 min) by adjusting pH of liq. to 4.8 ± 0.1 with 1N HCl (ca 1 mL). Centrif. sample 10 min at 1500 × *g* (3000 rpm) without refrigeration. Pour off and discard supernate.

I. Elution of Viruses from Precipitate

Add 4 mL 0.2M Na_2HPO_4 (ca 23°) and 1 mL freshly prepd 10% Cat floc to pellet (10 g) and suspend pellet by vortex-mixing 30 s. Centrf. suspension (pH 7.5 ± 0.2) 20 min at 10 000 × *g* (8000 rpm) at 10°.

J. Plaque Assay of Eluate

Pipet supernate (ca 10 mL) onto two 150 sq. cm BGM cell monolayers previously rinsed with 50 mL MEMH adjusted with 7.5% $NaHCO_3$ to pH 7.0. Discard pellet. Incubate inoculated cell monolayers 2 h at 36°. Tilt culture flasks at least twice during incubation to redistribute eluate over cell monolayers; then overlay monolayers as described below.

K. Overlay Procedure

Pour tempered plaque agar into 36° medium, and mix by inverting flask few times. Pour mixt. (60 mL) into cell monolayer flask onto side opposite cell sheet, and rotate flask so that agar flows over monolayer once and then covers cells. Let overlay agar solidify at room temp. (ca 23°). Cover flasks with cloth to exclude light during solidification (ca 15 min). In-

cubate 7 days at 36°, agar side up, in dark incubator. Mark plaques and count as they appear.

Ref.: JAOAC **68,** 884(1985).

SOMATIC CELLS

973.68* Somatic Cells in Milk

Optical Somatic Cell Counting (OSCC) Method I

First Action 1973
Final Action 1980
Surplus 1989

See **46.152–46.160**, 14th ed.

978.25* Somatic Cells in Milk

Optical Somatic Cell Counting (OSCC) Method II

First Action 1978
Final Action 1980
Surplus 1989

See **46.161–46.170**, 14th ed.

978.26 Somatic Cells in Milk

Optical Somatic Cell Counting (OSCC) Method III

First Action 1978
Final Action 1979

A. Principle

Fresh or preserved milk samples are automatically sampled at 40°, mixed with buffer and dye, and stirred. Portion of mixt. is transferred to rotating disk which serves as object plane for microscope. Xe arc lamp excites somatic cell nuclei-dye complex to emit fluorescent light, and energy emitted by each nucleus is measured as elec. pulse.

B. Apparatus

Optical somatic cell counter.—Fossomatic (manufactured by Foss Food Technology Corp.), consisting of heating coils, rotating table, stirrer, syringes for delivering buffer and dye, rotating disk, microscope equipped to detect fluorescence, and totalizing circuit and printer (*see* Fig. **978.26**).

C. Reagents

(**a**) *Ethidium bromide dye soln.*—(*1*) *Stock soln.*—0.1%. Dissolve 1.00 g ethidium bromide (Aldrich Chemical Co., Inc., or equiv.) in 1 L H_2O by heating to 40–50° and mixing thoroly. Stock soln is stable 60 days in light-proof, air-tight bottle. (*2*) *Working soln.*—0.002%. Dil. 20 mL dye stock soln to 1 L with KH phthalate buffer soln, (**c**), and mix thoroly.

(**b**) *Rinsing liquid.*—(*1*) *Stock soln.*—1% Triton X-100 (Rohm & Haas Co., or equiv.). Dissolve 10 mL Triton X-100 in 1 L H_2O. Stock soln is stable 25 days in air-tight container. (*2*) *Working soln.*—Add 10 mL stock soln to 25 mL NH_4OH (1 + 3), dil. to 10 L with H_2O, and mix thoroly.

(**c**) *Potassium hydrogen phthalate buffer soln.*—0.025*M*. Dissolve 51.0 g KH phthalate and 13.75 g KOH in 10 L H_2O by heating to 50° and mixing thoroly. Add 10 mL 1% Triton X-100, (**b**)(*1*), and again mix thoroly. Store ≤7 days in air-tight container.

D. Analytical System

Two fl oz (60 mL) milk sample is heated to 40°, placed on self-feeding rack, and stirred to ensure even cell distribution just before 200 μL milk is withdrawn. Sample is combined with 1.800 mL 60° buffer soln and 2.000 mL 60° dye soln. Final mixt. is stirred continuously until 20 μL is spread 10 μm deep on edge of rotating disk, 0.5 mm wide along effective length of 3500 mm. Field is viewed with 15× microscope objective. Cell-dye complex is excited by filtered blue light (400–570 nm) from Xe lamp to emit red fluorescence, and filtered

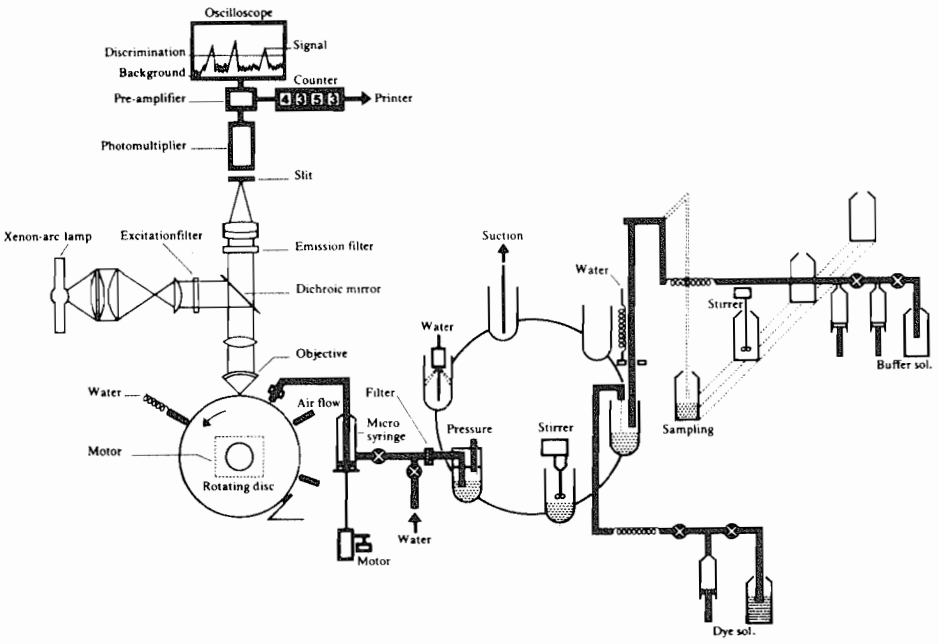


FIG. 978.26—Optical somatic cell counter flow diagram (Fossomatic) (Method III)

fluorescence (590–700 nm) over background (removed by discriminator) is sensed by photomultiplier. Each pulse is transformed and reading of total cells/20 μL is digitized on display as well as on printer. Rinsing liq. is used to flush system between milk samples to ensure no carryover effect of sample.

Somatic cells/mL milk = No. pulses × 1000

E. Standardization

Perform direct somatic cell counts (DMSCC) on 3 std milk samples within range 300,000–2,000,000 cells/mL as in **973.68** or **978.25**. (Before analysis, obtain 3 subsamples of each std to avoid excessive reheating.) To arrive at optimum discriminator setting, compare stds over ≥5 discriminator settings having increments of 0.25–0.5 between settings. Choose initial setting near previous operating point and additional settings to provide ≥1 set of readings above and ≥1 below apparent optimum. Optimum is setting at which deviations of Fossomatic readings from those of stds are minimal, with 1 of opposite sign from rest. Check instrument every 700–800 samples or after each 4 hr of operation against std milk samples preserved with 0.05% $K_2Cr_2O_7$.

Ref.: JAOAC **61,** 779(1978).

980.33* Somatic Cells in Milk

Membrane Filter-Deoxyribonucleic Acid (MF-DNA) Method

First Action 1980
Final Action 1981
Surplus 1989

See **46.176–46.180**, 14th ed.

SPECIAL REFERENCE

FDA Bacteriological Analytical Manual (BAM) 6th edition (1984) AOAC, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301.