Validación de un método de cultivo rápido para *Salmonella*: Oxoid Salmonella Precis

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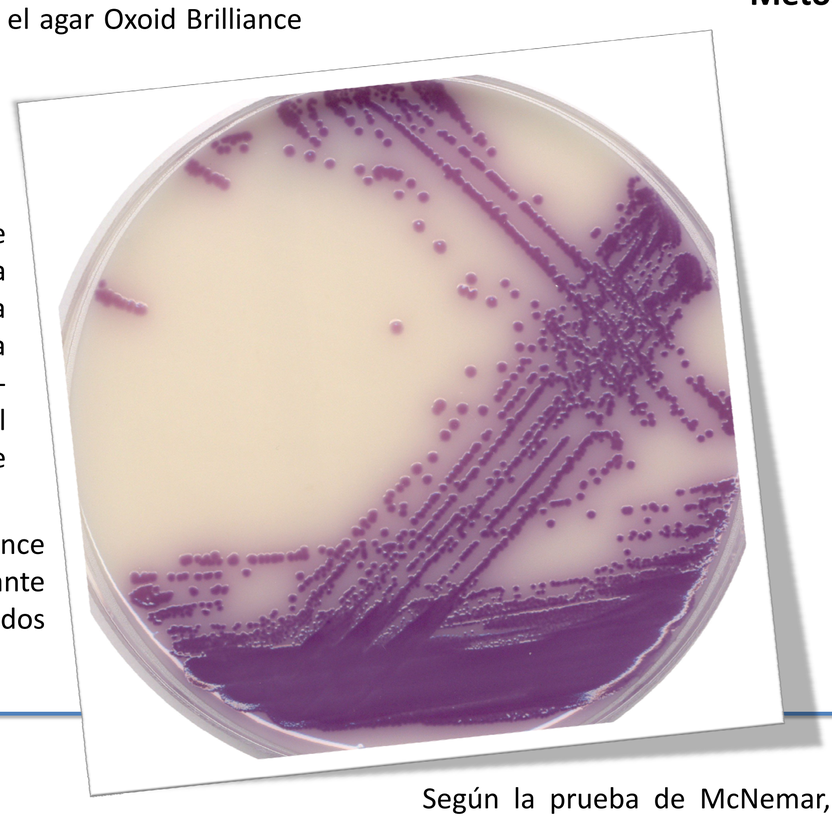
Introducción

La salmonelosis continúa siendo una significativa preocupación de salud pública a nivel global. Los métodos tradicionales para la detección de *Salmonella* spp. son lentos, tardando hasta 4-5 días. Oxoid Salmonella Precis es un avanzado método rápido de cultivo, que contempla un único enriquecimiento en el caldo ONE Broth-Salmonella seguido de un único subcultivo en un solo medio cromogénico, el agar Oxoid Brilliance Salmonella

El caldo ONE Broth-Salmonella es un medio altamente nutritivo para la recuperación y crecimiento de *Salmonela* spp. que inhibe el crecimiento de organismos competitivos.

El agar Brilliance Salmonella es el primero de una nueva clase de medios cromogénicos que incorpora la tecnología Inhibigen, la cual mejora la recuperación de *Salmonella* spp. reduciendo la flora acompañante. Los cromógenos dirigidos frente a la actividad C8-esterasa propia de *Salmonella* spp. y frente a la actividad β-glucosidasa, no presente en *Salmonella* spp., ayudan a una fácil identificación y diferenciación mediante la producción de colonias de vivos colores.

Las colonias características de *Salmonella* spp. e el agar Brilliance Salmonella pueden ser fácil y rápidamente confirmadas mediante una aglutinación en látex o bien mediante los métodos confirmatorios descritos en la norma ISO 65796:2002[1].



Material y métodos

Todos los experimentos fueron llevados a cabo según las especificaciones técnicas indicadas en la norma de validación ISO 16140:2003[2], comparando el método Salmonella Precis frente al método ISO 6579:2002.

Método Salmonella Precis

Día 0: Enriquecimieno
25g o ml de muestra
225ml de caldo ONE Broth-Salmonella

Incubar 16-20h a 42°C

Día 1: Pase a placa
Inocular una sóla placa de Brilliance Salmonella usando un asa de 10µL

Incubar 22-26h a 37°C

Día 3: Resultados
Si la hay, seleccionar una colonia púrpura y testarla con Oxoid Salmonella Latex Test. Alternativamente se pueden emplear los métodos descritos en la norma ISO 6579:2002.

Resultados

Se determinó la precisión relativa, el límite de detección, la practicabilidad, la precisión, la sensibilidad y la especificidad para ambos métodos. También se realizaron un estudio interlaboratorios para evaluar la repetibilidad y reproducibilidad.

Límites de detección

Seis pares de matriz/cepa con cuatro niveles de inoculación, incluyendo seis repeticiones, se pusieron a prueba. Los límites de detección relativos fueron calculados de acuerdo con la prueba de Spearman-Kärber[3] (ver tabla 1)

Matriz / cepa	Límite de detección relativo (UFC/25 g o 25mL) según la prueba de Spearman-Kärber	
	Método de referencia	Método alternativo
Pavo crudo / S. Typhimurium	0,267 [0,109 – 0,659]	0,521 [0,309 – 0,878]
Leche cruda/ S. Anatum	0,440 [0,274 – 0,707]	0,516 [0,321 – 0,838]
Ensalada / S. Enteritidis	0,138 [0,042 – 0,451]	0,160 [0,047 – 0,548]
Huevos crudos / S. Enteritidis	0,444 [0,173 – 1,136]	0,395 [0,154 – 1,012]
Galletas para perros / S. Anatum	0,281 [0,109 – 0,725]	0,188 [0,080 – 0,442]
Agua de proceso / S. Give	0,707 [0,274 – 1,826]	0,355 [0,207 – 1,378]

Tabla 1.Límite de detección relativo del método Salmonella Precis

Inclusividad y exclusividad

Se emplearon 53 cultivos puros de *Salmonella* spp de 38 serovariedades diferentes y 40 cepas no-Salmonella. Todas las *Salmonella* spp fueron detectadas por el método Salmonella Precis, con la excepción de una cepa de S. Dublin. Es conocido que algunas cepas de S. Dublin se caracterizan por tener una baja actividad C8-esterasa[4]. Todas las cepas no-*Salmonella* fueron inhibidas o diferenciadas por el método Salmonella Precis. Dos cepas no-*Salmonella*, una de *Enterobacter sakazakii* y otra de *Citrobacter diversus*, dieron colonias púrpuras típicas de *Salmonella* spp. en el agar Brilliance Salmonella, pero sin embargo fueron descartadas con la prueba confirmatoria de látex y por las descritas en la norma ISO 6579:2002

Sensibilidad, especificidad y exactitud relativas

Se emplearon 7 tipos de matrices: carne, productos lácteos, hortalizas, marisco, huevos, piensos y muestras ambientales, analizándose un total de 424 muestras. De ellas, 216 fueron positivas con al menos uno de los dos métodos. El 33,3% de las muestras estaban contaminadas de forma natural, mientras que el resto fueron dopadas con 0 , 5 y 25 UFC/g

Las sensibilidades, especificidades y exactitudes relativas fueron determinadas para cada método (Tabla 2).

	Látex	Tradicional	Estudio interlabarotarios
Exactitud relativa %	90,6	91,0	99,7
Especificidad relativa %	92,0	92,0	100
Sensibilidad relativa %	88,9	89,9	100

Tabla 2. sensibilidades, especificidades y exactitudes relativas

Según la prueba de McNemar, χ^2 fue igual a 0,105, por lo tanto, y con un nivel de probabilidad del 5%, no hubo ninguna diferencia significativa entre los métodos,.

Para evaluar la repetibilidad y reproducibilidad de los resultados, se organizó un ensayo interlaboratorios en el que participaron 13 laboratorios. Se analizaron ocho muestras no contaminadas y 16 muestras artificialmente contaminadas con el método de referencia y el método alternativo. La repetibilidad, reproducibilidad y odds ratio de ambos métodos se muestran en la tabla 3.

	Método	grado de contaminación (UFC/g)		
		0	5	25
Repetibilidad %	referencia	100	100	100
	alternativo	98	100	100
Reproducibilidad %	referencia	100	100	100
	alternativo	99	100	100
Odds ratio	referencia	1	1	1
	alternativo	1	1	1

Tabla 3. Repetibilidad, reproducibilidad y odds ratio

Practicabilidad

La practicabilidad fue definida como el tiempo que se tarda en analizar 30 muestras. En la tabla 4 se muestra el tiempo de manipulación mediante ambos métodos. Además, no se observaron diferencias significativas en los resultados si se realizó el pase a placa desde el caldo justo después de la incubación o después a partir de caldo refrigerado después de la incubación.

Muestras	Tiempo total(minutos)	
	Método de referencia	Método alternativo
Negativas	8,2	3,3
Positivas	14,2	4 (látex)
		5,8 (test de confirmación ISO 6579)

Tabla 4. Tiempo de manipulación de 30 muestras

Conclusiones

El método Salmonella Precis muestra sensibilidad, especificidad y exactitud equivalentes al método ISO 6579:2002. Los límites de detección relativos son también equivalentes. El estudio interlaboratorios muestra claramente que Salmonella Precis es un método preciso.

De acuerdo con las norma ISO 16140:2003, el método Salmonella Precis representa un valioso método alternativo para la detección de *Salmonella* spp. en alimentos, piensos y muestras medioambientales. El método Salmonella Precis también ofrece importantes ahorros económicos, reduciendo al mínimo el tiempo de obtención de resultados y el número de operaciones necesarias. Los estudios de flujo de trabajo demuestran una reducción de 2 a 3 veces en el tiempo de manipulación utilizando el método Salmonella Precis en comparación con el método de referencia ISO.

1. ISO 6579:2002: Microbiology of food and animal feeding stuffs. Horizontal method for detection of *Salmonella* spp.
2. ISO 16140:2003: Microbiology of food and animal feeding stuffs. Protocol for the validation of alternative methods
3. Statistical Techniques in Bioassay; Z. Govindarajulu; 2001
4. A novel chromogenic ester agar medium for detection of Salmonellae. Cooke VM et al. *Appl Environ Microbiol*. 1999 Feb ; 65(2): 807-12

Thermo SCIENTIFIC

RAPID TWO DAY ISOLATION OF SALMONELLA USING SINGLE SELECTIVE ENRICHMENT AND BRILLIANCE SALMONELLA AGAR

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ABSTRACT

Salmonella is a gram-negative, rod-shaped, motile bacterium with a widespread occurrence in animals, especially in poultry and swine. The most common food sources contaminated with *Salmonella* are poultry, beef, pork, eggs, and fresh produce[1]. Detection is critical as *Salmonella* infections cause an estimated 1.4 million human illnesses and 400 deaths annually in the United States[2]. It has been reported an infectious dose of as low as 17 cells may cause infection[3]. The data presented from this study describes the evaluation of Oxoid ONE Broth-Salmonella and *Brilliance*™ Salmonella Agar chromogenic medium as a detection method for *Salmonella* in ground beef, ground chicken, lettuce, shrimp, and shell eggs. The selected foods were inoculated with *Salmonella* serovars at levels ranging from low (1 colony forming unit (CFU)/25g) to high (1.1 CFU/g) and analyzed for growth.

The method was also evaluated for inclusivity using multiple *Salmonella* serovars (n=102) from various sources. Exclusivity testing was performed using closely related bacterial species (n=30) to determine the specificity of the described method. Overall, when compared to the standard USDA and FDA reference methods there was no significant difference in sensitivity between *Brilliance* Salmonella Agar and the standard reference method. However, when Oxoid ONE Broth-Salmonella was used as the enrichment step prior to plating on *Brilliance* Salmonella Agar, the time to detection was reduced from 3-4 days to 2 days. For the inclusivity study, 98 *Salmonella* serovars grew on *Brilliance* Salmonella Agar. For the exclusivity study, 30 non-*Salmonella* spp. were tested to determine the specificity of the described method and only one, *Cronobacter sakazakii*, demonstrated typical growth on the chromogenic medium, but only when not enriched in ONE Broth-Salmonella prior to plating.

INTRODUCTION

Brilliance Salmonella Agar is a novel chromogenic medium for the detection and identification of *Salmonella* spp. in food. When used in conjunction with Oxoid ONE Broth-Salmonella it reduces *Salmonella* detection time from 3-4 days to 2 days. This study evaluated and compared the performance of the Salmonella Rapid Culture method to the reference U.S. Department of Agriculture-Food Safety Inspection Service (USDA/FSIS) and U.S. Food and Drug Administration's Bacteriological Analytical Manual (FDA/BAM) methods for the detection of *Salmonella* spp. in raw ground beef, ground chicken, lettuce, shrimp, and shell eggs. Results demonstrate the Salmonella Rapid Culture method to be superior to the reference methods in incubation time and ease of identification.

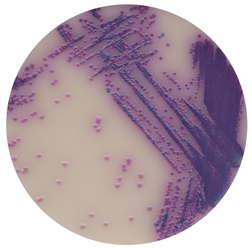
METHOD COMPARISON

Ground beef, ground chicken, lettuce, shrimp, and shell eggs were inoculated with approximately 1 CFU/ 25g and allowed to equilibrate per AOAC instructions. Side-by-side samples were set up and run using Oxoid ONE Broth-Salmonella/*Brilliance* Salmonella Agar chromogenic medium and the appropriate FDA/USDA reference for each matrix. Three different lots of Oxoid ONE Broth-Salmonella/Brilliance Salmonella Agar were evaluated. Stability and lot-to-lot variation were evaluated simultaneously. Organisms were initially grown in BHI (brain-heart infusion broth) and incubated overnight. Inoculation levels of Salmonella serovars Typhimurium and Enteritidis were approximately 1-10 CFU into 225ml of ONE Broth. A non-*Salmonella* organism (E. coli O157:H7) was inoculated into 225ml of Oxoid ONE Broth-Salmonella at a concentration achieved with overnight growth in BHI.

INCLUSIVITY: 102 *Salmonella* (table 3) strains were cultured in Oxoid ONE Broth-Salmonella and plated on *Brilliance* Salmonella Agar according to manufacturer's instructions.

EXCLUSIVITY: All strains were cultured overnight in BHI Strains were then plated on *Brilliance* Salmonella Agar and incubated per manufacturer's instructions.

LOT TO LOT AND STABILITY: Ruggedness : Organisms were initially grown in BHI and incubated overnight. Inoculation levels of *Salmonella* serovars Typhimurium and Enteritidis were approximately 1-10 CFU into 225ml of Oxoid ONE Broth-Salmonella. A non-*Salmonella* organism (E. coli O157:H7) was inoculated into 225ml of Oxoid ONE Broth-Salmonella at a concentration achieved with overnight growth in BHI. Variations were introduced as follows:



Ruggedness parameters tested:

- Incubation temperature of Oxoid ONE Broth-Salmonella: 40, 42, 44°C.
- Incubation temperature of *Brilliance* Salmonella Agar plates: 33, 35, 37, 39, 41°C
- Incubation time of *Brilliance* Salmonella Agar plates: 21, 24, 27 hrs.

RESULTS

The results obtained for ground beef, ground chicken, lettuce, shrimp, and shell eggs demonstrate that the Oxoid ONE Broth- Salmonella/*Brilliance* Salmonella Agar are equivalent to the reference method in the foods tested (table 1). When specificity was evaluated 98/102 *Salmonella* serovars were identified using this method (table 3) and 30/30 of the non- *Salmonella* showed no growth or atypical growth. The three different lots of Oxoid ONE Broth-Salmonella/*Brilliance* Salmonella Agar evaluated gave equivalent results (table 2). Variations in incubation time and temperature of the Oxoid ONE Broth-Salmonella/Brilliance Salmonella Agar did not effect the performance of the method.

Table 1: Method Comparison

				REFERENCE METHOD	TEST KIT	
INOCULATING ORGANISM	LEVEL	MPN/25g	NO. TEST PORTIONS	POSITIVE	PRESUMP. POSITIVE	
Salmonella Muenchen	Low	0.9	20	10	8	8
	Control	0	5	0	0	0
Salmonella Montevideo	Low	2.3	20	17	15	15
	Control	0	5	0	0	0
Salmonella Heidelberg	Low	5.75	20	15	14	14
	Control	0	5	0	0	0
Salmonella Cholersuis	Low	<0.75	20	9	10	10
	Control	0	5	0	0	0
Salmonella Typhimurium	Low	0.9	20	8	11	11
	Control	0	5	0	0	0

Table 2: Ruggedness and Lot to Lot Stability

	Lot 1:08A14-1	Lot 2: 08B06-1	Lot 3 08B13-1
	EXPIRE: Broth:2-11-08 Plates:1-28-08	EXPIRE: Broth:3-5-08 Plates:2-27-08	EXPIRE: Broth:2-13-08 Plates:2-13-08
Salmonella Typhimurium	5/5 positive	5/5 positive	5/5 positive
Salmonella Enteritidis	5/5 positive	5/5 positive	5/5 positive
E. coli O157:H7	5/5 negative	5/5 negative	5/5 negative

References: 1. Foley, S.L., A.M. Lynne, and R. Nayak. (2008).*Salmonella* challenges: prevalence in swine and poultry and potential pathogenicity of such isolates. *J. Anim. Sci.* 86 (14 Suppl):E149-62. Epub (2007) Oct. 2. Voetsch A.C., T.J. Van Gilder, F.J. Angulo, *et al.* (2004). FoodNet estimate of the burden of illness caused by nontyphoidal *Salmonella* infections in the United States. *Clin. Infect. Dis.;* **38** *(* Suppl. 3):S127--34. 3. Blaser, M.J., and L.S. Newman. (1982). A Review of Human Salmonellosis: I. Infective Dose *Reviews of Infectious Diseases,* Vol. 4, No. 6 pp. 1096-1106.

Table 3: Inclusivity

GENUS	SEROVAR	RESULT	GENUS	SEROVAR	RESULT
Salmonella	4,27:undetermined	Typical Growth	*Salmonella*	jena	Typical Growth
Salmonella	4,5,12: i:-	Typical Growth	*Salmonella*	Kahla	Typical Growth
Salmonella	4,5,12:b:- variant L(+)	Typical Growth	*Salmonella*	Kentucky	Typical Growth
Salmonella	4,5,12:i:-	Typical Growth	*Salmonella*	Kitenge	Typical Growth
Salmonella	6,7:k:-	Typical Growth	*Salmonella*	Kunzendorf	Typical Growth
Salmonella	abaetetuba	Typical Growth	*Salmonella*	Litchfield	Typical Growth
Salmonella	Abony	Typical Growth	*Salmonella*	London	Typical Growth
Salmonella	abortusovis	No growth*	*Salmonella*	Maarssen	Typical Growth
Salmonella	adelaide	Typical Growth	*Salmonella*	Maartensdijk	Typical Growth
Salmonella	Agona	Typical Growth	*Salmonella*	Maastricht	Typical Growth
Salmonella	Albany	Typical Growth	*Salmonella*	Mbandaka	Typical Growth
Salmonella	Anatum	Typical Growth	*Salmonella*	Meleagridis	Typical Growth
Salmonella	Arizonae	Typical Growth	*Salmonella*	Menden	Typical Growth
Salmonella	bareilly	Typical Growth	*Salmonella*	michigan	Typical Growth
Salmonella	berta	Typical Growth	*Salmonella*	Minnesota	Typical Growth
Salmonella	blockley	Typical Growth	*Salmonella*	Montevideo	Typical Growth
Salmonella	bongori	Typical Growth	*Salmonella*	Muenchen	Typical Growth
Salmonella	Braenderup	Typical Growth	*Salmonella*	muenster	Typical Growth
Salmonella	Brandenburg	Typical Growth	*Salmonella*	Newington	No growth*
Salmonella	Bredeney	Typical Growth	*Salmonella*	Newport	Typical Growth
Salmonella	Breukelen	Typical Growth	*Salmonella*	Ngili	Typical Growth
Salmonella	california	Typical Growth	*Salmonella*	Ochsenzoll	Typical Growth
Salmonella	Cerro	Typical Growth	*Salmonella*	Ohio	Typical Growth
Salmonella	chaco	Typical Growth	*Salmonella*	oranienberg	Typical Growth
Salmonella	Chailey	Typical Growth	*Salmonella*	panama	Typical Growth
Salmonella	Chester	Typical Growth	*Salmonella*	Paratyphi A	Typical Growth
Salmonella	Cholerasuis	Typical Growth	*Salmonella*	Paratyphi B var L(+) tartrate +	Typical Growth
Salmonella	Cubana	Typical Growth	*Salmonella*	paratyphi C	No growth*
Salmonella	Derby	Typical Growth	*Salmonella*	Parera	Typical Growth
Salmonella	Diarizonae Lactose (+) H2S (-)	Typical Growth	*Salmonella*	Phoenix	Typical Growth
Salmonella	Dublin	Typical Growth	*Salmonella*	Pomona	Typical Growth
Salmonella	Enteritidis	Typical Growth	*Salmonella*	Poona	Typical Growth
Salmonella	essen	Typical Growth	*Salmonella*	pullorum	Typical Growth
Salmonella	Etterbeek	Typical Growth	*Salmonella*	Putten	Typical Growth
Salmonella	Ferlac	Typical Growth	*Salmonella*	Reading	Typical Growth
Salmonella	Florida	Typical Growth	*Salmonella*	San Diego	Typical Growth
Salmonella	gallinarum	Typical Growth	*Salmonella*	Senftenberg	Typical Growth
Salmonella	give	Typical Growth	*Salmonella*	simsbury	Typical Growth
Salmonella	Hadar	Typical Growth	*Salmonella*	Sloterdijk	Typical Growth
Salmonella	Harmelen	Typical Growth	*Salmonella*	St. Paul	Typical Growth
Salmonella	Heerlen	Typical Growth	*Salmonella*	stanley	Typical Growth
Salmonella	Heidelberg	Typical Growth	*Salmonella*	tallahassee	Typical Growth
Salmonella	Hilversum	Typical Growth	*Salmonella*	Tennessee	Typical Growth
Salmonella	Hooggraven	Typical Growth	*Salmonella*	typhi	No growth*
Salmonella	houten	Typical Growth	*Salmonella*	Typhimurium	Typical Growth
Salmonella	Illinois	Typical Growth	*Salmonella*	Typhimurium Var Copenhagen	Typical Growth
Salmonella	indiana	Typical Growth	*Salmonella*	Typhimurium Var O:5-	Typical Growth
Salmonella	indica	Typical Growth	*Salmonella*	typhisuis	Typical Growth
Salmonella	Infantis	Typical Growth	*Salmonella*	Urbana	Typical Growth
Salmonella	Inverness	Typical Growth	*Salmonella*	Vellore	Typical Growth
Salmonella	Javiana	Typical Growth	*Salmonella*	Worthington	Typical Growth

* S. Newington, typhi (6439) and paratyphi C showed typical growth on *Brilliance* Salmonella Agar when plated directly from blood agar (no growth in ONE Broth directly). *S. abortusovis* appeared to grow in ONE Broth but did not grow on *Brilliance* Salmonella Agar when plated.

CONCLUSIONS

This method comparison evaluation of the Salmonella Rapid Culture Method (Oxoid ONE Broth-Salmonella/*Brilliance* Salmonella Agar) clearly demonstrated that this method is equivalent to the USDA/FSIS reference method for the detection and presumptive identification of *Salmonella* spp. at spiked levels ranging from low (1 colony forming unit (CFU)/25g) to high (1.1 CFU/g) in ground beef, ground chicken, lettuce, shrimp, and shell eggs. In addition, the Salmonella Rapid Culture method was found to be superior to the reference method in incubation time and ease of identifying typical colonies. The inclusivity data demonstrated that the Salmonella Rapid Culture method (Oxoid ONE Broth Salmonella/*Brilliance* Salmonella Agar) detected essentially all species and serovars of *Salmonella* tested. The exclusivity data confirmed that the Salmonella Rapid Culture method was able to discriminate *Salmonella* spp. from non-*Salmonella* micro-organisms. Lot-to-lot comparability and stability data along with the ruggedness data verified that the Salmonella Rapid Culture method was robust and can provide reproducible results over a range of culture conditions (time and temperature).