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Evaluation of New Culture Media for Rapid Detection and Isolation of Salmonellae in Foods

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Conventional methods for *Salmonella* detection in foods can require up to 6 and at least 4 days. We have observed that the total analysis time can be reduced to 48 h by using Salmosyst broth as a liquid medium for both preenrichment and selective enrichment and Rambach agar (RA), a new selective plate medium. In samples of artificially contaminated ground beef *Salmonella enteritidis* was detected at a concentration of 0.4 CFU/g (10 CFU/25 g) by both a conventional method and the new method. Of 519 samples of foods for sale, 38 were *Salmonella* positive by both methods while 471 were negative. Nine samples which were negative by the conventional method were positive by the Salmosyst-RA method, while one sample positive by the first method was negative by the last. Therefore, the Salmosyst-RA method showed 97.9% sensitivity compared with the 81.2% sensitivity of the conventional method. The new method was also highly specific (98% specificity) in presumptive identification of *Salmonella* colonies. Furthermore, a 6-h preenrichment in Salmosyst broth has been proved sufficient for the repair of heat-injured *Salmonella* cells and for subsequent recovery by selective enrichment. In conclusion, the Salmosyst-RA method shows several advantages over both conventional and rapid noncultural methods: (i) only two media are required instead of the five media for conventional methods; (ii) in real time it is comparable to other rapid noncultural methods, which require 30 to 31 h; (iii) it is highly sensitive and specific; and (iv) it allows the isolation of *Salmonella* strains which can be characterized by appropriate phenotypic and genotypic typing methods for epidemiological investigations.

Food-borne salmonellosis remains a major public health problem (14). Although the genus *Salmonella* contains more than 2,300 different serovars (17), only a relatively restricted number belonging mainly to the species *Salmonella enterica* subsp. *enterica* are responsible for the great majority of human infections (2, 13). All these serovars are primarily pathogenic for animals, so the most common sources of *Salmonella* infections for humans are contaminated foods such as eggs, poultry, meat, and meat products (14). In the last decade a dramatic increase in infections caused by *S. enteritidis* has been registered in several countries in Europe as well as in North and South America (19). Eggs and poultry meat have been recognized as the major vehicles of human infections because of epizootics in fowl (1, 21).

Conventional methods for *Salmonella* detection in foods are time-consuming. They require at least 4 days in the case of noncontaminated products and 5 to 6 days for contaminated samples (12). In recent years some more rapid methods have been developed. They are based on different principles, such as immunoimmobilization (7), enzyme immunoassays (6, 22), DNA probes (4, 20), immuno-PCR (9), and hydrophobic grid membrane filters (8). Using Salmosyst broth as a selective enrichment medium, some methods can reduce the total analysis time to 30 to 31 h (9, 22). However, these methods are not able to furnish *Salmonella* cultures for further studies of epidemiological interest.

Recently Rambach agar (RA) (18), a new selective plate medium for *Salmonella* isolation, was described. The medium uses the ability of salmonellae to produce acid from propylene glycol for their differentiation from other enteric bacteria. In addition, the presence of the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) allows detection of β-D-galactosidase production by other enterobacteria. *Salmonella* cultures are positive only in the first reaction and grow as bright red colonies, while *Escherichia coli* and other coliform bacteria are positive only for β-D-galactosidase and appear as blue colonies. *Proteus* spp. are negative for both reactions, so their colonies are colorless. *Citrobacter* colonies appear violet from the combination of colors resulting from the two reactions.

Our preliminary studies with Salmosyst broth and RA indicated that the total analysis time can be reduced to 48 h for both negative and positive food samples (15, 16). The present study was undertaken to evaluate the specificity and sensitivity of this culture method for isolation of salmonellae from foods.

MATERIALS AND METHODS

Media and reagents. All the culture media and reagents were commercial products. Tryptic soy broth, tryptic soy agar (TSA), buffered peptone water (BPW), Müller-Kauffmann tetrathionate broth (M-KTB), desoxycholate citrate lactose agar as modified by Hynes (DCL), and Kligler iron agar were from Oxoid. Selenite cystine broth (SCB) and brilliant green agar (BGA) were from Difco Laboratories. Salmosyst and RA were from Merck. Biochemical identification of cultures was made by the API 20E system (bioMerieux), and serological identification was made with *Salmonella* polyvalent and monovalent anti-O and anti-H sera (either Pasteur Diagnostics or Biogenetics).

Test organisms. Recently isolated *S. enteritidis*, *S. typhimurium*, *S. virchow*, and *S. newport* strains were used. Ten milliliters of tryptic soy broth was inoculated from a stock TSA slant and incubated for 24 h at 37°C. Fresh cultures were prepared by inoculating 10-ml aliquots of tryptic soy broth with 0.1 ml of the 24-h broth cultures and incubating them for 4 h at 37°C. The number of viable salmonellae per ml was then estimated by the direct surface plate count procedure. Decimal and fivefold dilutions were prepared in BPW, and counts were done with four plates of TSA for each dilution incubated at 37°C for 48 h. Each developed colony was assumed to have grown from one viable unit, i.e., 1 CFU.

Artificially contaminated food samples. *Salmonella*-negative ground beef with

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TABLE 1. *S. enteritidis* detection by the conventional method and the new method in artificially contaminated food samples

CFU of *S. enteritidis*/g of seed inocula	Detection of *S. enteritidis* by:				
	M-KTB plus:		SCB plus:		Salmosyst-RA
	DCL	BGA	DCL	BGA	
0.4	+	+	−	−	+
2	+	+	+	−	+
10	+	+	+	+	+

TABLE 2. *Salmonella* detection by the conventional method and the new method from naturally contaminated food samples

Product	No. of samples analyzed	No. of positive samples (%) by:	
		Conventional method	New method
Poultry	85	18 (21.2)	26 (30.5)
Meat[a]	246	14 (5.7)	14 (5.7)
Eggs	188	7 (3.7)	7 (3.7)
Total	519	39 (7.5)	47 (9.0)

[a] Beef (n = 103), pork (n = 35), and horse meat (n = 108).

a total aerobic plate count (37°C) of approximately 10^4 CFU/g was used for preparation of contaminated samples. Three ground beef samples of 25 g each were homogenized with 225 ml of BPW for 1 min in a stomacher. To the three samples were then added 250 CFU (10 CFU/g), 50 CFU (2 CFU/g), and 10 CFU (0.4 CFU/g) of *S. enteritidis*. Three other ground beef samples of 25 g each were homogenized with 225 ml of Salmosyst broth and inoculated as described above.

Naturally contaminated foods. A total of 519 samples of food on sale was examined. They consisted of chicken parts (n = 85), beef (n = 103), pork (n = 35), horse meat (n = 108), and eggs (n = 188). The bacteriological analysis started on the same day of drawing. The whole content of eggs drawn aseptically was examined.

Analysis of food samples. Aliquots of 50 g of meat were taken from each sample and minced; 25 g was homogenized for 1 min in a stomacher with 225 ml of BPW and then preenriched by incubation for 24 h at 37°C, while 25 g was homogenized with 225 ml of Salmosyst broth and then preenriched by incubation for 6 h at 37°C. The whole contents of two eggs were homogenized for 30 s in a stomacher and then divided in two aliquots of 50 g each for preenrichment with 450 ml of BPW and 450 ml of Salmosyst broth.

After the preenrichment period, 10 ml of BPW was transferred to 100 ml of M-KTB for selective enrichment by incubation for 24 and 48 h at 43°C, while 10 ml was transferred to 100 ml of SCB and incubated for 24 and 48 h at 37°C. Two plates of DCL and two plates of BGA were inoculated from the above-mentioned enriched media, as prescribed by the International Organization for Standardization methods for detection of salmonellae (12). All the plates were incubated for 24 h at 37°C. Five suspect colonies from each plate were cultured on Kligler iron agar and eventually submitted to full biochemical and serologic identifications by API 20E and anti-*Salmonella* sera.

For the selective Salmosyst enrichment, 10 ml of preenrichment broth base was supplemented with one selective supplement tablet and incubated for 18 h at 37°C. Following incubation, a loopful of selective broth culture was streaked onto a plate of RA. Red colonies that developed after 24 h of incubation at 37°C (five colonies for each plate) were directly submitted to slide agglutination with polyvalent anti-*Salmonella* sera and to biochemical identification as described above.

Evaluation of efficiency of preenrichment and enrichment media in heat-injured *Salmonella* recovery. *Salmonella* cultures were heat injured at 56°C for 30 min, washed, and resuspended in BPW or in Salmosyst preenrichment broth (SPB) to a density of 10^3 CFU/ml as described by Chen et al. (3). Aliquots of these suspensions were decimally diluted, respectively, in BPW and in SPB in tubes at average cell densities of 10^3, 10^2, 10^1, and 10^0 CFU/ml. All the tubes were incubated for 6 h at 37°C for preenrichment. After preenrichment, triplicate 1-ml aliquots from each tube were mixed with 1 ml of either BPW (control tubes) or twice-concentrated Salmosyst enrichment broth (SEB), SCB, or M-KTB. The BPW and SEB tubes were incubated for 18 h at 37°C, while the SCB tubes were incubated for 24 h at 37°C and the M-KTB tubes were incubated for 24 h at 43°C. Tubes were examined for the presence of salmonellae by streaking a loopful from each tube onto plates of TSA, RA, DCL, and BGA and incubating the plates at 37°C for 24 h.

RESULTS

In the samples of artificially contaminated meat, *S. enteritidis* was detected at a lowest concentration of 0.4 CFU/g (10 CFU/25 g) after enrichment in M-KTB and Salmosyst and at a concentration of 2 CFU/g (50 CFU/25 g) after enrichment in SCB (Table 1).

Table 2 shows the results obtained by the conventional method and the new method for the detection of salmonellae in 519 food samples. Thirty-eight samples were *Salmonella* positive both by the conventional method and by the Salmosyst-RA method. A total of 471 samples were *Salmonella* negative by both methods. Nine samples which were negative by the conventional method were positive by the Salmosyst-RA method, while one sample positive by the first method was negative by the last. On the basis of these results, the sensitivity of the Salmosyst-RA method was 97.9%, compared with a sensitivity of 81.2% for the conventional method.

Red colonies on RA were observed from 48 samples. A total of 235 colonies (5 colonies from each sample) were directly identified as salmonellae by slide agglutination with polyvalent anti-*Salmonella* sera and then confirmed by API 20E and complete serological (O and H antigen) identification. Five red colonies from a poultry sample were not agglutinated by polyvalent anti-O sera. They were all identified as *Citrobacter freundii* on the basis of their biochemical characteristics, although they were *o*-nitrophenyl-β-D-galactopyranoside negative for the lack of β-D-galactosidase. This biochemical anomaly was responsible for the development of red color on RA just like *Salmonella* colonies. These results show the high specificity (97.9%) of RA for presumptive identification of *Salmonella* colonies.

In Table 3 are listed the serovars identified from positive samples. A total of 18 serovars was identified. The same serovars were found in 33 samples positive by both methods. *S. virchow* (four isolates), *S. enteritidis* (one isolate), *S. obogu* (one isolate), *S. escanaba* (one isolate), and *Salmonella* serovar II 4,12:b:− (two isolates) were identified from nine food samples positive only by the rapid method, while *S. enteritidis* was iden-

TABLE 3. *Salmonella* serovars isolated by the conventional method and the new method

Serovar	Antigen formula	No. of isolates by:	
		Conventional method	New method
Salmonella serovar II 4,12:b:−	4,12:b:−	2	4
S. schleissheim	4,12:b:−	1	1
S. sandiego	4,12:e,h:e,n,z_{15}	1	1
S. typhimurium	1,4,12:i:1,2	2	2
S. brandenburg	4,12:l,v:e,n,z_{15}	1	1
S. coeln	4,12:y:1,2	3	2
S. montevideo	6,7:g,m,s:−	1	1
S. escanaba	6,7:k:e,n,z_{15}	0	1
S. virchow	6,7:r:1,2	3	8
S. obogu	6,7:z_4,z_{23}:1,5	0	1
S. planckendael	6,7:z_4,z_{23}:1,6	1	1
S. muenchen	6,8:d:1,2	1	0
S. haardt	8:k:1,5	2	0
S. hadar	6,8:z_{10}:e,n,x	1	1
S. enteritidis	9,12:g,m:−	14	14
S. muenster	3,10:e,h:1,5	1	0
S. anatum	3,10:e,h:1,6	7	8
S. london	3,10:l,v:1,6	0	1

TABLE 4. Efficiency of different preenrichment and enrichment media for heat-injured *Salmonella* recovery

Serovar	Preenrichment medium	Enrichment medium	Recovery[a]			
			10^3	10^2	10^1	10^0
S. enteritidis	BPW	BPW	3	3	3	0
		SEB	3	2	0	0
		SCB	3	3	0	0
		M-KTB	3	0	0	0
	SPB	BPW	3	3	3	2
		SEB	3	3	3	1
		SCB	3	3	3	0
		M-KTB	3	0	0	0
S. typhimurium	BPW	BPW	3	3	0	0
		SEB	3	3	0	0
		SCB	3	3	0	0
		M-KTB	3	0	0	0
	SPB	BPW	3	3	3	0
		SEB	3	3	3	0
		SCB	3	3	3	0
		M-KTB	3	0	0	0
S. virchow	BPW	BPW	3	3	3	0
		SEB	3	3	0	0
		SCB	3	3	3	0
		M-KTB	3	0	0	0
	SPB	BPW	3	3	3	3
		SEB	3	3	2	0
		SCB	3	3	3	3
		M-KTB	3	3	1	0
S. newport	BPW	BPW	3	3	3	0
		SEB	3	3	0	0
		SCB	3	3	3	0
		M-KTB	3	3	0	0
	SPB	BPW	3	3	3	0
		SEB	3	3	2	0
		SCB	3	3	3	0
		M-KTB	3	3	2	0

[a] Number of tubes confirmed positive on TSA from samples containing 10^3, 10^2, 10^1, or 10^0 CFU/ml prior to preenrichment.

tified from the sample that was positive only by the conventional method. From each of two samples two different serovars (*S. typhimurium* and *S. muenchen* from one sample and *S. virchow* and *S. haardt* from the other) were isolated by the conventional method, while only one serovar (*S. typhimurium* and *S. virchow*, respectively) was isolated by the rapid method. Three further samples were positive for *S. haardt*, *S. muenster*, and *S. coeln* by the conventional method and for *S. virchow*, *S. london*, and *S. anatum*, respectively, by the rapid method.

Because short periods of preenrichment have been reported to provide insufficient time for the repair of stressed or injured cells (5), we examined if a 6-h preenrichment in SPB is satisfactory for the full recovery of heat-injured salmonellae during selective enrichment. Table 4 shows the development of heat-injured *Salmonella* cells of four different serovars in the selective enrichment media after short (6-h) preenrichment in BPW and SPB. The best recovery was obtained after Salmosyst preenrichment, as shown by the higher number of positive tubes observed with all the selective enrichment media. Of the three selective enrichment media assayed, M-KTB gave recovery of the least number of *Salmonella* cells after preenrichment either in BPW or in SPB. No differences were observed in the number of positive cultures on selective solid media (RA, DCL, and BGA) with respect to those observed on TSA plates.

DISCUSSION

The combined utilization of Salmosyst and RA for food analysis shows several advantages. First, only two media are required, a liquid medium for both preenrichment and selective enrichment (Salmosyst) and a solid medium for selective isolation (RA), instead of the five media required by the conventional method. Moreover, the total time needed to accomplish the analysis is reduced from 4 to 6 days to 48 h. In fact, the preenrichment in Salmosyst broth and the selective enrichment are limited, respectively, to 6 and 18 h, while a further 24-h period is enough for the incubation of inoculated RA plates for selective isolation. In real time this cultural method is comparable to other rapid methods, such as enzyme-linked immunosorbent assay (22) and magnetic immuno-PCR assay (9), which require 30 to 31 h. These methods, in fact, require 2 workdays, so that the ascertainment of a negative or positive result can be utilized in any case on day 3.

The analysis of artificially contaminated ground beef samples revealed high sensitivity for both conventional and new cultural methods, which were both able to detect at least 0.4 CFU of *S. enteritidis* per g. With naturally contaminated foods the new method showed a higher sensitivity (97.9%) than the conventional method (81.2%). The same serovars were isolated by both methods from the great majority of positive samples, although a few differences have been observed, probably because of the variable sensitivity of some serovars to the inhibitory characteristics of the different selective media. The superiority of Salmosyst broth for *Salmonella* preenrichment with respect to BPW has been demonstrated also by the dilution endpoint method that has been proposed for the evaluation of new selective media (3). The results of the assay with pure cultures of different serovars demonstrate that a 6-h preenrichment in Salmosyst broth is sufficient for the repair of heat-injured *Salmonella* cells and for subsequent selective enrichment. SEB has been proved more sensitive than M-KTB and almost as sensitive as SCB for the recovery of the repaired *Salmonella* cells. In conclusion, all the experimental data on artificially and naturally contaminated foods and on pure cultures of heat-injured *Salmonella* cells show that a 6-h preenrichment followed by an 18-h selective enrichment in Salmosyst offers at least the same sensitivity as 24 h of preenrichment followed by 24 h of enrichment in traditional media.

Further advantages are offered by the use of RA because red colonies of salmonellae are very easy to recognize even when other enterobacteria are preponderantly developed. A limitation to the sensitivity of this medium could derive from the peculiarities of some *Salmonella* serovars which are inactive against propylene glycol or produce β-D-galactosidase (10, 11, 15). Nevertheless, these serovars have a negligible epidemiological impact, because they are very rarely or not at all encountered in human infections (2, 13).

In our experience RA was also proved to be highly specific. In one sample only we observed false-positive red colonies of β-D-galactosidase-negative *C. freundii*. This can be considered an exceptional occurrence in light of other experimental results which gave 100% specificity assaying a great number of bacterial strains belonging to different enterobacterial species (10, 11).

Finally, a further advantage of the described rapid cultural method in comparison with other noncultural rapid methods derives from the isolation of *Salmonella* strains in pure culture. This is of capital importance from the epidemiological point of view. The full characterization of the isolated strains by appropriate typing methods (biochemical and phage typing, antibi-

otic resistance pattern, plasmid pattern, and ribotyping) is of great value in epidemiological investigations.

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