

Comparison of Enrichment and Plating Media for Recovery of Virulent Strains of *Yersinia enterocolitica* from Inoculated Beef Stew

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ABSTRACT

Five plating agar media were evaluated for their ability to recover pure cultures of virulent strains of *Yersinia enterocolitica* serotypes O:3, O:8 and O:5,27. Cellobiose-arginine-lysine and bismuth sulfite agars were unproductive at 32°C but gave quantitative recovery with 48 h of incubation at 22°C. Adjustment of the pH of bismuth sulfite agar to 7.4 made the medium inhibitory. MacConkey, DHL and cefsulodin-irgasan-novobiocin agars gave quantitative recovery with 24 h of incubation at 32°C. Four preenrichment media incubated at four different temperatures, three selective enrichment media incubated at 22°C, and the five plating media were evaluated for their ability to recovery *Y. enterocolitica* from beef stew seeded with a background of ten other gram-negative bacteria. None of the plating media was superior for recovery; however, cefsulodin-irgasan-novobiocin agar showed the highest confirmation rate for presumptive colonies. Buffered-sorbitol-bile broth was inferior to richer media such as trypticase soy broth for preenrichment. Of the three selective enrichment media examined, only bile-oxalate-sorbose broth was found useful, especially for strains of serotype O:8 which could be recovered after 1 d of preenrichment and 3 d of selective enrichment at 22°C. Strains of serotypes O:8 and O:3 were recovered when two cells with 10⁷ cells of ten other gram-negative bacteria were added to 10 g of beef stew following preenrichment in trypticase soy broth at 22°C for 7 d and selective enrichment in bile oxalate-sorbose broth at 22°C for 3 to 5 d. Strains of serotype O:5,27 were more difficult to recover even with longer enrichment times. These studies indicated that the most comprehensive enrichment system for recovery of *Y. enterocolitica* from foods is preenrichment in trypticase soy broth at 22°C for 1 d and 2 to 4°C for 4 to 7 d followed by selective enrichment in bile-oxalate-sorbose broth at 22°C for 3 to 5 d and isolation on cefsulodin-irgasan-novobiocin agar.

A number of different enrichment procedures and agar plating media have been used for the isolation of *Yersinia enterocolitica* from foods. Phosphate-buffered saline (PBS) has been the most popular for "cold" enrichment. Mehlman et al. (8) modified PBS by addition of 1% sorbitol and 0.15% bile salts. Weagant and Kaysner (18) recently recommended supplementation of PBS with 0.5% peptone for better growth of *Y. enterocolitica*. van Pee and

Stragier (15) suggested that a richer medium like trypticase soy broth was superior for cold enrichment. Vidon and Delmas (16) used PBS with 2% peptone and cycloheximide for cold enrichment of milk samples.

Only a few selective enrichment media have been developed for recovery of *Y. enterocolitica*. Selenite broth was used by Lee et al. (7) and Caprioli et al. (1). Vidon and Delmas (16) developed a selective enrichment designated PST for use following cold enrichment of milk samples. Modified Rappaport broth described by Wauters et al. (17) is highly selective for strains of serotypes O:3 and O:9, which are commonly associated with human illness in Europe; but is highly inhibitory for strains of serotype O:8, common in North America, and O:5,27, found in many countries. Modified Rappaport broth has been useful for recovery of strains of serotype O:3 when used for selective enrichment following cold preenrichment in PBS (10). Two new media have recently been described for isolation of *Y. enterocolitica* from foods with a two-step procedure: (a) yeast-extract-rose-bengal broth used for preenrichment at 4 or 10 °C and (b) bile-oxalate-sorbose broth used for selective enrichment at 22°C (11).

Several traditional enteric agar plating media have been used for isolation of *Y. enterocolitica* such as salmonella-shigella (SS), MacConkey, Drigalski, lactose-sucrose-urea (LSU), desoxycholate-citrate (DCA) and bismuth sulfite. Harmon et al. (5) have reported one of the few studies available comparing the performance of plating media for recovery of *Y. enterocolitica* from foods. Their study concluded that cefsulodin-irgasan-novobiocin (CIN) agar was the most effective among selective-differential plating media for recovery of *Y. enterocolitica* from artificially inoculated ground pork homogenates.

There has been considerable progress in the ability to distinguish between virulent and avirulent strains of *Y. enterocolitica*. Virulence in *Y. enterocolitica* is dependent on the presence of a plasmid (Vwa) that also codes for autoagglutination at 35°C (6), which provides a simple indirect test for identifying virulent strains (14). Reported studies on isolation methods have rarely considered virulence in the strains selected for comparing recovery efficiencies.

The study reported here was undertaken to compare the performance of four different preenrichment media which were incubated at four different temperatures; three selective enrichment media all incubated at 22°C; and five plating media, for recovery of virulent strains of *Y. enterocolitica* serotypes O:8, O:3 and O:5,27 from canned beef stew artificially seeded with a background flora composed of ten other gram-negative bacteria.

MATERIALS AND METHODS

Y. enterocolitica test strains

Six strains of *Y. enterocolitica* were selected for the major part of this study: E661 (O:8), E663 (O:8), E739 (O:3), E752 (O:3), E758 (O:5,27) and E771 (O:5,27). Two additional strains, E665 (O:8) and E750 (O:21) were added for some media evaluations. All strains were originally isolated from humans, and represent, with the exception of E750, serotypes that are commonly associated with human illness. All cultures were obtained from Dr. Sandu Toma, Canadian National Reference Service for *Yersinia*, Toronto, Ontario, Canada. Each strain was positive for autoagglutination (6), a test which has been correlated with the presence of the Vwa virulence plasmid and pathogenicity (4). Strain E661 had previously been examined for other markers of virulence including calcium dependency (4), guinea pig conjunctivitis (12), and mouse diarrhea and lethality (13,14). Strains E661 and E663 were previously shown to be infective for HeLa cells (2).

All test strains were preserved in phosphate-buffered glycerol-peptone solution at -20°C. Subcultures were prepared on trypticase soy agar with 0.6% yeast extract (TSY) or brain heart infusion (BHI) agar, and incubated at room temperatures (RT) for 48 h. Cell suspensions and dilutions were prepared in phosphate-buffered saline pH 7.6 (PBS). Periodically the subculture was tested for autoagglutination to verify that the Vwa plasmid had been retained.

Gram-negative bacteria

A variety of foods were enriched in peptone water (0.1%) at room or refrigeration temperatures. The enrichments were streaked onto MacConkey agar and colonies selected at random for subculture and identification. A library of various gram-negative bacilli was thus assembled and preserved at -20°C in buffered glycerol-peptone solution. Twenty cultures divided into two groups of ten each (I and II) were chosen for seeding beef stew samples. Group I organisms are identified in Table 7 and Group II organisms in Table 8.

Enrichment media

Four preenrichment media were selected for this study: (a) phosphate-buffered saline with 1.0% sorbitol and 0.15% bile salts (BSB) (8); (b) PSB as described by Vidon and Delmas (16); (c) trypticase soy broth (TSB;BBL); and (d) yeast extract-rose-bengal (YER) broth (11). Three selective enrichment broths were chosen for investigation: (a) PST broth as described by Vidon and Delmas (16); (b) modified Rappaport broth without carbenicillin (MRB) (17); and (c) bile-oxalate-sorbose (BOS) broth (11). A modification of BOS prepared by addition of 2.5% sodium chloride (BON) was included in one experiment. PST broth was prepared as described (16), the pH adjusted to 8.3, and the solution was autoclaved. Because the azide reacted with brilliant green to form a milky solution, these ingredients were added as filter-sterilized stock solutions just before use. Any attempt to adjust the pH of MRB resulted in precipitation. To eliminate the precipitate which formed after autoclaving, the magnesium chloride was autoclaved separately in 300 ml of distilled water and the solutions were mixed after cooling.

Plating media

DHL agar was prepared from basic ingredients according to the following formulation (per L): trypticase (BBL), 10 g; peptone No. 3 (Difco), 10 g; beef extract (Difco), 3 g; lactose, 10 g; sucrose, 10 g; L-cysteine, 0.2 g; sodium citrate, 1 g; sodium desoxycholate, 0.5 g; sodium thiosulfate, 2 g; ferric ammonium citrate, 1 g; agar, 15 g; and neutral red 0.3 mg/ml, 4.0 ml. The medium was boiled to dissolve all ingredients but not autoclaved.

Cellobiose-arginine-lysine (CAL) agar (Scott Laboratories) was prepared without pH adjustment, as was bismuth sulfite agar after initial studies found a deleterious effect from altering the pH. MacConkey (Mac) (Difco), cefsulodin-irgasan-novobiocin (CIN) (Oxoid) and DHL agars were adjusted to pH 7.3 to 7.4 after cooling to 45-50°C. All prepared plating media were held under refrigeration for at least 1 d before use and never for longer than 14 d. Each batch of plating medium was examined for recovery ability using spread plate inoculation with a pure culture suspension of *Y. enterocolitica*. Mac agar was included in each comparison as a control.

Inoculated foods

The evaluation of isolation methods for *Y. enterocolitica* using naturally contaminated foods has several drawbacks: (a) a food with a reasonable probability of containing virulent forms of all types of *Y. enterocolitica* is not available; (b) foods commonly contain avirulent and related forms of bacteria that interfere with the recovery of either endogenous or seeded virulent forms of *Y. enterocolitica*; (c) naturally contaminated foods do not permit control of the ratio of competitive background organisms to *Y. enterocolitica*; and (d) the background flora in naturally contaminated foods may be limited in both variety and type. To overcome these obstacles, canned beef stew was selected as a representative food and was inoculated with one strain of *Y. enterocolitica* and a group of ten other gram-negative bacteria previously isolated from foods. The densities were determined beforehand so that the ratio of background to *Y. enterocolitica* was known in each case.

A homogenate was prepared with canned beef stew and a selected preenrichment medium (1:10 ratio) and then distributed in 100-ml portions, i.e., 10 g of food. *Y. enterocolitica* was inoculated at about 10 cells per g of food, i.e., 100 cells per enrichment, and the composite of ten gram-negative bacteria at about 10⁶ cells per g of food, i.e., 10⁷ cells per enrichment. After incubation of the preenrichment, selective enrichments were inoculated at a ratio of 0.1 ml per 10 ml.

RESULTS

Agar plating media

Various combination and preparations of the five plating media were compared for recovery of *Y. enterocolitica* from suspensions of pure cultures in PBS. Incubation of CAL at 32°C and pH adjustment of bismuth sulfite (BiS) agars were counter-productive (Table 1). Both CAL and BiS agars gave quantitative recovery if incubated at 22°C for 48 h, and if pH adjustment of the BiS agar was not attempted (Table 2).

TABLE 1. Comparative performance of plating media for recovery of *Y. enterocolitica*.

Test strain	Mean colony count (n = 3)			
	Mac ^a (32°C, 24 h)	CAL (32°C, 48 h) ^b	BiS (22°C, 72 h) ^c BBL	Difco
E661	120	88 ^d	91 ^d	72 ^d
E663	106	100	101	95
E752	105	10 ^d	111	105
E758	77	22 ^d	69	61
E739	80	16 ^d	78	71
E771	80	19 ^d	59	46 ^d
All strains	95	45 ^d	85	75 ^d

^aMac, MacConkey; CAL, cellobiose-arginine-lysine; BiS, bismuth sulfite.

^bNo growth after 24 h.

^cPinpoint colonies only after 48 h. BiS pH adjusted to 7.4.

^dSignificantly different from Mac agar by Student's t test for independent means ($\alpha = 0.05$).

DHL agar required only 24 h of incubation at 32°C for quantitative recovery, and autoclaving of CIN agar was not detrimental (Table 3). Mac, DHL and CIN agars gave quantitative recovery with 24 h of incubation at 32°C (Table 4), with the exception of recovery of strain E665 (O:8) on CIN agar. This inhibition of certain strains of serotype O:8 on CIN agar had been previously observed. Some conflicting results suggested that the diluent, which was PBS in all of these experiments, might be a factor. Two strains of *Y. enterocolitica* O:8 and one of serotype O:3 were suspended in four diluents, held at room temperature for 30 min, and then plated on CIN and TSY agars. The results (Table 5) confirmed that the diluent influenced quantitative recovery of certain strains of serotype O:8 on CIN agar. Saline (0.85%) appeared to be the diluent of choice.

During the enrichment studies described below, each of the plating media was scored regarding the presence of colonies that morphologically resembled *Y. enterocolitica* and required confirmation. Two presumptive colonies were selected from each plate for identification. The results (Table 6) showed that the major advantage of CIN agar was not necessarily selectivity but differentiation. The frequency with which confirmation was required of colonies that resembled but were not *Y. enterocolitica* was lower with CIN than the other agar media.

Cold enrichment

It has frequently been stated that cold enrichment is productive for recovery of *Y. enterocolitica* because the organism "grows better" at low temperatures. In fact, many other gram-negative bacteria are able to grow at refrigeration temperatures equally as well (Tables 7 and 8). The benefit of low temperature appears to be in reducing a temperature-dependent antagonism by certain other gram-negative bacteria (Table 9). The reduced population of *Y. enterocolitica*, that would be even smaller if the ratio of antagonist to *Y. enterocolitica* was greater, prevents recovery

ery by direct streaking onto an agar plating medium. (Count of each organism in mixed cultures was achieved by use of carbenicillin and novobiocin for selection.)

TABLE 3. Comparative performance of plating media for recovery of *Y. enterocolitica*.

Test strain	Mean colony count (n = 3) ^a			
	BHI ^b (Control)	DHL	CIN	
			Boiled	Autoclaved
E661	76	77	70	70
E663	55	60	51	51
E739	75	75	69	67
E752	83	81	73	80
E758	99	90	84 ^c	90
E771	112	108	102	106
All strains	83	82	75	77

^aPlates incubated at 32°C for 24 h. BiS (BBL) with pH adjusted to 7.4 showed no growth at 32°C and only pinpoint colonies at 22°C after 48 h incubation.

^bBHI, brain heart infusion; CIN, cefsulodin-irgasan-novobiocin.

^cSignificantly different from BHI agar by Student's t test for independent means ($\alpha = 0.05$).

TABLE 4. Comparative performance of plating media for recovery of *Y. enterocolitica*.

Test strain	Mean colony count (n = 3) ^a				
	BHI ^b (Control)	Mac	DHL	CIN	
				Oxoid	Difco
E661	178	190	168	183	201
E663	236	237	252	244	250
E665	107	91	104	42 ^c	36 ^c
E739	308	284	251	274	282
E750	247	236	263	220	216
E752	154	154	172	185	185
E758	341	300	273	277	292
E771	200	251 ^c	251 ^c	219	237
All strains	222	218	217	204	213

^aPlates incubated at 32°C for 24 h.

^bBHI, brain heart infusion; Mac, MacConkey; CIN, cefsulodin-irgasan-novobiocin.

^cSignificantly different from BHI by Student's t test for independent means ($\alpha = 0.05$).

TABLE 5. Influence of diluent on quantitative recovery of *Y. enterocolitica* on CIN agar.

Diluent ^a	Percent recovery ^b		
	E661 (O:8)	E739 (O:3)	E665 (O:8)
PBS, pH 7.6	106	106	37 ^c
PBS + 0.02% peptone, pH 7.6	85	91	30 ^c
Dulbecco's PBS	121	98	59 ^c
NaCl, 0.85%	103	86	80

^aCell suspension prepared in diluent held at room temperature for 30 min before plating.

^bRelative to recovery on trypticase soy-yeast extract agar. Media incubated at 32°C for 22 h.

^cSignificantly different from recovery on trypticase soy-yeast extract agar by Student's t test for independent means ($\alpha = 0.05$; n = 3).

TABLE 2. Comparative performance of plating media for recovery of *Y. enterocolitica*.

Test strain	Mean colony count (n = 3) ^a		
	BHI ^b (Control)	CAL	BiS ^c (Difco)
E661	114	101	112
E663	80	69	71
E739	73	65	70
E752	64	66	66
E758	123	105	112
E771	97	71 ^d	90
All strains	92	80	87

^aPlates incubated at 22°C for 51 h.

^bBHI, brain heart infusion; CAL, cellobiose-arginine-lysine; BiS, bismuth sulfite.

^cBoiled medium cooled to 45°C had pH of 7.64. pH not adjusted.

^dSignificantly different from BHI agar by Student's t test for independent means ($\alpha = 0.05$).

Recovery from inoculated beef stew

Selective enrichment in PST and MRB following preenrichment in any of four different media at four temperatures for various time periods was unsuccessful for recovery of *Y. enterocolitica* O:8 (E661) (Table 10). A single day of preenrichment at 22°C followed by selective enrichment in BOS for 3 d was adequate for recovery of this

TABLE 6. Confirmation rate of plating media for *Y. enterocolitica*.

	CIN ^a	DHL	Mac	BiS	CAL
No. of plates with presumptive colonies requiring confirmation ^b	68	45	61	35	113
No. of plates yielding <i>Y. enterocolitica</i>	50	21	22	13	27
Percent confirmation	74	47	36	37	24

^aCIN, cefsulodin-irgasan-novobiocin; Mac, MacConkey; BiS, bismuth sulfite; CAL, cellobiose-arginine-lysine.

^bThe total number of plates used of each medium was not equal. Plates were streaked from enrichments of foods inoculated with one strain of *Y. enterocolitica* and ten strains of other gram-negative bacteria.

strain. There was no apparent superiority of one plating medium over the others. As noted previously, the benefit of CIN agar was primarily in differentiation which reduced the necessity of confirming presumptive colonies that were not *Y. enterocolitica*.

Three selective enrichment media and three plating media were evaluated for recovery of *Y. enterocolitica* O:3 (E739) from inoculated beef stew (Table 11). MRB was unsuccessful with the exception of two occasions. This was contrary to previous experience that showed this medium to be excellent for recovery of strains of serotype O:3 (10). However, in this instance the preparation procedure was modified by autoclaving the magnesium chloride solution separately and mixing the two cooled solutions, a technique that eliminated precipitation. Autoclaving the complete medium produces a heavy precipitate. An attempt to modify MRB by addition of yeast extract (25 mg/L) and sodium pyruvate (50 mg/L) resulted in recovery on only one occasion. Unlike the results with serotype O:8, a longer preenrichment period in richer media (PSB, TSB, YER) and 5 d selective enrichment in BOS were required for recovery of serotype O:3. CAL agar appeared slightly less productive than CIN and DHL agars in this experiment.

Four different selective enrichment media, i.e., BOS, MRB, MRB with yeast extract and sodium pyruvate added and BOS with 2.5% sodium chloride added (BON), were

TABLE 7. Growth of pure cultures of gram-negative bacteria in four enrichment media at three temperatures.

Organism	Time (d) to turbidity ^a											
	BSB ^b			PSB			TSB			YER		
	22	10	2	22	10	2	22	10	2	22	10	2
	(°C)			(°C)			(°C)			(°C)		
<i>Y. enterocolitica</i>												
O:8 (E661)	2	6	9	1	2	5	1	2	5	1	2	5
O:8 (E663)	2	6	9	1	3	5	1	2	5	1	2	5
O:3 (E739)	4	9	9	1	3	7	1	3	6	1	3	5
O:3 (E752)	2	5	9	1	3	6	1	2	5	1	2	4
O:5,27 (E758)	4	8	9	2	4	9	1	4	8	1	4	7
O:5,27 (E771)	ND ^c	6	NG	2	4	9	1	4	8	1	4	7
<i>Acinetobacter calcoaceticus</i> var. <i>anitratus</i> ^d	4	8	NG	1	4	NG	1	3	4	2	4	NG
<i>Proteus vulgaris</i>	4	12	NG	1	5	NG	1	5	NG	1	6	NG
<i>Klebsiella pneumoniae</i>	4	4	9	1	3	8	1	3	6	1	3	6
<i>Pseudomonas fluorescens</i>	2	4	5	1	2	4	1	2	4	1	2	4
<i>Pseudomonas aeruginosa</i>	2	4	9	1	3	7	1	3	6	1	3	6
<i>Enterobacter agglomerans</i>	2	4	7	1	3	5	1	2	4	1	2	5
<i>Serratia rubidea</i>	2	4	9	1	3	8	ND	3	6	1	3	6
<i>Citrobacter freundii</i>	2	5	NG	1	4	NG	1	4	21	1	4	9
<i>Aeromonas hydrophila</i>	2	5	9	1	3	8	1	3	7	1	3	6
<i>Providencia alcalifaciens</i>	4	9	NG	1	4	NG	1	4	NG	1	4	NG

^aInitial inoculum 10⁴ to 10⁵ cells per 10 ml of medium.

^bBSB, buffered-sorbitol-bile; TSB, trypticase soy broth; YER, yeast extract-rose-bengal.

^cND, no data; NG, no growth after 22 d of incubation.

^dGram-negative organisms identified in text as Group I.

examined for recovery of *Y. enterocolitica* O:5,27 (E758). As observed with the test strain of serotype O:3 (E739), recovery was achieved with BOS only after longer preenrichment in richer media (Table 12).

Three additional strains representing the same serotypes were inoculated into beef stew with a second group of ten

TABLE 8. Growth of gram-negative bacteria in trypticase soy broth at 10 and 2°C.

Organism ^a	Time (d) to turbidity ^b	
	10°C	2°C
<i>Klebsiella pneumoniae</i>	3	NG ^c
<i>Aeromonas hydrophila</i>	2	4
<i>Pseudomonas aeruginosa</i>	3	5
<i>Pseudomonas putida</i>	2	3
<i>Enterobacter agglomerans</i>	3	9
<i>Acinetobacter calcoaceticus</i> var. <i>lwoffii</i>	3	6
<i>Pseudomonas fluorescens</i>	2	3
<i>Citrobacter freundii</i>	3	17
<i>Providencia rettgeri</i>	3	18
<i>Providencia alcalifaciens</i>	3	NG

^aGram-negative bacteria identified as Group II.

^bInitial inoculum 10⁴ to 10⁵ cells per 10 ml of medium.

^cNG, no growth after 22 d of incubation.

gram-negative bacteria. Preenrichment was completed in TSB at 10 and 2°C, and BOS was used for selective enrichment. Strains E663 (O:8) and E752 (O:3) were recovered with the same efficiency. Strain E771 (O:5,27), however, could not be recovered (Table 13).

Recovery of injured *Y. enterocolitica*

Three strains of *Y. enterocolitica* were suspended individually in beef stew and frozen at -20°C for 10 d. After thawing at room temperature for 3 h, dilutions were made to inoculate a beef stew homogenate prepared with TSB. Based on differences in counts on BHI and CIN agars,

TABLE 9. Antagonism of gram-negative bacteria to growth of *Y. enterocolitica* in mixed cultures.

Culture conditions ^a	Density of <i>Y. enterocolitica</i> with:		
	Alone	<i>Serratia rubidea</i> (220:1) ^b	<i>Citrobacter freundii</i> (620:1)
32°C, 24 h	1.8 × 10 ⁷	1.8 × 10 ⁴	1.9 × 10 ³
22°C, 24 h	2.4 × 10 ⁷	6.5 × 10 ⁵	1.4 × 10 ⁵
22°C, 48 h	1.0 × 10 ¹⁰	1.5 × 10 ⁵	1.8 × 10 ⁵
10°C, 96 h	1.0 × 10 ¹⁰	1.2 × 10 ⁸	7.2 × 10 ⁸

^aTrypticase soy broth incubated statically.

^bInitial ratio of this organism to *Y. enterocolitica*.

TABLE 10. Recovery of *Y. enterocolitica* from inoculated beef stew^a.

Preenrichment			Recovery after selective enrichment in BOS at 22°C on ^b :				
Medium	Temp (°C)	Time (d)	CIN ^c	DHL	Mac	BiS	CAL
BSB	32	1	++ ^d	+	+	—	—
		2	++	+++	+	+++	++
		7	+++	+++	+	++	+
	10	3	+++	+++	+++	+++	+++
		7	+++	+++	+++	+++	+++
	2	7	+++	+++	+++	+++	+++
PSB	32	1 ^e	+	+	+++	—	+++
		2	+	++	+	—	—
		7	+++	+++	+++	+++	+++
	10	3	+++	+++	+++	+++	+++
		7	+++	+++	+++	+++	+++
	2	7	+++	+++	+++	+++	+++
TSB	32	1 ^e	—	—	+++	—	++
		2	+++	+++	+++	—	+++
		7	+++	++	+++	++	+
	10	3	+++	+++	+++	+++	+++
		7	+++	+++	+++	+++	+++
	2	7	+++	+++	+++	+++	+++
YER	32	1 ^e	+	+	+++	—	+++
		2	+++	++	+	+++	—
		7	+	+	+	—	++
	10	3	+++	+++	+++	+++	+++
		7	+++	+++	+++	+++	+++
	2	7	+++	+++	+++	+++	+++

^a*Y. enterocolitica* (E661, O:8) inoculated at 6.4 cells/g and Group I organisms at 5 × 10⁵/g.

^bSelective enrichment in PST for 2 and 4 d, and in MRB for 4 d, were unsuccessful with preenrichments shown and with preenrichment at 10°C for 5 d and 2°C for 10 d.

^cCIN, cefsulodin-irgasan-novobiocin; Mac, MacConkey; BiS, bismuth sulfite; CAL, cellobiose-arginine-lysine; BSB, buffered-sorbitol-bile; TSB, trypticase soy broth; YER, yeast extract-rose-bengal.

^dRelative density of presumptive colonies on isolation agar medium.

^eBOS incubated 5 d; all other results for 3 d of incubation.

there was little cell injury produced by freezing. Nevertheless, the experiment was continued to confirm the high sensitivity of TSB preenrichment and BOS selective enrichment for recovery of serotypes O:8 and O:3 (Table 14). Strains E771 (O:5,27) was, in contrast, recovered only at the highest cell density and with the longest preenrichment period.

A second attempt to produce cell injury by heating was more successful. Total cell densities were higher in the inoculated food in this experiment; however, the majority of the inoculated population represented injured cells. For example, the lowest density of strain E661 was 23 cells/g of food, but the degree of injury was 91%. Therefore, the density of uninjured cells was only 2 per g. All three test strains were recovered (Table 15). Strain E758, a second

culture of serotype O:5,27, was also recovered but only after preenrichment for 7 d and selective enrichment for 5 d.

DISCUSSION

PSB, TSB and YER, representing nutritionally richer media, were better for preenrichment than BSB. This agrees with the observation of van Pee and Stragier (15), who found tryptone-soya broth to be better than phosphate buffer for cold enrichment. Since TSB is readily available in dehydrated form, it would appear to be the medium of choice for preenrichment of foods for isolation of *Y. enterocolitica*.

TABLE 11. Recovery of *Y. enterocolitica* from inoculated beef stew^a.

Preenrichment			Recovery after selective enrichment in BOS at 22°C for 5 d on:		
Medium	Temp (°C)	Time (d)	CIN ^b	DHL	CAL
BSB	22	1	—	—	—
	10	3	—	—	—
	2	7	— ^c	—	—
PSB	22	1	—	—	—
	10	3	—	—	—
	2	7	+++	+++	—
TSB	22	1	—	—	—
	10	3	—	—	++
	2	7	+++	+++	— ^c
YER	22	1	—	—	—
	10	3	—	—	—
	2	7	+++	+++	—

^a*Y. enterocolitica* (E739, O:3) at 10/g and Group I organisms at 1.6×10^6 /g.

^bCIN, cefsulodin-irgasan-novobiocin; CAL, cellobiose-arginine-lysine; BSB, buffered-sorbitol-bile; TSB, trypticase soy broth; YER, yeast extract-rose-bengal.

TABLE 12. Recovery of *Y. enterocolitica* from inoculated beef stew^a.

Preenrichment			Recovery after selective enrichment in BOS at 22°C for 5 d on ^b :		
Medium	Temp (°C)	Time (d)	CIN ^c	DHL	CAL
BSB	22	1	—	—	—
	10	3	—	—	—
	2	7	—	—	—
PSB	22	1	—	+	—
	10	3	++	+++	+
	2	7	+++	+++	+++
TSB	22	1	—	—	—
	10	3	+++	+++	—
	2	7	+++	+++	+++
YER	22	1	—	—	—
	10	1	—	—	—
	2	7	++	+++	+++

^a*Y. enterocolitica* (E758, O:5,27) at 8.7/g and Group I organisms at 3.4×10^6 /g.

^bSelective enrichment in MRB and BOS with 2.5% NaCl was unsuccessful.

^cCIN, cefsulodin-irgasan-novobiocin; CAL, cellobiose-arginine-lysine; BSB, buffered-sorbitol-bile; TSB, trypticase soy broth; YER, yeast extract-rose-bengal.

TABLE 13. Recovery of *Y. enterocolitica* from inoculated beef stew^a.

Preenrichment in TSB		Direct recovery on CIN agar			Recovery after selective enrichment in BOS at 22°C			
Temp (°C)	Time (d)	E663 (O:8)	E752 (O:3)	E771 (O:5,27)	Enrichment time (d)	Recovery on CIN agar		
10	3	—	—	—	3	+++	—	—
					5		+++	—
2	4	—	—	—	3	+++	+	—
					5			—
	7	+	—	—	3	+++	—	—
					5		+++	—
	10	—	—	—	3			—
	14	—	—	—	5			—

^a*Y. enterocolitica* E663 (O:8), 23/g; E752 (O:3), 14/g; E771 (O:5,27), 33/g; and Group II organisms, 7.8×10^5 /g of food.

The performance of any available selective enrichment medium varies greatly with the serotype of *Y. enterocolitica*. One of the difficulties in the development of a reliable procedure for isolation of *Y. enterocolitica* has been in treating the various types as if they were physiologically and biochemically identical. This is not the case, and it may be that no system will ever be suitable for recovery of all varieties of *Y. enterocolitica* that are human pathogens.

The PST broth described by Vidon and Delmas (16) for recovery of *Y. enterocolitica* from raw milk was not a useful enrichment medium for the virulent strains used in this study. MRB, contrary to previous experiences, could not recover strains of serotype O:3. This failure may have been the consequence of a modification in preparation of the medium adopted in order to eliminate precipitation after autoclaving. Addition of yeast extract and pyruvate to MRB was not beneficial.

BOS broth was the only useful medium for selective enrichment, particularly for strains of serotype O:8. Only 1 d preenrichment in TSB at 22°C and 3 d selective enrichment in BOS were required for recovery of two strains of serotype O:8. Strains of serotype O:3 required slightly longer enrichment times with a lower temperature preferable for preenrichment. Strains of serotype O:5, 27 do not

grow well in BOS, and could be recovered only with a long preenrichment period at 2°C.

Based on the results from these studies, the most comprehensive procedure for recovery of *Y. enterocolitica* from foods appears to be preenrichment in TSB at 22°C for 1 d and at 2 to 4°C for at least 4 but preferably 7 d. BOS broth (10 ml) inoculated with 0.1 ml of preenrichment should be transferred to CIN agar after both 3 and 5 d of incubation at 22 to 25°C. This system does not, however, guarantee recovery of all strains of serotype O:5,27. None of the other enrichment media evaluated was able to recover this serotype either.

A reliable enrichment system for recovery of all pathogenic forms of *Y. enterocolitica* from foods is not yet available. A good system should be able to recover all pathogenic forms with equal reliability and within a reasonable time period. Problems of antagonism by certain gram-negative bacteria in foods expressed at higher temperatures, and the physiological-biochemical variability among pathogenic strains of *Y. enterocolitica*, are difficult obstacles in developing such an isolation procedure. In the meantime, it appears that BOS broth is one of the better selective enrichment media available, especially for strains of the common North American serotype O:8, and should be considered in evaluations of other isolation procedures.

TABLE 14. Recovery of *Y. enterocolitica* from frozen inoculated^a beef stew^b.

Time (d) of:		Recovery at density (cells/g) of:								
Preenrichment (TSB, 2°C)	Selective enrichment (BOS, 22°C)	E663 (O:8)			E752 (O:3)			E771 (O:5,27)		
		15	1.5	0.15	16	1.6	0.16	10	1.0	0.1
4	3	—	+	—	+	—	—	—	—	—
	5	+++		—		+++	—	—	—	—
7	3			++			—	—	—	—
	5			++			+	—	—	—
10	3			+			—	—	—	—
	5			+++			—	—	—	—
14	3							—	—	—
	5							+	—	—

^aBeef stew inoculated with *Y. enterocolitica* at three densities and stored at -20°C for 10 d. Thawed at room temperature for 3 h and densities determined by plate count.

^bInoculated after thawing with Group II organisms at 1×10^6 /g.

TABLE 15. Recovery of heat-injured^a *Y. enterocolitica* from inoculated beef stew^b.

Time (d) of:		Recovery at density (cells/g) of:								
Preenrichment (TSB, 2°C)	Selective enrichment (BOS, 22°C)	E661 (O:8) (91%) ^c			E752 (O:3) (76%)			E758 (O:5,27) (90%)		
		2300	230	23	960	96	9.6	8800	880	88
4	3	+++	+++	++	+++	—	+	—	—	—
	5					+++	+++	—	—	—
7	3							—	—	—
	5							+++	+++	+

^aPBS suspension exposed to 56°C for 10 min then cooled rapidly.

^bInoculated with Group II organisms at 1.3×10^6 /g and one strain of *Y. enterocolitica*.

^cPercent injury calculated from difference in counts on TSY and CIN agars incubated at 32°C for 24 h. Density shown based on TSY counts.

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REFERENCES

1. Caprioli, T., A. J. Drapeau, and S. Kasatiya. 1978. *Yersinia enterocolitica*: serotypes and biotypes isolated from humans and the environment in Quebec, Canada. *J. Clin. Microbiol.* 8:7-11.
2. Devenish, J. A., and D. A. Schiemann. 1981. HeLa cell infection by *Yersinia enterocolitica*: evidence for lack of intracellular multiplication and development of a new procedure for quantitative expression of infectivity. *Infect. Immun.* 32:48-55.
3. Dudley, M. W., and E. B. Shotts, Jr. 1979. Medium for isolation of *Yersinia enterocolitica*. *J. Clin. Microbiol.* 10:180-183.
4. Gemski, P., J. R. Lazere, and T. Casey. 1980. Plasmid associated with pathogenicity and calcium dependency of *Yersinia enterocolitica*. *Infect. Immun.* 27:682-685.
5. Harmon, M. C., C. L. Yu, and B. Swaminathan. 1983. An evaluation of selective differential plating media for the isolation of *Yersinia enterocolitica* from experimentally inoculated fresh ground pork homogenate. *J. Food Sci.* 48:6-9.
6. Lair, W. J., and D. C. Cavanaugh. 1980. Correlation of autoagglutination and virulence in yersiniae. *J. Clin. Microbiol.* 11:430-432.
7. Lee, W. H., M. E. Harris, D. McClain, R. E. Smith, and R. W. Johnston. 1980. Two modified selenite media for the recovery of *Yersinia enterocolitica* from meats. *Appl. Environ. Microbiol.* 39:205-209.
8. Mehlman, I. J., C. C. G. Aulisio, and A. C. Sanders. 1978. Problems in the recovery and identification of *Yersinia* from food. *J. Assoc. Off. Anal. Chem.* 61:761-771.
9. Schiemann, D. A. 1979. Synthesis of a selective agar medium for *Yersinia enterocolitica*. *Can. J. Microbiol.* 25:1298-1304.
10. Schiemann, D. A. 1980. Isolation of toxigenic *Yersinia enterocolitica* from retail pork products. *J. Food Prot.* 43:360-365.
11. Schiemann, D. A. 1982. Development of a two-step enrichment procedure for recovery of *Yersinia enterocolitica* from food. *Appl. Environ. Microbiol.* 43:14-27.
12. Schiemann, D. A., and J. A. Devenish. 1980. Virulence of *Yersinia enterocolitica* determined by lethality in Mongolian gerbils and by the Serény test. *Infect. Immun.* 29:500-506.
13. Schiemann, D. A., and J. A. Devenish. 1982. Relationship of HeLa cell infectivity to biochemical, serological, and virulence characteristics of *Yersinia enterocolitica*. *Infect. Immun.* 35:497-506.
14. Schiemann, D. A., J. A. Devenish, and S. Toma. 1981. Characteristics of virulence in human isolates of *Yersinia enterocolitica*. *Infect. Immun.* 32:400-403.
15. van Pee, W., and J. Stragier. 1979. Evaluation of some cold enrichment and isolation media for the recovery of *Yersinia enterocolitica*. *Antonie van Leeuwenhoek J. Microbiol. Serol.* 45:465-477.
16. Vidon, D. J. M., and C. L. Delmas. 1981. Incidence of *Yersinia enterocolitica* in raw milk in Eastern France. *Appl. Environ. Microbiol.* 41:355-359.
17. Wauters, G. 1973. Improved methods for the isolation and the recognition of *Yersinia enterocolitica*. *Contrib. Microbiol. Immunol.* 2:68-70.
18. Weagant, S. D., and C. A. Kaysner. 1983. Modified enrichment broth for isolation of *Yersinia enterocolitica* from nonfood sources. *Appl. Environ. Microbiol.* 45:468-471.
19. hot-boning on selected beef muscles. *Proc. European Meat Workers Conf.*, Vol. II:40-44.
20. Marshall, N. 1960. Electronic cookery of top round of beef. *J. Home Econ.* 52:31-34.
21. McCrae, S. E., and P. C. Paul. 1974. Rate of heating as it affects the solubilization of beef muscle collagen. *J. Food Sci.* 38:18-22.
22. Montgomery, T. H., C. B. Ramsey, and R. W. Lee. 1977. Microwave and conventional precooking of hot and cold processed pork loins. *J. Food Sci.* 43:310-314.
23. Roberts, P. C. B., and R. A. Laurie. 1974. Effects on bovine 1. dorsi muscle of conventional and microwave heating. *J. Food Technol.* 9:345-349.
24. Schmidt, G. R., and K. V. Gilbert. 1970. The effect of muscle excision before the onset of rigor mortis on the palatability of beef. *Food Technol.* 5:331-335.
25. Stiffler, D. M., E. E. Ray, and R. M. Harp. 1978. Effects of electrical stimulation on beef muscle pH and tenderness. *Proc. West. Sec. ASAS.* 29:151-154.
26. Streit, R. H., H. W. Ockerman, and V. R. Cahill. 1977. Maintenance of beef tenderness by inhibition of rigor mortis. *J. Food Sci.* 42:583-587.
27. Voris, H. H., and F. O. Van Dyne. 1979. Low wattage microwave cooking of top round roasts: Energy consumption, thiamine content and palatability. *J. Food Sci.* 44:1447-1450.

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