JOURNAL OF CLINICAL MICROBIOLOGY, June 1987, p. 1068–1073
0095-1137/87/061068-06$02.00/0

Vol. 25, No. 6

New Selective Agar Medium for Isolation of Virulent *Yersinia enterocolitica*

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Received 24 November 1986/Accepted 24 February 1987

A selective agar medium for isolation of virulent *Yersinia enterocolitica* (VYE agar) was developed for the rapid and accurate isolation of virulent *Y. enterocolitica* from environmental samples highly contaminated with environmental *Yersinia* organisms, as well as for isolation from clinical specimens. VYE agar provided a quantitative recovery of 51 different strains of virulent *Y. enterocolitica* at 32°C after incubation for 24 h. The cefsulodin, irgasan, josamycin, and oleandomycin content of the medium resulted in a high selectivity, and the mannitol and esculin content provided some differentiation. The greatest advantage of VYE agar is that virulent *Y. enterocolitica*, which forms red colonies, is easily differentiated from most environmental *Yersinia* organisms and other gram-negative bacteria, which form dark colonies with a dark peripheral zone as a result of esculin hydrolysis. Use of VYE agar led to a high recovery of *Y. enterocolitica* biotype 3B serotype O:3 strains from experimentally inoculated meat samples, compared with use of CIN agar. Biotype 2 serotypes O:5,27 and O:9 and biotype 1 esculin-negative serotypes O:4,32, O:8, O:13a,13b, O:18, O:20, and O:21 (American types) were readily differentiated from other environmental organisms able to grow on VYE agar. Epidemiological studies on *Y. enterocolitica* should be greatly facilitated by the use of this selective agar medium.

Different agar plating media have been used to isolate *Yersinia* spp. including *Yersinia enterocolitica*, a recognized enteric pathogen (11), from various specimens (7, 8, 13, 16). Schiemann (12) developed a selective agar medium, cefsulodin-irgasan-novobiocin (CIN) agar, specifically for the isolation of *Y. enterocolitica*. Many investigators reported that CIN agar was the most effective of the selective differential plating media for the recovery of *Y. enterocolitica* from clinical specimens (7), animals (1, 17), food (7), and water (14).

The frequency of isolation of *Y. enterocolitica* serotype O:3 from patients showed a tendency to increase during our recent 7-year study in Japan (6). In particular, of *Y. enterocolitica* serotype O:3, the new biotype 3B bacteriophage type 2 strain has been recovered from clinical samples, samples from pigs, and pork, at an increasing rate since 1972 (2, 4–6). However, it was demonstrated that most of the strains of *Y. enterocolitica* biotype 3B serotype O:3 were inhibited when inoculated onto CIN medium (3). Fukushima and Gomyoda (3) found that CIN medium has not been adequately evaluated for the recovery of *Y. enterocolitica* biotype 3B serotype O:3 isolated from clinical specimens and from meat.

Head et al. (8) reported that a minor weakness of CIN medium is that colonies of *Citrobacter freundii*, *Serratia liquefaciens*, and *Enterobacter agglomerans*, if present on this medium, may not be reliably differentiated from colonies of *Y. enterocolitica*. Moreover, Fukushima and Gomyoda (3) found that although colonies of *Y. enterocolitica* serotype O:3 strains on CIN agar had a deep-red center with a sharp border surrounded by a translucent zone, the center color of *Y. enterocolitica* biotype 2 serotypes O:5,27 and O:9 and biotype 1 serotype O:8 resembled that of environmental *Yersinia* spp., especially *Yersinia intermedia*, on CIN agar. After 48 h of incubation, the color of the *Y. enterocolitica* serotype O:3 colonies faded, and *Y. enterocolitica* biotype 2 serotypes O:5,27 and O:9 and biotype 1 serotype O:8 were undistinguishable from environmental *Yersinia* spp., especially in samples contaminated with *Y. intermedia*.

For these reasons, I undertook the development of a selective agar medium for the isolation of known virulent *Y. enterocolitica* (i.e., biotype 4 serotype O:3; biotype 3B serotype O:3 [6]; biotype 2 serotypes O:5,27 and O:9; and biotype 1 serotypes O:4,32, O:8, O:13a,13b, O:18, O:20, and O:21 [9]).

MATERIALS AND METHODS

The composition and suppliers of the constituents of VYE medium are as follows (in grams per liter of distilled water): Bacto-Peptone, 17; proteose peptone, 3; sodium deoxycholate, 1 (all from Difco Laboratories); mannitol, 10; esculin, 1; ferric citrate, 0.5; sodium chloride, 1 (all from Wako Pure Chemical Industries Ltd.); neutral red bacteriological, 0.03; crystal violet bacteriological, 0.001 (both from Difco); agar bacteriological (Waco), 13.5; supplemented with irgasan DP300 as the 2,4,4′-trichloro-2′-hydroxy diphenyl ether (CIBA-GEIGY [Basel] Ltd.), 0.004; cefsulodin (Takeda Chemical Industries Ltd.), 0.004; oleandomycin (Sigma Chemical Co.), 0.01; and josamycin (Wako), 0.02. The medium constituents were suspended in 1 liter of distilled water, the pH of the medium was adjusted to 7.4 with 1 N NaOH, and the medium was autoclaved for 15 min at 15 lb pressure (121°C). After the medium had been cooled in a water bath to about 45°C, the supplements (cefsulodin, irgasan, josamycin, and oleandomycin) were added. The medium was immediately poured into sterile petri dishes, allowed to solidify, and kept at 4°C in a refrigerator until use. Trypticase soy agar (BBL Microbiology Systems), MacConkey agar (Nissui Pharmaceutical Co., Tokyo, Japan), and CIN agar (Difco) were also prepared.

The 76 *Yersinia* strains and 43 strains of other gram-negative bacteria used in these studies are listed in Table 1. All strains except for American type strains of *Y. enterocolitica* and *Y. pseudotuberculosis* were from my stock culture collection; *Y. pseudotuberculosis* was a gift from M. Tsubokura, Department of Veterinary Microbiology, Faculty of Agriculture, Tottori University, Tottori, Japan, and one biotype 1 serotype O:8 strain was provided by T.

TABLE 1. Colony appearance of *Yersinia* spp. and other gram-negative bacteria on VYE agar after 24 h at 32°C

Organism	No. tested	% Recovery[a] on[b]:		Avg diam of colony (mm) on[b]:	
		VYE	CIN	VYE	CIN
Y. enterocolitica[c]					
Biotype 4 serotype O:3	15	87	84	1.5	1.6
Biotype 3B serotype O:3	15	92	41	1.4	1.2 (10) and <0.4 (5)
Biotype 2 serotypes O:5,27 and O:9	12	80	72	1.4	1.6
Biotype 1 (esculin-negative American strains) serotypes O:4,32, O:8, O:13a,13b, O:18, O:20, and O:21	9	79	66	1.9	2.3
Biotype 1 (esculin positive) serotypes O:6, O:7,8; O:13,7, and O:14	5	92	78	1.9	2.6
Y. intermedia	5	70	79	2.0	2.6
Y. frederiksenii	5	87	82	2.1	2.8
Y. kristensenii	5	68	109	2.2	2.3
Y. pseudotuberculosis	5	0.3	83	0.9 (4) and NG[d] (1)	0.7
C. freundii	5	71	75	2.5	2.9
C. diversus	1	68	54	3.0	3.3
E. agglomerans	2	72	86	2.5	2.4
E. aerogenes	4	0	0	NG	NG
E. cloacae	2	0	0	NG	NG
Escherichia coli	4	0	0	NG	NG
Hafnia alvei	2	0	0	NG	NG
K. oxytoca	1	0	6×10^{-7}	NG	0.5
K. pneumoniae	2	0.3	0.2	1.4	1.4
Proteus mirabilis	2	0	0	NG	NG
Proteus vulgaris	1	0	0	NG	NG
S. liquefaciens	2	83	86	2.0	3.1
S. marcescens	1	100	84	1.6	2.4
P. aeruginosa	5	12 (1) and 0 (4)	14 (1) and 0 (4)	2.2 (1) and NG (4)	2.1 (1) and NG (4)
P. maltophilia	2	81	72	1.2	1.4
P. fluorescens	2	1	1	<0.4	<0.4
P. putida	1	167	167	1.0	1.2
P. stutzeri	1	0	0	NG	NG
Acinetobacter sp.	2	0	0	NG	NG
Flavobacterium sp.	1	0	0	NG	NG

[a] The recovery rate is expressed as a percentage of the mean count of colonies on Trypticase soy agar.
[b] Number of tested strains is given in parentheses.
[c] Serotypes O:5,27 (10 strains), O:8 (3 strains), O:9, O:13,7, and O:13a,13b (two strains each) and O:6, O:7,8, O:14, O:18, O:20, O:21, and O:4,32 (one strain each) were tested.
[d] NG, No growth on tested medium.

Maruyama, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan. Other American type strains of *Y. enterocolitica* were provided by G. Wauters, Unité de Microbiologie, Faculté de Medicine, Université Catholique de Louvain, Louvain, Belgium. Stock cultures were maintained at 6°C in nutrient agar stabs.

The ability of VYE and CIN agars to support the growth of *Yersinia* spp. and other gram-negative bacteria was determined. Test strains were subcultured in Trypticase soy broth at 25°C for 48 h. Inocula for this study consisted of suspensions of a pure culture containing approximately 10^8 CFU per ml. Colony counts of these strains on each test medium were made by a modification of the drop technique of Miles et al. (10); namely, 0.04 ml of each suspension diluted serially 10-fold to 10^{-7} with Bacto-Peptone broth (Difco) was placed on each test medium. The plates were examined for the colony characteristics, size, and color of the test organisms, and the number of colonies on each test medium was determined after 24 h of incubation at 32°C. The recovery ratio was expressed as a percentage of the mean count of colonies on Trypticase soy agar. I also attempted to determine the selectivity and differentiating ability of VYE medium for the isolation of virulent *Y. enterocolitica* strains from an inoculum containing environmental *Yersinia* organisms commonly found in meat samples. Mixtures of 10^7 CFU of *Y. enterocolitica* biotype 4 serotype O:3, biotype 3B serotype O:3, biotype 2 serotype O:5,27, and biotype 1 serotypes O:8 and O:13a,13b with 10^7 CFU of *Y. enterocolitica* biotype 1 and *Y. intermedia* were plated on VYE and CIN agars.

Comparison of selective media for recovery of *Yersinia* spp. from clinical specimens and pig feces. The ability of VYE and CIN agars to support the growth of *Yersinia* spp. was determined by using clinical specimens and pig feces. Feces from 18 patients and 5 pigs infected with *Y. enterocolitica* biotype 4 serotype O:3 (13 patients), biotype 3B serotype O:3 (4 patients and 5 pigs), and biotype 2 serotype O:5,27 (1 patient) were used. The fecal specimens from the 18 patients were stored in Cary-Blair transport medium (BBL Microbiology Systems) at −70°C for several months after sampling. Pig feces were collected from a pig farm on the day they were used. The recovery ratio was expressed as a percentage of the mean count of colonies on MacConkey agar.

I also wished to determine the effectiveness of VYE agar for isolating virulent *Y. enterocolitica* organisms from experimentally inoculated fecal materials and meat (beef, pork, and chicken). Each of 12 feces samples from healthy persons was suspended 1:10 with 0.067 M phosphate buffer solution (pH 7.6), and each of 9 ground-meat samples (3 each of beef, pork, and chicken) obtained from three local grocery stores was suspended 1:5 with phosphate buffer solution. Suspensions of *Y. enterocolitica* biotype 4 serotype O:3, biotype 3B

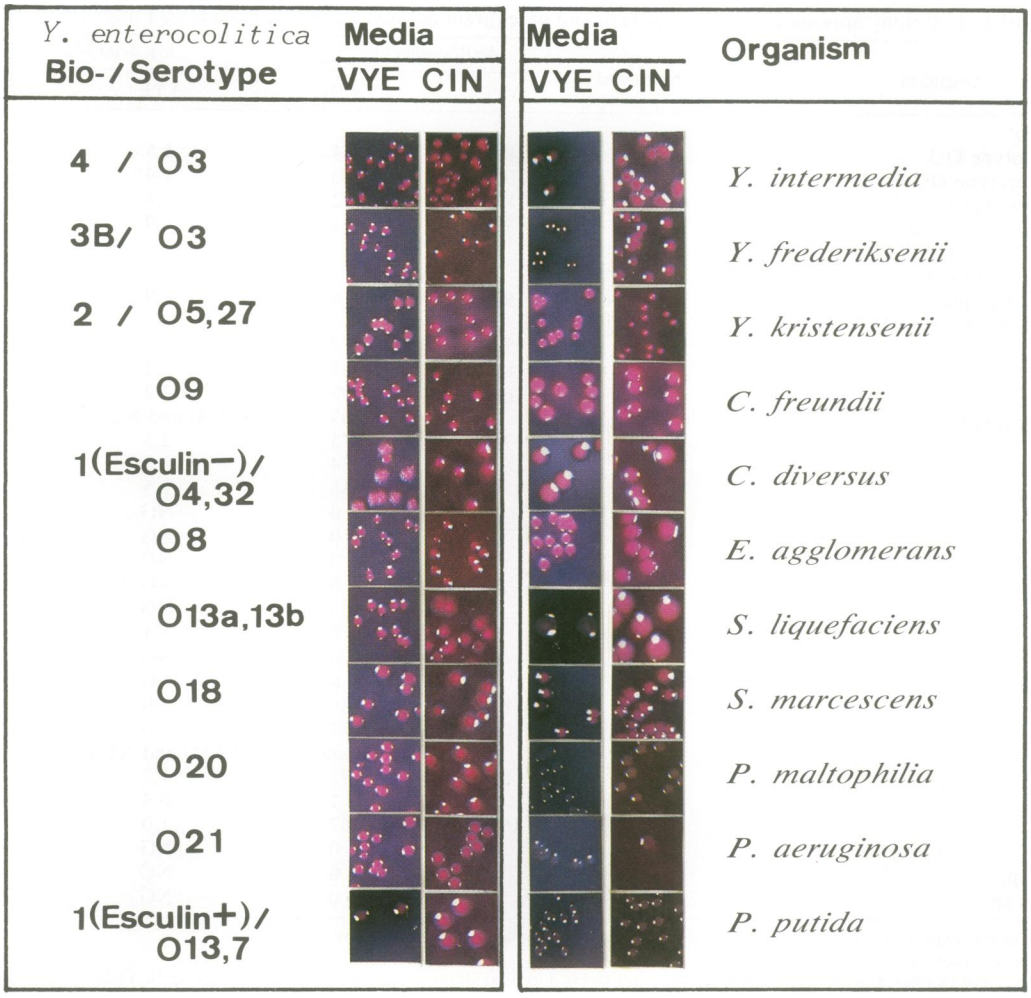

FIG. 1. Appearance of *Y. enterocolitica* and other gram-negative bacteria on VYE and CIN agars after 24 h of incubation at 32°C.

serotype O:3, biotype 2 serotype O:5,27, biotype 1 serotypes O:8 and O:13a,13b (0.1 ml in 0.85% saline) subcultured in Trypticase soy broth at 25°C for 48 h were inoculated into the above specimens. These inocula yielded 10^5 *Y. enterocolitica* organisms per g of feces and 10^4 *Y. enterocolitica* organisms per g of meat. Each of two media (VYE and CIN agars) was inoculated by being streaked with 1 loopful (1 μl) of each fecal suspension without a direct alkali treatment (2) and 1 loopful of each meat suspension, with or without a direct alkali treatment, and were then incubated at 32°C for 24 h.

RESULTS

Evaluation of selective media. VYE agar was evaluated with 76 different strains of *Yersinia* organisms (Table 1). The recovery ratios and colony size of these strains on VYE and CIN agars are shown in Table 1. The results show that recovery on VYE and CIN agars was comparable to that on Trypticase soy agar for all strains, except *Y. enterocolitica* biotype 3B serotype O:3 and *Y. pseudotuberculosis*. A recovery ratio of 92% for *Y. enterocolitica* biotype 3B serotype O:3 strains was observed with VYE agar, compared with 41% for *Y. enterocolitica* biotype 3B serotype O:3 strains on CIN agar. A recovery ratio of 0.3% was observed for *Y. pseudotuberculosis* strains on VYE agar, compared with 83% for *Y. pseudotuberculosis* strains on CIN agar.

The virulent *Y. enterocolitica* colonies were slightly larger on VYE agar than on CIN agar, especially those of *Y. enterocolitica* biotype 3B serotype O:3 strains. The environmental *Yersinia* colonies were smaller on VYE agar than on CIN agar. The appearance of *Yersinia* colonies on VYE agar was distinct from that of colonies on CIN agar (Fig. 1). Representative isolates of known virulent bioserogroups of *Y. enterocolitica* and *Y. kristensenii*, which showed red colonies, the result of mannitol fermentation and esculin nonhydrolysis, could be differentiated from environmental *Yersinia* organisms (*Y. enterocolitica*, *Y. intermedia*, and *Y. frederiksenii*), which showed dark-red colonies with a dark peripheral zone, the result of mannitol fermentation and esculin hydrolysis.

The selectivity of VYE agar is shown by the results presented in Table 1. All 43 strains of gram-negative bacteria used to evaluate the selectivity, representing 10 different genera, grew well on Trypticase soy agar, while only 5 genera (19 strains) showed growth on VYE agar after 24 h at 32°C, and 5 genera (20 strains) grew on CIN agar under the same conditions. Most of the other gram-negative bacteria that were able to grow on VYE agar were easily differentiated from virulent *Y. enterocolitica* colonies. *C. freundii*, *C. diversus*, and *E. agglomerans* colonies, which are mannitol

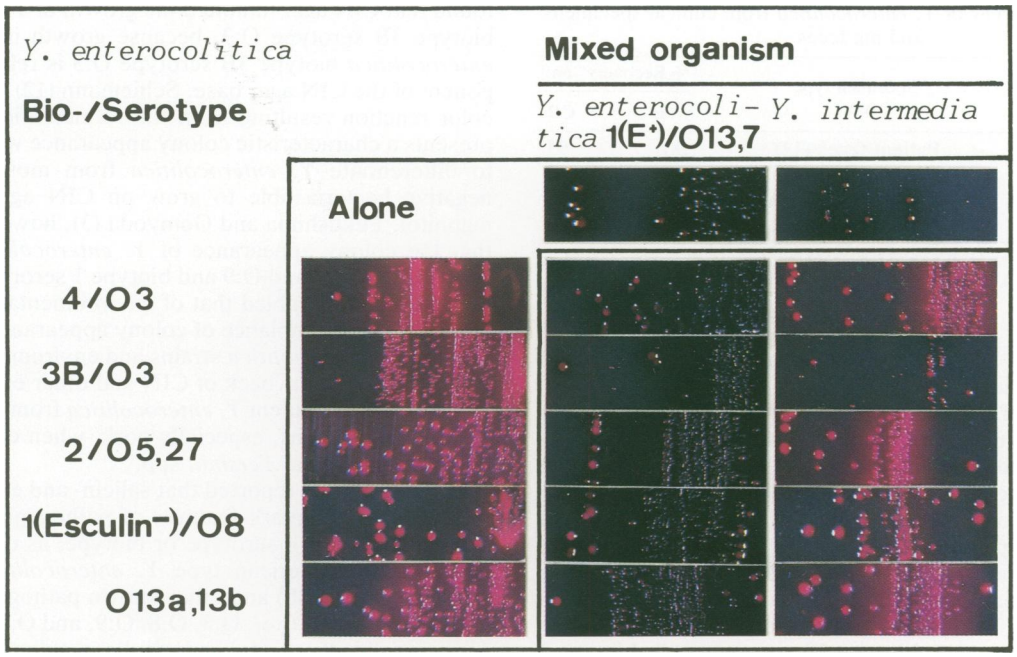

FIG. 2. Selectivity and differentiation of VYE agar by isolation of virulent *Y. enterocolitica* strains from mixtures with environmental *Yersinia* spp. E^+, Esculin positive.

positive and esculin negative, could be easily differentiated from virulent *Y. enterocolitica* colonies by their large size. Other esculin-positive gram-negative bacteria which grew on VYE agar had a dark zone around the colony. *Klebsiella pneumoniae*, *S. liquefaciens*, and *S. marcescens* colonies were dark pink or violet as a result of the mannitol fermentation, and *Pseudomonas* colonies were transparent, in the absence of the mannitol fermentation.

Figures 2 and 3 show the colony growth of *Y. enterocolitica* biotype 4 serotype O:3, biotype 3B serotype O:3, biotype 2 serotype O:5,27, and biotpye 1 serotypes O:8 and O:13a,13b on VYE and CIN agars when mixtures of those virulent *Y. enterocolitica* strains with *Y. enterocolitica* biotype 1 (avirulent serotype O:13,7) and *Y. intermedia* were streaked on VYE and CIN agars and then incubated at 32°C for 24 h. The virulent *Y. enterocolitica* strains that were able to form red colonies on VYE agar were easily differentiated from environmental *Y. enterocolitica* strains. Moreover, the growth of *Y. intermedia* in mixed cultures with virulent *Y. enterocolitica* strains on VYE agar was inhibited to a greater

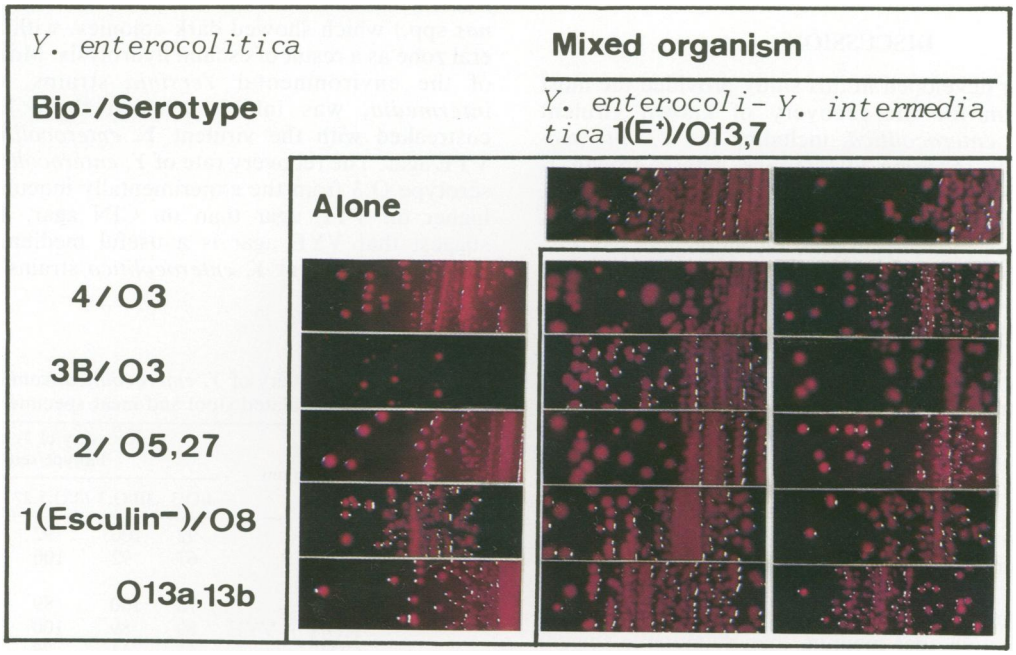

FIG. 3. Selectivity and differentiation of CIN agar by isolation of virulent *Y. enterocolitica* strains from mixtures with environmental *Yersinia* spp. E^+, Esculin positive.

TABLE 2. Recovery of *Y. enterocolitica* from clinical specimens and pig feces

Biotype/serotype of *Y. enterocolitica*	Specimen type (no.)	% Recovery[a] on:	
		VYE	CIN
4/O:3	Patient feces (13)	90	95
3B/O:3	Patient feces (4)	80	62
	Pig feces (5)	96	75
2/O:5,27	Patient feces (1)	38	40

[a] The recovery rate is expressed as a percentage of the mean count of colonies on MacConkey agar.

extent than was a pure culture on VYE agar or the mixed culture on CIN agar.

Comparison of selective media for recovery of virulent *Y. enterocolitica* from naturally infected and experimentally inoculated specimens. Depending on the selective medium (VYE or CIN agar), the recovery rate of *Y. enterocolitica* biotype 4 serotype O:3, biotype 3B serotype O:3, and biotype 2 serotype O:5,27 from infected human feces and *Y. enterocolitica* biotype 3B serotype O:3 from infected pig feces was comparable to that on MacConkey agar for all samples (Table 2). The recovery rate of *Y. enterocolitica* biotype 4 serotype O:3, biotype 3B serotype O:3, biotype 2 serotype O:5,27, and biotype 1 serotypes O:8 and O:13a,13b from experimentally inoculated human feces and meat (beef, pork, and chicken) samples differed little between cultures on VYE and CIN agar, except for the recovery rate of *Y. enterocolitica* biotype 3B serotype O:3 from meat (Table 3). However, the virulent *Y. enterocolitica* strains with red colonies on VYE agar were more easily differentiated from environmental *Y. enterocolitica* and other gram-negative bacteria than on CIN agar. Although the meat samples were contaminated with 10^5 to 10^7 CFU of environmental *Y. enterocolitica* organisms, *Y. enterocolitica* biotype 3B serotype O:3 strains on VYE agar were recovered more frequently than on CIN agar, with or without a direct alkali treatment.

DISCUSSION

The VYE agar developed in this study provided the most effective medium for the recovery of known virulent biogroups of *Y. enterocolitica*, including the *Y. enterocolitica* biotype 3B serotype O:3 strains whose growth is inhibited on CIN agar (3), after 24 h of incubation at 32°C. VYE agar with mannitol and esculin and the four antibiotics cefsulodin, irgasan, josamycin, and oleandomycin has several distinct advantages over other enteric agars for the isolation of virulent *Y. enterocolitica* strains, especially *Y. enterocolitica* biotype 2 serotypes O:5,27 and O:9 and American type serotypes. The greatest advantage of VYE agar is that the virulent *Y. enterocolitica* strains with red colonies on VYE agar are easily differentiated from most environmental *Yersinia* spp. and other gram-negative bacteria, which showed pink or dark-red colonies or transparent colonies with a peripheral dark zone as the result of esculin hydrolysis. The second advantage is the remarkable inhibition of the growth of environmental *Yersinia* spp., especially *Y. intermedia* cultured with virulent *Y. enterocolitica*, on VYE agar.

Many investigators (1, 7, 8, 14, 17) reported that CIN agar was a more effective agar medium than salmonella-shigella and MacConkey agars for isolation of *Y. enterocolitica* from various specimens. Fukushima and Gomyoda (3), however, found that CIN agar inhibited the growth of *Y. enterocolitica* biotype 3B serotype O:3, because growth inhibition of *Y. enterocolitica* biotype 3B serotype O:3 is related to a component of the CIN agar base. Schiemann (12) reported that a color reaction resulting from the fermentation of mannitol presents a characteristic colony appearance which can serve to differentiate *Y. enterocolitica* from most other gram-negative bacteria able to grow on CIN agar with added mannitol. Fukushima and Gomyoda (3), however, indicated that the colony appearance of *Y. enterocolitica* biotype 2 serotypes O:5,27 and O:9 and biotype 1 serotype O:8 strains on CIN agar resembled that of environmental *Yersinia* spp. strains. This resemblance of colony appearance between the virulent *Y. enterocolitica* strains and environmental *Yersinia* spp. is a major drawback of CIN and other enteric agars for the isolation of virulent *Y. enterocolitica* from environmental and food specimens, especially pork, when cocontaminated with environmental *Yersinia* spp.

Toma et al. (15) reported that salicin- and esculin-negative reactions are hallmark features of pathogenic *Y. enterocolitica*, regardless of serotype or biotype, as deduced from a study of the American type *Y. enterocolitica* (biotype 1 serotype O:13a,13b) and other known pathogenic *Y. enterocolitica* serotypes (i.e., O:3, O:8, O:9, and O:5,27). On VYE agar containing mannitol and esculin, a color reaction resulting from fermentation of mannitol and from nonhydrolysis of esculin presents a characteristic colony appearance which can be used to differentiate virulent *Y. enterocolitica* strains from most environmental *Yersinia* spp. and other gram-negative bacteria able to grow on VYE agar. Virulent *Y. enterocolitica*, *Y. kristensenii*, *C. freundii*, *C. diversus*, and *E. agglomerans*, which ferment mannitol but do not hydrolyze esculin, showed red colonies. The virulent *Y. enterocolitica* strains are easily differentiated from *C. freundii*, *C. diversus*, and *E. agglomerans* by colony size and from *Y. kristensenii* by results of the autoagglutination test and sucrose fermentation. VYE agar was highly selective after only 24 h of incubation at 32°C, especially against the bacteria (*Y. enterocolitica* biotype 1, *Y. intermedia*, *Y. frederiksenii*, *Serratia* spp., *K. pneumoniae*, and *Pseudomonas* spp.) which showed dark colonies, with a dark peripheral zone as a result of esculin hydrolysis. Moreover, growth of the environmental *Yersinia* strains, especially *Y. intermedia*, was inhibited when these bacteria were costreaked with the virulent *Y. enterocolitica* strains on VYE agar. The recovery rate of *Y. enterocolitica* biotype 3B serotype O:3 from the experimentally inoculated meat was higher on VYE agar than on CIN agar. These findings suggest that VYE agar is a useful medium not only for isolation of virulent *Y. enterocolitica* strains from environ-

TABLE 3. Recovery of *Y. enterocolitica* from experimentally inoculated stool and meat specimens

Sample (no.)	Medium	% Recovery of *Y. enterocolitica* biotype/serotypes:				
		4/O:3	3B/O:3	2/O:5,27	1/O:8	1/O:13a,13b
Stool (12)	VYE	92	100	92	100	100
	CIN	67	92	100	92	100
Meat (9)	VYE	78	100	89	78	78
	KOH + VYE	89	89	100	44	100
	CIN	67	11	78	78	89
	KOH + CIN	100	0	89	67	100

mental samples highly contaminated with environmental *Yersinia* spp. but also for application to clinical specimens.

ACKNOWLEDGMENTS

I thank M. Tsubokura, T. Maruyama, and G. Wauters for kind provision of the *Yersinia* strains and M. Ohara for reading the manuscript.

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