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Isolation of *Yersinia enterocolitica* from Raw Milk

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Four enrichment procedures were used for examining 131 raw milk samples for the presence of *Yersinia enterocolitica*. Forty-two isolations were obtained from 19 pooled- (31.1% positive) and 10 individual-producer samples (14.3% positive). Enrichment by Butterfields phosphate buffer incubated at 4°C for 14 days and then inoculation of modified Rappaport broth incubated at 23°C for 5 days produced the greatest number of isolations. The majority of isolates were biotype 1, and many were atypical from clinical isolates in being rhamnose positive (47.6%), citrate positive (16.7%), and lactose positive (26.2%). Thirteen isolates were serotypable, belonging to seven different O serotypes, with O:5 occurring most frequently.

Yersinia enterocolitica, a name applied by Frederiksen in 1964 (11) to an organism previously identified as *Bacterium enterocoliticum* (28), *Pasteurella pseudotuberculosis* type b (8), and *Pasteurella* X (13), has been recognized within a relatively short period as a major cause of acute gastroenteritis, mesenteric lymphadenitis, and terminal ileitis. The organism is usually isolated from feces and less frequently from appendix, mesenteric lymph nodes, abscesses, blood, urine, and from asymptomatic carriers. A review of the bacteriology of *Y. enterocolitica* has been prepared by Sonnenwirth (29), and a comprehensive review has recently been published by Bottone (2).

Swine are the only well-recognized animal reservoir of *Y. enterocolitica*, commonly harboring serotype O:3 which is also frequently associated with human illness (8, 10, 20, 25, 33, 35, 39, 41). *Y. enterocolitica* has been isolated from many other species of animals, but they are mostly types not frequently associated with human illness (4, 15, 21-23, 34). *Y. enterocolitica* isolations from water (G. P. Jansen and T. N. Saari, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, Q68, p. 272; 32, 36) and food (12, 15) are in most cases biochemically and serologically different from those isolated from humans. However, waterborne transmission of human yersiniosis has been described (17, 18). Only one documented foodborne outbreak has been reported (6), despite the fact that outbreaks have occurred in which a common vehicle was likely (40) and that the major route of transmission of this zoonotic organism has been suggested as food (30).

There have been a limited number of reports on the isolation of *Y. enterocolitica* from cows. Zen-Yoji (41) examined 200 cecal and 197 mesenteric lymph node specimens from cattle at an abattoir in Japan with completely negative results. A year later, however, Inoue and Kurose (15) obtained nine isolations from 115 cow intestinal content specimens. These conflicting results may derive from the type of specimens examined, since other workers have also observed lower isolation rates for mesenteric lymph nodes over cecal content specimens (5, 41). Wauters et al. (39) obtained only three isolates from mesenteric lymph node and fecal specimens from 103 cows (serotypes O:5 and O:6), and Esseveld and Goudzwaard (10) reported only two isolations from 386 cows (serotypes O:6 and O:7). Ahvonen et al. (1) reported completely negative results in examination of 669 fecal specimens from cows for *Y. enterocolitica*. Leistner et al. (20) found a much higher incidence in Germany with 11% of cow fecal specimens positive for *Y. enterocolitica* and 21% positive for *Y. enterocolitica*-like organisms.

There are also very few reports on the isolation of *Y. enterocolitica* from cow's milk. Pohl and Fameree (26) tested milk from 50 cows with mastitis and found all samples negative for *Y. enterocolitica*. The investigation of a suspected foodborne outbreak of yersiniosis in Montreal in 1976 included isolation of the organism from raw milk (14). In 1975 the Canadian National Reference Service for *Yersinia* received two isolates of *Y. enterocolitica* from a Toronto hospital that had been isolated from pasteurized milk (nontypable) and from egg nog made with milk (serotype O:5). The only reported foodborne outbreak of yersiniosis was traced to choc-

olate milk that contained the same serotype (O:8) as that isolated from the patients (6).

The isolation of *Y. enterocolitica* from both clinical specimens and environmental samples is greatly improved by the use of enrichment before plating on selective media (22, 24, 35). Incubation of fluid enrichment media at 4°C up to 21 days has been especially productive (9, 15, 33). Wauters (37; G. Wauters, Ph.D. thesis, Université Catholique de Louvain, Louvain, Belgium, 1970) first described the use of a modification of Rappaport broth, previously developed for *Salmonella* (27), for isolation of *Y. enterocolitica*. Both Wauters and later Lee (19) found that certain serotypes of *Y. enterocolitica* could not be recovered from this enrichment medium. The recently published *Compendium of Methods for the Microbiological Examination of Foods* (31) describes two enrichment methods for *Y. enterocolitica*: (i) phosphate buffer, 0.067 M, pH 7.6, incubated at 4°C for 14 or 21 days; and (ii) modified Rappaport broth inoculated with 0.1 ml of the sample homogenate, and from the phosphate buffer after cold enrichment, then incubated at 25°C for 48 h.

In this paper we describe the isolation of *Y. enterocolitica* from raw milk, using enrichment by modified Rappaport broth, Butterfields phosphate buffer at 4°C, and Rappaport broth after prior enrichment of the milk sample in either phosphate buffer at 4°C or cooked meat at room temperature. A description of biotypes and serotypes isolated from raw milk is presented.

MATERIALS AND METHODS

Milk samples. Raw milk samples originating from individual producers in Southern Ontario and delivered to the Toronto Public Health Laboratory for routine bacteriological examinations were examined for the presence of *Y. enterocolitica*. A total of 131 samples were tested, 61 consisting of pools prepared with 4 to 20 (usually 20) individual-producer samples, and 70 consisting of individual-producer samples without pooling. One milliliter of the pooled or individual sample was transferred to 10 ml of enrichment media for examination.

Enrichment methods. Four types of enrichment procedures were evaluated: (i) modified Rappaport broth (MRB) (31) incubated at room temperature (23 ± 1°C) for 5 days; (ii) Butterfields phosphate buffer (PB), pH 7.2 (31), incubated at 4°C for 14 days; (iii) MRB inoculated with 1 ml of PB (incubated at 4°C for 14 days) and incubated at 23°C for 5 days (PB+MRB); and (iv) MRB inoculated with 1 ml of cooked-meat broth (incubated at 23°C for 28 days) and incubated at 23°C for 5 days.

Isolation and identification. The selective plate media used for isolation were MacConkey agar with Tween 80 and modified deoxyribonuclease agar as described by Lee (19). Media were incubated at 23°C for 48 h. Colonies resembling *Y. enterocolitica* were fished to triple sugar iron slants, and, if a typical reaction was obtained (i.e., acid/acid without gas or H_2S), the organisms were further confirmed by biochemical tests (see Table 2). Serotyping was done by slide agglutination, using 34 absorbed and unabsorbed O antisera (38) prepared in rabbits in our laboratory.

RESULTS

Forty-two isolates of *Y. enterocolitica* from different milk samples or different types from the same sample were obtained from 131 raw milk samples. Nineteen pooled- (31.1%) and 10 individual-producer samples (14.3%) yielded *Y. enterocolitica*. Twenty-four samples were positive by enrichment with PB + MRB compared to six with MRB alone, eight with PB alone, and three with cooked meat plus MRB (Table 1). In only five cases did a sample fail to yield *Y. enterocolitica* by enrichment with PB+MRB but did so by another enrichment method.

Enrichment with cooked meat at 23°C for 28 days was also attempted, but because selective media were frequently overgrown with background flora this method was dropped. The use of MRB in conjunction with cooked meat eliminated some of the interfering background flora, but, as shown in Table 1, yielded *Y. enterocolitica* from only three samples.

Y. enterocolitica was recovered from 20 enrichment media by Tween 80-supplemented MacConkey agar alone, from an additional 15 enrichments by modified deoxyribonuclease agar alone, and from 7 enrichment media by both selective media. This demonstrates the value in utilizing more than one selective plate medium for recovery, as is the common practice for isolation of other enteric pathogens.

Biochemical reactions for all isolates are shown in Table 2. Those isolates that were serotypable are listed in Table 3 along with those biochemical reactions that were variable in these isolates. All typable isolates were positive for indole, sucrose, xylose, salicin, and esculin.

DISCUSSION

The largest number of isolations of *Y. enterocolitica* from raw milk samples was obtained by using modified Rappaport broth inoculated with the phosphate buffer-cold enrichment and then incubated at 23°C for 5 days. This combination of enrichments is similar to the use of a nonselective pre-enrichment followed by a selective enrichment for isolation of other enteric pathogens such as *Salmonella*. What remains unanswered is whether this two-step enrichment technique will recover those strains of *Y. enterocolitica* that cannot be recovered by Rappaport broth directly (19, 37; Wauters, Ph.D. thesis, 1970).

TABLE 1. *Isolation of Y. enterocolitica from raw milk by four enrichment procedures*

Positive sample no.	Enrichment procedure[a]			
	Rappaport (23°C, 5 days)	Buffer (4°C, 14 days)	Buffer (4°C, 14 days)/ Rappaport (23°C, 5 days)	Cooked meat (23°C, 28 days)/ Rappaport (23°C, 5 days)
1	+			
2			+	
3		+		
4	+		+	
5			+	
6				+
7			+	+
8			+	
9		+	+	
10			+	
11			+	
12			+	
13	+	+	+	
14		+	+	
15		+	+	
16			+	
17	+		+	
18				+
19			+	
20			+	
21		+	+	
22		+	+	
23			+	
24			+	
25	+		+	
26			+	
27	+			
28		+	+	
29			+	
Totals	6	8	24	3

[a] +, Positive recovery of *Y. enterocolitica* by this enrichment procedure.

Thirty-seven of the 42 isolates (88.1%) were lecithinase positive, thus resembling Wauters biotype 1 (Ph.D. thesis, 1970). Twenty of the 42 isolates (47.6%) were rhamnose positive, a biochemical characteristic of many environmental strains. However, Chester and Stotzky (7), Bottone and Robin (3), and Bottone (2) have described the isolation of rhamnose-positive strains from a variety of human infections. These isolates were citrate and lactose positive (delayed), whereas most of the rhamnose-positive milk isolates were citrate negative (70.0%) and lactose negative (80.0%). Three rhamnose-positive, lactose- and citrate-negative isolates from humans having the same serotype as those isolated from milk (O:6,30;21;7,13) have been re-

TABLE 2. *Biochemical reactions for Y. enterocolitica isolates obtained from raw milk*

Biochemical test[a]	No. tested	No. positive	% Positive
Beta-galactosidase			
36°C	41	40	98
22°C	42	42	100
Motility			
36°C	42	1	2
22°C	42	42	100
Indole, 30°	42	40	95
Nitrate reductase	42	39	93
Voges-Proskauer, 22°C	42	38	91
Lecithinase, 22°C	42	37	88
Urease	42	41	98
Citrate, 22°C	42	7	17
Lysine decarboxylase	42	0	0
Ornithine decarboxylase, 22°C	42	42	100
Arginine dihydrolase	18	0	0
Rhamnose	42	20	48
Lactose	42	11	26
10% Lactose purple	42	27	64
Sucrose, 22°C	42	42	100
Xylose, 22°C	42	42	100
Salicin	42	42	100
Dextrose	42	42	100
Arabinose	30	30	100
Malonate	41	0	0
Dulcitol	41	0	0
Maltose	29	29	100
Esculin hydrolysis	42	42	100
Mannitol	42	42	0

[a] All biochemical tests completed at 36°C unless otherwise noted.

TABLE 3. *Y. enterocolitica serotypes isolated from raw milk*

Sample no.	Serotype O:	Variable biochemical reactions		
		Rhamnose	Lactose	Nitrate reductase
4	14	+	−	+
7	7,8	+	−	+
9	5	−	−	+
10	5	−	−	+
13	6,30	−	−	+
16	4,33	−	−	+
21	6,30	−	−	+
22	15	−	−	+
23	15	−	−	+
25/1	5	−	+	−
25/2	5	−	+	+
27	5	−	+	−
28	21	+	−	+

ceived by the Canadian National Reference Service for *Yersinia*.

A significant proportion of the milk isolates were rhamnose negative (52.4%). Most of these isolates were also citrate negative (95.5%), presenting a biochemical pattern more agreeable with clinical isolates. Ten of the 13 isolates that were serotypable were rhamnose negative. However, one was atypically lactose positive, and two were lactose positive and nitrate reductase negative.

Thirteen of the 42 isolates (31%) were serotypable and represented 7 different serotypes, with O:5 as the most common (Table 2). Serotype O:6,30, which was isolated twice, is the third most common human serotype in Canada (34). Christenson (E. H. Christenson and G. P. Jansen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, C43 p. 42) has reported four isolations of serotype O:6,30 from clinical cases of infection in Wisconsin. All of the serotypes isolated from raw milk have been reported from humans. Subsequent to compilation of these data, we obtained two isolations of serotype O:7,13 from raw milk, a type also associated with human illness.

Whereas raw milk may contain many so-called "environmental strains" of *Y. enterocolitica*, it also yields biotypes and serotypes that have been associated with human illness. The association is obviously not as strong as that for swine, where serotype O:3 is predominant in both animal reservoir and human illness. It is always possible, however, that any description of the presence and distribution of *Y. enterocolitica* types is influenced by the methodology, especially enrichment methods, and the skill of the investigating laboratory, and that the true picture may be quite different.

Our survey revealed a surprisingly high incidence of *Y. enterocolitica* in raw milk. The origin of the organism could be either the animal or the environment. Even a slight contamination of milk with *Yersinia* could ultimately result in high cell densities since milk is a good growth medium and *Y. enterocolitica* is able to multiply at refrigeration temperatures. When we related our isolations to the milk gel index, an indirect measure of leukocytes similar to the Wisconsin mastitis test and which is completed routinely on all raw milk samples received by our laboratory, no difference was observed between mean milk gel index scores for positive and negative samples. However, when isolations were related to the plate loop count, the mean count for samples positive for *Y. enterocolitica* was 54,000/ml compared to 20,000/ml for negative samples. Though this difference is not large, it does suggest, nevertheless, that insanitary conditions and poor temperature control, which can allow for contamination and multiplication of other bacteria, also contribute to the presence of *Y. enterocolitica*. It further suggests that the organism does not originate from an infected animal but, more likely, from the environment, which may include another animal reservoir.

There is no reason to doubt that adequate pasteurization of milk will destroy *Y. enterocolitica*. There is, however, the possibility of inadequate pasteurization or recontamination with raw milk containing *Y. enterocolitica* that could subsequently multiply under refrigerated storage. The consumption of raw milk and the manufacture of cheese or other dairy products from raw milk are practices that further allow for the transmission of human yersiniosis by this vehicle.

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