Short communication

Comparison of four methods for isolation of *Yersinia enterocolitica* from raw and pasteurized milk from northern Iran

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Abstract

Four methods for isolation of *Yersinia enterocolitica* from raw and pasteurized milk from northern Iran were compared. Three hundred and ten raw milk samples were collected from tanks on their arrival at various central dairies, and 40 pasteurized milk samples were collected from tanks on their arrival at a manufacturing plant. Each sample was examined for the presence of *Y. enterocolitica* by (1) direct culture; (2) enrichment in double-strength buffered peptone water at 4 °C for 1 month; (3) enrichment in modified Rappaport medium at room temperature for 72 h after a preenrichment in double-strength peptone water at 4 °C for 1 month; and (4) enrichment in a medium containing sucrose, tris (hydroxymethyl) aminomethane, sodium azide, and ampicillin at 28 °C for 48 h after a preenrichment in double-strength peptone water at 4 °C for 1 month. All samples and enrichments were spread on MacConkey agar plus calcium chloride and Tween 80, *Yersinia* selective agar, and Hektoen medium plus ampicillin. Five samples (1.6%) of raw milk but no pasteurized milk samples were positive for *Y. enterocolitica*. No *Y. enterocolitica* were recovered by methods 1 or 2. *Y. enterocolitica* were recovered from 2 samples by method 3 followed by culture on *Yersinia* selective agar, and from 5 samples by method 4 followed by culture on Hektoen medium plus ampicillin. The isolates were biotype 1A or 1B, serotype O:7-13 or O:9 and phage type X\(_{10}\) or X\(_{p}\). All isolates were resistant to ampicillin and amoxicillin, and sensitive to tetracycline, streptomycin, chloramphenicol, and trimethoprim–sulfamethoxazole.

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1. Introduction

Consumption of raw or inadequately pasteurized milk has been associated with outbreaks of enteric infections (Tacket et al., 1984; Barrett, 1986; Ackers et al., 2000). *Yersinia enterocolitica* can grow to large numbers at refrigeration temperatures, so milk contaminated with that organism could become a significant health risk for consumers (Hughes, 1979; Greenwood and Hooper, 1990; Greenwood et al., 1990; Feng and Weagant, 1994; Jayarao and Henning, 2001). *Y. enterocolitica* is well recognized as a cause of enteritis in children, (Kohl et al., 1977; Marks et al.,...
1980; Hoogkamp-Korstanje and Stolk-Engelaar, 1995), but yersiniosis may be relatively infrequent in Iran (Haghighi and Vahdat, 1977; Bashiribod, 1989). This microorganism has been isolated in different countries from water and various foods including meats, shellfish, vegetables, and milk (Langeland, 1983; Desmasures et al., 1997; Uraz and Yucel, 1999; Szabo et al., 2000; Langiano et al., 2002).

Environmental isolates of *Y. enterocolitica* have been recovered from nature and numerous animal sources (Gugnani, 1999). These environmental strains are devoid of common virulence-linked properties and are generally regarded as nonpathogenic. However, *Y. enterocolitica* lacking the virulence plasmids and strains not usually associated with infection have been implicated in foodborne transmission of yersiniosis contracted from foods (Lian et al., 1987; Grant et al., 1998; Gugnani, 1999).

Most laboratories do not routinely screen for *Yersinia* species, which may explain its infrequent identification. The organism grows poorly on the *Salmonella–Shigella* agar commonly used in many clinical laboratories for the isolation of stool pathogens, and also grows poorly at the usual incubation temperature of 37 °C (Tsubokura, 1989). *Yersinia* does grow well on MacConkey agar, but at routine incubation temperatures forms only very small colonies which are easily overlooked or overgrown by competing flora (Tsubokura, 1989). Use of a selective growth medium is recommended to overcome these problems. The most widely used is cefsulodin–irgasan–novobiocin (CIN) agar, which inhibits the growth of competing flora and gives a characteristic colony morphology (Head et al., 1982).

Because of the uncertainty about appropriate procedures to use with milk, four methods for isolation of *Yersinia enterocolitica* from raw and pasteurized milk were compared.

### 2. Materials and methods

Three hundred and ten raw milk samples were collected directly from tanks on their arrival at the various central dairies in Behshahr city, and 40 pasteurized milk samples were collected from tanks on their arrival at the only manufacturing plant in the region. The pasteurized milk had been subjected to a high temperature short time (HTST) treatment of 72 °C for 15–20 s. The samples were analyzed for the presence of *Y. enterocolitica*. Four methods were used: (1) direct culture; (2) enrichment in double-strength buffered peptone water (BPW; peptone 20 g, sodium chloride 10 g, disodium hydrogen phosphate 7 g, potassium dihydrogen phosphate 3 g, distilled water to 1 l, pH 7.2 ± 0.2) at 4 °C for 1 month; (3) preenrichment in BWP at 4 °C for 1 month, then transfer of 1 ml to modified Rappaport (MR) medium without carbenicillin, followed by incubation at room temperature for 72 h (BPW-MR); (4) preenrichment in BWP at 4 °C for 1 month, then transfer of 1 ml to sucrose tris sodium azide ampicillin (STSA) medium [peptone 1 g, tris (hydroxymethyl) aminomethane 3 g, brilliant green 0.0125 g, sodium azide 0.192 g, and ampicillin 0.005 g per liter pH 8.3] followed by incubation at 28 °C for 48 h (BPW-STSA; Vidon and Delmas, 1981). *Y. enterocolitica* were isolated on three culture media: MacConkey agar supplemented with calcium chloride at 0.2 g/l and Tween 80 at 10 ml/l, *Yersinia* selective agar (Wauters et al., 1987), and Hektoen agar with of ampicillin at 5 mg/l (Vidon and Delmas, 1981). All plates were incubated at 28 °C for 48 h. All isolates were examined for Gram staining, oxidase and catalase, motility at 22 and 37 °C, Kligler’s iron agar reaction, urease, indole production, citrate utilization, O-nitrophenyl-β-D-galactoside hydrolysis, acid reaction with methyl red, acetoin production at 22 and 37 °C, production of each of arginine dihydrolase, ornithine decarboxylase and lysine decarboxylase, nitrate reduction, and the ability to produce acid from each of glucose, ribose, man­nose, manitol, D-trehalose, L-rhamnose, sucrose, D-melibiose, and D-raffinose. The identity of the isolates was further confirmed by the use of the API 20 E identification system (bio Merieux, Marcy L’ Etoile, France).

Antimicrobial susceptibility tests were performed at 28 °C on Mueller–Hinton agar by the disk diffusion method (Jorgensen et al., 1999). All isolates were examined for their susceptibilities to ampicillin (10 μg), amoxicillin (20 μg), tetracycline (30 μg), streptomycin (10 μg), chloramphenicol (30 μg), trimethoprim–sulfamethoxazole (1.25/23.75 μg). Antibiotic disks were obtained from bio-Merieux.

The significances of differences between the different recovery methods were determined by a two-
tailed chi-square distribution analysis with Yates’ correction at the 95% level of significance, using SPSS/PC 11.0 software (SPSS Chicago, IL).

3. Results

Five raw milk samples (1.6%) were positive for Y. enterocolitica. No Y. enterocolitica was isolated from pasteurized milk. Seven strains of Y. enterocolitica were recovered from the five samples, 5 by enrichment in BWP-STSA followed by culture on Hektoen medium with 5 mg of ampicillin, and 2 by enrichment in BWP-MR followed by culture on Yersinia selective agar. No Y. enterocolitica was recovered by the other methods. However, the differences among the isolation methods were not statistically significant.

All Y. enterocolitica isolates were biotype 1 (Table 1). The most frequent serotype was O:9. Phage types were X_2 and X_9. All isolates were resistant to ampicillin, and sensitive to tetracycline, streptomycin, chloramphenicol and trimethoprim–sulfamethoxazole.

4. Discussion

This is the first report of the isolation of Y. enterocolitica from milk in Iran. The frequency of Y. enterocolitica isolation was lower than that reported for other studies of raw milk (Vidon and Delmas, 1981; Hamama et al., 1992; Desmasures et al., 1997). Y. enterocolitica biotype 1, Yersinia intermedia and Yersinia kristensenii are usually environmental organisms that are not associated with human gastrointestinal infections. However, recent data suggest that some of these organisms may cause disease by virtue of virulence factors distinct from those of other Y. enterocolitica (Sulakvelidze, 2000). Two of the Y. enterocolitica isolates belonged to biotype-1B and were pyrazinamidase positive and proline peptidase, salicin and esculin negative, which indicates possible pathogenicity. (Wauters et al., 1987; Grant et al., 1998). The majority of the seven strains belonged to the 9 and 7, 13 serogroup, which is different from the serogroup of 3, 8, 5, 10K, and 6, which

<table>
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<th>No.</th>
<th>Characteristics</th>
<th>Phage type</th>
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<tbody>
<tr>
<td>1</td>
<td>1A Nonagglutinable</td>
<td>X_2</td>
</tr>
<tr>
<td>2</td>
<td>1B O:9</td>
<td>X_3</td>
</tr>
<tr>
<td>3</td>
<td>1B O:9</td>
<td>X_2</td>
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<td>4</td>
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<td>X_1</td>
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<tr>
<td>5</td>
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<td>X_1</td>
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<tr>
<td>6</td>
<td>1A Nonagglutinable</td>
<td>X_1</td>
</tr>
<tr>
<td>7a</td>
<td>1A O:9</td>
<td>X_2</td>
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* Lipase negative; pyrazinamidase negative.
30 reported in other studies (Vidon and Delmas, 1981; Greenwood and Hooper, 1990; Ostroff et al., 1994; Verhaegen et al., 1998; Ackers et al., 2000). The phage type of the isolates and their antimicrobial susceptibilities were similar to those found by others (Vidon and Delmas, 1981; Tzzelepi et al., 1999).

The results of this investigation indicate that enrichment in BWP-STSA and culture on supplemental Hektoen agar is an appropriate procedure for recovery of Y. enterocolitica from milk. Use of that sensitive, selective, and differential procedure for the isolation of Y. enterocolitica would improve recovery rates, enhance the accuracy of results, and lead to a better understanding of the epidemiology of the organism.

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