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Prevalence and Characteristics of Yersinia Enterocolitica and Yersinia Pseudotuberculosis from Raw Milk Supplied in Tehran

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ABSTRACT

Background: Foodborne diseases are caused by eating contaminated food. *Yersinia* species are among the bacteria involved in food contamination, such as meat, poultry, vegetables, and milk. This study aims to investigate the prevalence and characteristics of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* from raw milk in Tehran. **Methods:** In this study, 360 samples of raw milk were collected from farms around Tehran and examined according to the FDA method. Then, 25 ml of milk samples were added to 225 ml of Peptone Sorbitol Bile Broth and kept for 10 days at 4 °C for enrichment. After 10 days, 0.5 ml of the sample was added to 0.5 ml of 0.72% KOH solution and 0.54% NaCl. After 30 sec, it was cultured in the selected Cefsulodin-Irgasan-Novobiocin agar (CINagar) medium to remove the normal flora. The plates were stored for 48 h at 28 °C. The suspected Bull's eye colonies were purified and phenotyping tests were carried out on the selected colony. The 20 E API kits were used for confirming identification. **Results:** From 360 raw milk samples, 4 *Yersinia* isolates (1.1%) including one *Y. pseudotuberculosis* (0.27%) and three *Y. enterocolitica* (0.83%) were isolated. In addition to *Yersinia*, other bacteria such as *Klebsiella*, *Serratia*, *Citrobacter*, and Providencia were isolated from milk samples. **Conclusion:** The findings showed that clean tap water and healthy cattle in livestock can be effective in preventing Yersinia contamination. Lack of personal and environmental hygiene could cause food contamination by *Yersinia* and other intestinal bacteria leading to gastrointestinal infections.

Keywords: *Yersinia enterocolitica*; *Yersinia pseudotuberculosis*; *Milk; Diarrhea*

Introduction

Milk is mainly made up of water in which a wide range of nutrients including vitamins, proteins, fats, and carbohydrates are suspended. These rich nutritional contents and production and processing methods in commercial milk production make it susceptible to contamination by pathogenic microbes that can cause disease in humans. Therefore, milk is known as an effective means of

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transmitting disease-causing agents to humans (Muehlhoff *et al.*, 2013). Bacterial agents may contaminate milk at various stages of preparation, processing, and distribution. Bacterial contamination may be caused by cow's udder, storage, milk collection materials, various additives added to dairy products, and livestock workers. *Staphylococcus aureus*, *Salmonella* spp., *Listeria monocytogenes, Escherichia coli O157: H7*, *Campylobacter*, and *Yersinia* spp. are the main microbiological hazards associated with raw milk consumption (McAuley *et al.*, 2014, Owusu-Kwarteng *et al.*, 2020). Preventing the growth of contaminated bacteria in milk includes limiting the level of contamination, cooling immediately after milking, and maintaining a refrigerated storage temperature. Bacterial constraints mainly include cleaning, disinfecting, and drying the cow's udder and udder before milking, and using healthy milking equipment. Removing solid milk from milk containers is very important in controlling psychrophilic bacteria (van den Brom *et al.*, 2020). Milk pasteurization has been used as the most effective method to reduce the risk of infection and the spread of disease. Although pasteurized milk is expected to last 14 to 20 days, the shelf life of pasteurized milk stored at room temperature depends on the efficiency of the pasteurization process (Garedew *et al.*, 2012, Hahn, 1996). The microbiological status of raw milk is affected by several factors including animal health status, farm management practices, environmental health, and inappropriate temperature control (Martin *et al.*, 2018).

Yersinia is a foodborne pathogen that has at least 16 species. Three species of *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* are the most important pathogenic species of Yersinia. These bacteria are among the gram-negative, facultative anaerobic bacteria, and psychrophilic bacteria in the Enterobacteriaceae family. These bacteria are widely isolated from soil, water, and food, including milk, meat, and cause common diseases between humans and animals (Fredriksson-Ahomaa and Korkeala, 2003, Jamali *et al.*, 2015, Rahman *et al.*, 2011, Soltan Dallal and Salmanian, 1996).

Y. enterocolitica is the cause of several human diseases including diarrhea, appendicitis, mesenteric lymphadenitis, arthritis, and sepsis. *Y. pseudotuberculosis* causes tuberculosis-like symptoms in animals and causes mesenteric lymphadenitis and diarrhea in humans. Also, *Yersinia s*pecies are cold-resistant agents that can grow at 4 °C and can be used as risk factors for cold chain food transfer (Huovinen *et al.*, 2010, Rivas *et al.*, 2021, Soltan Dallal and Moezardalan, 2004).

Pathogenic serotypes and biotypes of *Y. enterocolitica* serogroups O:3 (biogroup 4), O:5,27 (biogroups 2 and 3), O:8 (biogroup 1B), and O:9 (biogroup 2) are globally widespread (Soltan Dallal *et al.*, 2017a). In Japan, O:3/3 has increased as the most important cause of Yersiniosis (Fukushima *et al.*, 2011). In Europe, more than 90% of Yersiniosis agents are O: 3 and O: 9 serotypes. This bacterium is normally excreted from animals and is present in smaller amounts in food (Bottone, 2015, Syczyło *et al.*, 2018). In East Asia, varieties 1b, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b were isolated from patients with systemic infection and fever, and almost all isolated strains were able to secrete YPMa super antigenic toxin (Fukushima *et al.*, 2011, Kameyama *et al.*, 2016). In Europe, strains 1a, 1b, and 3 were isolated from patients with gastroenteritis (Espenhain *et al.*, 2019, Rosner *et al.*, 2013).

One of the factors that transmit *Yersinia* to humans is milk. Milk in various forms including raw milk, yogurt, cheese, cream, buttermilk, ice cream, cocoa milk, skim milk powder, and low-lactose milk are in the food basket of households. The use of this valuable and high-consumption product requires careful and sensitive monitoring and observation, since this important and valuable food product can easily transmit dangerous diseases to humans. Therefore, sensitivity and accuracy require high attention (Górska-Warsewicz *et al.*, 2019, Rahman *et al.*, 2011).

In many countries, milk and its products make up an important part of the human diet. By subsidizing dairy products, the consumption of

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such products in the household nutrition basket will increase. The history of milk subsidies and school milk programs in industrialized countries is about a century old. In all European and American countries, subsidies are paid to milk producers. In Western European and North American countries, the per capita consumption of milk and its products, which are converted into liquid milk, varies between 300 and 490 kg per year, and this source provides more than 85% of the calcium needed by consumers (Raghiante *et al.*, 2018, Soltan-Dallal *et al.*, 2004).

According to statistics, more than 60% of the country milk passes through health channels and is supplied to the consumer market. However, a large part of the produced milk is consumed without passing through these channels, which greatly increases the risk of foodborne illnesses, especially biological factors. Some areas are used for processing under traditional conditions or in a non-pasteurized or even non-pasteurized form. Therefore, the possibility of contamination with pathogens is common, which prevents the spread of foodborne illness in the population (Al-Jawaldeh *et al.*, 2020, Msalya, 2017, Rahimi *et al.*, 2014). This study aimed to investigate the prevalence and characteristics of *Y. enterocolitica and Y. pseudotuberculosis* from raw milk supplied in Tehran.

Materials and Methods

Sampling: In this descriptive cross-sectional study, sampling was performed in cold seasons of the year, since *Yersinia* grows better in cold weather. Sampling was performed from autumn to winter 2018. In total, 360 samples of raw milk from farms around Tehran were prepared in closed containers under sterile conditions. They were then transferred to a microbiology laboratory in the Department of Food Microbiology at Tehran University of Medical Sciences, according to the Iranian National Standard No. 164 (milk and its products test methods, ISIRI, 2003), and the Iranian National Standard No. 3549 (milk protection conditions after milking, ISIRI, 1995). Sample transportation took place in cold chain conditions. Samples without numbering label in the open container that were not taken by cold chain method and more than twelve hours had been passed from milking were excluded. The samples were tested immediately upon arrival.

Isolation of Yersinia: The samples were tested for the presence or absence of the genus *Yersinia a*ccording to the FDA method (Ahmed *et al.*, 2019).

Enrichment: In this study, 25 ml of milk sample was mixed with 225 ml of Peptone Sorbitol Broth (PSB) in sterile glass containers. After homogenization, they were kept in the refrigerator for 10 days. After 10 days, the samples were taken out of the refrigerator and an alkaline treatment method was performed to remove milk flora (Ahmed *et al.*, 2019). In this method, KOH-sensitive bacteria were killed. The streaking culture was performed on Cefsulodin-Irgasan-Novobiocin agar (CIN agar, Merck, Germany). The samples were stored at 25 °C for 24 to 48 h (Ahmed *et al.*, 2019). Differentiation of Yersinia growth on CIN agar medium was done based on mannitol fermentation, staining, and production of colonies with a red center and a white border resembling a cow's eye. In this medium, the presence of sodium deoxycholate and violet crystal prevents the growth of gram-positive and many gram-negative bacteria (**Figure 1**). After 48 h, suspicious colonies (colonies with red center and white border) were isolated and biochemical tests of O-Nitrophenyl-β-D-galactopyranoside (ONPG), Kligler's Iron Agar (KIA, Ornithine Decarboxylase Test (ODC), and urease test (Merck, Germany) were performed. The motility test on the Sulphide Indole Motility(SIM) environment was checked. All species of Yersinia except Yersinia pestis are immobile at 37 °C and 25 °C. The API 20E kit manufactured by the French company BioMérieux was used for final identification (Soltan Dalal *et al.*, 2009).

Also, to determine the biotypes of *Yersinia enterocolitica*, xylose fermentation test, trehalose fermentation test, lipase test, Bile Esculin test, Voges–Proskauer (VP) test, and Indol fermentation

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test were used. To determine the serotype, MAST antiserum (England) was used according to the manufacturer's instructions (Soltan Dallal *et al.*, 2017a).

Isolation of other species of Enterobacteriaceae: To isolate other species of Enterobacteriaceae, all gram-negative bacteria were isolated during the study period on general media identified to the species level based on gram stain and biochemical tests (Ewing, 1968, Soltan Dallal *et al.*, 2017b).

Results

In terms of livestock age, 105 (29.16%) samples aged 2-3 years, 140 (28.88%) 3-4 years, and 115 (31.94%) samples aged over 4 years, respectively. Also, in terms of the type of livestock, 247 (68.61%) samples were small and 113 (31.38%) samples were domestic.

Regarding milking type, 155 (43.05%) samples were milked by milking equipment and 205 (56.94%) samples were manually milked. Regarding the type of drinking water, 101 (28.05%) samples consumed tap water, 94 (26.11%) samples running water, and 165 (45.83%) samples consumed well water. The sampled cows included 300 (83.33%) crossbred, 41(11.38%) native cows, and 19 (5.27%) purebred cows. In this study, of 360 samples of raw milk tested, one (0.27%) sample was infected with *Y. pseudotuberculosis* and three (0.83%) samples were infected with *Y. enterocolitica*. The identification was confirmed by the API20E kit. The influential parameters in *Yersinia* identification are given in **Table 1**.

In addition, out of three samples of isolated *Y. enterocolitica*, 1 sample biotype 3, 1 sample biotype 1A, and 1 sample biotype 1B were identified (**Table 2**).

Of 360 milk samples apart from *Yersinia* (61.7%), other bacteria including 32 (8.9%), Klebsiella *pneumonia*, 50 (13.9%) *Klebsiella oxytoca*, 60 (16.7%) *Citrobacter freundii* 52 (14.4%) *Serratia liquefaciens*, 3 (0.83%) *Serratia nematodiphilia*, and 25 (6.9%) *Providencia rettgeri* were isolated from CIN agar medium (**Figure 2**).

Table 1. Impact of environmental conditions on *Yersinia* isolation.

Environmental condition	*Yersinia entercolitica*	*Yersinia pseudotuberculosis*
Water type		
Tap water	0	0
Running water	1	1
Well water	2	0
Livestock type		
Small	1	1
Domestic	2	0
Type of milk preparation		
By machine	1	1
Manual	2	0
Type of cow		
Hybrid	1	0
Domestic	2	1
Purebred	0	0
Age (Year)		
2-3	1	0
3-4	1	1
> 4	1	0

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Table 2. Biotyping tests of Y. entercolitica.

Biochemical test	6	5	4	3	2	1B	1A
lipase	-	-	-	-	-	+	+
Aesculin/salicin(24 h)	-	-	-	-	-	-	+/-
Indole	-	-	-	-	(+)	+	+
Xylose	+	V	-	+	+	+	+
Trehalose	+	-	+	+	+	+	+
Pyrazinamidase	+	-	-	-	-	-	+
d- glucosidase	-	-	-	-	-	-	+
VP	-	(+)	+	+/-[c]	+	+	+

V: Variable; VP: Voges-Proskauer; [c]: Biotype of serotype O:3 found in Japan.

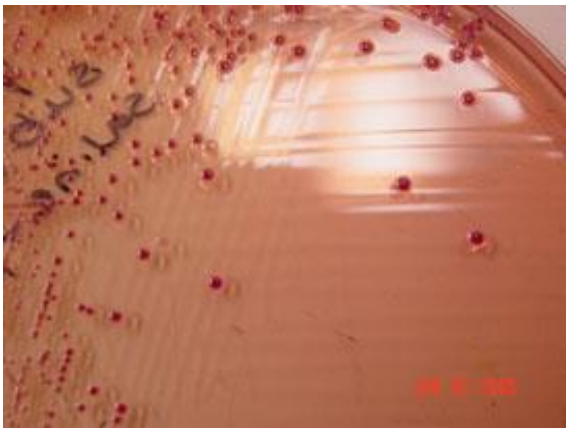

Figure 1. *Yersinia* colonies on the CIN agar medium.

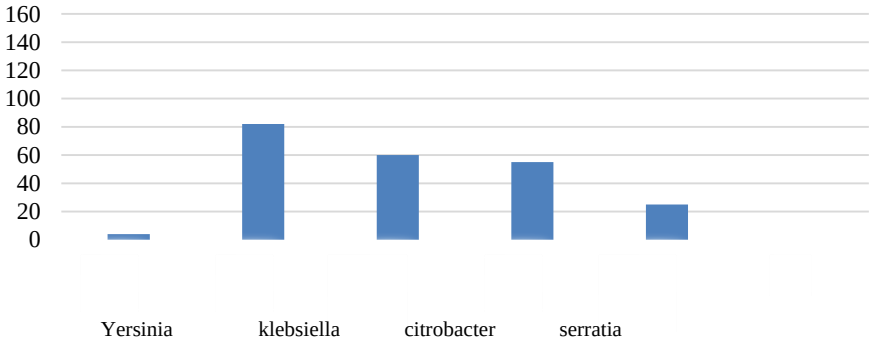

Figure 2. The frequency of gram-negative bacteria isolation in milk samples

Discussion

From a total of 360 raw milk samples taken during the cold seasons in 1994, 3 strains of *Y. enterocolitica* (0.83%) and 1 strain of *Y. pseudotuberculosis* (0.27%) were isolated. *Y. frederiksenii*, *Yersinia kristensenii*, *Yersinia Intermedia*, and *Yersinia aldovae* were not separated. In these experiments, the species of *Serratia*, *Citrobacter*, *Klebsiella*, and *Providence* were repeatedly isolated. In the authors' previous study (Soltan Dallal and Moezardalan, 2004) on the contamination of raw milk with *Yersinia enterocolitica* in Iran, the prevalence of *Y. enterocolitica* was 1.6%, which was very close to the prevalence of the present study. In a study from Chaharmahal Bakhtiari, 200 samples of raw milk

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and 200 samples of pasteurized milk were prepared by random cluster sampling and *Yersinia enterocolitica* was isolated in 6 cases (3%) of raw milk and 2 cases (1%) of pasteurized milk (Sharifzadeh *et al.*, 2004). In a study by Hanifian and Khani in Tabriz, using PCR and tracing of *ail* and *virf* genes on pasteurized milk, *Yersinia enterocolitica* was reported (Hanifian and Khani, 2012)). Due to the sensitivity of *Yersinia* to high temperatures during pasteurization, the importance of secondary contamination of milk after pasteurization is notable. The enrichment of the samples was similar to the present study and the PSBB medium was used at a ratio of 25 by 225 ml. Only one case of *Yersinia enterocolitica* was isolated by culture method, while 16 cases were isolated by PCR method. This explains the high sensitivity difference of PCR. Although there is no difference between live and dead bacteria in PCR, the presence of *Yersinia enterocolitica* DNA in pasteurized milk is notable (Hanifian and Khani, 2012). In 2015, Jamali *et al.* conducted a study on 446 raw milk of cows, sheep, and goats in the Varamin region. They isolated 29 strains of *Yersinia*, 19 strains of which were *Yersinia enterocolitica* with biotypes of 1A / O: 9, 1B / O: 8, and 4 / O; 3. No isolates of *Yersinia pseudotuberculosis* were obtained (Jamali *et al.*, 2015). In the present study, 1 *Yersinia pseudotuberculosis* isolate was isolated from raw milk. Unlike *Yersinia pseudotuberculosis*, many studies have been performed on the isolation of *Yersinia enterocolitica* from raw milk. In another study by Tavassoli et al., in 2019, 200 traditional kinds of cheese were randomly collected from northeastern Iran (100 samples from Khorasan Razavi and 100 samples from Golestan) and 100 samples of raw milk from Mashhad. In total, 38 *Yersinia enterocolitica* were obtained from cheese and 33 isolates from raw milk. All isolates belonged to two biotypes of 1A and 1B (Tavassoli *et al.*, 2019). In a study conducted in Egypt by Darwish et al., 27 *Yersinia intermedia*, 13 *Yersinia kristensenii*, and 3 *Yersinia frederiksenii* were separated. Examination of their working method shows that enrichment was similar to the current study, but their selected environments included CIN agar, McConkey, and Blood agar (Darwish *et al.*, 2015). In 2013, Bernardino-Varo *et al.* studied the prevalence and diversity *of Yersinia* species from 1,300 samples of raw milk collected from stables in Mexico City. A total of 454 samples were declared positive; this number included 44.25% of *Yersinia enterocolitica*, 18.26% of *Yersinia kristensenii*, 13.65% of *Yersinia intermedia*, 14.85% of *Y. frederiksenii*, and *Yersinia aldovae* 9.14% (Bernardino-Varo *et al.*, 2013). The high prevalence of Yersinia in this study might be due to the stable environment, which may be in the process of increasing *Yersinia* pollution from the workplace, especially if the stables were shared with pigs that are *Yersinia* reservoirs. In a study in Egypt, *Yersinia enterocolitica* was isolated from 10% of raw milk and dairy samples (Ahmed *et al.*, 2019). The highest separation rates were from raw milk with 22%, followed by 12%, 4%, and 2% of fermented milk, pasteurized milk, and cheese, respectively.

Conclusion

The results of this study highlight the role of milk as an important factor in the transmission of potentially pathogenic strains of *Y. enterocolitica* and *Y. pseudotuberculosis.* The findings show that tap water is effective in preventing *Yersinia*contamination. Also, the presence of purebred cattle in livestock can be effective in preventing *Yersinia* contamination.Furthermore, the presence of O9, O: 8, O: 5 pathogenic serotypes in milk should increase attention to *Yersinia* in milk tests.

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Authors' contributions

Soltan Dallal MM designed the research; Mobassei B conducted the research; Sharifi Yazdi

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S analyzed the data; Sharifi Yazdi S, Mirbagheri Z wrote the manuscript; Soltan Dallal MM had primary responsibility for final content. All authors read and approved the final manuscript.

Conflict of interest

The authors declared that there is no conflict of interest.

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