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Isolation and identification of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* from cow raw milk in Tehran

Abstract

Aims and background: *Yersinia* is a cocobacilliary gram-negative bacteria which can cause yersiniosis in all age groups. This study was carried out to investigate the presence of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* in raw milk of livestock farms in Tehran.

Materials and methods: Three hundred and sixty raw milk samples were collected from livestock farms in Tehran during 6 months. *Y. pseudotuberculosis* and *Y. enterocolitica* were isolated and identified according to the FDA guidelines. After treatment with low temperature (10°C for 10 days) and alkaline treatment (using KOH), the samples were cultured in the selective medium named CIN agar at 25°C. After incubation, colonies similar to the bull eye were chosen and characterized using phenotypic tests and ultimately confirmed by the API-20E strip test (Biomerieux, France) and detection of *ail* and *inv* genes by PCR. Serotyping and bio typing tests carried out on *Y. enterocolitica* isolates.

Results: Of 360 samples, a total of four isolates were identified as *Yersinia* species, including three *Y. enterocolitica* and one *Y. pseudotuberculosis*.

Conclusion: Due to the presence of *Y. pseudotuberculosis* and *Y. enterocolitica* in raw milk, it is suggested that the food and drug control laboratories, milk factories and other researchers pay more attention to these bacteria.

Keywords: raw milk, Yersinia enterocolitica, Yersinia pseudotuberculosis, cow, alkaline

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Mohammad Mehdi Soltan Dallal,[1,2] Milad Abdi,[3,4] Ramin Mazaheri Nezhad Fard,[1,5] Behzad Mobasseri[2]

[1]Food Microbiology Research Center, Tehran University of Medical Sciences, Iran
[2]Division of Food Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Iran
[3]Department of Microbiology, Faculty of Medicine, Iran University of Medical Sciences, Iran
[4]Student research committee, Faculty of Medicine, Iran University of Medical Sciences, Iran
[5]Division of Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Iran

Correspondence: Mohammad Mahdi Soltan Dallal, Division of Food Microbiology, Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran, Tel 98-21-42933082, Email msoltndallal@gmail.com

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Introduction

Due to the increasing population, providing adequate and safe food is one of the most important issues in different countries of the world. In addition to the food deficiency, inappropriate sanitation in different steps of production to consumption process of these substances leads to a reduction in the amount of food. So, not only the providing of food and its products in terms of quantity, but also the providing of healthy and safe food is very important.[1] Milk is one of the most consuming dairy products and essential food. Healthy and safe milk will help the health of the community, hence, ignoring this point may endanger the community health as well as the health economy. Due to the importance of this subject, rapid and accurate identification of contamination of raw milk is important.[2] The contamination of both raw and pasteurized milk is one of the problems that may occur for many reasons even in advanced countries. In addition to the total number of bacteria as an indicator for health of milk, detection of pathogenic bacteria in milk is important.[3,4] Raw milk and its products which are contaminated by microorganisms, can cause important diseases. Among the microbial contaminants of milk, various bacteria such as *Coxiella burnetii*, *Escherichia coli*, *Listeria monocytogenes*, *Mycobacterium paratuberculosis* and *Yersinia enterocolitica* can be mentioned.[5–7] The genus *Yersinia* is particularly important due to the ability to grow at low temperature (4°C). *Yersinia* after *Salmonella*, *Campylobacter* and *Shigella* is considered as the most common pathogen isolated from human feces. *Yersinia* cause various clinical disorders such as enterocolitis, Pseudo-appendicitis, extra-intestinal infections, and bacteremia.[8,9] *Yersinia* is a psychrotrophic gram negative coccobacillus bacterium which contains three main species including *Yersinia pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*.[10,11] Due to the growth at 4°C, *Yersinia* is a potential risk factor for food cold chain.[11,12] Unpasteurized milk and dairy products are potentially contaminated with this bacterium which can be transmitted to humans.[5] Due to the lack of sufficient study on cow milk contamination by *Yersinia* spp., especially in Iran, the aim of this study was to isolation and identification of *Y. enterocolitica* and *Y. pseudotuberculosis* from raw milk.

Materials and methods

Sample collection and phenotypic identification

Three hundred and sixty samples of raw milk were collected from livestock farms around Tehran during cold seasons of 2015–2016 (autumn and winter). The samples were transported to microbiology laboratory of School of Public Health of Tehran University of Medical Sciences in closed containers under sterile and cold conditions according to the National Standards of Iran No. 3549 (milk protection conditions after milking)[13] and No. 164 (milk and its products- raw milk- characteristics and testing methods).[14] The samples were enriched in phosphate buffered saline (PBS), and 25ml of milk samples was added to 225 ml of PBS and placed in a refrigerator for 10 days. After cold treatment, alkaline treatment was carried out in order to eliminate KOH susceptible bacteria especially normal flora of the milk. To performing such a treatment, 500µl of samples was

mixed with 500µl of 0.5% KOH solution and 0.5% NaCl for 30 s. Subsequently, streak culture was carried out on selective medium CIN agar (Merck, Germany) and incubated for 24–48 h at 25°C.[5,11] After incubation, suspicious colonies (colonies with red center and white margin or bulls eye appearance) were chosen. Desired colonies were surveyed by phenotypic and biochemical tests such as O-nitrophenyl-β-D-galactopyranoside (ONPG), ornithine decarboxylase (ODC), urease and motility. For final approval, the API-20E (BioMerieux, France) strip test was used.[12] To determine the biotypes of *Y. enterocolitica*, fermentation of xylose and trehalose as well as lipase, bile-esculin, Voges–Proskauer (VP) and indole tests were evaluated. Furthermore, serotyping was carried out using anti-serum (MAST, UK) according to the manufacturer's instructions.[15]

PCR assay

The DNA of *Yersinia* isolates was extracted using boiling method from purified cultures.[16] In short, using sterile loop a few colonies of bacteria were suspended in sterile distilled water in 1.5 ml micro tube, was placed in a boiling water container for 20 min. After centrifugation for 5 min, supernatant was removed and finally concentration of DNA was measured with Nano Drop 3300 (Thermo Fisher Scientific, USA). The primers which used for the *ail* and *inv* genes are listed in Table 1. The PCR reaction was performed in a total volume of 25µl containing 12.5µl of Master Mix, 8.5µl distilled water, 2µl DNA, 1µl forward primer and 1µl reverse primer. PCR reactions were carried out in a Master cycler gradient (Eppendorf, Germany). Table 2 shows appropriate time and temperature for PCR steps. *Y. pseudotuberculosis* PTTC 1244 and *Y. entrocolitica* ATTC 11010 were used as positive control strains. After amplification, to analyze the PCR products, electrophoresis was carried out using a 1% agarose gel containing safe stain and a TBE 0.5× for electrophoresis buffer as well as a 100-bp ladder and a voltage of 80–100 V (Table 1&2).

Table 1 Primers used in the current study and their sequences

Gene	Primer	Sequence
ail	Forward	5'-TAATGTGTACGCTGCGAG-3'
	Reverse	5'-GACGTCTTACTTGCACTG-3'
inv	Forward	5'-CGGTACGGCTCAAGTTAATCTG-3'
	Reverse	5'-CCGTTCTCCAATGTACGTATCC-3'

Table 2 PCR conditions in the current study

Gene	Step	Temperature (°C)/Time (min/sec)	Gene	Step	Temperature (°C)/Time (min/sec)
ail	Initial denaturation	95 / 3	inv	Initial denaturation	95 / 3
	denaturation	95 / 30		denaturation	95 /30
	annealing	54 / 40		annealing	62 / 40
	extension	72 / 1		extension	72 / 1
	Final extension	72 / 5		Final extension	72/ 5

Results

Phenotypic evaluation

Of 360 samples, a total of four isolates were identified as *Yersinia* species (1.1%), including three *Y. enterocolitica* (0.83%) and one *Y. pseudotuberculosis* (0.27%). Figure 1&2 shows the result of the API-20E strip test which was used for confirmation of both species. Two isolates of *Y. entrocolitica* were belonged to 1A biotype and one was 3 (Figure 1&2), (Table 3).

Also, result of serotyping of *Y. enterocolitica* isolates showed that two isolates were belonged to O:5 serotype and one was O:9.

Figure 1 API-20E biochemical tests for *Y. enterocolitica*.

Figure 2 API-20E biochemical tests for *Y. pseudotuberculosis*.

Table 3 Biotyping results of *Y. entrocolitica* isolates

No.	Xylose	Trehalose	Bile-esculin	Voges–Proskauer	Indole	Lipase	Biotype
1	+	+	-	+	-	-	3
2	+	+	+	+	+	+	1A
3	+	+	+	+	+	+	1A

PCR assay

PCR amplification products indicated that *Y. pseudotuberculosis* included *inv* gene, while only one isolate of *Y. enterocolitica* included *ail* gene (Figure 3&4).

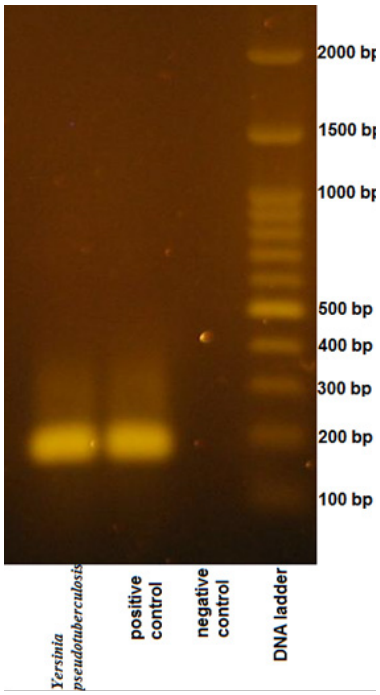

Figure 3 Amplification products from PCR assay for the detection of *inv* in *Y. pseudotuberculosis* (product size of 183bp).

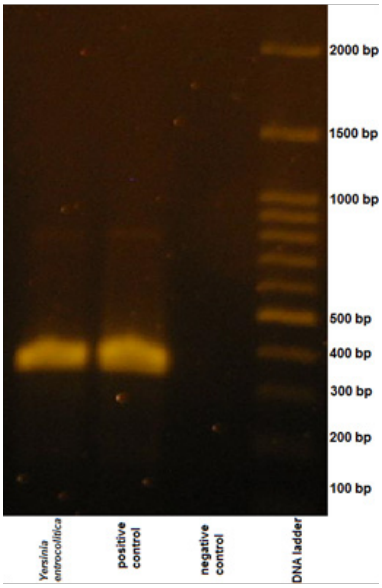

Figure 4 Amplification products from PCR assay for the detection of *ail* in *Y. enterocolitica* (product size of 351bp).

Discussion

Of 360 raw milk samples taken during the cold seasons of 2015-2016, three isolates of *Y. enterocolitica* and one isolate of *Y. pseudotuberculosis* were obtained. A similar study was carried out in Finland in 2014 by Parn et al. during which an outbreak of *Y. pseudotuberculosis* serotype O:1 occurred following consumption of raw milk which created fever and gastroenteritis in 44 persons.[17] A similar study was carried out in Finland in 2014;[17] during which, an outbreak of *Y. pseudotuberculosis* serotype O:1 occurred following consumption of raw milk which created fever and gastroenteritis in 44 people. In 2015, Jamali et al,[18] carried out a study on 446 raw milk samples of cattle, sheep and goats in the Varamin area. Twenty-nine isolates of *Yersinia* were obtained, of which 65.5% were *Y. entrocolitica*, 31% *Y. frederiksenii,* and 3.4% *Y. kristensenii*. Nineteen isolates of *Y. enterocolitica* were belonged to 1A/O:9, 1B/O:8 and 4/O:3 serotypes, no isolates of pseudotuberculosis were obtained.[18] In 2015, Jamali et al. carried out a study on 446 raw milk samples of cattle, sheep and goats in the Varamin area. Its results showed that, 29 isolates of *Yersinia* were obtained, of which 65.5% were *Y. entrocolitica*, 31% *Y. frederiksenii*, and 3.4% *Y. kristensenii*. Nineteen isolates of *Y. enterocolitica* were belonged to 1A/O:9, 1B/O:8 and 4/O:3 serotypes, no isolates of pseudotuberculosis were achieved.[18] In the present study, *Y. pseudotuberculosis* was isolated from raw milk. The first report of contamination of raw milk with *Y. enterocolitica* in Iran was presented by Soltan Dallal et al. on 310 raw milk samples in Behshahr town.[5] The first report of contamination of raw milk with *Y. enterocolitica* in Iran was presented by Soltan Dallal et al,[5] on 310 raw milk samples in Behshahr City. During this study, the prevalence of *Y. enterocolitica* was 1.6%, which is close to prevalence of present study. In another study, Sharifzadeh et al,[19] isolated six *Y. enterocolitica* isolates from 400 samples of raw and pasteurized milk in 2004. Their findings and prevalence were similar to our study.[19] In another study, six *Y. enterocolitica* isolates were isolated from 400 samples of raw and pasteurized milk in 2004.[19] Their findings and prevalence were similar to the present study. The physiological characteristics of *Yersinia* are effective in growth and expression of virulent genes and bacterial toxin.[20] Although *Yersinia* is sensitive to pasteurization heat, but in the study of Pagan et al. in 1999, six strains of *Y. enterocolitica* was inoculated to the milk, after pasteurization three strains of them were survived.[20] Although *Yersinia* is sensitive to pasteurization heat, but in another study,[21] six strains of *Y. enterocolitica* was inoculated to the milk, after pasteurization three strains of them were survived. In another study, Soltan Dallal et al,[20] showed that both pH and temperature influence growth but only temperature affects virulence.[21] In other study,[22] the obtained results showed that both pH and temperature of *Yersinia* influence growth but only temperature affects virulence. The heat-resistant enterotoxin is produced at 4°C and 25°C, but it is not possible to produce at 37°C. These results emphasize the importance of temperature on the expression of virulence and enterotoxin genes. *Yersinia* contamination occurs primarily by lactating animal and secondary by environment and water. However, livestock as a source of *Yersinia* has an important role, but water as carrier is important and can lead to the transfer of *Yersinia* species to milk and dairy

products, especially in rural and nomadic areas. Cheyne reported the presence of *Y. entrocolitica* and other atypical *Yersinia* species, such as *Y. frederiksenii, Y. kristensenii,* and *Y. intermedia* in surface water.[23] Soltan Dallal & Salmanian[22] isolated *Y. entrocolitica* strains from drinking and surface water in Kan region of Tehran.[24] Milk is a nutritious and popular food; therefore, bacteriological quality control of raw and pasteurized milk at production, collection and processing stages is necessary. The microbial and chemical changes of raw milk can interfere with processing stages which reduces shelf-life of dairy products, and consequently endanger the health of consumers and community. Presentation of *Yersinia* as one of the most important food-borne agents along with ability to grow at room and refrigerator temperatures and production of enterotoxin, makes it an important issue which needs to be paid more attention to improving food safety.[2,22]

Conclusion

This study recommends the importance of scientific follow-up in routine laboratory experiments to assess dairy samples. Furthermore, milk samples must bacteriologically be tested by food and drug control laboratories. Results of the current study indicate the importance of milk pasteurization in dairy industries due to the presence of *Y. pseudotculosis* and *Y. enterocolitica* as indicated by this study.

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Conflicts of interest

Author declares that there are no conflicts of interest.

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