



Contamination Rate of Bovine Raw Milk with *Yersinia enterocolitica* Biotypes in Mashhad, Iran

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ABSTRACT

Background: Yersiniosis is an important foodborne zoonotic disease worldwide. Gastroenteritis and septicemia are the primary forms of yersiniosis. *Yersinia enterocolitica* is a causative organism, which is frequently isolated from foods such as milk. This study aimed to evaluate the contamination rate of bovine raw milk with *Y. enterocolitica* and the biotyping of the isolated organisms during January-July 2017 in Mashhad, Iran.

Methods: In total, 100 bovine raw milk samples were collected. The samples were enriched in peptone sorbitol broth. After alkali treatment, the enriched samples were streaked into cefsulodin-irgasan-novobiocin agar plates. Suspected *Y. enterocolitica* colonies were tested by the amplification of the 16s rRNA gene to confirm the isolated *Y. enterocolitica*. The confirmed isolates were biotyped using biochemical tests.

Results: The results showed that 33% of the bovine raw milk samples were contaminated with *Y. enterocolitica*. Biotype 1A was predominant (26%), while the pathogenic biotypes 1B (6%) and 5 (1%) were also detected.

Conclusion: According to the results, the isolation of pathogenic *Y. enterocolitica* from bovine raw milk is a public health hazard due to its presence in dairy products.

1. Introduction

Yersinia spp. is a member of *Enterobacteriaceae* family and currently consists of 17 species, three of which are pathogenic to humans, including *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, and *Yersinia pestis* [1, 2]. *Yersinia enterocolitica* is an important foodborne pathogen, which causes severe diseases in humans [3]. Acute gastroenteritis is the most common form of the disease

caused by this organism. Mesenteric lymphadenitis mimicking appendicitis and septicemia are the other forms of the disease [3]. *Yersinia* is widely distributed in nature and has been detected in various foods [4, 5]. *Y. enterocolitica* is a psychrophilic organism, which could replicate at refrigeration temperature. Therefore, the microbe could survive in foods and liquids for a long period and cause a significant public health issue [3].

Foods with animal origin are associated with high risk of



gastrointestinal diseases caused by *Y. enterocolitica* in humans [6, 7]. Milk and dairy products are among the most frequently consumed of animal foods. The presence of *Y. enterocolitica* in milk and dairy products has been investigated in several studies in Iran and other countries [4, 6-8]. Milk is considered as a complete food (especially for children) because of various nutrients like proteins, minerals, and vitamins as well as a proper medium for growth of microorganisms.

Therefore, the hygienic quality and safety of this product are crucial. Foodborne diseases caused by bacterial, viral, and parasitic microorganisms are highly prevalent health issues [9]. The consumption of contaminated raw milk leads to the outbreak of several gastrointestinal infections worldwide [10, 11]. Drinking raw milk is a routine habit in Iran, especially in rural areas. In this regard, some cases of diarrhea caused by *Y. enterocolitica* have been studied in Iran [12].

The European Food Safety Authority (EFSA) recommends biotyping to determine the pathogenicity of isolated organisms [13]. *Y. enterocolitica* consists of six biotypes, including 1A, 1B, 2, 3, 4, and 5. Some biotypes of *Y. enterocolitica* (1B, 2, and 5) are human and animal pathogens. Biotype 1A is often considered as a non-pathogenic strain, while some virulence markers have recently been attributed to the strains of this biotype [14].

The presence of numerous background microbiota alongside the non-pathogenic *Yersinia spp.* and the low concentration of pathogenic strains (especially in food samples) have complicated the isolation process which leads to the underestimated prevalence of pathogenic *Y. enterocolitica* in food [15]. In addition, the biochemical confirmation of organisms and biotyping require several biochemical tests and reagents [16]. Polymerase chain reaction (PCR) assays are commonly used to confirm *Y. enterocolitica*. Molecular assays are rapid, highly specific, sensitive, and able to overcome the limitations of conventional methods in the identification of foodborne pathogens [16]. In this regard, several studies have been done to determine the contamination rate of *Y. enterocolitica* in different foods (meat, chicken, and dairy products) [4-8, 15, 17, 21-27].

This is the first study conducted on assessing the prevalence of the pathogenic strains of *Y. enterocolitica* in raw milk in Mashhad, Iran.

The present study aimed to evaluate the contamination rate of *Y. enterocolitica* in fresh bovine milk with culture as confirmed by PCR and the biotyping of the isolated organism in Mashhad, Iran.

2. Materials and Methods

2.1. Sample collection

100 bovine raw milk samples were randomly collected twice from seven milk collection centers (MCC) in Mashhad,

Iran during January-July 2017. In each MCC, the samples were obtained from 13-15 milk boxes of different farms. Milk was agitated thoroughly before sampling, and 50 mL milk was collected from each milk box in sterilized tubes. It was immediately transferred to a laboratory and preserved in hygienic conditions in an icebox.

2.2. Sample Enrichment

For enrichment, 10 mL of each milk sample and 90 mL of peptone sorbitol broth (Sigma-Aldrich, Taufkirchen, Germany) were agitated for five min and incubated for 48 h at the temperature of 25°C in a shaker incubator (Thermo Fisher Scientific, Schwerte, Germany) [18].

2.3. Isolation and Identification of *Y. enterocolitica*

At this stage, 0.5 mL of each enriched sample was thoroughly mixed with 4.5 mL of potassium hydroxide (KOH 0.25%) for 20 sec and cultured onto *Yersinia* selective agar plates (CIN agar; Merck KGaA, Darmstadt, Germany), which was supplemented with cefsulodin-irgasan-novobiocin antibiotics (Merck KGaA, Darmstadt, Germany) [18]. The agar plates were incubated at the temperature of 30°C for 24-48 h. Small colonies (diameter: 1-2 mm) with a deep red center and a sharp border surrounded by a clear colorless zone with complete edges were selected from each CIN plate. Afterwards, gram-negative bacilli were assessed using oxidase and catalase tests. The colonies containing no cytochrome c oxidase that were able to dissociate H₂O₂ were selected for the other phenotypic tests, including urease and indole tests. In addition, suspected colonies that used urea as a source of nitrogen and were able to convert tryptophan into indole were presumptively regarded as *Y. enterocolitica* and selected for DNA extraction [18].

2.4. DNA Extraction and PCR Assay

DNA was extracted from the purified presumptive colonies using the boiling method [19]. The suspected colonies were dissolved in deionized distilled water and boiled for 10 min at the temperature of 95°C. Following that, the boiled samples were centrifuged, and the supernatant containing DNA was transferred to another tube. PCR assay was carried out for the amplification of 16s rRNA with the final volume of 20 µl, containing one µl (10 picomols) of a forward primer (5'-AATACCGCATAACGTCTTCG-3') and a reverse primer (5'-CTTCTTCTGCGAGTAACGTC-3') (Macrogen, Seoul, South Korea) [20], two µl of the DNA template, 10 µl of a master mix (Ampliqon, Odense, Denmark), and six µl of nuclease-free deionized distilled water. A thermal cycler (Techne, Wiesbaden, Germany) was programmed, as follows: 94°C for five min for initial denaturation in 36 cycles (denaturation at 94°C for 45 sec, annealing at 26°C for 45 sec and at 72°C for 45 sec for extension). In addition, the final extension step

was carried out at the temperature of 72°C for seven min. *Y. enterocolitica* ATCC 9610 and nuclease-free deionized water were used as the positive and negative controls, respectively [8]. At the next stage, 1.5% agarose gel was prepared, and four µl of the PCR products (330 bp) were loaded into a well. The gel was pre-stained using the Green-Viewer and photo-documented under UV light (Thermo Fisher Scientific, Schwerte, Germany).

2.5. Biotyping

For the biotype determination of the isolates, the esculin hydrolysis (Merck KGaA, Darmstadt, Germany), indole production of tryptophan, lipid hydrolysis, acid, and gas production of sorbose (Merck KGaA, Darmstadt, Germany) were investigated. The acid production of salicin, trehalose, and xylose was also examined [21]. Moreover, phenol red broth (Merck KGaA, Darmstadt, Germany) was prepared, and 10% salicin, trehalose, and xylose were added to the broth to dissolve the colonies. Finally, the tubes were incubated at the temperature of 30°C for 24 h [21].

3. Results and Discussion

According to the conventional culture method, 35 out of 100 raw milk samples (35%) were suspected of *Y. enterocolitica*. In the subsequent molecular detection, the gene-specific 16s rRNA to *Y. enterocolitica* was amplified in 33 out of 35 contaminated samples (Figure 1). Among 100 raw milk samples, 26 cases were contaminated with biotype 1A, while six samples were contaminated with biotype 1B, and one sample was contaminated with biotype 5 of *Y. enterocolitica*. Table 1 shows the biotype determination of the *Y. enterocolitica* isolates. Table 2 shows the distribution of the biotypes of *Y. enterocolitica* in the sampling sites.

To the best of our knowledge, such a high rate of positive results (33%) has not been previously reported in Iran, which might be due to the enrichment medium used in the current research or the geographical region of the study. In a similar

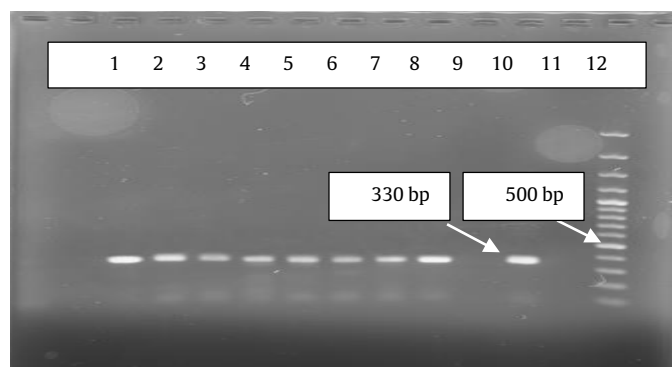


Figure 1: PCR Amplification of 16s rRNA gene of *Y. enterocolitica* (Lane 1-8: positive sample, lane 9: negative control, lane 10: positive control, lane 12: 100 base-pair plus DNA ladder)

Table 1: Determination of *Y. enterocolitica* biotypes by method of Ye *et al.* (2016)

Biotype	1A	1B	2	3	4	5
Esculin hydrolysis	+	-	-	-	-	-
Indole production	+	+	(+)	-	-	-
Lipase	+	+	-	-	-	-
Sorbose fermentation	+	+	+	+	+	-
Salicin	+	-	-	-	-	-
Trehalose	+	+	+	+	+	-
Xylose	+	+	+	+	-	V

(Delayed reaction; V: Variable reactions).

study conducted in the north of Iran (Behshahr), 1.6% of bovine raw milk samples were observed to be positive in terms of *Y. enterocolitica* based on the culture method which is lower than our findings [7]. In another study, Rahimi *et al.* (2014) reported that based on the culture method, 20 out of 237 raw milk samples of cow, sheep, goat, and camel were contaminated with *Yersinia* spp. On the other hand, 12 out of 237 samples were observed to be infected with *Y. enterocolitica* in Isfahan, Iran [22], while the PCR assay confirmed *Y. enterocolitica* in 4.21% of the samples [22]. *Y. enterocolitica* was also detected in 5.8% of raw cow milk in Varamin, Iran by the culture method [4]. Notably, the mentioned studies have used different enrichment media compared with the current research.

Alavi *et al.* (2018) investigated the raw milk of sheep and goat regarding the presence of *Y. enterocolitica* in Shahrekord, Iran. According to the obtained results, 9 out of 84 sheep milk samples were contaminated with *Y. enterocolitica* based on the culture assay, and all the isolates were confirmed by the PCR assay. However, the organism was not detected in 16 goat milk samples [23]. In another research, Hanifian and Khani (2012) reported the prevalence of virulent *Y. enterocolitica* in raw cow milk in the north-west of Iran (East Azerbaijan province). They found that 2.26% of bulk raw milk samples were contaminated with *Y. enterocolitica* based on the culture and PCR assays [8].

In Mexico City, *Yersinia* spp. was isolated from 454 out of 1300 raw cow milk samples which were collected from stables. Among the isolated *Yersinia* genus, 44.25% were observed contaminated with *Y. enterocolitica* [24]. *Y. enterocolitica* was reported to be the most common isolated species from raw cow milk in Ankara, Turkey. It was reported that the organism was isolated from 47.3% of the raw cow milk samples using the culture method and alkali treatment before streaking on the CIN agar [25]. The reported rates in the mentioned studies are higher compared to our findings.

According to a review in this regard, some studies have suggested that alkali treatment could increase the isolation rate of *Y. enterocolitica*, while other findings seem to contradict [16].

Table 2 :Distribution of *Y. enterocolitica* biotypes in sampling sites as confirmed by PCR

Sampling site	Number of samples	1A	1B	2	3	4	5	Total
MCC 1	13	3	1	0	0	0	0	4
MCC 2	15	4	2	0	0	0	0	6
MCC 3	15	6	1	0	0	0	1	8
MCC 4	14	2	0	0	0	0	0	2
MCC 5	13	5	1	0	0	0	0	6
MCC 6	15	2	1	0	0	0	0	3
MCC 7	15	4	0	0	0	0	0	4
Total	100	26	6	0	0	0	1	33

According to a review in this regard, some studies have suggested that alkali treatment could increase the isolation rate of *Y. enterocolitica*, while other findings seem to contradict. [16]. the discrepancies in the reported contamination rates may be due to the isolation procedure with or without alkali treatment.

In the present study, biotype 1A was detected in most of the isolates (26 out of 33), which is consistent with the previous studies in this regard [4, 7, 24]. Furthermore, two pathogenic biotypes of 1B and 5 were isolated in the current research. Similarly, Jamali *et al.* (2015) reported that 15.8% of the isolated *Y. enterocolitica* belonged to biotype 1B in Varamin, Iran [4]. Other related studies have also isolated this biotype from animals and foods of animal origin [26, 27]. Some researchers have detected other pathogenic biotypes in milk as well [4, 8, 24].

Contamination of raw milk with *Y. enterocolitica* may be associated with poor hygienic practices in milk production. Therefore, taking effective sanitary measures for proper milking management is essential to control this pathogen at the farm level. The primary sources of the growth, propagation, and survival of *Y. enterocolitica* are the consumption of raw unpasteurized milk and dairy products that are produced from raw milk or using non-hygienic methods. As a result, measures such as sanitary milking, using pasteurized milk and dairy products, and protecting milk and dairy products from dust and insects are the optimal approaches to prevent *Y. enterocolitica* infections [22].

Considering the presence of pathogenic biotypes in raw milk, pasteurization of milk before consumption and production of other dairy products is a critical measure. Further studies are needed to determine the pathogenic biotypes, serotypes, and virulent genes of *Y. enterocolitica* isolates.

4. Conclusion

According to the results, bovine raw milk was contaminated with the pathogenic biotypes of *Y. enterocolitica*. Therefore, the consumption of raw milk and the dairy products might pose the potential risk of yersiniosis and cause a public health issue for food safety agencies.

Further investigations are required regarding the prevalence of the virulent genes and serotypes of *Y. enterocolitica*, as well as the emerging drug resistance in order to enable the detection of foods with a significant risk of consumption and adopting the most effective strategies against yersiniosis.

Authors' Contributions

F.M., performed the sampling, bacterial culture, and PCR assay and drafted the manuscript; T.Z., contributed to the research design, provided important intellectual content for the experiment, coordinated the study, and drafted the manuscript; A.J., supervised and contributed to the research design, coordinated the study, and drafted and reviewed the manuscript. All the authors read and approved the final manuscript.

Conflicts of Interest

The Authors declare that there is no conflict of interest.

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