Prevalence, characterization, and antimicrobial resistance of *Yersinia* species and *Yersinia enterocolitica* isolated from raw milk in farm bulk tanks

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ABSTRACT

The aims of this study were to investigate the prevalence and to characterize and determine the antibiotic resistance of *Yersinia* spp. isolates from raw milk. From September 2008 to August 2010, 446 raw milk samples were obtained from farm bulk milk tanks in Varamin, Iran. *Yersinia* spp. were detected in 29 (6.5%) samples, out of which 23 (79.3%), 5 (17.2%), and 1 (3.4%) were isolated from cow, sheep, and goat raw milk, respectively. The most common species isolated was *Yersinia enterocolitica* (65.5%), followed by *Yersinia frederiksenii* (31%), and *Yersinia kristensenii* (3.4%). Of the 19 *Y. enterocolitica* isolates, 14 (73.7%) were grouped into bioserotype 1A/O:9, 4 (21.1%) belonged to bioserotype 1B/O:8, 1 (5.3%) belonged to bioserotype 4/O:3, and 1 isolate (biotype 1A) was not typable. All the isolates of biotypes 1B and 4 harbored both the *ystA* and *ail* genes. However, all the isolates of biotype 1A were only positive for the *ystB* gene. The tested *Yersinia* spp. showed the highest percentages of resistance to tetracycline (48.3%), followed by ciprofloxacin and cephalothin (each 17.2%), ampicillin (13.8%), streptomycin (6.9%), and amoxicillin and nalidixic acid (each 3.4%). All of the tested isolates demonstrated significant sensitivity to gentamicin and chloramphenicol. Recovery of potentially pathogenic *Y. enterocolitica* from raw milk indicates high risks of yersiniosis associated with consumption of raw milk.

Key words: *Yersinia* spp., virulence gene, bioserotype, antimicrobial resistance, raw milk

INTRODUCTION

The genus *Yersinia* is gram-negative and belongs to the family of *Enterobacteriaceae*. It comprises at least 16 species. Among them, *Yersinia pestis*, *Yersinia pseudotuberculosis*, and *Yersinia enterocolitica* are con-

sidered the most important human pathogenic species (von Tils et al., 2012). *Yersinia enterocolitica* is an important foodborne pathogen in humans and is primarily transmitted through consumption of contaminated pork, milk, or water (Thoerner et al., 2003).

Milk is an important source of nutrients for humans (Sharma and Joshi, 1992). Several outbreaks of enteric infections caused by consumption of contaminated raw milk have been documented (Tacket et al., 1984; Barrett, 1986; Ackers et al., 2000). *Yersinia enterocolitica* is able to survive at refrigeration temperature and, consequently, consumption of contaminated milk with the pathogen could be considered a public health risk. Although, *Y. enterocolitica* is well known as a cause of yersiniosis in children (Hoogkamp-Korstanje and Stolk-Engelaar, 1995), the infection has been found infrequently in Iran, where pork is never used (Bashiribod, 1989). This microorganism is ubiquitous in nature and has been isolated from different types of foods, animals, and environments (Fredriksson-Ahomaa and Korkeala, 2003).

Although, numerous *Y. enterocolitica* biotypes have been isolated from nature, only a few are human pathogens (Weagant and Feng, 2001). *Yersinia enterocolitica* includes nonpathogenic biotype 1A (Bottone, 1997) and pathogenic biotypes 1B, 2, 3, 4, and 5 (Thoerner et al., 2003). Pigs are the main reservoirs and vehicles for *Y. enterocolitica* transmission (Fredriksson-Yhomaa et al., 2006), but virulent strains have been also detected in milk and dairy products (Soltan-Dallal et al., 2004; Hanifian and Khani, 2012). The presence of virulence plasmids was applied for the evaluation of the pathogenicity of *Y. enterocolitica* isolates (Platt-Samoraj et al., 2006). Virulence genes, such as *ail* and *yst*, are located on the bacterial chromosome (Miller et al., 1989; Grant et al., 1998; Gierczynski, 2000). The Ail protein is encoded by the *ail* gene and only occurs in pathogenic *Y. enterocolitica*; it contributes in bacterial adhesion to the host cell as well as intensifies resistance to the bactericidal effects of complement (Mors and Pai, 1980; Miller et al., 1989; Miller et al., 1990; Bleves and Cornelis, 2000; Gierczynski, 2000; Thoerner et al.,

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2003). Moreover, the *yst* gene, which encodes the thermostable enterotoxin Yst protein, facilitates the invasion of the pathogen into tissues (Robins-Browne et al., 1979; Ibrahim et al., 1997; Ramamurthy et al., 1997; Gierczynski, 2000; Revell and Miller, 2001). The YstA and YstB are produced by pathogenic and nonpathogenic *Y. enterocolitica*, respectively (Mikulskis et al., 1994; Ramamurthy et al., 1997; Singh and Virdi, 2004). However, studies on the prevalence and resistance profiles of *Yersinia* spp. in raw milk in Iran are lacking (Hanifian and Khani, 2012) and no clear picture of the risks associated with consumption of raw milk exists. Hence, the current study was carried out to investigate the prevalence and to characterize and determine the antimicrobial resistance status of *Yersinia* spp. isolates from raw milk in Tehran province, Iran.

MATERIALS AND METHODS

Sample

A total of 446 raw milk samples, including 240 cow milk samples, 165 sheep milk samples, and 41 goat milk samples, were aseptically collected from farm bulk milk tanks in Varamin, Tehran province, Iran, from September 2008 to August 2010. Transportation of the samples to the laboratory was done in ice boxes within 4 h after collection (Jamali et al., 2013).

Isolation and Detection

Isolation and detection of *Yersinia* spp. were done according to Yucel and Ulusoy (2006). Five milliliters of each sample was added to 45 mL of Tris-buffered peptone water (10 g of peptone, 12.1 g of Tris methylamine, 5 g of sodium chloride, pH 8.4) and the samples were incubated for 3 wk at 4°C. One milliliter of each cold enriched broth was treated with 9 mL of a 0.25% KOH solution (0.25% KOH in 0.5% NaCl) to repress background microbiota at 7, 14, and 21 d. Then, a loopful of the mixture was streaked on cefsulodin-irgasan-novobiocin agar (Oxoid, Basingstoke, UK) and incubated for 24 h at 30°C. The presumptive isolates were examined for biochemical tests as reported by Greenwood and Hooper (1989), Feeley and Schiemann (1984). The isolates were further identified by using the API 20E according to the guideline of the manufacturer (bioMérieux, Marcy l'Etoile, France).

Biotyping and Serotyping

The *Y. enterocolitica* isolates were biotyped by discriminatory tests (lipase, xylose, indole, esculin, trehalose, and salicin; Wauters et al., 1987). The serotype of

the isolates was also determined by using commercial serum agglutinant O-Antisera (Bio-Rad, Marnes-la-Coquette, France).

Detection of Virulence Genes by Multiplex PCR

Detection of the *ail*, *ystA*, and *ystB* genes was carried out using a multiplex PCR as described by Platt-Samoraj et al. (2006).

Antimicrobial Susceptibility Testing

The Kirby-Bauer disc diffusion method on Mueller Hinton agar (Oxoid) was applied to determine the antimicrobial susceptibility as described in Clinical and Laboratory Standards Institute (CLSI, 2006). The panel of applied antimicrobial agents and concentrations was as follows: trimethoprim (15 µg), ciprofloxacin (5 µg), tetracycline (15 µg), gentamicin (10 µg), nalidixic acid (30 µg), streptomycin (30 µg), chloramphenicol (30 µg), ampicillin (30 µg), amoxicillin (30 µg), and cephalothin (30 µg).

Statistical Analysis

Chi-squared analysis was applied to analyze the relationship among contaminated samples and various types of raw milk. The statistical and Chi-squared analyses were done via SPSS 18.0 (SPSS Inc., Chicago, IL). A *P*-value <0.05 was used for statistical significance.

RESULTS

In the present study, 6.5% (29 out of 446) of the tested raw milk samples harbored *Yersinia* spp., and the prevalence of *Y. enterocolitica*, *Y. frederiksenii*, and *Y. kristensenii* were 65.5, 31, and 3.4%, respectively. Among the 446 samples, 23 (9.6% of cow milk samples), 5 (3% of sheep milk samples), and 1 (2.4% of goat milk samples) cow, sheep, and goat raw milk samples were contaminated with *Yersinia* spp. (Table 1). A significant difference was found between contaminated raw cow milk samples with *Yersinia* spp. and other kinds of raw milk (*P* < 0.05).

The 19 *Y. enterocolitica* isolates from raw milk samples were distributed into biotype 1A (78.9%), biotype 1B (15.8%), and biotype 4 (5.3%). The tested *Y. enterocolitica* isolates were further grouped into serotypes O:9 (bioserotype 1A/O:9; 73.3%), O:8 (bioserotype 1B/O:8; 15.8%), O:3 (bioserotype 4/O:3; 5.3%), and 1 isolate (biotype 1A) was not typable (Table 2).

The *ystA* gene was observed in all the isolates having bioserotypes 1B/O:8 or 4/O:3. However, the *ail* gene

Table 1. Prevalence of *Yersinia* spp. in raw milk samples (values in parentheses indicate % of samples positive within each type of milk)

Item	No. of samples	<i>Yersinia</i> spp.	<i>Yersinia enterocolitica</i>	<i>Yersinia frederiksenii</i>	<i>Yersinia kristensenii</i>
Cow milk	240	23 (9.6%)	14 (5.8%)	8 (3.3%)	1 (0.4%)
Sheep milk	165	5 (3%)	4 (2.4%)	1 (0.6%)	—
Goat milk	41	1 (2.4%)	1 (2.4%)	—	—
Total	446	29 (6.5%)	19 (4.3%)	9 (2%)	1 (0.2%)

was only detected from biotype 4/O:3 and all isolates of *Y. enterocolitica* biotype 1A harbored only the *ystB* gene (Table 2).

The resistance profiles of *Yersinia* spp. in our study are shown in Table 3. The results showed that 17 isolates (58.6%) of *Yersinia* spp. indicated significant resistance to 1 or 2 of the tested antimicrobial agents. Moreover, multi-drug resistance was observed in 2 of the *Yersinia* spp. isolates (6.9%; Figure 1). The isolates of *Yersinia* spp. were resistant to tetracycline (48.3%), ciprofloxacin, and cephalothin (each 17.2%). All the *Yersinia* spp. isolates were susceptible to gentamicin and chloramphenicol.

DISCUSSION

Yersiniosis is a zoonotic disease with a wide distribution and a known public health significance; it is one of the most frequently reported zoonosis in the European Union (EFSA, 2007). Although raw milk and home-made dairy products, such as unripened traditional cheese, are commonly consumed in Iran, there are few

studies on the prevalence and characterization of *Y. enterocolitica* in raw milk (Hanifian and Khani, 2012).

Among the different types of raw milk samples analyzed in the present study, the highest percentage of *Y. enterocolitica* (5.8%) was observed in cow milk. The pathogen was isolated from 2.3% of raw cow milk in northern Iran (Soltan-Dallal et al., 2004). In another study, Hanifian and Khani (2012) detected the virulent *Y. enterocolitica* in 7.6% of raw cow milk samples in northwest Iran. *Yersinia enterocolitica* was also isolated from cow, sheep, and goat raw milk in other countries (Yucel and Ulusoy, 2006; El-Aal and Atta, 2009; Schoder et al., 2010; Bernardino-Varo et al., 2013). Four percent of raw milk cheese samples were contaminated with *Y. enterocolitica* in Morocco (Hamama et al., 1992). The frequency of *Y. frederiksenii* (2%) and *Y. kristensenii* (0.2%) isolation in our study was lower as compared with other studies of raw milk in Turkey and Mexico by Yucel and Ulusoy (2006) and Bernardino-Varo et al. (2013). Inadequate hygiene in dairy farms, particularly during milking, could probably be a source of *Yersinia* spp. in raw milk (Hanifian and Khani, 2012). The pres-

Table 2. Biotyping, serotyping, and PCR analysis of *ail*, *ystA*, and *ystB* genes in isolates of *Yersinia enterocolitica*

Isolation code	Milk type	Biotype	Serotype	<i>ail</i>	<i>ystA</i>	<i>ystB</i>
Y-C-1	Cow	1A	O:9	—	—	+
Y-C-2	Cow	1A	O:9	—	—	+
Y-C-3	Cow	1B	O:8	—	+	—
Y-C-4	Cow	1A	O:9	—	—	+
Y-C-5	Cow	1A	Not typable	—	—	+
Y-C-6	Cow	1A	O:9	—	—	—
Y-C-7	Cow	1A	O:9	—	—	+
Y-C-8	Cow	1A	O:9	—	—	+
Y-C-9	Cow	1B	O:8	—	+	—
Y-C-10	Cow	1B	O:8	—	+	—
Y-C-11	Cow	1A	O:9	—	—	+
Y-C-12	Cow	4	O:3	+	+	—
Y-C-13	Cow	1A	O:9	—	—	+
Y-C-14	Cow	1A	O:9	—	—	+
Y-S-1	Sheep	1A	O:9	—	—	+
Y-S-2	Sheep	1A	O:9	—	—	+
Y-S-3	Sheep	1A	O:9	—	—	+
Y-S-4	Sheep	1A	O:9	—	—	—
Y-G-1	Goat	1A	O:9	—	—	+

Table 3. Antimicrobial resistance profiles of *Yersinia* spp. isolated from raw milk samples (values in parentheses indicate % of isolates resistant to each agent)

Antimicrobial agent	<i>Yersinia</i> spp. (29)	<i>Yersinia enterocolitica</i> (19)	<i>Yersinia frederiksenii</i> (9)	<i>Yersinia kristensenii</i> (1)
Tetracycline	14 (48.3%)	10 (52.6%)	4 (44.4%)	—
Chloramphenicol	—	—	—	—
Amoxicillin	1 (3.4%)	1 (5.3%)	—	—
Ampicillin	4 (13.8%)	3 (15.8%)	1 (11.1%)	—
Gentamicin	—	—	—	—
Streptomycin	2 (6.9%)	2 (10.5%)	—	—
Ciprofloxacin	5 (17.2%)	5 (26.3%)	—	—
Nalidixic acid	1 (3.4%)	1 (5.3%)	—	—
Cephalothin	5 (17.2%)	3 (15.8%)	1 (11.1%)	1 (100%)
Trimethoprim-sulfonamide	2 (6.9%)	2 (10.5%)	—	—

ence of *Yersinia* spp. in our study concurred with the previous findings and further confirmed the yersiniosis risks associated with consumption of unpasteurized milk.

In the present study, 3 bioserotypes (1A/O:9, 1B/O:8, and 4/O:3) were identified and, among them, 1A/O:9 was the most predominant *Y. enterocolitica* bioserotype. The high percentage rate of bioserotype 1A/O:9 in raw milk is congruent with earlier findings in Iran (Soltan-Dallal et al., 2004) and other countries (Bernardino-Varo et al., 2013). Although *Y. enterocolitica* biotype 1A, *Y. frederiksenii*, and *Y. kristensenii* are considered as environmental organisms that are nonpathogenic, these organisms could generate infection by virtue of virulence factors distinct from those of other *Y. enterocolitica* (Sulakvelidze, 2000). The pathogenic bioserotypes 1B/O:8 and 4/O:3 of *Y. enterocolitica* were only isolated from cow raw milk in the present study. These pathogenic bioserotypes were also isolated from animal

and animal food origin in previous studies (Bonardi et al., 2013; Tan et al., 2014).

The YstA and Ail proteins are important factors for *Y. enterocolitica* virulence. Hence, the existence of the *ystA* and *ail* genes, which encode these proteins, are used as appropriate pathogenicity markers of *Y. enterocolitica* isolates (Miller et al., 1989; Grant et al., 1998; Gierczynski, 2000; Thoerner et al., 2003). All the *Y. enterocolitica* isolates from raw milk samples were examined for the presence of the genes by PCR. The *ail* and *ystA* genes were detected in all isolates of bioserotypes 1B/O:8 and 4/O:3, which confirms previous findings (Grant et al., 1998; Thoerner et al., 2003; Platt-Samoraj et al., 2006; Bancarz-Kisiel et al., 2012). Conversely, all 15 isolates of biotype 1A harbored the *ystB* gene. In the previous studies, the *ystB* gene was found in 100, 87.3, and 80% of *Y. enterocolitica* biotype 1A isolated from aborted fetuses (Platt-Samoraj et al., 2006), feces of animals (Grant et al., 1998), and

Figure 1. Percentage resistance of *Yersinia* spp. isolated from raw milk against selected antibiotics..

humans, food, and the environment (Thoerner et al., 2003), respectively.

High resistance of *Yersinia* isolates to tetracycline in our study is in agreement with earlier findings (Subha et al., 2009). However, in another study, isolates of *Yersinia* were susceptible to tetracycline (Pham et al., 1995). Furthermore, high resistance of isolated *Yersinia* spp. from poultry to cephalothin and ampicillin was also observed in previous studies in Tehran Province, Iran (Soltan-Dallal et al., 2010; Jamali et al., 2014). A variety of antimicrobial agents are used against microbial infection and as growth promoters in animals. Excessive administrations of these agents have recently led to an upward trend in the incidence of antimicrobial-resistant isolates of microorganisms (Jamali et al., 2013). Tetracycline is commonly applied in animal food to control and treat infectious diseases in dairy farms in Iran. Hence, the significant resistance rates of the pathogen to tetracycline might be attributed to widespread application of the antibiotic (Jamali et al., 2013). Conversely, the presence of antimicrobial-resistant *Yersinia* in foods, especially in milk and the potential transmission of the pathogen with contaminated food, could be a public health concern for the consumers.

In summary, recovery of potentially pathogenic *Y. enterocolitica* from raw milk samples showed that consumption of raw milk may be a potential risk of yersiniosis. Therefore, further investigations on the prevalence of *Y. enterocolitica* as well as emerging antibiotic resistance are needed to enable the recognition of foods with significant risks and also to ensure effective treatments against yersiniosis.

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