PREVALENCE OF *YERSINIA ENTEROCOLITICA* IN RAW SMALL RUMINANT MILK IN SHAHREKORD, IRAN

S. M. ALAVI¹, E. RAHIMI² & E. TAJBAKHSH³

¹Graduated of Veterinary Medicine; ²Department of Food Hygiene, Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran; ³Department of Microbiology, Faculty of Basic Sciences, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran

Summary

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Yersinia enterocolitica is a common food-borne pathogen involved in yersiniosis among humans. The present study aimed to isolate virulent *Y. enterocolitica* from small ruminant milk in Shahrekord, Iran. One hundred raw milk samples of 84 sheep and 16 goats were collected from different parts of Shahrekord, during fall 2015. The samples were studied by microbial method and the positive-culture samples were investigated using PCR techniques. According to the results, 9% of all samples (sheep milk) were positive after microbial culture; subsequently 5% of positive samples were confirmed as O:3-positive bioserotypes. After gene detection, the *ail* gene was found in 4 isolates, the *yadA* gene in 3, the *virF* and *ystA* genes in 2 *Y. enterocolitica* strains. Isolation of *Y. enterocolitica* from raw milk suggested a high risk of yersiniosis associated with consumption of raw sheep milk.

Key words: goat, milk, sheep, *Yersinia enterocolitica*

INTRODUCTION

Yersinia enterocolitica is a small rod-shaped Gram-negative coccobacillus psychrotrophic enterobacterium, isolated from a variety of environmental sources, foods, and human clinical samples (Thornier *et al.*, 2003; Soltan-Dallal *et al.*, 2004; Myers *et al.*, 2006; Lambertz *et al.*, 2008). *Y. enterocolitica* is well known as a cause of yersiniosis in humans (Bernardino-Varo *et al.*, 2012). The most common clinical manifestation of yersiniosis is self-limiting gastroenteritis; however

the extraintestinal manifestations and post infectious sequelae such as reactive arthritis and erythema nodosum occur as well (Elisa *et al.*, 2010). *Y. enterocolitica* can grow to large numbers at refrigeration temperatures, so milk contaminated with that organism could become a significant health risk for consumers (Hanifian & Khani, 2012; Jamali *et al.*, 2013; 2015).

Most laboratories do not routinely screen for *Yersinia* species, which may explain its infrequent identification (Sol-

tan-Dallal *et al.*, 2004). The efficiency of PCR method is much more reliable than that of the culture method in detection of the virulent *Y. enterocolitica*. Direct isolation, even on selective medium is time-consuming yet seldom successful. Besides, there is no comprehensive culture method which could recover all pathogenic strains. Therefore, the low rates of isolation of pathogenic *Y. enterocolitica* in natural samples may be due to the limited sensitivity of culture methods (Rahimi *et al.*, 2014). In Iran the consumption of raw milk has traditionally been a very common practice. However, very little information is available on the prevalence of *Y. enterocolitica* in dairy products.

The aim of this study was to determine the prevalence of *Yersinia enterocolitica* in raw small ruminant milk in Shahrekord, Iran.

MATERIALS AND METHODS

Sample collection

A total of 100 raw milk samples, including 84 sheep milk samples, and 16 goat milk samples, were obtained from sheep and goat pens in various parts of Shahrekord, Iran, from October 2015 to November 2015. Each sample was collected in

clean, dry and sterile sampling bottle labelled to identify the source, site, and date of sampling. Transportation of the samples to the laboratory was done in ice boxes within 4 h after collection (Jamali *et al.*, 2013).

Culture methods

Aliquots of 500 µL of each milk sample were aseptically transferred into 5 mL *Yersinia* Enrichment Broth and the samples were incubated for 48 h at 29 °C. Then, a loopful of the mixture was streaked on Cefsulodin-Irgasan-Novobiocin Agar plates (CIN) and incubated for 24 h at 37 °C. The presumptive isolates were examined for response to biochemical tests such as Gram reaction, oxidase and catalase tests, indole production, etc.

PCR protocol

Purification of DNA from bacterial colonies was achieved using a genomic DNA purification kit (Fermentas, Germany) according to the manufacturer's instructions. The PCR reaction was carried out by thermal cycler (Eppendorf AG, Hamburg, Germany) under the following conditions: an initial denaturation at 94 °C for 4 min, 35 cycles of 94 °C for 30 s (denaturation), 60 °C for 1 min (annealing) and

Table 1. Primers used for detection of the various virulence genes of *Y. enterocolitica*

Primer sequence (5'-3')	Primer name	Gene	Amplicon length (bp)	Reference
CTTCAGATACTGGTGTCTGCTGT	yadA1	<i>yadA</i>	849	Saberianpour <i>et al.</i> , 2014
GAAGTCTTGAATCCCTGAAAACCG ACTCGATGATAACTGGGGAG	YC2 Ail1	<i>ail</i>	170	Collee <i>et al.</i> , 1998
CCCCCAGTAATCCATAAAGG AATGCTGTCTTCATTTGGAGCA	Ail2 Pr2a	<i>ystA</i>	145	Lambertz <i>et al.</i> , 2008
TCATGGCAGAACAGCAGTCAG ACTCATCTTACCATTAAGAAG	virf1 virf2	<i>virF</i>	590	Carter, 1994

Table 2. Prevalence of *Yersinia enterocolitica* in raw milk samples, serotyping, and PCR analysis of *ail*, *yadA*, *virF* and *ystA* genes in isolates of *Y. enterocolitica*

Samples	No. of samples	<i>Yersinia enterocolitica</i>	O:3 Sero-type	<i>Virulence genes</i>			
				<i>Ail</i>	<i>yadA</i>	<i>virF</i>	<i>ystA</i>
Sheep milk	84	9	5	4	3	2	2
Goat milk	16	–	–	–	–	–	–
Total	100	9	5	4	3	2	2

72 °C for 1 min (extension), followed by a final extension at 72 °C for 5 min (Thisted Lambertz & Danielsson-Tham, 2005). Detection of the *ail*, *yadA*, *ystA*, and *virF* genes was carried out using a multiplex PCR as described by Platt-Samoraj *et al.* (2006).

To specifically amplify the *Y. enterocolitica* O:3 serotype, a set of primers, (Yer O3-F:CGCATCTGGGACACTAATTCG) and (Yer O3-R:ACGAATTCCATCAAAACCACC), was used for detecting *rFbc* gene (Rahimi *et al.*, 2014). The primers of the various virulence genes used in the study are given in Table 1. The PCR products (454 bp) were visualised by agarose gel electrophoresis followed by staining of the agarose gel (1%) (Invitrogen, California, USA) with ethidium bromide (0.5 mg/mL) (Merck, Darmstadt, Germany). A 100-bp DNA ladder (Fermentas, St. Leon-Rot, Germany) was used to determine the size of PCR product.

RESULTS

After culturing on selective medium, 9 sheep milk samples (9%) positive for *Y. enterocolitica* were detected. No *Y. enterocolitica* was isolated from goat milks. After PCR examination, the genes of *Y. enterocolitica* were isolated from the positive-culture samples and all of these samples were confirmed. Subsequently,

5% of positive samples were confirmed as O:3-positive bio-serotypes (Table 2).

For gene detection, *ail*, *yadA*, *inv*, *ystA*, and *virF* were PCR-amplified and individual amplified fragments were subjected to agarose gel electrophoresis. According to the results, the *ail* gene was found in 4 isolates, the *yadA* gene in 3, the *virF* and *ystA* genes in 2 of *Y. enterocolitica* strains (Fig. 1).

Fig. 1. Agarose gel electrophoresis of polymerase chain reaction products (genes from *Yersinia enterocolitica*) amplified with the multiplex polymerase chain reaction method. Gene biotypes: *ail*, 170 bp; *yadA*, 849 bp; *ystA*, 145 bp; *virF*, 590 bp. M: 100 bp ladder, 1: negative control, 2, 3 and 5: positive samples, 4: positive control.

DISCUSSION

Milk has exceptional nutritional value, as the most complete and balanced food, exclusively consumed by humans in their very early life and excellent at any age (Giannino *et al.*, 2009). Several studies indicated that yersiniosis in humans was caused by consumption of contaminated food. In this study, *Y. enterocolitica* was isolated from 9 (9%) of the 100 milk samples tested (Table 2).

In this study, the prevalence of *Y. enterocolitica*, O:3 serotype, was 5%. Similar results were reported in different parts of Iran; Isfahan, Iran (5.07%) (Rahimi *et al.*, 2014), Varamin, Iran (6.5%) (Jamali *et al.*, 2015), northern-west of Iran (7.62%) (Hanifian & Khani, 2012).

In the present study sheep milk samples showed the prevalence of *Y. enterocolitica* contamination (9 out of 100; 9%). No *Y. enterocolitica* was isolated from goat milks. In comparison to goats, sheep usually have a mass of fat attached to the back of their rump and it is in touch with faecal contamination. Thus sheep milk would be contaminated by faeces during milking. This contamination is similar or lower and higher than that observed in the survey previously conducted in Iran and other countries on several kinds of dairy products. In Turkey, 47.3% prevalence of *Y. enterocolitica* contamination was observed (Yucel & Ulusoy, 2006). In Mexico, Bernardino-Varo *et al.* (2012) reported a 34.92% prevalence of *Y. enterocolitica* contamination. In our study 9 sheep milk samples (9%) were positive for *Y. enterocolitica*. In Iran, Jamali *et al.* (2015) showed that *Y. enterocolitica* contamination of sheep milk samples was 3% and that of goat milk samples – 2.4%.

Y. enterocolitica is one of the most common causes of foodborne gastroenteritis in western and northern Europe.

The incidence is also increasing in the United States and Canada, although this may be a result of improved surveillance and detection methods (Weynants *et al.*, 1996; Lamps *et al.*, 2001). Bacteriological examination and bio- and serotyping, commonly applied in diagnostics of infections with *Y. enterocolitica*, are time consuming and labour intensive, while at the same time they do not clearly identify pathogenic strains. Molecular methods, such as the multiplex PCR, which is particularly useful in showing the presence or absence of fragments of several genes, were used in this study for determining the prevalence of virulence genes in *Y. enterocolitica* (Weynants *et al.*, 1996). The pathogen was isolated from 13.6% of frozen dairy products in China (Ye *et al.*, 2014) which is in agreement with our study. In the present research, 9% of total samples including sheep milks were positive after microbial culture; subsequently 5% of positive samples were confirmed as O:3-positive bioserotypes. In a study performed by Jamali *et al.* (2015), the *ystA* gene was observed in all the isolates having bioserotypes 1B/O:8 or 4/O:3. However, the *ail* gene was only detected from biotype 4/O:3 and all isolates of *Y. enterocolitica* biotype 1A harboured only the *ystB* gene. Hanifian & Khani (2012) reported 2.26% isolation of the *ail* gene of raw milks and cheese samples. In this research the *ail* gene was found in 4 isolates, the *yadA* gene in 3, the *virF* and *ystA* genes in 2 of *Y. enterocolitica* strains. In Iran (Shahrekord), Saberianpour *et al.* (2014) detected 65 contaminated samples among the 300 samples of chicken meat purchased from different markets. In our study, 9 samples of sheep milk were positive for *Y. enterocolitica* strains.

Among the serogroups that cause disease in humans, O:3, O:8, O:9, and O:5,27 are the most prevalent. Strains belonging to bioserotype O:3 are the most frequently detected pathogenic *Y. enterocolitica* over the world (Garzetti *et al.*, 2014). In the United States but also in Europe, *Y. enterocolitica* O:3 is the serotype most frequently implicated in disease (Bernardino-Varo *et al.*, 2012). In our research, a specific detection was obtained with *rFbC* primers, which included a PCR product of the expected size exclusively with pathogenic *Y. enterocolitica* of serotype O:3.

Virulence genes, such as *ail* and *yst*, are located on the bacterial chromosome. The Ail protein is encoded by the *ail* gene and occurs only in pathogenic *Y. enterocolitica*; it helps the bacterial adhesion to the host cell as well as intensifies resistance to the bactericidal effects of complement (Hanifian & Khani, 2012; Jamali *et al.*, 2015). Moreover, the *yst* gene, which encodes the thermostable enterotoxin Yst protein, facilitates the invasion of the pathogen into tissues. The YstA and YstB are produced by pathogenic and nonpathogenic *Y. enterocolitica* (Saberianpour *et al.*, 2014). In a research performed by Saberianpour *et al.* (2014), multiplex PCR assay results showed that chromosomal virulence genes included *inv* (100%), *ail* (50%) and *ystA* (51.85%), and plasmid-encoded virulence factors included *yadA* (44.74%) and *virF* (35.18%). In another study, the *ail* gene was found in 100% of pathogenic *Y. enterocolitica* strains and the *yadA* gene in only 86% of pathogenic *Y. enterocolitica* strains, but neither of the genes were detected in non-pathogenic strains of *Yersinia* spp. (Blais & Phillippe, 1995). In this research, multiplex PCR test results showed the *ail* gene in 4 (4%) isolates, the *yadA* factor in

3 (3%), the *virF* and *ystA* genes in 2 (2%) of *Y. enterocolitica* strains.

These results show that sheep milk and its traditional products can be a risk factor for public health of humans. There are not precise data on the consumption of raw small ruminant's milk or on the frequency of this organism. Furthermore, no routine search for this bacterium is performed, making it impossible to know its real epidemiological relevance and the potential health risk for the population consuming raw milk. For these reasons and because there has been scant research related to food contamination by *Y. enterocolitica*, this work sought to demonstrate the presence of this bacterium in raw milk of sheeps and goats in Shahrekord and to find the most abundant bioserotypes in these samples. This study showed that the prevalence rate of virulent *Y. enterocolitica* was relatively high and demonstrated the potential for the transmission of the bacterium to humans via the consumption of raw and non-pasteurised milk and dairy products.

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Correspondence:

Prof. Ebrahim Rahimi
Department of Food Hygiene,
Faculty of Veterinary Medicine,
Shahrekord Branch, Islamic Azad University,
Shahrekord, Iran
Tel: +98 38 33361060, Fax: +98 31 36259809
Email: ebrahimrahimi55@yahoo.com;
rahimi@iaushk.ac.ir