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Detection of major food-borne pathogens in raw milk samples from dairy bovine and ovine herds in Iran

Fakhri Haghi [a], Habib Zeighami [a,*], Ghazal Naderi [b], Ali Samei [b], Shekoufeh Roudashti [b], Shahin Bahari [b], Paniz Shirmast [b]

[a] Department of Microbiology, Zanjan University of Medical Sciences, Zanjan, Iran
[b] Student Research Center, Zanjan University of Medical Sciences, Zanjan, Iran

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ABSTRACT

Food safety has emerged as an important global issue with international trade and public health implications. Bacterial pathogens are major etiological agents of diseases related to the consumption of dairy products and represent a major public health problem in developing countries. Fast and accurate diagnosis of food-borne pathogens using molecular methods such as polymerase chain reaction is very important for a positive outcome of eradication programs. A total of 60 individual raw milk samples were randomly collected from 4 dairy bovine and ovine herds and investigated the presence and the frequency of *Listeria monocytogenes*, *Campylobacter jejuni*, *Coxiella burntii*, *Mycobacterium tuberculosis* complex and *Brucella* spp. Overall, 36 (60%) milk samples were positive for the presence of at least one selected food-borne pathogens. The most prevalent pathogen in milk samples was *Brucella* spp. (53.3%), followed by *M. tuberculosis* complex (13.3%) and *C. burnetii* (11.6%). No *L. monocytogenes* and *C. jejuni* were detected from any of the milk samples in our study. *C. burnetii* was detected with slightly higher frequency in bovine samples (8.3%) than in ovine milk samples (3.3%). Moreover, nine (14.9%) bovine milk samples were contained simultaneously more than one pathogen. These evidences reinforce the need to optimize quality programs of dairy products, to intensify the sanitary inspection of these products and the necessity of further studies on the presence of these pathogens in milk and milk products.

1. Introduction

The traditional consumption of homemade dairy products composed of raw milk poses a serious public health threat (Di Pinto et al., 2006). Bacterial pathogens are major etiological agents of diseases related to the consumption of dairy products, accounting for 90% of all cases (Marília Masello Junqueira et al., 2013). Among the bacterial pathogens, *Brucella* spp., *Listeria monocytogenes*, *Campylobacter jejuni*, *Coxiella burntii*, *Staphylococcus aureus*, *Salmonella* spp. and *Mycobacterium tuberculosis* complex are the most important food-borne pathogens and represent a major public health problem worldwide. Although, governmental surveillance of milk pasteurization and sanitation in dairy processing plants was performed in Iran for many years, direct sale of unpasteurized milk and dairy products from producers to the consumer is not uncommon in many regions including Zanjan province. Therefore, it is essential to gather information about microbial risk factors and hazards associated with raw milk production. Risk assessment and microbial monitoring will continue to play important role in quality assurance of milk and milk-related products (Xiaofeng et al., 2007; Konosonoka et al., 2012). The culture based approaches for diagnosis of these pathogens are quite laborious and many times remain inconclusive (Singh et al., 2012). However, PCR has been increasingly used for the rapid, sensitive, direct and specific detection of foodborne pathogens (Khan et al., 2013).

Brucellosis is the most widespread zoonotic disease transmitted from animals by direct contact with animal products or through consumption of unpasteurized milk and milk products (Tina et al., 2013; Gupta et al., 2006; Kamal et al., 2013). Brucellosis causes infertility and abortion in bovines and undulant fever in humans (Mukherjee et al., 2007). Although eradication programs are being applied in several countries, brucellosis remains a public health problem with severe economic consequences in developing countries (Jafar et al., 2015).

C. jejuni is a leading cause of acute bacterial gastrointestinal infection worldwide (Feizabadi et al., 2007). *Campylobacter* associated gastroenteritis is thought to occur through zoonotic transmission, being acquired from exposure to tainted food and/or

* Corresponding author. Fax: +98 2433449553.
E-mail address: zeighami@zums.ac.ir (H. Zeighami).

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contaminated drinking water. Several food-borne outbreaks have been associated with the consumption of unpasteurized milk (Josiane da et al., 2012).

L. monocytogenes is an important agent of food-borne diseases and listeriosis is associated with the highest case mortality rate of 30% approximately, unlike infection with other common food-borne pathogens, such as *Salmonella*, which rarely results in fatalities (Khan et al., 2013). Milk plays important role in *L. monocytogenes* epidemiology (Konosonoka et al., 2012).

C. burnetii is the causative agent of Q fever, an important zoonotic disease with worldwide distribution. Cats and farm animals (cattle, sheep and goat) are identified as sources of human infection (Rahimi et al., 2010). *C. burnetii* is mainly shed during and after parturition or abortion in birth products but shedding also occurs in urine, faeces, vaginal mucus and milk (Van den Brom et al., 2013). Humans are usually infected by inhalation of aerosol and dust containing *C. burnetii* in a contaminated environment. Unpasteurized milk or milk products may contain virulent *C. burnetii* and Q fever can be transmitted through consumption of these products (Khalili et al., 2015).

Tuberculosis in cattle, the most important known source of human food-borne tuberculosis is predominantly associated with *M. tuberculosis* complex (Messelhäusser et al., 2011). These microorganisms are highly able to survive in milk and transmitted by milk and dairy products (Marília Masello Junqueira et al., 2013).

The objective of the present study was to investigate the presence and the frequency of major food-borne pathogens *L. monocytogenes*, *C. jejuni*, *C. burntii*, *M. tuberculosis* complex and *Brucella* spp. in raw milk samples collected from 4 dairy herds in Zanjan, Iran.

2. Materials and methods

2.1. Collection of milk samples

In this cross-sectional study, a total of 60 individual unpasteurized milk samples (one sample per animal) including 38 bovine and 22 ovine samples were collected from 4 dairy farms in different rural areas in Zanjan, Iran from June to August 2014. The animals whose milk samples collected for this study were clinically healthy and the milk samples showed physical (color, pH, and density) consistency. Milk samples were taken under forceful hygienic conditions and immediately transported to the laboratory in a cooler with ice packs and stored at −20 °C.

2.2. Reference strains

The following reference strains were used as positive controls: *L. monocytogenes* ATCC 7644, *C. jejuni* ATCC 27853, Bacillus Calmette-Guerin (BCG) strain ATCC 27289, *Coxiella burnetii* Nine Mile phase I/RSA 493 and *Brucella abortus* 544 (ATCC23448).

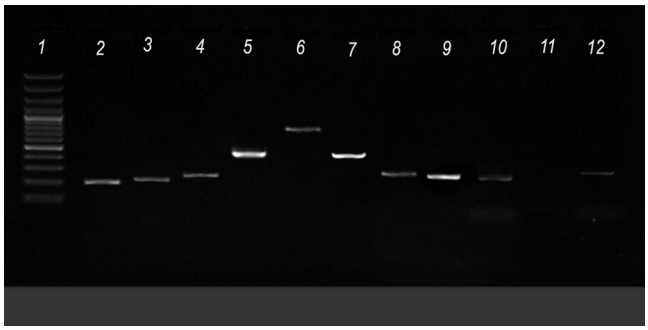

Fig. 1. PCR amplification of reference strains and milk samples. M: molecular marker (100 bp DNA Ladder), Lane 1: *Listeria monocytogenes* ATCC 7644 (*prfA*), Lane 2: *Brucella abortus* 544 (ATCC23448) (*bcsp31*), Lane 3: Bacillus Calmette-Guerin (BCG) strain ATCC 27,289 (*IS6110*), Lane 4: *Coxiella burnetii* Nine Mile phase I/ RSA 493 (*com2*), Lane 5: *Ca mpylobacter jejuni* ATCC 27,853 (*hipO*), Lane 6-12: positive and negative milk samples

2.3. Extraction of genomic DNA from milk samples

DNA extraction and purification was performed using the protocol described previously (Gupta et al., 2006). Briefly, 50-ml frozen milk samples were thawed at room temperature and centrifuged at 12,000 × *g* for 5 min. After removing the cream and milk layers, the precipitate was mixed with 100 μl of TE buffer (1 mM EDTA and 10 mM Tris–HCl; pH 7.6). To that mixture, 100 μl of 24% sodium dodecyl sulfate was added as a denaturing agent. The mixture was incubated at 100 °C for 10 min and then cooled on ice. Proteinase K (650 μg/mL) was added and the mixture was kept at 37 °C for 1 h. The cell debris was removed by precipitation with 5 M NaCl and hexadecyltrimethylammonium bromide-NaCl (CTAB-NaCl) solution at 65 °C for 10 min. Deoxyribonucleic acid was extracted by standard methods with a phenol–chloroform–isoamyl alcohol mixture (25:24:1), and then precipitated with isopropanol, washed with ethanol, and dried under vacuum. The DNA pellet was dissolved in 30 μl of sterile distilled water and stored at −20 °C until further use.

2.4. Detection of pathogenic bacteria by PCR

All milk samples were screened for direct detection of *L. monocytogenes*, *C. jejuni*, *Brucella* spp., *C. burnetii* and *M. tuberculosis* complex using the primers listed in Table 1. PCR assays were performed using the protocols described previously (Mukherjee et al., 2007; Carvalho et al., 2014; Abd El-Malek et al., 2010) for the detection of the following markers: *IS6110* (specific insertion sequence of *M. tuberculosis* complex); *bcsp31* (genus specific *Brucella* cell surface

Table 1
Primers used in this study.

Bacterial pathogen	Target gene	Primer sequence (5′ → 3′)	Amplicon size (bp)	Ref.
C. burnetii	com1	AGTAGAAGCATCCCAAGCATTG TGCCTGCTAGCTGTAACGATTG	501	Zhang et al. (1998)
	com2	GAAGCGCAACAAGAAGAACAC TTGGAA GTTATCACGCAGTTG	438	Zhang et al. (1998)
L. monocytogens	prfA	TCATCGACGGCAACCTCGG TGAGCAACGTATCCTCCAGAGT	217	Abd El-Malek et al. (2010)
Brucella spp.	bcsp31	TGGCTCGGTTGCCAATATCAA CGCGCTTGCCTTTCAGGTCTG	223	Mukherjee et al. (2007)
C. jejuni	hipO	GAAGAGGGTTTGGGTGGTG AGCTAGCTTCGCATAATAACTTG	735	Ghorbanalizadgan et al. (2014)
M. *tuberculosis* complex	IS6110	CGTGAGGGCATCGAGGTGGC GCGTAGGCGTCGGTGACAAA	245	Carvalho et al. (2014)