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PREVALENCE AND ANTIMICROBIAL RESISTANCE OF *LISTERIA* SPECIES ISOLATED FROM TRADITIONAL DAIRY PRODUCTS IN CHAHAR MAHAL & BAKHTIYARI, IRAN

E. RAHIMI[1], H. MOMTAZ[2], A. SHARIFZADEH[2], A. BEHZADNIA[3], M. S. ASHTARI[4], S. ZANDI ESFAHANI[4], M. RIAHI[5] & M. MOMENI[5]

[1]Departments of Food Hygiene; [2]Department of Microbiology; [4] Undergraduate student of Veterinary Medicine, College of Veterinary Medicine, [3]Graduate student of Food Sciences and Technology, College of Agriculture; [5]Food Research Center, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran

Summary

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A surveillance study was carried out to determine the prevalence of *Listeria* spp. in traditional dairy products in Chahar Mahal & Bakhtiyari province, Iran. From February 2009 to February 2010, a total of 290 samples of various traditional dairy products were obtained from randomly selected retail stores located in 6 major cities of the province. Using cultural method, 21 samples (7.2%) were found positive for *Listeria* spp. The highest prevalence of *Listeria* was found in traditional ice-cream (16.7%), followed by cheese (15.0%), butter (7.5%), and kashk (2.2%) samples. The overall prevalence of *Listeria* was 7.2%, in which *L. innocua* was the most commonly recovered species (66.6%); the remaining isolates were identified as *L. monocytogenes* (23.8%), *L. murrayi* (4.8%) and *L. seeligeri* (4.8%). All 5 *Listeria* strains identified as *L. monocytogenes* were also positive using polymerase chain reaction (PCR). Susceptibilities of the 21 strains to nine antimicrobial drugs were determined using the disk diffusion assay. All isolates were resistant to one or more antimicrobial agents. Six strains (28.6%) were resistant to a single and 5 strains (23.8%) showed resistance to two antimicrobial agents. Multi-drug resistance was established in 23.8% of *Listeria* strains. Resistance to nalidixic acid was the commonest finding (85.7%), followed by resistance to penicillin (47.6%), and tetracycline (33.3%). The results provide information about the contamination levels of traditional dairy products in one of the provinces of Iran and highlight the emergence of multi-drug resistant *Listeria* in the environment.

Key words: antimicrobial resistance, dairy products, Iran, *Listeria* spp.

INTRODUCTION

Listeria species are ubiquitous bacteria, well adaptable in the environment, in animal and vegetable foods. The genus *Listeria* comprises seven species. Six of them (*L. grayi, L. innocua, L. ivanovii, L. welshimeri, L. murrayi* and *L. seeligeri*) are not usually pathogenic for humans, while *L. monocytogenes* is considered one of the major foodborne pathogens that can induce listeriosis in humans and animals (McLauchlin, 1997). Human listeriosis is associated with consumption of contami-

nated milk, soft cheese, undercooked meat, and unwashed raw vegetables and cabbage (Oliver *et al.,* 2005; Aygun & Pelivanlar, 2006; Colak *et al.*, 2007). It may range from mild flu-like sickness to severe manifestations. Groups at highest risk are pregnant women, neonates, adults with underlying disease, elderly and immunocompromised individuals (McLauchlin *et al.,* 2004).

The excessive use of antimicrobials has led to antibiotic resistance and particularly multiresistance, which are important public health concerns since they may cause failure of therapeutic treatment. Furthermore, antimicrobials used as growth promoters in animal feed have resulted in the dissemination of antimicrobial-resistant bacteria into the environment (Jansen *et al.,* 2003). Monitoring the antimicrobial resistance of *L. monocytogenes* in humans and animals is important to control the use of antimicrobial agents and prevent the spread of multi-drug resistant bacteria (Harakeh *et al.,* 2009).

Currently, there is limited information regarding the prevalence and antimicrobial susceptibility patterns of *Listeria* spp. in foods in Iran. Therefore, the present study was undertaken to determine the prevalence and the antimicrobial resistance rate of *Listeria* strains isolated from traditional dairy product samples in Chahar Mahal & Bakhtyari province, Iran.

MATERIALS AND METHODS

Food samples

A total of 290 samples of various traditional dairy products (Table 1) were obtained from randomly selected retail stores located in 6 major cities of the Chahar Mahal & Bakhtiyari province from February 2009 to February 2010. This province is located in the central and southern part of Iran with about 850,000 inhabitants. All samples were immediately transferred to the Food Microbiology lab in the Islamic Azad University, Shahrekord Branch, in portable insulated cool boxes. The samples were analysed on the day they were collected.

Isolation and identification of Listeria

The samples were analysed for the presence of *Listeria* spp. using the selective enrichment and isolation protocol recommended by the United States Department of Agriculture (McClain & Lee, 1988). Twenty-five grammes of each sample were aseptically taken, blended for 2 min in 225 mL of *Listeria* enrichment broth (UVM I) (Merck, Germany) and incubated at 37 °C for 24 h. One mL of primary enrichments was transferred to 9 mL of UVM II (Frazer broth) (Merck, Germany) and incubated at 37 °C for 24 h. Secondly enrichments were streaked onto Oxford and Palcam agars (Merck, Germany) and incubated at 35 °C for 48 h. The plates were examined for typical *Listeria* colonies (black colonies with black sunken centres) and at least 3 suspected colonies were subcultured on Trypton Soy agar supplemented with 0.6% of yeast extract (TSAYE) (Merck, Germany) and incubated at 37 °C for 24 h. All the isolates were subjected to standard identification and biochemical tests including Gram staining, catalase test, motility at 25 °C and 37 °C, acid production from glucose, mannitol, rhamnose, xylose, α-methyl-D-mamoside, and nitrate reduction, hydrolysis of esculin, MR/VP test, ß-haemolytic activity, and CAMP test (Aygun & Pehlivanlar, 2006).

DNA extraction and PCR condition

Only *L. monocytogenes* isolates identified by bacteriological methods were tested by

PCR as described previously (Zhou & Jiao, 2005). Briefly, 1 mL of pure culture of *L. monocytogenes* was centrifuged at 13,000×*g* for 5 min at room temperature. The DNA was then extracted using a genomic DNA purification kit (Fermentas, GmbH, Germany, K0512) according to the manufacturer's protocol. Oligonucleotide primers for the PCR assay were selected based on the published nucleotide sequence of the *actA* gene (Cai *et al.*, 2002). The pair of primers 01 (5′-GCT GATTTAAGAGATAGAGGAACA-3′) and 02 (5′-TTTATGTGGTTATTTGCT GTC-3′) were used to amplify an 827 bp DNA fragment that corresponds to the 3′ end region of *actA* gene. A 25 µL aliquot of PCR buffer contained 22 µL of PCR supermix (0.2 µL of each primer at 12.5 µM, 2.5 µL of 10 × PCR buffer, 1.0 µL of 25 mM $MgCl_2$, 1.0 µL of 1 mM of dNTPs mix, 0.1 µL of 3 U/mL Taq DNA polymerase in 17 µL of ddH_2O). Three µL of each supernatant were added to the PCR mix. Thermocycling conditions included an initial hold of 2 min at 94 °C, a denaturation step at 95 °C for 10 s, annealing at 60 °C for 30 s and a 30 s extension at 72 °C for a total of 40 cycles. A final hold at 4 °C followed a final extension at 72 °C for 10 min. Amplification reactions were carried out using a DNA thermal cycler (Master Cycler Gradiant, Eppendrof, Germany). The PCR products were examined by electrophoresis in a 1.5% agarose gel, stained with 1% ethidium bromide, and examined under UV illumination. In the present study, DNA extracted from *L. monocytogenes* (laboratory control strain) and DNase free water were used as positive and negative controls, respectively.

Antimicrobial susceptibility testing

Susceptibilities of 21 *Listeria* isolates from 21 samples were determined for 9 antimicrobial drugs using the disk diffusion assay. Antimicrobial susceptibility testing was performed by the Kirby-Bauer disc diffusion method using Mueller-Hinton agar (HiMedia Laboratories, Mumbai, India, MV1084) supplemented with 5% defibrinated sheep blood, according to the Clinical and Laboratory Standards Institute (CLSI, 2006). The antimicrobial agents tested and their corresponding concentrations were as followed: nalidixic acid (30 µg), ciprofloxacin (15 µg), erythromycin (15 µg), tetracycline (15 µg), gentamicin (10 µg), vancomycin (30 µg), ampicillin (10 µg), chloramphenicol (30 µg), and penicillin (10 U/IE). After incubation at 42 °C for 48 h in a microaerophilic atmosphere, the susceptibility of *Listeria* spp. to each antimicrobial agent was measured and the results were interpreted in accordance with interpretive criteria provided by CLSI (2006). *Staphylococcus aureus* and *Escherichia coli* were used as quality control organisms in antimicrobial susceptibility determination.

Statistical analysis

Data were transferred to a Microsoft Excel spreadsheet (Microsoft Corp., Redmond, WA, USA) for analysis. Using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA), a chi-square test and Fisher's exact two-tailed test analysis was performed and differences were considered significant at values of $P<0.05$.

RESULTS

In the present study, a total of 290 samples of various traditional dairy products collected in Chahar Mahal & Bakhtyari province of Iran were tested for *Listeria* species (Table 1).

Table 1. Prevalence of *Listeria* spp. in traditional dairy products in Iran

Type of food	Number of samples	Number (%) of *Listeria* spp.	Number (%) of *L. monocytogenes*	Number (%) of *L. innocua*	Number (%) of *L. seeligeri*	Number (%) of *L. murrayi*
Cheese *	60				–	–
Ice-cream	48	8 (16.7) [a]	–	8 (16.7)	–	–
Yogurt	55	– [b]	–	–	–	–
Doogh **	42	– [b]	–	–	–	–
Butter	40	3 (7.5) [a, b]	–	2 (5.0)	1 (2.5)	–
Kashk ***	45	1 (2.2) [b]	–	–	–	1 (2.2)
Total	290	21 (7.2)	5 (1.7)	14 (4.8)	1 (0.3)	1 (0.3)

*Made from raw sheep or cow milk; **A dairy product prepared by beating unflavoured yogurt until smooth, and then diluting with water to a consistency similar to whole milk; ***A dairy product prepared by prolonged boiling of yogurt. Values in the same column with different superscripts (a, b) are significantly different ($P<0.05$).

Table 2. Antimicrobial resistance profiles of *Listeria* isolated from traditional dairy products in Iran, according to the species

Antimicrobial agent	*Listeria* spp. (n=21)	*L. monocytogenes* (n=5)	*L. innocua* (n=14)	*L. seelegari* (n=1)	*L. murrayii* (n=1)
Ampicillin	4 (19.0%)	2 (40.0%)	–	1 (100%)	1 (100%)
Chloramphenicol	3 (14.3%)	1 (20.0%)	2 (14.9%)	–	–
Ciprofloxacin	6 (28.6%)	1 (20.0%)	4 (28.6%)	1 (100%)	–
Erythromycin	4 (19.0%)	3 (60.0%)	–	–	1 (100%)
Gentamicin	–	–	–	–	–
Nalidixic acid	18 (85.7%)	3 (60.0%)	13 (92.9%)	1 (100%)	1 (100%)
Penicillin	10 (47.6%)	3 (60.0%)	7 (50.0%)	–	–
Tetracycline	7 (33.3%)	3 (60.0%)	4 (28.6%)	–	–
Vancomycin	–	–	–	–	–
Resistance to 1 antimicrobial	6 (28.6%)	2 (40.0%)	4 (28.6%)	–	–
Resistance to 2 antimicrobials	5 (23.8%)	1 (20.0%)	4 (28.6%)	–	–
Resistance to 3 antimicrobials	5 (23.8%)	–	3 (21.4%)	1(100%)	1(100%)
Resistance to > 4 antimicrobials	5 (23.8%)	2 (40.0%)	3 (21.4%)	–	–

Using cultural techniques, 21 of 290 samples (7.2%) were positive for *Listeria* spp. The highest prevalence of *Listeria* was found in traditional ice-cream (16.7%), followed by cheese (15.0%), butter (7.5%), and kashk (2.2%) samples. The overall prevalence of *Listeria* was 7.2%, in which *L. innocua* was the most prevalent species recovered (66.7%); the remaining isolates were *L. monocytogenes* (23.8%), *L. murrayi* (4.8%) and *L. seeligeri* (4.8%). All 5 *Listeria* strains identified as *L. monocytogenes* were also positive using polymerase chain reaction (PCR). There were significant differences ($P<0.05$) in the level of contamination with *Listeria* between different traditional dairy products.

Susceptibilities of 21 strains were determined for nine antimicrobial drugs using the disk diffusion assay (Tables 2 and 3). All isolates were resistant to one or more antimicrobial agents. Six strains (28.6%) were resistant to a single and 5 strains (23.8%) showed resistance to two antimicrobial agents. Multi-drug resistance was found in 47.6% of *Listeria* strains. Resistance to nalidixic acid was most commonly encountered (85.7%), followed by resistance to penicillin (47.6%), and tetracycline (33.3%).

DISCUSSION

Traditional dairy products in Iran are produced in small productive centers mostly located in urban areas and distributed unpacked. These products may be produced from unpasteurized milk. In the present study, 7.2% of the traditional dairy product samples examined were positive for *Listeria* spp. This is in agreement with the results reported by El-Sharef *et al.* (2006) and Arslan & Özdemir (2008). Among all dairy products tested in this study, traditional ice-cream and cheese had the highest incidence of *Listeria* spp. These microorganisms, being able to grow at 4 ^{o}C make contamination of ice cream a frequent finding (Warker *et al.,* 2000, Akman *et al.,* 2004). The prevalence of *Listeria* spp. in ice-cream samples found in this study is comparable with the results reported by Warker *et al.* (2000), Jalali & Abedi (2008) and Rahimi *et al.* (2010). However, Beak *et al.* (2000) and El-Sharef *et al.* (2006) reported higher incidence rate of *L. monocytogenes* (4 and

Table 3. Antimicrobial resistance profiles of 21 *Listeria* spp. strains isolated from traditional dairy products in Iran in the disk diffusion assay

Antimicrobial agent	Number of isolates (%)		
	Resistant	Intermediate	Susceptible
Ampicillin	2 (9.5%)	2 (9.5%)	17 (81.0%)
Chloramphenicol	1 (4.8%)	2 (9.5%)	18 (85.7%)
Ciprofloxacin	5 (23.8%)	1 (4.8%)	15 (71.4%)
Erythromycin	3 (14.3%)	1 (4.8%)	17 (81.0%)
Gentamicin	–	–	21 (100%)
Nalidixic acid	14 (66.7%)	4 (19.0%)	3 (14.3%)
Penicillin	9 (42.9%)	1 (4.8%)	11 (52.4%)
Tetracycline	4 (19.0%)	3 (14.3%)	14 (66.7%)
Vancomycin	–	–	21 (100%)

6.2%, respectively). In a similar study by Abrahao *et al.* (2008) on cheese and ice cream in the State of Parana, Brazil, all 60 tested ice cream samples were found to be negative for *Listeria* spp. Whereas *L. innocua* was found to a large extent in the analysed ice cream samples, *L. monocytogenes* was not encountered. It should be mandatory to perform routine controls to screen ice cream samples for detection of *Listeria* spp.

Contamination of different type of cheese with *Listeria* spp. has been also reported. The incidence of *Listeria monocytogenes* in soft and semi-soft cheese varied from 0.0% to 46.00% in Chile (Cordano & Rocourt, 2001), Iran (Jalali & Abedi, 2008; Rahimi *et al.* 2010; Mahmoodi, 2010), Turkey (Colak *et al.,* 2007; Arslan & Özdemir, 2008), Canada (Farber *et al.,* 1987), Norway (Rorvik & Yndestad, 1991), and Portugal (Pintado *et al.,* 2005). In a study in Noorabad, Iran, *L. monocytogenes* was detected in 3.3% and 6.7% of white cheese samples that were collected from two traditional dairy manufacturers (Mahmoodi, 2010). In another study conducted in Isfahan province of Iran, *Listeria* spp. was identified in 18.9% of 90 cheese samples in which *L. innocua* was detected in 8.2% and *L. monocytogenes* was detected in 10.0% of samples (Rahimi *et al.,* 2010). Silva *et al. (*1998) examined 103 samples of 3 different types of cheese. Of the analysed samples, 11 (10.7%) were contaminated with *L. monocytogenes*, 13 (12.6%) with *L. innocua*, 6 (5.8%) with *L. grayi*, and 1 (1%) with *L. welshimeri*. Also, Pintado *et al.* (2005) reported that 47 of 63 soft cheese samples made from raw sheep milk were positive for *Listeria* spp., in which 29 samples (46%) were positive for *L. monocytogenes* and 18 samples (29%) were positive for *L. innocua*. Similarly, in a study in Turkey, Arslan & Özdemir (2008) isolated *Listeria* spp. in 33.1% of homemade white cheese samples. In another study conducted in the Isfahan province of Iran, *Listeria* spp. and *L. monocytogenes* were identified in 18.9% and 10.0% of 90 cheese samples (Rahimi *et al.,* 2010).

In the present study, no *Listeria* isolate was detected in yogurt and doogh and only 1 kashk and 3 traditional butter samples were positive for *Listeria* spp. These results are consistent with those reported by Aygun & Pehlivanlar (2006) and Jalali & Abedi (2008). Survival of *Listeria* spp. in foods depends on the sample acidity; the bacteria disappear when the pH falls to 3.5 (Cottin *et al.,* 1990). Furthermore, it has been shown that bacteriocins produced by the lactic acid bacteria reduce significantly the number of *L. monocytogenes* in yogurt (Benkerrom *et al.,* 2003). The absence of *Listeria* spp. in yogurt, doogh and kashk samples and the low prevalence of *Listeria* spp. in butter in our study could possibly be attributed to the acidity of these products; however, is also could be due to the boiling step during their processing.

It is interesting to note that *L. innocua* was the predominant isolate among *Listeria* species in our study (Table 1). *L. innocua* was found as the commonest *Listeria* species isolated from milk and dairy products by other investigators (Jalali & Abedi, 2008; Dhanshree *et al.,* 2003).

The results of antimicrobial susceptibility testing in the present study indicate a high resistance of *Listeria* spp. to nalidixic acid, penicillin, tetracycline, and ciprofloxacin, and to a lesser extent to erythromycin, ampicillin, and chloramphenicol. These results are comparable to those reported by Pesavento *et al.* (2009) from Italy, Arslan & Özdemir (2008) from Turkey, and Rahimi *et al.* (2010) from

Iran. The results of antimicrobial resistance found in this study are correlated to antibiotics that are being used to treat infection in food animals in Iran.

In conclusion, the presence of *Listeria* spp. has been shown in variety of traditional dairy products in Iran. The results of this study indicate the potential risk of infection with *Listeria* in people consuming these products. Further extensive prevalence studies on the occurrence of *Listeria* spp. among farmers, milk-processing workers, and veterinarians, and on possible dangers of dairy products will be needed to elucidate the epidemiology of listeriosis in Iran.

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Correspondence:

Ebrahim Rahimi,
Department of Food Hygiene,
College of Veterinary Medicine,
Shahrekord Branch, Islamic Azad University,
Shahrekord-Iran
e-mail: ebrahimrahimi55@yahoo.com