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Occurrence and Antibiotic Resistance of *Listeria monocytogenes* in Retail Minced Beef Distributed in Ahvaz, South-West of Iran

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Abstract

Background: *Listeria monocytogenes* is one of the most important food-borne bacteria causing septicemia, meningitis and encephalitis in humans. The objective of this study was to evaluate the occurrence and antibiotic resistance of the bacterium in retail minced beef in Ahvaz, South-West of Iran.

Methods: In this survey, 150 samples of minced beef were randomly obtained from retail butcheries in Ahvaz, Iran and tested for presence of *L. monocytogenes*. The procedure was one-step enrichment in *Listeria* enrichment broth followed by plating on oxford agar. Isolated colonies were subjected to subsequent biochemical tests and polymerase chain reaction (PCR) assay using the target *iap-P60* gene encoding P60 protein. Susceptibility of the isolates to various antibiotics was investigated by Kirby-Bauer disk diffusion method. The results were analyzed by chi-square test and fisher's exact test using SPSS 16.0 software.

Results: The incidence of *Listeria* spp. was 2.7% (4 of 150 samples) and only one sample (0.66%) was contaminated to *L. monocytogenes*. Statistical analysis showed no significant difference in prevalence of *Listeria* between various regions (p>0.05). The isolate was resistant to streptomycin and showed an intermediate susceptibility to tetracycline and penicillin. However, it was sensitive to other tested antibiotics.

Conclusion: Our findings showed the presence of antibiotic resistant *L. monocytogenes* strain among beef samples in this region of Iran and so, indicated the potential risk for public health from consumption of raw or undercooked beef which may increase the possibility of acquisition of resistance to antibiotics.

Introduction

Listeria monocytogenes, the most important species of *Listeria* spp., is commonly present in food, water, feed, soil and sewage. This food-borne pathogen causes acute complications such as septicemia, meningitis and encephalitis in humans especially infants, pregnant women, the elderly people and also immune-compromised persons (Boughattas and Salehi, 2014; Drevets and Bronze, 2008; Rahimi et al., 2012a; Rahimi et al., 2012b). *Listeria* can grow at low temperatures with an optimum pH requirement of 5.4-9.6 (Jalali and Abedi, 2008; McLauchlin et al., 2004).

Antimicrobial resistance of pathogenic microorganisms is a worldwide public health concern because of increasing global trade and travelling (Doyle et al., 2013). *L. monocytogenes* is naturally susceptible to various anti-

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biotics targeting Gram-positive bacteria (Charpentier and Courvalin, 1999). It has been indicated that clinical strains of *L. monocytogenes* are sensitive to a wide range of antibiotics. Typically, invasive infections have been treated by combination of ampicillin or amoxicillin and gentamicin (Gómez et al., 2014).

According to the high reported mortality rate of *L. monocytogenes* (about 30%), the presence of this bacterium in food is considered as one of the major health problems (Pesavento et al., 2010). A wide range of food types has been implicated in transmission including meat, dairy, fish and vegetable products. The occurrence of *Listeria* spp. as well as *L. monocytogenes* in meat and raw meat products has been investigated in several countries (Karakolev, 2009; Marian et al., 2012; Ozbey et al., 2013; Wieczorek et al., 2012). To the best of our knowledge, few studies regarding the prevalence and antimicrobial susceptibility of *L. monocytogenes* in foodstuff in Iran has been documented (Jalali and Abedi, 2008; Rahimi et al., 2012a; Rahimi et al., 2012b). The present study was conducted to determine the occurrence and antimicrobial resistance of *L. monocytogenes* isolated from raw minced beef in Ahvaz, South-West of Iran.

Materials and methods

Sample collection

In this cross-sectional survey, during a six month period (January 2013–July 2014) a total of 150 samples of minced beef (250 g) were obtained from 67 retailer shops located in North, South, West, East and central regions of Ahvaz city (South-West of Iran). Samples were put in cold box and immediately transferred to the laboratory and then, they were microbiologically analyzed on same day.

Microbial culture

In the first step, samples were analyzed for the detection of *Listeria* spp. using enrichment, selective and isolation protocol, recommended by the US Food and Drug Administration (Lovett, 1989). For each sample, individually 25 g was aseptically removed, blended in 225 ml of *Listeria* enrichment broth (Merck, Germany), and incubated at 35 °C for 48 h. Enrichment cultures were streaked onto oxford agar (Merck, Germany) plates, incubated at 35 °C for 48 h, and examined for *Listeria* (black colonies with black halo). All suspected *Listeria* colonies were subjected to some standard biochemical tests, including catalase test and a motility test at 25 °C. For further confirmation, other biochemical reactions containing acid production from maltose, mannitol, rhamnose and xylose, β-haemolytic activity on 5% sheep blood agar (Merck, Germany) and MRVP test were performed.

PCR assay

In the next step and for final confirmation of *L. monocytogenes*, the colonies were tested by PCR assay. Template DNA was obtained by boiling method from a pure culture of the suspected isolate, which was grown in Tripticase Soy Broth (TSB; Merck, Germany) at 30 °C for an overnight. Briefly, the overnight culture was centrifuged, the pellet was resuspended in 1 ml of deionized water (dH_2O), and the sample was boiled at 100 °C for 10 min. After heating step, the obtained suspension was centrifuged at 14000 rpm for 10 min, and then the supernatant was used as the PCR template. Also, the DNA of standard *L. monocytogenes* (ATCC 7644) and distilled water were used as positive and negative controls, respectively. Each PCR tube contained 50 µl of reaction mixture, consisting of PCR buffer (10X, 5µl), $MgCl_2$ (50 mM, 1.5 µl), Taq DNA polymerase (5U/µl, 0.5 µl), dNTPs Mix (10 mM, 1 µl), primers (100 pmol/µl, 1 µl each), dd H_2O (36 µl) and 5 µl (100 ng) of template DNA. The used primers (Table 1) were P60-Protein-coding gene (*iap-P60*) according to Kim et al. (2007). The cycling conditions used in the thermal cycler (Bioer, China) were as follows: first denaturation at 94 °C (3 min, 1 cycle), denaturation at 94 °C for 45 s, annealing at 60 °C for 45 s and extension at 72 °C for 1 min. After 35 cycles, a final cycle comprised a 5 min extension step at 72 °C. The amplified PCR products were detected by agarose gel electrophoresis (Paya Pajoohesh Pars, Iran), stained, and visualized under UV light illumination (Kiagen, Iran).

Antimicrobial susceptibility testing

According to the method of CLSI (2006), antimicrobial susceptibility tests were carried out using Kirby-Bauer disk diffusion method. The tested antimicrobial agents were ampicillin, amikacin, erythromycin, streptomycin, penicillin, tetracycline, gentamicin, chloramphenicol, cotrimoxazole and vancomycin. A swab was taken from each bacterial suspension (1×10^7 CFU/ml) and stroked trolley on Mueller-Hinton agar (Merck, Germany), and then antibiotic discs (Padtan Teb, Iran) were placed on the agar. After incubation at 35 °C for 24 h, the diameter of inhibition zone was measured for each antibiotic. Then, the isolates were classified as resistant, intermediate (reduced susceptibility) or sensitive.

Statistical analysis

The results were analyzed by chi-square test and fisher's exact test using SPSS 16.0 software. Mean values

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were considered statistically different at 95% confidence levels.

Results

Out of 150 examined minced beef samples, on oxford agar, black colonies were observed on 68 culture plates, with similarity to *Listeria* spp. colonies. By biochemical tests, four samples (2.7%) were positive for *Listeria* spp. and the rest were retained negative. Among the positive samples, *L. monocytogenes*, *L. innocua* and *L. grayi* were isolated from 1 (0.66%), 1 (0.66%) and 2 (1.33%) samples, respectively. Statistical analysis showed no significant difference in prevalence of *Listeria* spp. between different regions (p>0.05). Positive *L. monocytogenes* isolate was confirmed by the PCR assay. Fig. 1 shows the results on agarose gel electrophoresis for the isolate, positive and negative control samples.

Data revealed that the only detected isolate of *L. monocytogenes* was resistant to streptomycin and intermediate resistant to penicillin and tetracycline. However, it was sensitive to other tested antibiotics.

Table 1: Primers used for detection of *L. monocytogenes* in this study

Target gene	Primer sequence	Product size (bp)	Reference
iap	Forward: 5'-CTGGCACAAAATTACTTACAACGA-3´ Reverse: 5'-AACTACTGGAGCTGCTTGTTTTTC-3´	454	Kim et al. (2007)

Table 2: Antibiotic-resistant *L. monocytogenes* isolated from meat products in the previous studies

Antibiotic	Sample type	Reference
Ampicillin	crude meat + meat products	Pesavento et al. (2010)
	minced beef + beef burger	Marian et al. (2012)
	ready-to-eat foods	Domenech et al. (2014)
	ready-to-eat foods	Shi et al. (2015)
Gentamycin	crude meat + meat products	Pesavento et al. (2010)
Tetracycline	crude meat + meat products	Pesavento et al. (2010)
	ready-to-eat foods	Domenech et al. (2014)
Streptomycin	poultry carcasses	Antunes et al. (2002)
	ready-to-eat foods	Kovacevic et al. (2012)
Gentamicin	minced beef + beef burger	Marian et al. (2012)
Chloramphenicol	meat products	Yücel et al. (2005)
	minced beef + beef burger	Marian et al. (2012)
	ready-to-eat foods	Kovacevic et al. (2012)
Cephalothin	ready-to-eat foods	Domenech et al. (2014)
	ready-to-eat foods	Shi et al. (2015)
Co-trimoxazole	ready-to-eat foods	Domenech et al. (2014)
Clindamycin	ready-to-eat foods	Kovacevic et al. (2012)
	ready-to-eat foods	Shi et al. (2015)
Gentamicin	poultry meat	Alonso-Hernando et al. (2012)
Vancomycin	foodstuffs	Conter et al. (2009)
	raw meat + retail foods	Pesavento et al. (2010)
Penicillin G	retail foods	Walsh et al. (2001)
Amikacin	ready-to-eat foods	Kovacevic et al. (2012)

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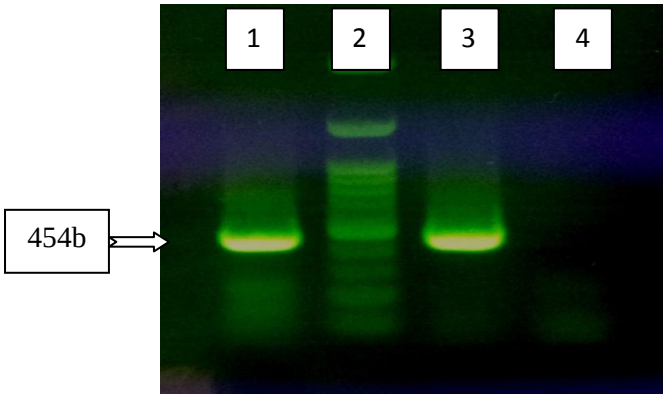


Fig. 1: PCR results on agarose gel electrophoresis. Lane 1: positive control (*L. monocytogenes* ATCC 7644); Lane 2: 100 bp DNA ladder; Lane 3: *L. monocytogenes* isolate; Lane 4: negative control

Discussion

In this study, *L. monocytogenes* was found in 0.7% of the analyzed minced beef samples. The existence of the bacterium in raw meats poses a health threat only when the meat is consumed raw or undercooked. Many incidence of listeriosis have been resulted from consumption of contaminated foodstuffs such as dairy, meat, vegetables, seafood, etc. (Rahimi et al., 2012a; Rahimi et al., 2012b). Presence of *L. monocytogenes* in raw meat increases the risk of listeriosis in people who consume undercooked meat. So, to ensure food safety, this pathogen should be absent in 25 g of foodstuff (Rantsiou et al., 2008). Overall, 2.7% (4 of 150) of all minced beef samples in the present work were contaminated with *Listeria* spp. and only in one sample, *L. monocytogenes* was detected. It is noticeable that the presence of other *Listeria* spp. may indicate the presence of more numbers of *L. monocytogenes*. Since both species share ecological niches, the presence of *L. innocua* could be an indicator of probable contamination with *L. monocytogenes* (Jinneman et al., 1999). Thus, when looking for sources of *L. monocytogenes*, the presence of other species especially *L. innocua* could be managed as equally significant. Importantly, in our survey different *Listeria* species were isolated from different butcheries across the city. Statistical analysis showed no significant difference in prevalence of *Listeria* between different regions. Therefore, it could be suggested that the source of contamination may be the main abattoir of Ahvaz (where in most of the annual slaughtered cattle are introduced), not butchery environment.

There are several reports regarding to isolation of *Listeria* spp. and *L. monocytogenes* from raw meat and meat products all over the world. For example, in Bulgaria, 786 samples (containing 505 samples of fresh meat and 281 samples of raw-dry and raw-smoked sausages) were analyzed for *Listeria* spp. From beef and pork samples, 7.7%, 0.6%, 4.6% and 0.8% were contaminated to *L. monocytogenes*, *L. ivanovii*, *L. innocua* and *L. welshimeri*, respectively (Karakolev, 2009). In another study in Malaysia, *L. monocytogenes* was detected in 8.57% of meat product samples (Marian et al., 2012). According to Wieczorek et al. (2012), it was found that 44 out of 406 hide samples (10.8%) were contaminated with *L. monocytogenes*, whereas 10 (2.5%) corresponding bovine carcasses were positive for this pathogen in Poland. There are limited data regarding prevalence of *L. monocytogenes* in minced beef consumed in Iran. Jalali and Abedi (2008) found that *Listeria* spp. were detected in 6.7% of meat and meat product samples, in 1.3% of diary samples, in 1.2% of vegetable samples and in 12% ready-to-eat samples in Isfahan, Iran. Rahimi et al. (2012b) reported that out of 1107 different meat samples collected in Iran, 141 samples (12.7%) were positive for *Listeria* spp. The most common recovered species was *L. innocua* (75.9%) followed by *L. monocytogenes* (19.1%). Our findings are not in agreement with the high prevalence of *Listeria* spp. in raw meat reported by previous Iranian researchers. It could be due to season, geographic conditions, sanitations in abattoir, methodological differences, etc. The number of *L. monocytogenes* is affected by the ability of the microorganisms to survive and grow in different types of food products. Low number of *Listeria* isolates in this survey may also be due to real low incidence in the products or presence of live-injured bacterial cells (LIBC) which cannot grow properly on culture media. This is a big concern in public health regarding presence of LIBC because they may be undetectable by regular culture methods but potentially are pathogenic under favorable conditions (Marian et al., 2012).

It is known that meat and meat products may be infected at slaughterhouse due to cross-contamination occurred during evisceration, slicing, mincing and other processing stages. Meat and meat products are stored under refrigeration and the absence of competitive microorganisms along with suitable water activity and pH values of the food allow this psychrotolerant pathogen to grow in high levels (Vitas and Garcia-Jalon, 2004). For preparing minced meat, the release of blood and meat juices during cutting, deboning and grinding of meat also favor growth of *Listeria* and may cause increase in contamination during the processing of raw meat products (Rahimi et al., 2012b; Vitas and Garcia-Jalon, 2004).

The results of the antimicrobial susceptibility tests indicated intermediate susceptibility of the isolated *L. monocytogenes* to penicillin and tetracycline. The bacterium also showed resistance to streptomycin and susceptibility to other antibiotics. These results are in

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agreement with previous works (Altuntas et al., 2012; Pesavento et al., 2010; Walsh et al., 2001) that reported *L. monocytogenes* isolates from meat products were resistant to streptomycin, penicillin and tetracycline. According to previous studies, *L. monocytogenes* isolated from meat and foodstuffs and their antibiotic resistance are indicated in Table 2. Considering the increasing number and type of new antibiotic resistant strains of *L. monocytogenes* reported worldwide, it seems that this bacterium probably obtains various antibiotic resistance genes by horizontal gene transfer, some of which may come from the commensal microorganisms found in foods (Lungu et al., 2011). On the other hand, genetic mutation in the new strains is possible, too. Although *L. monocytogenes* strains with resistance to one or more antibiotics have been isolated from food source but generally resistance to antibiotics commonly used to treat listeriosis such as ampicillin, amoxicillin with or without gentamicin or trimethoprim-sulfamethoxazole (co-trimoxazole) has rarely been observed (Gómez et al., 2014).

Conclusion

Our findings show the presence of antibiotic resistant *L. monocytogenes* strain among beef samples in this region of Iran and so, indicate the potential risk for public health from consumption of raw or undercooked beef which may increase the possibility of acquisition of resistance to antibiotics.

Conflicts of interest

The authors indicated that there is no conflict of interest in this research.

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