Occurrence and antimicrobial susceptibility of *Listeria monocytogenes* isolates from retail raw foods

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Abstract
The occurrence and counts of *Listeria monocytogenes* were investigated in a total of 526 retail raw food samples. All *L. monocytogenes* isolates were further analyzed by serotyping and antimicrobial susceptibility assays. The molecular basis of tetracycline resistance of each isolate and the genetic relatedness were determined. *L. monocytogenes* isolates were found in 12.4% (65/526) of the samples, with counts below 10^2 CFU/g. *L. monocytogenes* was most commonly isolated from pork (20%, 20/100), seafood (13.8%, 15/109), chicken (13.2%, 14/106), and beef (10.3%, 11/107). In addition, *L. monocytogenes* was also detected in 4.8% (5/104) of raw mutton samples. Four serogroups were identified among the 65 *L. monocytogenes* isolates, with serogroups 1/2a-3a (60%) and 4b-4d-4e (24.6%) being dominant. Most *L. monocytogenes* isolates were resistant to cefotaxime (54.6%), fosfomycin (51.5%), and clarithromycin (36.4%). Some isolates showed intermediate resistance to streptomycin (12.1%), norfloxacin (13.6%), ciprofloxacin (13.6%), and nitrofurantoin (9.1%). Multiple resistances were observed in 72.3% of isolates. Genetic relatedness analysis revealed that there were no prominent associations between specific food types, serotypes, antimicrobial susceptibility profiles and Pulsed-field gel electrophoresis (PFGE) patterns. In addition, these isolates were multiresistant and belonged to the epidemiologically important serotypes 1/2a and 4b, implying a potential public health risk.

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1. Introduction

*Listeria monocytogenes* is a gram-positive, facultative intracellular pathogen that can cause serious human and animal infections, including abortion and septicemia (Rocourt & Cossart, 1997). Epidemiological studies have demonstrated that *L. monocytogenes* is an important foodborne pathogen (Farber & Peterkin, 1991). Foodborne listeriosis linked to *L. monocytogenes* was first reported in 1981 (Schlech et al., 1983). Since then, several outbreaks of foodborne listeriosis associated with this bacterium have been documented all over the world (Denny & McLauchlin, 2008; Warriner & Namvar, 2009). *L. monocytogenes* isolates associated with outbreaks of listeriosis have been detected in various kinds of products including dairy products, raw meat, vegetables and seafood (Bell & Kyriakides, 2005; Schlech, 2000; Yücel, Çitak, & Önder, 2005). The high occurrence of *L. monocytogenes* in foods (Farber et al., 1991; Jorgensen & Huss, 1998) and the high mortality rate (up to 30%) associated with listeriosis have contributed to *L. monocytogenes* being considered a pathogen of major concern (Jalali & Abedi, 2008).

Serogroup determination is usually used for the microbiological characterization and a rapid initial screening in epidemiological investigations of *L. monocytogenes*. Furthermore, serotyping is widely used for long-term microbiological surveillance of human listeriosis (Doumith, Buchrieser, Glaser, Jacquet, & Martin, 2004). To date, 13 serotypes of *L. monocytogenes* have been identified, but only serotypes 1/2a, 1/2b, 1/2c, and 4b are frequently isolated from foods and patients, with serotypes 1/2a, 1/2b, 3b, and 4b causing most cases of human listeriosis (Farber et al., 1991).

Currently, ampicillin or penicillin G combined with gentamicin is the reference therapy for human listeriosis, whereas trimethoprim-sulfamethoxazole, vancomycin and erythromycin...
are regarded as second-choice drugs to treat listeriosis in pregnant women (Hof, 2004). However, since the first isolation of a multi-resistant strain in France in 1988 (Poyart-Salmeron, Trieu-Cuot, Carlier, & Courvalin, 1990), the number of strains resistant to one or more antibiotics used for treating listeriosis has continually increased (Granier et al., 2011; Morvan et al., 2010; Yan et al., 2010). The levels of resistance vary and are influenced by antimicrobial use and geographical differences. Therefore, it is necessary to monitor the occurrence and the antibiotic susceptibility of \textit{L. monocytogenes} on a worldwide basis. China is one of the major producers and consumers of food-producing animals; however, there is a paucity of information on the prevalence and antimicrobial susceptibility of \textit{L. monocytogenes}.

Therefore, this study investigated the occurrence, the serotypes, the antimicrobial susceptibility, and the molecular basis of tetracycline resistance of \textit{L. monocytogenes} isolates from fresh raw foods.

2. Materials and methods

2.1. Samples, \textit{L. monocytogenes} detection and enumeration

Four hundred and sixty-three raw samples of pork (\(n = 100\)), beef (\(n = 107\)), mutton (\(n = 104\)), chicken meat (\(n = 106\)), and seafood (\(n = 109\)) were randomly collected from six cities in Heilongjiang province in the Northeast of China from 2008 to 2009. Detection and enumeration of \textit{L. monocytogenes} were carried out as recommended by ISO 11290-1 and 11290-2 methods, respectively (1996, 1998). All culture media and selective supplements were from Oxoid Ltd. (Oxoid, Hampshire, UK) unless otherwise mentioned.

\textit{L. monocytogenes} detection consisted of a resuscitation step in which 25 g of sample were transferred to sterile plastic bags containing 225 mL of half-Fraser (HF) broth without supplements, homogenized using a Stomacher 400-laboratory blender (Seward Medical, London, UK), and incubated at 20 °C for 1 h. After this resuscitation step, selective supplement SR-166 was added to the HF broth, following incubation at 30 °C for 72 h before the addition of the supplement SR-166, aliquots of HF broth were taken and submitted to serial decimal dilutions in 0.1 mL of loading buffer and separated on a 2% agarose gel in a TBE buffer (90 mM Trizma base, 90 mM boric acid, 2 mM EDTA, pH 8.3). The PCR products were visualized by ethidium bromide staining.

2.2. Serotyping

Strains identified as \textit{L. monocytogenes} were serotyped using a multiplex PCR method (Doumith et al., 2004), with some modifications. Briefly, each isolate was inoculated onto Trypticase Soy Agar (TSA; BD, Sparks, MD) supplemented with 0.6% of yeast extract, and the plates were incubated for 48 h at 37 °C. Three to five bacterial colonies from each purified isolate were emulsified in 50 µL of 0.25% sodium dodecyl sulfate and 0.05 N sodium hydroxide and heated at 99 °C for 15 min. Then, 100 µL of deionized distilled H₂O was added to the mixture, 1 µL of which was used as the PCR template. PCR was performed in a final volume of 20 µL containing 1 µL of template DNA and 10 µL of 2 × PCR Mix (MBI Fermentas). The five primer sets were added at the following final concentrations: 1 µM for \textit{lmo073}, ORF2819, and ORF2110; 1.5 µM for \textit{lmol}118; and 0.2 µM for \textit{prs}. PCR was performed with an initial denaturation step at 94 °C for 3 min; 35 cycles of 94 °C for 0.40 min, 53 °C for 1.15 min, and 72 °C for 1.15 min; and one final cycle of 72 °C for 7 min in a thermal cycler (Applied Biosystems). Five microliters of the reaction mixture was mixed with 3 µL of loading buffer and separated on a 1% agarose gel in a TBE buffer (90 mM Trizma base, 90 mM boric acid, 2 mM EDTA, pH 8.3). The PCR products were visualized by ethidium bromide staining.

2.3. Antimicrobial susceptibility testing

Susceptibility to the following antimicrobial agents was determined for all \textit{L. monocytogenes} strains by the microdilution broth method. The antibiotics used were (concentration range in µg/mL): ampicillin (0.03–32), penicillin (0.03–32), cefalozin (0.12–128), cephalothin (0.06–64), cefotaxime (0.12–128), fosfomycin (0.125–128), vancomycin (0.06–64), trimethoprim-sulfamethoxazole (0.6–640), nitrofurantoin (0.25–256), tetracycline (0.125–128), kanamycin (0.125–128), gentamicin (0.03–32), streptomycin (0.25–256), norfloxacin (0.03–32), ciprofloxacin (0.03–32), clari-thromycin (0.03–32), and erythromycin (0.06–64). The results were interpreted according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006a,b). Because there are no CLSI breakpoints for fosfomycin that are applicable to \textit{Listeria} or \textit{Staphylococcus}, the breakpoint for sensitivity was defined as ≤64 µg/mL, as reported by Troxler, von Graevenitz, Funke, Wiedemann, and Stock (2000). \textit{Staphylococcus aureus} ATCC 29213, \textit{Escherichia coli} ATCC 25922, and \textit{Enterococcus faecalis} ATCC 29212 were used as quality control strains.

2.4. Detection of tetracycline resistance determinants and the \textit{Tn916} transposon

Tetracycline-resistant and tetracycline-susceptible \textit{L. monocytogenes} strains were screened for the four tetracycline resistance genes. The tetracycline resistance determinants \textit{tet(L)}, \textit{tet(M)}, \textit{tet(K)} and \textit{tet(S)} were amplified by PCR with specific primers as previously described (Ng, Martin, Alfa, & Mulvey, 2001). To investigate whether the \textit{Tn916} transposon was present in the \textit{tet(M)}-positive isolates, the \textit{int-Tn} gene, which encodes for the integrase of \textit{Tn916}-\textit{Tn1545}, was also amplified as described by Morvan et al. (2010).

2.5. Pulsed-field gel electrophoresis (PFGE)

PFGE was carried out as described previously (Graves & Swaminathan, 2001) except that genomic DNA from all of the isolates was digested with Asc I. The electrophoretic parameters used were as follows: initial switch time, 4.0 s; final switch time, 40.0 s; run time, 22 h; angle, 120°; gradient, 6.0 V/cm; temperature, 14 °C; and ramping factor, linear. Xba I-digested plugs of Salmonella serovar Braenderup H9812 standards served as size markers. PFGE patterns were analyzed with BioNumerics version 5.1 (Applied Maths, Sint-Martens-Latem, Belgium). If a difference in the PFGE pattern was observed, a new pulstype was assigned. The definition of a PFGE cluster was based on a similarity cutoff of 100% (Latorre et al., 2009) using the Dice coefficient (with an optimization of 1% and a position tolerance of 0.5%–1%), and the relationships were
represented using UPGMA. All PFGE clusters were given in alphabetical order.

3. Results and discussion

*L. monocytogenes* was detected in each food categories tested. The overall incidence of the pathogen in all the samples was 12.4% (65/526). Among raw meat samples, raw pork samples had the highest occurrence of *L. monocytogenes* (20%, 20/100), followed by 13.2% (14/106) in raw chicken, 10.3% (11/107) in raw beef, and 4.8% (5/104) in raw mutton samples; these findings are similar to those of previous studies (Mayrhofer, Paulsen, Smulders, & Hilbert, 2004; Pesavento, Ducci, Nieri, Comodo, & Lo Nostro, 2010; Yücel et al., 2005). The presence of *L. monocytogenes* in raw meats could be the result of fecal contamination during evisceration, environmental contamination, or the actions of food handlers (Fenlon, Wilson, & Donachie, 1996; Skovgaard & Nørrung., 1989; Yücel et al., 2005). The overall prevalence of *L. monocytogenes* isolates in raw meats was 12% (50/417), which is higher than that found in other studies in China. Relative to these past results, an increasing in raw meats was 2.47% in 2001, 7.1% during 2003–2005, 6.28% during 2005–2007, 11.67% during 2006–2009, and 12% during 2008–2009 in the study (Chao, Zhou, Jiao, 2009; Yan, et al., 2010). Among the 109 seafood samples, 15 were positive for *L. monocytogenes*, equivalent to a contamination level of 13.8%. The prevalence of *L. monocytogenes* in other studies ranged from 5.2% to 39.0% in seafood samples (Gudbjörnsdóttir et al., 2004; Qian, & Xu, 2007; Yan et al., 2007; 2010; Yücel et al., 2005). This difference could be partly due to the sizes of samples, the sampling season, and the isolation methods (Hansen, Vogel, & Gram, 2006).

When these samples were carried out counting of *L. monocytogenes* by using the ISO 11290-2 enumeration method, none of them presented colonies in the OXO and Palcam agar plates. These results imply that these samples in which the presence of *L. monocytogenes* was detected but the pathogen could not be enumerated, the level of contamination was lower than 10^2 CFU/g, the detection limit of the method. Even the low level of contamination, the high prevalence of *L. monocytogenes* in these samples cannot be neglected. Although meat is normally eaten after cooking, we have not to underestimate the risk of listeriosis, due to the consumption of this type of food, because of, the worldwide increasing practice of eating raw food as "carpaccio", "sushi" and hamburger not totally cooked inside (Pesavento et al., 2010).

The antimicrobial susceptibility tests of *L. monocytogenes* isolates (Table 1) revealed that resistance to cefotaxime (54.6%) was the most prevalent type of resistance, followed by resistance to fosfomycin (51.5%) and clarithromycin (36.4%). It is not surprising that there were high rates of resistance among *L. monocytogenes* isolates to cefotaxime, fosfomycin, and clarithromycin in the study because *Listeria* spp. have been reported to be naturally resistant to, or to have an intermediate susceptibility to, most "modern" cephalosporins, fosfomycin, and macrolides (Troxler et al., 2000). However, unexpectedly, *L. monocytogenes* isolates showed high resistance to cephalothin (98.5%) and cefazolin (98.5%) in the study. The *Listeria* genus is also thought to be naturally susceptible to antibiotics active against gram-positive bacteria, including ampicillin or penicillin (combined with aminoglycosides), trimethoprim (alone or combined with sulfamethoxazole), tetracyclines, erythromycin, and gentamicin (Rota et al., 1996; Teuber, 1999; Yücel et al., 2005). The high susceptibility of isolates to ampicillin (97%), penicillin (95.4%), tetracyclines (95.5%), gentamicin (94%), erythromycin (95.5%), and trimethoprim-sulfamethoxazole (95.5%) was evident in the study (Table 1). Similar results were also observed in other reports (Conter et al., 2009; Yan et al., 2010). Fortunately, 94.6% of our *L. monocytogenes* isolates were vancomycin susceptible; this drug is one of the last therapeutic options for the treatment of human infections. However, some isolates showed intermediate resistance to streptomycin (12.1%), norfloxacin (13.6%), ciprofloxacin (13.6%), and nitrofurantoin (9.1%), which might be in part due to the excessive use of these antibiotics in veterinary medicine. Furthermore, the antimicrobial resistance of *L. monocytogenes* may be associated with the presence of a plasmid or may be determined by genes that are transferred by conjugation and mutational events in chromosomal genes (Harakeh et al., 2009; Poros-Gluchowska & Markiewicz, 2003). Since the first report of antibiotic-resistant strains of *L. monocytogenes* (Poyart-Salmeron et al., 1990), strains resistance to one or more agents have been found in food samples or other sources in recent years (Conter et al., 2009; Yan et al., 2010; Zhang et al., 2007). Likewise, in the present study, 72.3% (47) of isolates were multiresistant to at least 3 of the antibiotics tested, with resistance to 3, 4, 5, 6 and 7 antimicrobials agents being found in 12, 19, 13, 2, and 1 isolates, respectively. These findings suggest that *L. monocytogenes* is slowly becoming resistant to antibiotics (Conter et al., 2009). Thus, attention also needs to be focused on monitoring the antimicrobial resistance of *L. monocytogenes* in humans and animals to understand changes in the patterns of resistance to commonly used antimicrobials, to implement pro-active measures to control the use of antimicrobial agents and to prevent the spread of multi-drug resistant strains, which can have many undesired consequences (Harakeh et al., 2005).

Four different serogroups were identified among the 65 *L. monocytogenes* strains (Table 2). Of the 65 isolates, 39 (60%) of Table 2

<table>
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<th>Source</th>
<th>Number of serotype-positive isolates (%)</th>
<th>Total food isolates</th>
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<td></td>
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<td>Pork</td>
<td>11(57) 7(35) 0 2(10)</td>
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<tr>
<td>Chicken</td>
<td>11(78.5) 3(21.5) 0 0</td>
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<tr>
<td>Beef</td>
<td>7(63.6) 0 0 4(36.4)</td>
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<tr>
<td>Mutton</td>
<td>5(100) 0 0 0</td>
<td>5</td>
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<tr>
<td>Seafood</td>
<td>5(33.3) 6(40) 4(26.7) 0</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>39(60) 16(24.6) 4(6.2) 6(9.2)</td>
<td>65</td>
</tr>
</tbody>
</table>
**Fig. 1.** The dendrogram based on the UPGMA and pulsed-field gel electrophoresis profiles of 59 *L. monocytogenes* isolates.

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<th>Isolate no.</th>
<th>PFGE</th>
<th>Serotype</th>
<th>Sources</th>
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<tr>
<td>n11</td>
<td>AA</td>
<td>4b, 4d, 4e</td>
<td>pork</td>
</tr>
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</table>
belonged to serogroup 1/2a-3a, 16 (24.6%) belonged to serogroup 4b-4d-4e, 4 (6.2%) belonged to serogroup 1/2b-3b, and 6 (9.2%) belonged to serogroup 1/2c-3c. Serogroup 1/2a-3a was found in each type of food sample, serogroup 4b-4d-4e was commonly present in seafood (40%, 6/15) and pork samples (35%, 7/20). Because serotypes 3a, 3b, 4d, and 4e are relatively rare in foods, the isolates identified as serogroups (1/2a-3a), (1/2b-3b), and (4b-4d-4e) were presumably serotypes 1/2a, 1/2b, and 4b, respectively (Zhang et al., 2007). The high prevalence of serotype 1/2a in this study is in agreement with the results of other studies showing that serotype 1/2a is the most prevalent serotype in foods (Nucera et al., 2010; Yan et al., 2010; Zhang et al., 2007). Serotypes 1/2a and 4b are more often associated with cases of human listeriosis than other serotypes (Farber et al., 1991; Fugett, Schoonmaker-Bopp, Dumas, Corby, & Wiedmann, 2007), and these serotypes were common in food category included in this study. The presence of these important serotypes among such food items indicates that these foods may pose a potential public health risk, although a coincidence might also exist due to the limited number of isolates.

All isolates were tested for four tet genes, tet(K), tet(L), tet(S) and tet(M). The tet(M) gene was the only tet gene detected in the 3 tetracycline-resistant isolates, and this gene was not found in the tetracycline-susceptible *L. monocytogenes*. The 3 tetracycline-resistant isolates were also screened for the presence of tetracycline-resistance, but this gene was not detected in strains harboring tet(M). Therefore, the tetracycline resistance of *L. monocytogenes* observed in this study is not due to the acquisition of Tn916-Tn5545, but this gene was not detected in strains harboring tet(M). Therefore, the tetracycline resistance of *L. monocytogenes* observed in this study is not due to the acquisition of Tn916-related transposons (Poyart-Salmeron, Trieu-Cuot, Carrier, & Courvalin, 1989), in contrast to previously obtained results (Morvan et al., 2010; Poyart-Salmeron et al., 1992).

All 65 tested strains after digestion with Ascl I were analyzed for genetic relatedness by PFGE: 59 isolates were typable and had 43 different PFGE patterns, whereas in the case of 6 isolates, repeated attempts failed to obtain discrete PFGE patterns. In the dendrogram produced by the UPGMA algorithm, the isolates were clustered in 27 groups (A to AA) containing between 1 and 8 isolates each (Fig. 1). Clusters E (8 isolates) and N (8 isolates) were predominant and belonged to serotypes 1/2b-3b and 1/2a-3a, respectively. The same clones were found in distant related food samples such as chicken, beef and seafood. This suggests that a dominant clone is either widespread in this region or that contamination may have occurred at the market places (Filiousis, Johansson, Frey, & Perreten, 2009).

Analysis of antimicrobial susceptibility in combination with serotypes, PFGE patterns and sources showed that there were no correlations except among four isolates from seafood (nb002, nb004, nb007, and nb008); these isolates belong to cluster E1 and have identical antimicrobial susceptibility profiles (susceptible to all drugs tested other than cefotaxime). As in this study, associations between specific food types and specific serotypes, antimicrobial susceptibility profiles and PFGE patterns were not found for 90 *L. monocytogenes* isolates from raw meat products, cooked meat and seafood (Yan et al., 2010). The absence of such associations may be the result of the limited number of isolates under investigation; to better understand and control sources of *L. monocytogenes* contamination, a large number of isolates from broad geographical and source ranges should be included in further studies.

Our data showed that *L. monocytogenes* contamination occurred in raw meat and seafood; these isolates are increasingly resistant to one or more antibiotics, and our isolates belonged to serotypes 1/2a and 4b, which are associated with human listeriosis, suggesting that this pathogen may represent a potential danger to public health. Thus, there is great need for a surveillance programs in China to monitor epidemiological information on *L. monocytogenes* diffusion in different sources.

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