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Isolation and characterization of *Listeria monocytogenes* from the black-headed gull feces in Kunming, China

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ABSTRACT

Listeria monocytogenes is among the most important foodborne pathogens, which can be isolated from bird feces. The aim of this study was to examine the prevalence, genotypes, virulence factors and drug-resistance characteristics of *L. monocytogenes* isolated from the black-headed gull in Kunming, China. Serotypes and virulence genes were identified by polymerase chain reaction. Pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) methods were used to describe the genetic characteristics of all positive isolates. Furthermore, all *L. monocytogenes* isolates were tested for antimicrobial susceptibility by disk diffusion method. All isolates were positive for *inlA*, *inlB*, *plcB*, *prfA*, *iap*, *actA* and *hly* genes. Serotyping showed the isolates belonged to serotype 1/2b (2/9, 22%), 1/2c (3/9, 33%), and 4a (4/9, 44%). PFGE showed 4 different pulsotypes (PTs), and MLST differentiated the 9 isolates into 4 sequence types (STs), ST3 (1), ST5 (1), ST35 (3) and ST201 (4). The result of antimicrobial susceptibility revealed that all isolates were naturally sensitive to most antibiotics. The *L. monocytogenes* isolates had pathogenic potential. There is a potential infection risk for people who come to frequent contact with black-headed gulls in Kunming. Hence, it is necessary to conduct surveillance of *L. monocytogenes* in the migratory black-headed gulls, which would be valuable to prevent listeriosis in local residents and tourists.

Introduction

Listeria monocytogenes, a gram-positive bacterium, is among the most important foodborne pathogens due to the high mortality rate and severity of the infection. Previous studies show that *L. monocytogenes* can be isolated from a variety of bird feces, including black-headed gull [4,15]. *L. monocytogenes* is frequently isolated from dairy products and poultry [12], and also can be isolated from domestic and wild animals [5]. *L. monocytogenes* can cause invasive diseases in humans, especially people of low immunity, including the elderly, pregnant women and newborns [10]. The main clinical symptoms are meningitis, encephalomyelitis, and new born septicemia and infection may be fatal [14]. Previous studies had reported that listeriosis can also be caused by *Listeria ivanovii*, *Listeria innocua* and *Listeria seeligeri* [3,7]. Pathogenicity of *L. monocytogenes* is determined by many virulence factors [14], such as internalins (*inlA, inlB*), listeriolysinO (*hlyA*), actin (*actA*), phosphatidylinositol-phospholipase C (PI-PLC, *plcA*), Iap (invasion associated protein, *iap*) and virulence regulator (*prfA*) [9].

L. monocytogenes was divided into 13 different serotypes. Serotypes 1/2a, 1/2b, 1/2c and 4b strains are associated with human infections. Almost all major outbreaks of invasive listeriosis are due to serovar 4b strains [8,9]. *L. monocytogenes* is susceptible to many antibiotics, however, multi-drug resistant isolates have been reported [9].

Pulsed-field gel electrophoresis (PFGE) and Multilocus sequence typing (MLST) have been applied to epidemiological analysis of *L. monocytogenes*. They have been proved to be mature tools in analyzing the characterization of *L. monocytogenes* and were instrumental in establishing epidemiological data of strains, which was useful for comparison between different laboratories [7,8].

Migratory wild birds can carry pathogenic microorganisms that spread in the natural environment and into food processing environment [4]. Previous studies have shown that wild birds, including black-headed gull, are the ultimate source of influenza A viruses for domestic birds and mammals, including humans [11]. In addition to the virus, bacteria also widely spread in bird feces. Every year migratory black-headed gulls fly to Kunming to survive the winter

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(November–March), tens of thousands black-headed gulls gather in the Dianchi Lake. There is a harmonious coexistence between people and the black-headed gulls as local residents and tourists can come to contact with black-headed gulls easily. Around the Dianchi Lake feces of black-headed gull scatter on the ground where the visitors gather, which increase the risk of pathogen transmission. The aim of this study was to investigate the prevalence and molecular characteristics of *L. monocytogenes* isolates in black-headed gull feces, which would be valuable to evaluate the potential risk of *L. monocytogenes* infection for humans in China.

Materials and methods

Sample collection and bacterial isolation

A total of 895 black-headed gull fecal samples were randomly collected on the beach of Dianchi Lake, Kunming in March 2016. March was chosen as it generally is the last month of stay for the black-headed gulls, they generally stay from November to March every year. We reasoned that it would be the locally adapted microflora condition for the black-headed gulls and the risk assessment is more reflective of the local situation. The fresh fecal samples were collected by using sterile cotton swabs and stored in brain heart infusion (BHI) broth containing 20% glycerin at 4 °C, and transported to the laboratory for isolation of *Listeria*.

The *Listeria* strains were isolated according to ISO 11290 method with some modifications. One mL of fecal sample was added to 5 mL Half Fraser enrichment broths, and incubated at 30 °C for 24 h, then 1 mL Half Fraser cultures was added to 5 mL Fraser enrichment broths, and incubated at 37 °C for 48 h. Two loopful of growth from Fraser enrichment broths were used to streak on the Palcam selective agar, incubated at 37 °C for 24–36 h. Five suspected colonies of *Listeria* were picked out for each samples, and subcultured onto BHI agar for pure culture, followed by species identification.

Identification of Listeria, *serotypes and virulence genes*

PCR was used to identify *Listeria* genus. Nucleic acid was extracted by boiling method. Suspected colonies were purified and scraped from the solid medium, added to 200 μL sterile distilled water and fully suspended, heated for 10 min in boiling water, and centrifuged for 5 min at 13,000 rpm/min, and the supernatant was used to be DNA template. Identification of *Listeria* species was mainly based on species specific primers (Table 1). The primers were synthesized by Beijing TSINGKE Biological Technology. The reaction was in a volume of 20 μL containing Master Mix 10 μL, forward primer (10 μM) 1 μL, reverse primer (10 μM) 1 μL, DNA template 1 μL and sterile distilled water7 μL. The amplification reaction were performed at 94 °C for 4 min, 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min, for 32 amplification cycles, then at 72 °C for 10 min. *EGD-e* strain was used as positive control.

The serotype group of all *L. monocytogenes* isolates was identified by multiplex PCR, the five pairs of primers (Table 2) were added to the reaction as following concentrations: 1 μM for *lmo0737*, *ORF2819*, and *ORF2110*, 1.5 μM for *lmo1118*, and 0.2 μM for *prs*. The primers were synthesized by Beijing TSINGKE Biological Technology. Depending on the PCR result, serotypes could be divided into four groups. Then, the exact serotype was determined by the serum antibody from Denka Seiken Company, Japan.

Virulence genes (*inlA*, *inlB*, *prfA*, *iap*, *actA*, *plcB* and *hly*) among *L. monocytogenes* isolates were detected by PCR. Primer sequences of virulence genes was showed in Table 3. The PCR amplification included an initial denaturation step for 94 °C for 4 min, followed by 32 amplification cycles of 30 s at 94 °C, 30 s at 54 °C and 1 min at 72 °C, followed by a final extension step of 72 °C for 10 min. *EGD-e* strain was used as positive control.

Table 1
Specific primers of *Listeria* species identification.

Target gene	Primer sequences (5′-3′)	Direction	Amplicon size (bp)
prs	ACGCATGTTGTTCGCAC	F	101
	TGGAAGAGCGATGGAGTT	R	
Lmo0733	TGTCCAGTTCCATTTTTAAC	F	416
	TTGTTGTTCTGCTGTACGA	R	

F, forward; R, reverse.

Table 2
Primers used for identification of serogroups.

Target genes	Primer sequence (5′-3′)	Direction	Amplicon size (bp)
lmo0737	AGGGCTTCAAGGACTTACCC	F	691
	ACGATTTCTGCTTGCCATTC	R	
lmo1118	AGGGGTCTTAAATCCTGGAA	F	906
	CGGCTTGTTCGGCATACTTA	R	
ORF2819	AGCAAAATGCCAAAACTCGT	F	471
	CATCACTAAAGCCTCCCATTG	R	
ORF2110	AGTGGACAATTGATTGGTGAA	F	597
	CATCCATCCCTTACTTTGGAC	R	
prs	GCTGAAGAGATTGCGAAAGAAG	F	370
	CAAAGAAACCTTGGATTTGCGG	R	

F, forward; R, reverse.

Table 3
Primers used for detection virulence genes of *L. monocytogenes*.

Target genes	Primer sequence (5′-3′)	Direction	Amplicon size (bp)
inlA	CCGCACTCACTAACTTAGAG	F	580
	GTTGTTTCTTTGCCGTCCAC	R	
inlB	CCTAAACCTCCGACCAAACA	F	293
	CCATTTCGCGCTTCTCTATC	R	
prfA	CTCAAGCAGAAGAATTCA	F	695
	TCCCCAAGTAGCAGGACA	R	
iap	TTTGCTAAAGCGGGTATCTC	F	205
	AGCCGTGGATGTTATCGTAT	R	
actA	TGCATTACGATTAACCCCGACA	F	431
	AGGCTTTCAAGCTCACTATCCG	R	
plcB	AGTGTTCTAGTCTTTCCGG	F	792
	ACCTGCCAAAGTTTGCTGT	R	
hly	ACGCAGTAAATACATTAGTG	F	372
	AATAAACTTGACGGCCATAC	R	

F, forward; R, reverse.

Pulsed-field gel electrophoresis (PFGE) analysis

For PFGE analysis, all isolated strains were diverted to BHI agar for pure cultures. Scraped proper amount of bacteria from the culture medium with sterile swabs were suspended into 1 mL TE buffer (10 mm Tris-HCL: 1 mm EDTA, pH 8.0), and used the bioMerieuxVitek colorimeter to adjust the McFarland standard between 6.1–6.3. This study only used the *AscI* restriction enzymes, because of the *AscI* PFGE patterns was more distinct than the *ApaI* [12]. The prepared blocks were digested by 200 units of *AscI* following the PulseNet standard laboratory operating procedure for *L. monocytogenes* PFGE. Block I (first stage) electrophoresis parameter settings included initial switch time of 4 s, final switch time of 40 s, voltage of 6 V/cm, angle of 120° and run time of 22 h. *Salmonella Barender* H9812 was used as control strain by the same method. Gel images were captured by the GELDOC2000 of BIO-RAD. The finger printing of DNA were analyzed with the Bionumerics soft-

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