Prevalence of *Listeria monocytogenes* and other *Listeria* spp. in gulls feeding at a landfill in Zagreb, Croatia

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Abstract

Gulls, as migratory wild birds are known to spread different pathogens over long distances. The aim of this study was to access the prevalence of different *Listeria* species in a gull population feeding at a landfill in Zagreb. In total, 390 gulls of three species were sampled: Yellow-legged Gull, *Larus michahellis*; Black-headed Gull, *L. ridibundus* and Common Gull, *L. canus*. The most prevalent species was *Listeria innocua* (14.4%), while *L. monocytogenes* was found in 11.3% of tested samples. Other species were present in a smaller percentage; *L. welshimeri* (1.3%), *L. ivanovii* (0.5%) and *L. seeligeri* (0.3%). Serotyping of *L. monocytogenes isolates* was performed using molecular and conventional methods, and most isolates belonged to the 1/2a and 1/2b serotypes. To the best of our knowledge, this is the first report of the presence of *L. monocytogenes* and other *Listeria* spp. in wild birds in Croatia.

Key words: *wild birds; zoonosis; Listeria monocytogenes*

Introduction

The genus *Listeria* includes several species that are closely related both morphologically and biochemically. The majority of these Gram-positive, rod-shaped, facultative aerobic-anaerobic, non-spore forming, psychotropic and ubiquitous bacterial microorganisms are non-pathogenic. Only two members of this genus are considered pathogens, *Listeria monocytogenes* and *Listeria ivanovii*

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that cause infection in humans and animals, respectively (Seeliger and Jones, 1986; Guillet et al., 2010; Beye et al., 2016).

In view of public health, listeriosis caused by *Listeria monocytogenes* is of undisputed importance. This zoonosis is primarily reported as a food-borne disease. There are many paths by which *L. monocytogenes* enters into the food chain, as this species is omnipresent. However, infection is mainly linked with the consumption of contaminated ready-to-eat foods whose physicochemical characteristics allow the survival and growth of this pathogen to quantities sufficient to cause disease (EFSA and ECDC, 2021). Similar to other opportunistic infections, the occurrence, clinical symptoms and the outcome of listeriosis depends on the status of the host's immune system, virulence characteristics of the present bacterial strain and the ingested dose. While most people ingest it relatively frequently in small amounts and do not develop symptoms, individuals immunocompromised for any reason are prone to develop listeriosis (Swaminathan and Gerner-Smidt, 2007; Orsi et al., 2011). Two forms of listeriosis are significant: gastroenteritis, and invasive infection. Though this is a relatively rare disease, it is characterized by a high mortality rate, and therefore is placed at the top of the list of food-borne diseases with a fatal outcome (EFSA and ECDC, 2021).

A number of discriminatory subtyping methods of *L. monocytogenes* strains (PFGE; MLST; WGS) have been introduced for the purpose of epidemiological analyses, while serotyping remains significant as the first step of initial classification. Based on the combination of somatic and flagellar antigens, *L. monocytogenes* isolates are categorised into 13 different serotypes (Seeliger and Jones, 1986; Farber and Peterkin, 1991; Graves et al., 1999) and further distributed into at least four phylogenetic lineages (I, II, III, and IV) that are differentiated by the nucleotide sequences of three genes (*flaA*, *iap*, and *hly*) (Nightingale, 2010; Orsi et al., 2011; Zhang et al., 2016; Wang et al., 2019). While *L. monocytogenes* lineage II strains (serotype 1/2a) are more widespread in the environment and in foods, and cause listeriosis in animals and sporadically in humans, lineage I strains (serotypes 1/2b and 4b) are responsible for most of the invasive listeriosis outbreaks worldwide (Wiedmann et al., 1996; Orsi et al., 2011).

Listeria reservoirs include a variety of wild and domestic birds and other animals, in both the natural and the urban environment (Sotirios et al., 2007; Vivant et al., 2013). It is well documented how wild and migratory birds represent long-distance vectors for different types of microorganisms that can pose a risk to humans (Nuttall, 1997; Kapperud et al., 1998; Thornley et al., 2003; Ejidokun et al., 2006). However, the involvement of free-living birds in supporting reservoirs and in the transmission of *L. monocytogenes* to humans has not yet been fully elucidated (Hellström et al., 2007).

The objective of this study was to investigate the presence of *L. monocytogenes* and other *Listeria* species in gulls as one of the most common birds living and feeding in the urban environment through species identification and the detection of serotypes, in order to determine their prevalence and possible zoonotic potential as a risk to human health.

Materials and Methods

Sample collection, bacterial isolation and identification

During the winter months (November, December, January) of three consecutive years (2018, 2019, 2020) a total of 390 cloacal swabs were taken from the gulls captured using a cannon net at a landfill site in Zagreb (45.45 N, 16.01 E). Gulls

Table 1. Specific primers for the detection of target genes

Target gene	Primer	Sequence (5'-3')	Amplicon size (bp)	Characteristic to
prfA	LIP 1 LIP 2	GAT ACA GAA ACA TCG GTT GGC GTG TAA TCT TGA TGC CAT CAG G	274	*Listeria monocytogenes*
Prs	PRS 1 PRS 2	GCT GAA GAG ATT GCG AAA GAA G CAA AGA AAC CTT GGA TTT GCG G	370	*Listeria*
lmo0737	LMO0737 1 LMO0737 2	AGG GCT TCA AGG ACT TAC CC ACG ATT TCT GCT TGC CAT TC	691	1/2a-3a 1/2c-3c
lmo 1118	LMO1118 1 LMO1118 2	AGG GGT CTT AAA TCC TGG AA CGG CTT GTT CGG CAT ACT TA	906	1/2c-3c
orf 2819	ORF2819 1 ORF2819 2	AGC AAA ATG CCA AAA CTC GT CAT CAC TAA AGC CTC CCA TTG	471	1/2b-3b-4b 4ab-4d-4e-7
orf 2110	ORF2110 1 ORF2110 2	AGT GGA CAA TTG ATT GGT GAA CAT CCA TCC CTT ACT TTG GAC	597	4ab-4b-4d-4e

belonged to three species (Yellow-legged Gull, *Larus michahellis*; Black-headed Gull, *L. ridibundus* and Common Gull, *L. canus*), with Black-headed Gull as most abundant.

The detection and isolation of *Listeria* spp. was performed according to the method EN ISO 11290-1:2017. Immediately after swabbing, collected cloacal swabs were placed in tubes with half-strength Fraser broth (primary enrichment) (Merck), and transported at ambient temperature to the laboratory to start incubation within eight hours from sampling. After incubation at 30°C for 25 h ± 1 h, a loopful (10 µL) of the primary enrichment cultures were streaked on the surface of two selective plating mediums: Agar *Listeria* according to Ottaviani and Agosti (Oxoid), and Oxford agar (Bio-Rad). Further, 0.1 mL of the same broth cultures was added to tubes containing secondary enrichment medium (Fraser broth, Merck). The secondary enrichment medium was incubated for at 37°C 24 h ± 2 h, and the surface plating-out procedure was repeated on the same two selective media. Selective agars were incubated at 37°C for a total of 48 h ± 2 h.

All presumptive *Listeria* spp. colonies were isolated from each sample, and subcultured onto Tryptone Soya Yeast Extract Agar (TSYEA) plates (TSA; Oxoid/YE [0.6%]; Oxoid) for confirmation, and 5% (v/v) sheep blood agar plates (Oxoid) to test for a haemolytic reaction. Further characterization included Gram staining, catalase test, determination of motility at 25°C, utilization of rhamnose and xylose, and species-level identification using VITEK2 gram-positive (GP) identification cards (bioMérieux, France). Confirmed *L. monocytogenes* isolates were stored in 20% glycerol at -80°C for further assays. Quality control strains used during the study were *L. monocytogenes* (ATCC13932, ATCC19111) and *Listeria ivanovii* subsp. *ivanovii* (ATCC19119).

Serotyping

Serotyping of *L. monocytogenes* isolates was performed with the multiplex PCR method, developed by ANSES (Kerouanton et al., 2013), and a commercially available *Listeria* Antisera Set according to the manufacturer's instruction (Denka Seiken, Japan). Briefly, DNA extraction was performed on overnight cultured colonies on 5% sheep blood agar plates (Oxoid) suspended in 100 µL PCR clean water, boiled for 20 minutes and centrifuged at 14 000 g

for 1 minute. The supernatant was used in the PCR reaction. Amplification reactions were carried out on a PCRmax thermocycler. PCR products were run in 2% agarose gel and visualized by staining with ethidium bromide. Genoserotyped *L. monocytogenes* strains from the EURL-LM collection were used as positive controls for PCR serotyping, and ultra purified water as the negative control.

Conventional serotyping for *L. monocytogenes* was carried out using the commercially available Listeria Antisera (Denka Seiken) according to manufacturer's instructions for strains belonging to PCR serogroups IIa, IIb, IIc and IVb. (Table 1).

Results and Discussion

In the long-distance chain, wild birds, especially migratory birds such as gulls, are an important link in the transmission of various pathogenic microorganisms of public interest (Munster et al., 2007; Literak et al., 2010; Jurinović et al., 2014).

In this study, a total of 390 samples of gulls' cloacal swabs were analysed from 2018 to 2020 for the presence of *Listeria* spp. Among them, 97 (24.9%) samples showed growth of the presumptive colonies on both agars already after the first enrichment step, and a total of 108 *Listeria* strains were isolated from these samples (Table 2).

Of the total 144 cloacal swabs examined in 2018, 42 (29.2%) contained bacteria of the genus *Listeria*, and 49 bacterial strains were isolated. Of these isolates, 27 (55.1%) belonged to the species *L. innocua*, 21 (42.9%) to *L. monocytogenes*, and one (2.0%) was identified as *L. seeligeri*. The simultaneous presence of two different species was found in seven (17.0%) positive samples, with the combination of *L. innocua* and *L. monocytogenes* proven in six samples (14.3%), and of *L. monocytogenes* and *L. seeligeri* in one (2.0%) positive swab sample examined in 2018.

Table 2. Number of *Listeria* species isolated from 2018 to 2020

Species \ Month/Year	01/2018	02/2018	12/2018	No. of isolates
Listeria monocytogenes	10	5	6	21
Listeria innocua	15	8	4	27
Listeria seeligeri	1			1
No. of strains / No. of examined samples in 2018				49/144
Species \ Month/Year	**01/2019**	**02/2019**	**12/2019**	
Listeria monocytogenes	2	5	4	11
Listeria innocua	5	7	3	15
Listeria ivanovii	1		1	2
Listeria welshimeri		1	1	2
No. of strains / No. of examined samples in 2019				30/106
Species \ Month/Year	**01/2020**	**02/2020**	**12/2020**	
Listeria monocytogenes	5	5	2	12
Listeria innocua	8	5	1	14
Listeria welshimeri		1	2	3
No. of strains / No. of examined samples in 2020				29/140
Total No. of strains / No. of examined samples				108/390

In 2019, a total of 106 gulls' cloacal swabs were taken and examined, of which 27 (25.5 %) contained bacteria of the genus *Listeria*. The presence of *L. innocua* was found in 13 (48.1%) positive samples, *L. monocytogenes* in nine (33.3%), *L. ivanovii* in two (7.4%) and *L. welshimeri* in one (3.7%) sample. In the remaining two (7.4%) of the total number of positive swab samples, the simultaneous presence of different species was determined, where one was a combination of *L. innocua* and *L. monocytogenes* (3.7%) and the second contained a combination of three species *L. innocua*, *L. monocytogenes* and *L.* welshimeri (3.7%).

A total of 140 cloacal swabs were examined in 2020, of which 28 (20.0%) contained bacteria of the genus *Listeria*. Out of 29 isolates of *Listeria* spp., *L. innocua* was proven for 13 (46.4%) isolates, 11 strains (39.3%) belonged to *L. monocytogenes*, and three isolates were identified as *L. welshimeri* (10.7%). The presence of two different species - *L innocua* and *L. monocytogenes* - was detected in the remaining isolated strain (3.6%).

Among the 108 strains of isolated *Listeria* spp., the most prevalent species was *L. innocua* (56 isolates, 51.9%), followed by *L. monocytogenes* (44 isolates, 40.7%), *L. welshimeri* (5 isolates, 4.6%), *L. ivanovii* (2 isolates, 1.9%) and *L. seeligeri* (1 isolate, 0.9%).

The average occurrence of *L. monocytogenes* in gulls in this study was 11.3%, with the highest prevalence in 2018 (14.6%), and decreased occurrence in 2019 and 2020 (10.4% and 8.6%, respectively). These results are concordant with earlier studies in wild birds that reported a prevalence of *L. monocytogenes* from 0 to 36% (Yoshida et al., 2000; Hellström et al., 2007; Gan et al., 2019). Fenlon (1985) found a higher prevalence of *L. monocytogenes* in gulls feeding around sewage (15.2%) than those feeding elsewhere (4.5%), and our results from a landfill are in the middle of these values. While the prevalence of *L. monocytogenes* is similar to those from previous studies, *L. innocua* was dominant in our dataset with more than half of all isolates obtained and a prevalence of 14.4%, which is higher than reported by Fenlon (1985) and Duarte et al. (2002) who found it in 4.5% and 5.3% of gull samples, respectively, but in concordance with Quessy and Messier (1992) who found it in 13.6% of gull samples. It is estimated that more than 10,000 gulls feed at this landfill in Zagreb during the winter, and these gulls have been proven to original from throughout Europe (Jurinović, 2018). Therefore, this may be significant for the transmission of *Listeria* species.

Multiplex PCR was performed on serogroup 44 *L. monocytogenes* isolates, and the results showed that 43.2% of isolates belong to the 1/2a-3a serogroup, 43.2% to the 1/2b-3b-7 serogroup and 13.6% to the 4b-4d-4e serogroup. Conventional serotyping was also performed on these 44 isolates that were subtyped into three serotypes: serotypes 1/2a and 1/2b were predominant and each included 19 isolates (43.2%), while the six remaining strains (13.6%) belonged to serotype 4b. Table 3 shows the concordance in the classification of isolates into serotypes using PCR primers with the classification based on conventional serotyping for all 44 *L. monocytogenes* tested strains. With such a high proportion of serotypes potentially causing human disease, gulls feeding at this landfill site should be monitored for the presence of *Listeria* species.

To the best of our knowledge, this is the first report investigating the presence of *L. monocytogenes* and other *Listeria* spp. in wild birds in Croatia.

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Table 3. Correlation of results between conventional serotyping and multiplex PCR for *L. monocytogenes* tested strains

Serotype	No. of strains	Multiplex PCR fragment amplification						Multiplex PCR classification
		lmo1118 (906 bp)	*lmo0737* (691 bp)	*orf 2110* (597 bp)	*orf 2819* (471 bp)	*prs* (370 bp)	*prfA* (274 bp)	
1/2a	19	–	+	–	–	+	+	*L. monocytogenes* 1/2a-3a
1/2b	19	–	–	–	+	+	+	*L. monocytogenes* 1/2b-3b-7
4b	6	–	–	+	+	+	+	*L. monocytogenes* 4b-4d-4e

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Pojavnost bakterije *Listeria monocytogenes* i drugih vrsta roda *Listeria* u galebova koji se hrane na zagrebačkom odlagalištu otpada, Hrvatska

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Za galebove je kao divlje ptice selice poznato da mogu na velike udaljenosti širiti različite patogene. Cilj je ovog istraživanja bio odrediti prevalenciju različitih vrsta roda *Listeria* u populaciji galebova koji se hrane na Zagrebačkom odlagalištu otpada. Sveukupno je pretraženo 390 galebova triju vrsta: galeb klaukavac - *Larus michahellis*; riječni galeb - *L. ridibundus* i burni galeb - *L. canus*. Najzastupljenija vrsta bila je *L. innocua* (14,4 %), dok je *L. monocytogenes* dokazana u 11,3 % ispitanih uzoraka. Prisutnost ostalih vrsta zastupljena je u manjem postotku i to *L. welshimeri* (1,3 %), *L. ivanovii* (0,5 %) i *L. seeligeri* (0,3 %). Serotipizacija izolata *L. monocytogenes* provedena je molekularnim i konvencionalnim metodama, a većina izolata pripadala je serotipovima 1/2a i 1/2b. Prema našem saznanju, ova studija donosi prve rezultate istraživanja prisutnosti *L. monocytogenes* i drugih vrsta roda *Listeria* u divljih ptica u Hrvatskoj.

Ključne riječi: *divlje ptice, zoonoza, Listeria monocytogenes*