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Isolation of Listeria monocytogenes from peridomestic birds and captive wild animals

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Listeria monocytogenes is an important foodborne pathogen responsible for septicemia, meningitis and abortions. There are several animal reservoirs; however, the role of wild animals and peridomestic birds remains underestimated. We have screened 270 faecal samples of wild animals in captivity (18 species) and peridomestic birds (12 species). Listeria species were isolated from seven (6.66%) mammals and two (1.21%) birds. L. monocytogenes was isolated from barking deer, porcupine, pigeon and crow. Isolated L. monocytogenes were virulent strains of 4b serogroup. There is a need to explore the role of such non-conventional sources in the spread of L. monocytogenes in nature.

Keywords: Antibiotic sensitivity, birds, Listeria monocytogenes, serotyping, wild animals.

LISTERIA monocytogenes is an emerging foodborne pathogen recognized globally. Ecology of L. monocytogenes is atypical and has several animal and inanimate reservoirs in nature1. Listeria infection is mainly acquired through a variety of contaminated non-meat and meat foods. Manifestations of listeriosis both in animals and humans include septicemia, meningitis, abortion and still birth. Several virulence factors encoded by the hlyA, plcA, actA and iap genes play a significant role in the pathogenesis of L. monocytogenes infections2.

Even though several studies have demonstrated broader distribution of Listeria species throughout the natural environment, the role of wild animals and birds in the ecology and as reservoirs of L. monocytogenes is not clearly understood3. Limited studies on the detection of pathogenic bacteria including L. monocytogenes from wild animals and birds have been documented4-7. Studies on the detection of Listeria from sources other than food-processing environments may help reveal the population genetics and natural history of Listeria species4. Due to several anthropogenic consequences, emerging trends in the epidemiology of infectious diseases have been recorded8. Living in close proximity and sharing the same environment virtually creates zoonotic nidus. Thus, asymptomatic healthy carriers are the cause of concern. In India, adequate studies on the occurrence of L. monocytogenes from foods have been conducted9,10,11; however, studies on wild animals and birds are largely lacking. Listeria species may survive for a longer period in the soil and possibly get excreted in the faeces of carrier animals without any symptoms, and may cause infection to other animals as well as personnel working in the zoo and visitators. The present study was conducted to isolate L. monocytogenes in peridomestic birds and wild animals in captivity.

Fresh faecal samples (n = 270) comprising 105 from mammals and 165 from peridomestic birds were collected. Samples were collected from the nesting sites of birds (12 species) with sterile swab without touching or disturbing their habitat. Samples of wild animals (18 mammals) were collected from the Rajiv Gandhi Zoological Park and Wild Life Research Centre, Katraj, Pune, India. The birds included pigeon (n = 80), sparrow...
Listeria species were isolated by the ISO 11290/1997 method with desirable modifications\(^2\)\(^3\). The samples were enriched (two-step) followed by selective plating on PALCAM and chromogenic rapid \(L.\) \(m\)ono differential agar (HiMedia, India). Faecal samples were first enriched in half-strength Frazer broth and then in full-strength Frazer broth at 35°C for 24 h. Following enrichment, the culture was plated on selective agar and incubated at 37°C for 24–48 h. Five typical colonies showing dispersive black zone of aesculin hydrolysis on PALCAM and bluish-green colonies on chromogenic agar were subcultured for further confirmation. The isolates were characterized by Gram staining, catalase positivity and motility at 20–25°C. Further characterization was done by testing for methyle red-voges-Proskauer (MR-VP) reactions, CAMP test with \(S\). \(a\)phylococcus \(a\)ureus and \(R\). \(e\)quino \(e\)qui, reduction of nitrate, utilization of sugars (mannitol, xylose, rhamnose and \(a\)-methyl-D-mannopyranoside) followed by hemolysis on 5% ovine blood agar. \(L.\) \(m\)onocyto-

genesis (MTCC1143) was used as known positive culture during the study.

Detection of \(L.\) \(s\)pecies and \(L.\) \(m\)onocyto-
genesis was done using multiplex PCR assay\(^3\). Table 1 provides details on the oligonucleotides used. DNA was extracted by snap chilling method. Briefly, 0.5 ml pure bacterial culture was pelleted at 10,000 rpm for 5 min. The pellets obtained were resuspended in 100 \(\mu\)l nuclelease-free water and heated for 10 min in a water bath. The tubes were removed and snap-chilled immediately. Subsequently, PCR reactions were performed employing the supernatant as DNA template.

Multiplex PCR assay was performed using 25 \(\mu\)l reaction mixture containing 10\(\times\) PCR buffer, 1 \(\mu\)l dNTPs, 25 mM MgCl\(_2\), 50 \(\mu\)M primer set, 3\(U\) \(Taq\) DNA polymerase, DNA template (50 ng) and adjusted to 25 \(\mu\)l by nuclelease-free water. PCR cycling conditions were set as initial denaturation at 95°C for 5 min followed by denaturation at 95°C for 30 sec, annealing at 56°C for 1 min, extension at 72°C for 2 min and one cycle of final extension at 72°C for 8 min (40 cycles). Resultant products were resolved in 1% agarose gel with 10 \(\mu\)g/ml ethidium bromide (EtBr) visualized under gel documentation system.

The \(L.\) \(s\)pecies were serotyped using multiplex PCR\(^4\). The PCR reaction mixture contained 2.5 \(\mu\)l of PCR buffer (10\(\times\)), 2 mM dNTPs, 3 mM MgCl\(_2\), 0.3 \(\mu\)M each of primers, 2\(U\) \(Taq\) DNA polymerase, DNA template (50 ng) and finally adjusted to 25 \(\mu\)l volume by nuclelease-free water. The cycling conditions for PCR were initial denaturation at 94°C for 3 min, 35 cycles of 94°C for 40 sec, 53°C for 75 sec, and 72°C for 75 sec, and one final extension cycle at 72°C for 7 min. The resultant products were separated on 1.5% agarose gel followed by staining with EtBr (10 \(\mu\)g/ml).

The virulence-associated genes of \(L.\) \(m\)onocyto-
genesis were detected using multiplex PCR assay\(^5\)–\(^8\). The assay was performed in 50 \(\mu\)l volume with 5.0 \(\mu\)l of 10\(\times\) PCR buffer, 1 \(\mu\)l of 10 mM dNTPs, 4 \(\mu\)l 25 mM MgCl\(_2\), 100 nM of each primer, 1\(U\) \(Taq\) DNA polymerase, and DNA template (50 ng) and adjusted to 50 \(\mu\)l using nuclelease-free water. Multiplex PCR was performed with initial denaturation at 95°C for 2 min with further 35 cycles of denaturation at 95°C for 15 sec, annealed at 60°C for 30 sec and extended at 72°C for 90 sec followed by a final extension at 72°C for 10 min. After amplification, the products were electrophoresed in 1.5% agarose gel and visualized with EtBr (0.5 \(\mu\)g/ml) staining in gel documentation system.

Disc diffusion method employing Mueller Hinton agar was used for antimicrobial susceptibility testing\(^9\). Agar plates were inoculated with overnight-grown pure bacterial culture in BHI broth, antibiotic discs were placed on plates and incubated at 37°C for 24 h. Results were interpreted as resistant, intermediate and sensitive according to the manufacturer’s instructions (HiMedia Laboratories, Mumbai). The following antibiotics were used: amikacin (30 \(\mu\)g), ampicillin-sulbactum (10/10 \(\mu\)g), cefixime (5 \(\mu\)g), chloramphenicol (30 \(\mu\)g), erythromycin (10 \(\mu\)g), gentamicin (5 \(\mu\)g), kanamycin (30 \(\mu\)g), neomycin (30 \(\mu\)g), norfloxacin (10 \(\mu\)g), oxytetracycline (30 \(\mu\)g), penicillin-G (10 \(\mu\)i) and tetracycline (10 \(\mu\)g).

Detection of \(tetA\), \(tetB\) and \(tetC\) genes was done using PCR assay\(^9\). The assay was carried out in 25 \(\mu\)l volume containing PCR master mix (12.5 \(\mu\)l; Thermo Scientific), 1 \(\mu\)l of each primer, DNA template (3 \(\mu\)l) and nuclelease-free water to make up the volume. Primers used were: \(tetA\) (F-5’-GGCGGTCTTTTCTATCATGC-3’ and R-5’-CGGCAGGGCAGAAATGAGTA-3’), \(tetB\) (F-5’-CATC-AATTGCGCAGTCGGCAG-3’ and R-5’-GCGGCATATCCGTAGC-3’), \(tetC\) (F-5’-GCCGCGCCGAGGCGA-3’ and R-5’-TTGACGATCG-3’), and \(tecc\) (F-5’-GCTGTAGC-GAGAAT-3’ and R-5’-GCTGTAGC-GAGAAT-3’). Amplification of DNA was performed as follows: 95°C for 15 min of initial denaturation; 34 cycles of 94°C for 30 sec, 64°C for 90 sec, 72°C for 90 sec and final extension at 72°C for 15 min. Amplicons were electrophoresed in 1.5% agarose gel with EtBr (0.5 \(\mu\)g/ml) and visualized under gel documentation system.

Out of 270 faecal samples examined, \(L.\) \(s\)pecies were isolated from 7 (6.66%) mammals and 2 (1.21%) bird species. Among wild animals in captivity, \(L.\) \(s\)pecies were detected in seven animals: spotted deer
The virulence genes plcA, hlyA, actA and iap were found to be present in all the L. monocytogenes strains (Figure 3).

Isolates were sensitive to all the 12 antimicrobials tested by disc diffusion method, except one L. monocytogenes isolate from porcupine that was resistant to neomycin. L. monocytogenes strains were also screened for the presence of genotypic resistance to tetracycline; however, none of the isolates was positive for tetA, tetB and tetC genes.

L. monocytogenes serogroup 1/2a, 1/2b and 4b strains are largely implicated in human foodborne listeriosis outbreaks. Listeria species are saprophytes having environmental reservoirs and they may enter into the food chain through various routes. Infection of L. monocytogenes is mainly acquired through contaminated food; several studies have highlighted the incidence of this pathogen in foods and food-processing environment\textsuperscript{21,22}. However, not many studies have been conducted on non-conventional sources of L. monocytogenes like the role of wild animals and birds. It is also a poorly studied pathogen in the Indian context\textsuperscript{10}. Therefore the aim of the present study was to examine different captive wild animals and bird species for the detection of pathogenic serovars of L. monocytogenes. Faecal samples of all the mammalian species reared in the Rajiv Gandhi Zoological Park and Wildlife Research Centre, Pune were screened for L. monocytogenes. Simultaneously, faecal droppings of peridomestic birds were also screened. The occurrence of Listeria species (6.66\%) and L. monocytogenes (1.90\%)
was found to be low. The detection of virulent strains of 4b serogroup in barking deer (Muntiacus muntjak), porcupine (Hystrix indica), pigeon (Columbia livia) and crow (Corvus splendens) is a cause of concern. Prevalence of L. monocytogenes in captive wild animals\textsuperscript{4,6,25} and bird species\textsuperscript{4,6,25} has been studied earlier worldwide. Recently, the red deer has been reported to excrete L. monocytogenes and other zoonotic pathogens in the environment in Europe\textsuperscript{26}. In India, Listeria species have been detected in wild animals in captivity and birds\textsuperscript{4,7,27}. Faecal samples of 623 wild mammals were screened in Japan and were found to harbour Listeria species and L. monocytogenes in 6.1% and 1% samples respectively\textsuperscript{4}. L. monocytogenes was detected in Japanese monkey (1.25%), red fox (1.33%), raccoon dog (0.96%), Japanese marten (0.79%) and Sika deer (1.05%). In contrast, comparatively higher prevalence of L. monocytogenes (18.6%) in zoo animals of Taronga Zoological Garden, Australia was recorded\textsuperscript{21}. High detection rates of Listeria species (21%) and L. monocytogenes (7%) were recorded in faeces of healthy animals from Antwerp Zoo, Belgium\textsuperscript{22}. Fairly high prevalence of L. monocytogenes (10.57–18%) in zoo animals and birds was reported from India\textsuperscript{5,27}. Yadav et al.\textsuperscript{3} recovered Listeria species (5.36%) and L. monocytogenes (1.79%) from faecal samples of healthy wild animals and birds from Gujarat, India.

Screening of the 165 faecal samples from 12 birds species revealed 2 Listeria species, both being virulent L. monocytogenes serogroup 4b strains. Droppings of Indian pigeon and crow contained L. monocytogenes. This was detected in 13.4% birds from Japan earlier\textsuperscript{4}. In the present study, we could not detect Listeria species and L. monocytogenes in broiler-layer chickens, sparrows, Indian mynah, duck, turkey, guinea fowl and lovebirds. Earlier, L. monocytogenes was isolated from 36% faecal samples from wild birds of Helsinki region\textsuperscript{6} and the most common serotypes detected were 1/2a, followed by 4b and 1/2b. Healthy wild birds were found to carry L. monocytogenes in their intestinal contents with higher prevalence of L. monocytogenes at a landfill site than in urban areas\textsuperscript{5}.

Variations in the prevalence rates can be attributed to several factors like difference in feeding habitat and living environment. Detection of Listeria species, including virulent L. monocytogenes strains in captive wild animals and peridomestic birds is indicative of their role as reservoirs or carriers of this pathogen. Undoubtedly, L. monocytogenes is a soil-borne pathogen; how it has been detected in different animal reservoirs is debatable. Antibiotic resistance was not detected in any of the isolates, except one strain showing resistance against neomycin. Listeria species sensitive to ciprofloxacin, levofloxacin, amoxicillin, azithromycin and er不是很长的词性。L. monocytogenes is a soil-borne pathogen; how it has been detected in different animal reservoirs is debatable. Antibiotic resistance was not detected in any of the isolates, except one strain showing resistance against neomycin. Listeria species sensitive to ciprofloxacin, levofloxacin, amoxicillin, azithromycin and enrofloxacin, and resistant to gentamicin, oxytetracycline, penicillin-G, cephalaxin, and nalidixic acid have been observed earlier\textsuperscript{21}. In this study, isolates were sensitive to amikacin, ampicillin, sulfabactam, ceftriaxone, chloramphenicol, erythromycin, gentamicin, kanamycin, norfloxacin, oxytetracycline, penicillin-G and tetracycline. Tetracycline resistance has been extensively studied and shown to be widespread in bacterial populations. It has been widely used in animals as a therapeutic agent and animal growth promoter in the past. Thus we wanted to understand the diversity of tetracycline genes in L. monocytogenes. However, tetA, tetB and tetC genes could not be detected.

Although ubiquitous, the ecology of L. monocytogenes in outside environments is poorly understood\textsuperscript{5}. Studies on L. monocytogenes in faeces of zoo animals and birds from India are scanty and there is great dearth of research on understanding the molecular epidemiology and ecological niche of L. monocytogenes in diverse environments. The present study showed the presence of virulent L. monocytogenes in captive wild animals and peridomestic birds harbouring similar serotype as that reported earlier to be predominant in the Indian subcontinent\textsuperscript{28}. Further studies are warranted to understand the sources and diversity of Listeria species in newer niches.

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