Prevalence of *Listeria* species including *L. monocytogenes* from apparently healthy animals at Baroda Zoo, Gujarat State, India

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Abstract: Listeriosis is a infectious bacterial disease of domestic and wild animals and humans. A total of 56 faecal samples were collected from mammals and birds at Baroda Zoo, Vadodara, Gujarat State, India. Confirmation of the isolates was based on biochemical tests followed by phenotypic characterization by hemolysis on sheep blood agar, Christie Atkins Munch-Petersen (CAMP) test, phosphatidylinositol-specific phospholipase C (PI-PLC) assay and phosphatidylcholine-specific phospholipase C (PC-PLC) assay. The isolates were subjected to genotypic characterization with the help of polymerase chain reaction (PCR) assay for five virulence-associated genes, plcA, prfA, hlyA, actA and iap. *Listeria monocytogenes* isolates were further subjected to multiplex-PCR based serotyping. From 56 samples three (5.36%) were found positive for *Listeria* spp. of which one (1.79%) was identified as *L. monocytogenes* and two (3.57%) were identified as *L. innocua*. The isolate of *L. monocytogenes* was hemolytic, CAMP positive, PI-PLC positive, hlyA, pclA and prfA positive by PCR and turned out to be PC-PLC positive and was serotyped as 4b.

Keywords: CAMP, *Listeria monocytogenes*, PCR, serotyping, serovar, zoo animals.

INTRODUCTION

Listeriosis is an important bacterial disease of animals and a zoonosis with a broad distribution; *Listeria monocytogenes* is the major pathogen causing listeriosis and is of significant economic and health concern as it causes disease in a wide variety of animals including sheep, goats, cattle, buffaloes, dogs, horses, chickens, rabbits and also human beings (Katiyar 1960).

Information on the serovar allows discrimination between isolates belonging to an outbreak and those that are not part of the outbreak. All major outbreaks of listeriosis are caused by serovar 4b, which is primarily responsible for ruminant listeriosis (Rocourt & Seeliger 1985; Radostits et al. 1994). This strain is infrequent in foods compared to 1/2a strains (Buchrieser et al. 1993; Farber & Peterkin 1991). The procedure adopted to investigate outbreaks relies on serovar characterization for isolated strains.

Although 13 serovars are described for *L. monocytogenes*, at least 95% of the strains isolated from foods and patients belong to the serovars 1/2a, 1/2b and 4b (Seeliger & Hohne 1979; Tappero et al. 1995; Graves et al. 1999).

In Gujarat State, India, there has been no report of *Listeria* sp. from wild animals; although *L. monocytogenes* has been isolated from wild animals in Nagpur City in the neighbouring Maharasthra by Kalorey et

al. (2006). It is very important for public health to understand the prevalence of *Listeria* in wild animals, since wild animals whether captive or free-ranging may act as a reservoir for the disease. The purpose of this study was to determine the prevalence of *Listeria* sp. in apparently healthy zoo animals and to characterize the isolates phenotypically and genotypically.

MATERIALS AND METHODS

Bacteria: Strains of *L. monocytogenes* 4b (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), *Rhodococcus equi* (MTCC 1135), *Escherichia coli* (MTCC 443) were obtained from the Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. Different strains of *Staphylococcus aureus* (ATCC 25923), *Streptococcus agalactiae* (NCIM 2401), *Bacillus* spp. (ATCC 6638) and *Pseudomonas aeruginosa* (ATCC 27853) were obtained from the Department of Veterinary Microbiology, College of Veterinary Science & Animal Husbandry, Anand, India.

Samples: A total of 56 faecal samples were collected from mammals (9) and birds (47) of Baroda Zoo. The animals screened were Sambar (2), Chital (3), Black Buck (3) Nilgai (1) and that of birds were Love birds (5), Cockatoo (5), Rosella (2), Macaw (3), Dove (10), Emu (5), Conur (4), Koel (2), Cockatiel (5), Lori (6).

Isolation of *Listeria*: Isolation of listeriae from the faecal samples of the animals followed the method of the US Department of Agriculture (USDA) described by McClain & Lee (1998) with some modifications.

Samples were enriched by two-step enrichment in University of Vermont (UVM) medium -I and II. Each of the faecal swabs was aseptically transferred into 10ml UVM-I. In UVM-I medium the samples were incubated at $30^{\circ}C$ for 24hr. Then the samples were incubated in the UVM -II medium at $30^{0}C$ for up to seven days. After 24hr, 48hr and seven days of incubation samples were simultaneously streaked onto Dominguez-Rodriguez isolation agar (DRIA), PALCAM agar, and Oxford agar.

Confirmation of the isolates: Morphologically typical colonies were verified by Gram's staining, catalase reaction, tumbling motility at 20–25 ^{0}C, Methyl Red–Voges Proskauer (MR–VP) reactions,

nitrate reduction and fermentation of sugars (rhamnose, xylose, mannitol and α-methylD - mannopyranoside).

Phenotypic characterization

Haemolysis on sheep blood agar (SBA): All the *Listeria* isolates were tested for the type (α or β) and the degree (narrow or wider) of hemolysis on SBA. The isolates were streaked onto 7% SBA plates and incubated at $37^{0}C$ in a humidified chamber for 24hr. After that they were examined for haemolytic zones around the colonies. Interpretation of the haemolytic reaction was based on the formation of a typical wide and clear zone of haemolysis (β-haemolysis) representing *L. ivanovii* and formation of a narrow zone of haemolysis (α-haemolysis) representing *L. monocytogenes* or *L. seeligeri*.

Christie, Atkins, Munch-Petersen (CAMP) test: The standard strains of *Staphylococcus aureus* and *Rhodococcus equi* were grown overnight on SBA plates at $37^{0}C$ in a humidified chamber. The colonies were then also streaked onto freshly prepared 7 % SBA plates in a manner that the streaks were wide apart and parallel to each other. In between the parallel streaks of *S. aureus* and *R. equi* the *Listeria* isolates were streaked at 90^{0} angles and 3mm apart before incubating them at $37^{0}C$ for 24hr. In case of a CAMP positive reaction the synergistic effect of the haemolysins would lead to a wider zone of complete haemolysis between a *Listeria* strain and the *S. aureus* or *R. equi* strain. The *Listeria* isolates with CAMP-positivity against *S. aureus* were characterized as *L. monocytogenes* and those with CAMP positivity against *R. equi* were characterized as *L. ivanovii* .

Phosphatidylinositol-specific phospholipase C (PI-PLC) assay: All the phenotypically characterized *Listeria* isolates were assayed for PI-PLC activity following the method of Leclercq (2004) with certain modifications. *Listeria* isolates were incubated overnight on 7% SBA plates at $37^{0}C$ in a humidified chamber. All *L. monocytogenes* isolates were streaked on L. mono differential agar (Hi Media Ltd, Mumbai, India) in order to assess PI-PLC activity. The inoculated plates were incubated at $37^{0}C$ in a humidified chamber for 24hr. Light blue colonies showing formation of a halo around the inoculation site were considered positive.

Phosphatidylcholine-specific phospholipase C (PC-PLC) Assay: an egg-yolk opacity test was

Table 1. Details of primers for amplification of virulence marker associated genes of *L. monocytogenes*

Target gene	Primer Sequence		Product size (bp)	*Reference*
plc A	Forward	5'- CTG CTT GAG CGT TCA TGT CTC ATC CCC C - 3'	1484	Notermans et al. 1991a
	Reverse	5'- CAT GGG TTT CAC TCT CCT TCT AC - 3'		
prf A	Forward	5'- CTG TTG GAG CTC TTC TTG GTG AAG CAA TCG - 3'	1060	Notermans et al. 1991a
	Reverse	5'- AGC AAC CTC GGT ACC ATA TAC TAA CTC - 3'		
hly A	Forward	5'- GCA GTT GCA AGC GCT TGG AGT GAA - 3'	456	Paziak-Domanska et al. 1999
	Reverse	5'- GCA ACG TAT CCT CCA GAG TGA TCG - 3'		
iap A	Forward	5'- ACA AGC TGC ACC TGT TGC AG - 3'	131	Furrer et al. 1991
	Reverse	5'- TGA CAG CGT GTG TAG TAG CA -3'		
act A	Forward	5'- CGC CGC GGA AAT TAA AAA AAG A - 3'	839	Suarez et al.2001
	Reverse	5'- ACG AAG GAA CCG GGC TGC TAG - 3'		
prs	Forward	5'- GCT GAA GAG ATT GCG AAA GAA G-3'	370	Doumith et al. 2004
	Reverse	5'- CAA AGA AAC CTT GGA TTT GCG G -3'		

conducted to examine the PC-PLC activity of the isolates. Tryptic soy agar (Hi Media Ltd. Mumbai, India) plates were prepared with 2.5% egg-yolk emulsion (Hi Media Ltd. Mumbai, India) and 2.5 % NaCl and a pH of 6.5–7. *Listeria* isolates were streaked onto the agar surfaces and incubated at 37^0C for 36–72 hr. Formation of opaque zones surrounding the colonies was considered positive (Coffey et al. 1996).

Genotypic Characterization

Polymerase chain reaction (PCR) based detection of virulence-associated genes: The standard strain of *L. monocytogenes* (MTCC 1143) was incubated overnight in brain heart infusion at 37^0C. About 0.5ml of the culture was then centrifuged in a microcentrifuge (Sigma, USA) at 6000×g for 10 min. The recovered pellet was resuspended in 100μl of sterilized DNAse and RNAse-free milliQ water (Millipore, USA), heated in a boiling water bath for 10 min and then shock chilled in crushed ice. The obtained lysate (5μl) was used as a DNA template in PCR reaction mixture. For the amplification of the virulence associated genes of *L. monocytogenes,* hemolysin gene (hlyA), regulatory gene (prfA), Phosphatidylinositol phospholipase C gene (plcA), Actin gene (actA) and p60 gene (iap), primers synthesized by Sigma Aldrich, USA were used. Details about primers and PCR product sizes are shown in Table 1. PCR conditions followed methods described by multiple authors (Furrer et al. 1991; Notermans

et al. 1991a; Paziak-Domanska et al. 1999; Suarez & Vazquez-Boland 2001) with some modifications. Conditions affecting the sensitivity and specificity of the reaction were optimized. Such conditions are for example the concentrations of $MgCl_2$, primers and Taq DNA polymerase, the annealing temperatures (50–60^0C) and the number of cycles for amplification of the target gene.

Based on optimization trials, the standard PCR protocol for a 50μl reaction mixture included 5.0μl of 10×PCR buffer (100mm Tris–HCl buffer with pH 8.3 containing 500mM KCl, 15 mM $MgCl_2$ and 0.01% gelatin), 1μl of 10mM dNTP mix (a final concentration of 0.2mM; Sigma, USA), 4μl of 25mM $MgCl_2$ (a final concentration of 2 mM) and 10μM of a primer set containing forward and reverse primers at a concentration of 0.1μM of each primer, 1 U of Taq DNA polymerase (Sigma, USA), 5μl of cell lysate and sterilized milliQ water to make up the reaction volume.

The 0.2ml PCR tube containing the reaction mixture was tapped thoroughly with a finger and then flash spun in a micro centrifuge. The DNA amplification was performed in a Master Cycler Gradient Thermocycler with a preheated lid (Eppendorf, Hamburg, Germany). The cycling conditions for PCR included an initial denaturation at 95^0C for two minutes followed by 35 cycles each of 15s denaturation at 95^0C, 30s annealing at 60^0C and 90s extension at 72^0C, followed by a final extension of 10min at 72^0C and kept at 4^0C. All the five sets of primers for virulence-associated genes

Table 2. Details of primers for amplification of target genes of *Listeria monocytogenes* employed in serotyping PCR

Gene target	Primer Sequence		Product Size (bp)	Serovar specificity	Protein encoded by the target gene
lmo0737	F	5'- AGG GCT TCA AGG ACT TAC CC- 3'	691	1/2a	Unknown, no similarity
	R	5'- ACG ATT TCT GCT TGC CAT TC - 3'			
ORF2819	F	5'- AGC AAA ATG CCA AAA CTC GT - 3'	471	1/2b and 4b	Putative transcriptional regulator
	R	5'- CAT CAC TAA AGC CTC CCA TTG - 3'			
ORF2110	F	5'- AGT GGA CAA TTG ATT GGT GAA - 3'	597	4b	Putative secreted protein
	R	5'- CAT CCA TCC CTT ACT TTG GAC- 3'			
prs	F	5'- GCT GAA GAG ATT GCG AAA GAA G- 3'	370	All *Listeria* species	Putative phosphoribosyl pyrophosphate synthetase
	R	5'- CAA AGA AAC CTT GGA TTT GCG G -3'			

were amplified under similar PCR conditions and amplification cycles. The resulting PCR products were analyzed by agarose gel electrophoresis (1.5%; low melting temperature agarose L), stained with ethidium bromide (0.5μg/ml) and visualized by a UV transilluminator (UVP Gel Seq Software, England).

Multiplex PCR based serotype detection of *Listeria monocytogenes* isolates: The multiplex PCR assay was standardized for the detection of serovars 1/2a, 1/2b and 4b of *L. monocytogenes* following the methodology described by Doumith et al. (2004). The primers for detection of *L. monocytogenes* 0737 gene (lmo0737), transcriptional regulator gene (ORF2819), secreted protein gene (ORF2110) and phosphoribosyl pyrophosphate synthetase gene (prs) of *L. monocytogenes* used in this study were synthesized from Sigma Aldrich. Details of the primer sequences are shown in Table 2.

The PCR was set for 50μl reaction volume. For the detection of *L. monocytogenes* serotypes concentrations of molecular biologicals, annealing temperature and number of cycles for amplification of the target genes were varied until optimal conditions were determined. The optimal reaction mixture for PCR contained 5.0μl of 10x PCR buffer (), 1.5μl dNTP mix, 4μl of 25mM $MgCl_2$ and 100μM of forward and reverse primers for each serovar 1/2a, 1/2b and 4b (final concentration 0.1μM each) and 10μM of forward and reverse primers for each *Listeria* spp. (final concentration 0.1μM each), 2 units of Taq DNA Polymerase, 5μl of cell lysate and sterilized milliQ water to make up the reaction volume.

The 0.2ml PCR tube containing the reaction mixture was flash spun in a micro centrifuge. The reaction was performed in a Px2 Thermal cycler with a pre-heated lid (Thermo electronic corporation, USA). The cycling conditions included an initial denaturation for five minutes at 94⁰C followed by 35 cycles of denaturation for 30 seconds at 94⁰C, 75 seconds of annealing at 54⁰C and 75 seconds of extension at 72⁰C. It was followed by 10 minutes of extension at 72⁰C and was finally held for 30 minutes at 4⁰C. After the reaction, PCR products were kept at –20⁰C until further analysis by agarose gel electrophoresis.

RESULTS

Isolation of *Listeria monocytogenes*: From 56 samples, 3 (5.36%) were found positive for *Listeria* spp., of which 1 (1.79%) was identified as *L. monocytogenes* and 2 (3.57%) as *L. innocua.*

Phenotypic characters: One of the *Listeria* isolates was CAMP positive and showed the characteristic enhancement of haemolytic zone with *S. aureus.* Two of the isolates did not show enhancement of the hemolytic zone either with *S. aureus* or *R. equi.* The CAMP positive isolate was also found to be positive for PI-PLC and PC-PLC assay and was confirmed to be *L. monocytogenes.*

Genotypic characters: The standardized PCR allowed amplification of virulence associated genes of *L. monocytogenes* plcA, prfA, actA, hlyA and iap to their respective base pairs, 1484 bp, 1060 bp, 839 bp, 456 bp and 131 bp, and allowed visualization of each virulence associated gene, each gene represented by a single band in the corresponding region of the DNA ladder. The primers used in the PCR were specific to the target genes and all the five genes were detected in standard strains of *L. monocytogenes*, whereas none

of the genes was detected in the cultures of the other bacterial species cultures (*Staphylococcus aureus*, *Rhodococcus equi*, *Escherichia coli, Streptococcus agalactiae, Bacillus* spp. and *Pseudomonas. aeruginosa*).

All three *Listeria* isolates were subjected to standardized PCR for detection of five virulence-associated genes. Only one isolate, which has been confirmed as *L. monocytogenes,* showed amplification for four of the virulence-associated genes (hlyA, plcA, prfA and actA), the fifth gene (iap) was not detected. The isolates confirmed as *L. innocua* failed to amplify any of the five virulence-associated genes.

The multiplex PCR was standardized for detection of three major serotypes of *L. monocytogenes* 1/2a, 1/2b and 4b by targeting various genes like Imo0737, ORF2819, ORF2110 and prs which were coding unknown protein, putative transcriptional regulator, putative secreted protein and putative phosphoribosyl pyrophosphate. The isolate showed amplification of three molecular size bands 471 bp, 597 bp and 370 bp corresponding to the genes, ORF2819, ORF2110 and prs, respectively.

All the isolates biochemically identified as *Listeria*, including the two isolates identified as *L. inocua,* amplified 370 bp product corresponding to gene prs. This was used as an internal amplification control.

The single *L. monocytogenes* isolate was serotyped as 4b by multiplex PCR assay.

DISCUSSION

Prevalence of *Listeria* spp. and *L. monocytogenes* from zoo animals: In a zoo setting infectious diseases are the main cause for animal losses as well as a concern for public health. Infectious diseases are caused by bacteria, fungi, parasites, or viruses. Control of such infections is difficult because of the design and purpose of the zoo itself. In a modern zoo we strive to show the animal under natural conditions and it is impossible to provide pathogen free soil, water or air.

However, infectious diseases can be minimized with appropriate recognition of the problem and training of personnel to limit the spread of infectious organisms.

In the present study 5.36% of the animals checked were found positive for *Listeria* spp, of which 1.79%

was *L. monocytogenes* and 3.57% were *L. innocua*. This is a fairly low infection rate compared to the results of Arumugaswamy & Gibson (1999), who reported 18.6% of the animals excreting *L. monocytogenes* in Taronga Zoological Garden, New South Wales, Australia, and Bauwens et al. (2003), who reported 7.5% of all wild animals in zoos were carrying pathogenic *Listeria*. Faeces of healthy animals have often been reported to contain *L. monocytogenes* (Skovgaard & Morgen, 1988). In India, Kalorey et al. (2006) isolated *L. monocytogenes* from eight (16.0%) of 50 faecal samples of healthy captive wild animals.

The presence of *Listeria* sp. including *L. monocytogenes* without clinical signs in zoo animals may reflect that these animals were in the incubation period of a disease or they are not susceptible to the organism. *Listeria* sp. are ubiquitous in nature and are commonly found in the intestines of animals and humans without necessarily causing disease.

In a zoo setting, the keeping of many species of animals in a restricted area could lead to an increased number of clinically healthy carriers posing an infection risk to susceptible animals, personnel and visitors.

In domestic cattle the reported rate of *Listeria* spp. identified in the faeces was in the range of 3.1–45.8 % (Gronstel 1979; Loken et al. 1982). In comparison to farm animals the prevalence of *Listeria* sp. and *L. monocytogenes* in zoo animals found in our study is very low. This might be a result of individual care, isolation and better hygienic measures.

For domestic cattle the usual route of infection is via contaminated food. However, silage was not fed to the herbivores at the zoo. All *Listeria* in our study were found in samples from carnivores and here the raw meat products fed to the animals may be the source of infection.

The rate of isolation of *Listeria* sp., *L. monocytogenes* and *L. innocua* in the present study, was very low; this was probably due to several factors that were compounded by an already low incidence of the organism. One of the major factors was the extremely high microbial load of feces. Our findings thus were consistent with that of Siragusa et al. (1993) who reported that *L. innocua* was the most frequent species of *Listeria* isolated from cattle.

The PCR assay in for detection of virulence associated genes, the iap gene from the *L.*

monocytogenes isolate was not amplified whereas the other four virulence-associated genes showed specific amplification. The iap gene might be absent or there is mutation in primer binding region. However, this did not reflect in any of the biochemical or phenotypical reactions of the isolate. Nishibori et al. (1995) has shown that the PCR detection of only one virulence associated gene is not always sufficient to identify *L. monocytogenes*. We have shown that at least one of the virulence associated genes can miss without an alteration of the isolate. The minimal number of virulence associated genes, which allow for exact identification of *L. monocytogenes*, must therefore still be determined. Geographic differences in the global distribution of serotypes apparently exist, but our data is not sufficient to allow for major conclusions. Serotype 4b however, the serotype identified in our study, has been found to be the serotype predominantly responsible for the animal listeriosis and *Listeria* associated foodborne outbreaks. So this result is of critical importance for the further epidemiological investigations.

In conclusion, it is clear that wild animals can act as reservoirs or carriers of *L. monocytogenes*. *Listeria* species were detected in wild animals that moved broadly and that had homeranges overlapping with areas inhabited by humans.

For public health, it is important to clarify the epidemiological relationship between *L. monocytogenes* in wild animals and *L. monocytogenes* in human cases of listeriosis and food contamination. In a zoo setting, infection risks need to be considered not only among the various zoo animal populations, but also with regard to human contacts.

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