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OCCURRENCE OF *LISTERIA* SPECIES IN DIFFERENT CAPTIVE WILD ANIMALS OF NANDANKANAN ZOO, BARANGA, ODISHA, INDIA

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Abstract: *Listeria* species were isolated from faecal samples collected from different captive wild animals of Nandankanan Zoo, Baranga, Odisha, using selective enrichment medium. The isolates were characterized based on their cell morphology, biochemical and sugar fermentation characteristics as well as culture morphology. Further, in vitro and in vivo pathogenicity tests were carried out to assess the pathogenic potential of the isolates. *Listeria* were found in 24 (23.07%) of the total 104 faecal samples. *Listeria* were isolated from the samples of tiger, bear, hyena, leopard, zebra, elephant, jackal, lion, barking deer, porcupine, chital, monkey and wild boar. Out of the 24 *Listeria* isolates 11 were confirmed as *L. monocytogenes*. The other 13 isolates included *L. innocua*, *L. seeligeri*, *L. welshimeri* and *L. ivanovii*. The pathogenicity study revealed that only four isolates were pathogenic. Three of these were *L. monocytogenes* isolated from tiger, hyena and elephant and one was *L. ivanovii* isolated from leopard. Antibiotic sensitivity of the 24 isolates was high towards ciprofloxacin, levofloxacin, amoxicillin, azithromycin and enrofloxacin. The isolates showed resistance towards oxytetracyclin, gentamicin, cephadroxil, penicillin- G and nalidixic acid.

Keywords: Captive wild animals, *Listeria*, *L. monocytogenes*, *L. ivanovii*, Nandankanan Zoo, Prevalence.

Listeriosis is an important emerging zoonotic disease caused by pathogenic strains of *Listeria* species particularly *L. monocytogenes* and *L. ivanovii*. The disease is distributed worldwide and has been reported in countries of six continents. The significance of the pathogen lies in its ubiquitous nature (Low & Donachie

1997) and wide host range, which includes 40 mammals, 20 birds, crustaceans, ticks and fishes (Sonnenworth 1980). The organism is commonly found in water, soil, vegetative materials and faecal samples of animals (Sheehan et al. 1994). The pathogenic potential of *Listeria* is well known as it causes abortion, mastitis, infertility, encephalitis and septicaemia in animals, but its spectrum of diseases is much broader and ranges from asymptomatic infection and silent carriage to cutaneous lesions or infections like conjunctivitis, urethritis, endocarditis, pneumonia, pericarditis and disturbance in gait, followed by death (Malik et al. 2010).

The number of animals in the wild is decreasing rapidly and many of the animals living in zoos represent endangered species. Proper care and management of such animals must be of prime importance. There is a high risk of infection to the captive animals in zoos as large numbers of animals are kept in a restricted area (Bowens et al. 2003). *Listeria* spp. have the ability to survive for a long period in the soil and are also possibly excreted in the faeces of asymptomatic carrier animals and pose a great risk of infection to other animals, zoo keepers and also to the visitors. This study was undertaken to determine the occurrence of *Listeria*

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species in faecal samples of wild animal species in the Nandankanan Zoo, Baranga, Odisha, India.

Materials and Methods

Test strains: The standard strains of *Listeria monocytogenes* (MTCC 1143), *Listeria ivanovii* (MTCC 7056), *Staphylococcus aureus* (MTCC 1144) and *Rhodococcus equi* (MTCC 1135) were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The strains were tested for their purity as well as for their morphological and biochemical characteristics. The *L. monocytogenes* strain was also tested for its pathogenicity in vitro and in vivo. All the strains were maintained by sub culturing them in Brain Heart Infusion (BHI) at 15 days interval.

Collection of samples: One hundred and four faecal samples were either collected from an individual animal, if it was kept in a separate enclosure (noted as individual samples) or from several animals if they were housed together in one enclosure (noted as pooled samples). All the samples were collected in the morning hours non invasively (Table 1).

Isolation method and media used: *Listeria* were isolated per USDA method described by McClain & Lee (1988) with slight modifications. Therefore, the samples were enriched in a two-step enrichment procedure and were then plated onto a selective medium as follows.

The swabs were directly transferred into sterile test tubes containing University of Vermont Medium 1 (UVM-1, Hi-Media) and were incubated at 37°C for 24hr. 0.1ml of the enriched inoculum was then transferred to University of Vermont Medium 2 (UVM-2, Hi-Media) and incubated at 37°C for 48hr. The enriched medium was streaked on Polymyxin Acriflavin Lithium chloride Ceftazidime Esculin Mannitol agar (PALCAM, Hi-Media). The streaked plates were incubated at 37°C for 48hr. The typical grey-green colonies with black centre and a black halo around the colonies were presumptively identified to be *Listeria*.

Confirmation of the isolates: The isolates were characterized by cell morphology, Gram's staining, catalase test, oxidase test, tumbling motility at 20–25 °C, MR-VP test, DLABN test and fermentation of sugar [rhamnose, xylose, maltose and mannitol] (Seeliger & Jones 1986; Low & Donachie 1997). In vitro pathogenicity was tested with blood agar and Christie, Atkin, Munch-Petersen (CAMP) test with *Staphylococcus aureus* and *Rhodococcus equi*. In vivo pathogenicity was tested by inoculating mice and chicken embryos.

In vitro pathogenicity tests

Haemolysis on sheep blood agar (SBA) plate: *Listeria* isolates were tested for the type and degree of haemolysis on SBA. Detection of haemolysin allows to discriminate between virulent and avirulent *Listeria* species. The isolates were streaked onto SBA plates, incubated at 37°C in a humidified chamber for 24hr and examined for haemolytic zones around the colonies. The characteristic wide and clear zone of β -haemolysis suggested *L. ivanovii*, while a narrow zone of β -haemolysis was characteristic for *L. monocytogenes* (McKellar, 1994).

Christie, Atkin, Munch-Petersen (CAMP) test: CAMP test was conducted following the standards of the Bureau of Indian Standards (BIS 1994) with some modifications. The standard strains of *Staphylococcus aureus* and *Rhodococcus equi* were cultured for 24hr on SBA plates at 37°C. The cultured inoculates of each strain were streaked onto freshly prepared 7% SBA plates in parallel streaks with considerable space between streaks. Afterwards, the *Listeria* isolates were streaked onto these plates at 90° angle and 3mm apart from *S. aureus* and *R. equi* streaks. The inoculated plates were incubated at 37°C for 24hr. The plates were examined for enhancement of haemolytic zone; in case of a CAMP-positive reaction the haemolytic zone between a *Listeria* strain and the *S. aureus* or *R. equi* strain was enhanced due to the synergistic effect of their haemolysins. All the *Listeria* isolates with CAMP-positivity against *S. aureus* or *R. equi* were characterized as *L. monocytogenes* and *L. ivanovii* respectively (McKellar 1994).

In vivo pathogenicity tests

Mice inoculation test: The pathogenicity of the *Listeria* isolates was tested by inoculating mice according to the method described by Menudier et al. (1991) with suitable modifications. The test isolates of *Listeria* were grown on Brain Heart Infusion (BHI) slants at 37°C for 24hr. The bacterial growth was harvested with sterile normal saline solution (NSS) and the opacity of solution was adjusted to McFarland Nephelometric tube number 1. Mice of either sex weighing 18–20 g were inoculated intraperitoneally with a volume of 0.4ml of the solutions containing approximately 10⁷ colony forming units (cfu) of the test organism per ml. The inoculated mice were observed for mortality over a period of 72hr.

Chick embryo inoculation test: The pathogenicity of *Listeria* isolates was also tested by inoculating chicken embryos following the method described by Notermans et al. (1991). The blood vessel-free surface of the chorioallantoic membrane (CAM) of two precandled 10-day old embryonated chicken eggs was inoculated with

Table 1. Isolation of *Listeria* species from Faecal samples of different wild animals of Nandankanan Zoo, Barang, Odisha.

	Species	No. of samples Collected	No. of positive samples	<i>Listeria</i> species isolated
1	Tiger <i>Panthera tigris</i>	19	4	<i>L. monocytogenes</i> (3), <i>L. welshimeri</i> (1)
2	Leopard <i>Panthera pardus</i>	5	1	<i>L. ivanovii</i> (1)
3	Lion <i>Panthera leo</i>	8	2	<i>L. monocytogenes</i> (2)
4	Jackal <i>Canis aureus</i>	2	1	<i>L. monocytogenes</i> (1)
5	Hyena <i>Hyaena hyaena</i>	4	2	<i>L. monocytogenes</i> (2)
6	Zebra <i>Equus quagga</i>	4	2	<i>L. innocua</i> (1), <i>L. seeligeri</i> (1)
7	Elephant <i>Elephas maximus</i>	5	1	<i>L. monocytogenes</i> (1)
8	Porcupine <i>Hystrix indicus</i>	3	1	<i>L. innocua</i> (1)
9	Wild Boar <i>Sus scrofa</i>	1	1	<i>L. innocua</i> (1)
	<u>Bear</u> Himalayan Black Bear <i>Selenarctos thibetanus</i> ; Sloth Bear <i>Melursus ursinus</i>	13	2	<i>L. innocua</i> (2)
11	<u>Deer</u> Barking Deer <i>Muntiacus muntjak</i> ; Chital <i>Axis axis</i> Sambar <i>Rusa unicolor</i> ; Chousingha <i>Tetracerus quadricornis</i> ; Hog Deer <i>Hyelaphus porcinus</i> ; Barasinga <i>Rucervus duvaucelii</i>	18	5	<i>L. monocytogenes</i> (2), <i>L. seeligeri</i> (2), <i>L. innocua</i> (1)
12	<u>Primate</u> Rhesus Macaque <i>Macaca mulatta</i> ; Bonnet Macaque <i>Macaca radiata</i> ; Assam Macaque <i>Macaca assamensis</i> ; Baboon <i>Papio</i> ; Nilgiri Langur <i>Semnopithecus johnii</i> ; Patas Monkey <i>Erythrocebus pata</i>	9	2	<i>L. seeligeri</i> (1), <i>L. welshimeri</i> (1)
13	Blue Bull <i>Boselaphus tragocamelus</i>	3	0	-
14	<u>Ape</u> Orangutan <i>Pongo pygmaeus</i> ; Chimpanzee <i>Pan troglodytes</i>	4	0	-
15	Rabbit <i>Oryctolagus cuniculus</i>	2	0	-
16	Rhinoceros <i>Rhinoceros unicornis</i>	1	0	-
17	Hippopotamus <i>Hippopotamus amphibius</i>	3	0	-
	Total	104	24	<i>L. monocytogenes</i> (11), <i>L. Welshimeri</i> (2), <i>L. Ivanovii</i> (1), <i>L. innocua</i> (6), <i>L. Seeligeri</i> (4)

0.1ml of the test culture in BHI broth. Control eggs were inoculated with 0.1ml of BHI broth. All eggs were sealed with molten paraffin and were horizontally placed at 37°C for 3 days. The eggs were examined twice a day by transillumination for embryo death. A test isolate causing embryo mortality after 24 hours of inoculation was considered to be pathogenic.

Antibiotic sensitivity test: The antibiotic sensitivity of each of the *Listeria* isolates was tested applying the Bauer-Kirby diffusion method (Bauer et al. 1966) using antibiotic discs (Hi-Media). The concentrations of antimicrobial agents used were as follows: levofloxacin 5µg/disc, enrofloxacin 5µg/disc, ciprofloxacin 5µg/disc, penicillin-G 10units/disc, amoxicillin 10µg/disc, chloramphenicol 30µg/disc, ceftriaxone 30µg/disc, cephotaxime 30µg/disc, cephadroxil 30µg/disc, cephalixin 30µg/disc, gentamicin 10µg/disc, oxytetracycline 30µg/disc,

amikacin 30µg/disc, tobramycin 30µg/disc, azithromycin 30µg/disc, nalidixic acid 30µg/disc.

Results

Out of 104 examined faecal samples 24 (23.07%) samples were found to be *Listeria* positive. *Listeria* isolates were recovered from the faecal sample of tiger, bear, hyena, leopard, zebra, elephant, jackal, lion, barking deer, porcupine, chital, monkey and wild boar. Out of 24 *Listeria* isolates 11 were identified as *L. monocytogenes*, six as *L. innocua*, four as *L. seeligeri*, two as *L. welshimeri* and one *L. ivanovii* (Table 1). Only four of the *Listeria* isolates were found to be pathogenic. These were three isolates of *L. monocytogenes* which were from tiger, hyena and elephant and one isolate of *L. ivanovii* from leopard (Table 2).

The *Listeria* isolates were tested in vitro for their

Table 2. Differentiation and Pathogenicity test of *Listeria* isolates from faecal samples of wild animals in Nandankanan Zoo, Baranga, Odisha.

1	Animal source	Pathogenicity profile										Species identified
		Rhamnose	Xylose	Mannitol	Maltose	In vitro test				In vivo test		
						Hemolysis on SBA	CAMP test with S/R	ALOA		Mouse lethality test	Chick embryo lethality	
								Colour	halo			
2	3	4	5	6	8	9	10	11	12	13	14	
1	Tiger	+	-	-	+	+	+S	BG	+	+	+	<i>L. m</i>
2	Tiger	+	-	-	+	+	+S	BG	+	-	-	<i>L. m</i>
3	Tiger	+	+	-	+	-	ND	BG	-	ND	ND	<i>L. w</i>
4	Tiger	+	-	-	+	+	+S	BG	+	-	-	<i>L. m</i>
5	Leopard	-	+	-	+	+	+R	BG	+	+	+	<i>L. i</i>
6	Lion	+	-	-	+	+	+S	BG	+	-	-	<i>L. m</i>
7	Lion	+	-	-	+	+	+S	BG	+	-	-	<i>L. m</i>
8	Jackal	+	-	-	+	+	+S	BG	+	-	-	<i>L. m</i>
9	Hyena	+	-	-	+	+	+S	BG	+	+	+	<i>L. m</i>
10	Hyena	+	-	-	+	+	+S	BG	+	-	-	<i>L. m</i>
11	Zebra	-	-	-	+	-	ND	BG	-	ND	ND	<i>L. inn</i>
12	Zebra	-	+	-	+	+	+S	BG	-	ND	ND	<i>L. s</i>
13	Elephant	+	-	-	+	+	+S	BG	+	+	+	<i>L. m</i>
14	Porcupine	-	-	-	+	-	ND	BG	-	ND	ND	<i>L. inn</i>
15	Wild Boar	-	-	-	+	-	ND	BG	-	ND	ND	<i>L. inn</i>
16	Bear	-	-	-	+	-	ND	BG	-	ND	ND	<i>L. inn</i>
17	Bear	-	-	-	+	-	ND	BG	-	ND	ND	<i>L. inn</i>
18	Barking Deer	+	-	-	+	+	+S	BG	+	-	-	<i>L. m</i>
19	Barking Deer	+	-	-	+	+	+S	BG	+	-	-	<i>L. m</i>
20	Chital	-	+	-	+	+	+S	BG	-	ND	ND	<i>L. s</i>
21	Chital	-	-	-	+	-	ND	BG	-	ND	ND	<i>L. inn</i>
22	Chital	-	+	-	+	+	+S	BG	-	ND	ND	<i>L. s</i>
23	Monkey	+	+	-	+	-	ND	BG	-	ND	ND	<i>L. w</i>
24	Monkey	-	+	-	+	+	+S	BG	-	ND	ND	<i>L. s</i>

L. m - *L. monocytogenes*; *L. i* - *L. ivanovii*; *L. w* - *L. welshimeri*; *L. s* - *L. seeligeri*; *L. inn* - *L. innocua*; BG - Blue Green; SBA - Sheep blood agar; CAMP - Christie- Atkin-Munch- Petersen test; S/R - *Staphylococcus aureus* / *Rhodococcus equi*; ALOA - Agar *Listeria* according to Ottaviani and Agosti

sensitivity to different antibiotics. The obtained antibiogram revealed a high sensitivity towards ciprofloxacin and levofloxacin (100%). High sensitivity was also observed towards amoxicillin, azithromycin (87.5%) and enrofloxacin (79.1%). Moderate sensitivity was observed towards chloramphenicol (66.7%) and amikacin (58.3%). The isolates were resistant towards oxytetracyclin, gentamycin and cephadroxil (both 75%), penicillin-G and tobramycin (both 62.5%), cephotaxim and cephalexin (both 45.8%) and ceftriaxone (33.3%). All isolates were resistant to nalidixic acid.

Discussion

Listeria monocytogenes is ubiquitous in nature and

has been isolated from numerous sites, including soil, sewage water and decaying plant materials etc. Its viability is remarkable, with survival in soil or silage for more than two years. Studies have shown that about 50% of faecal samples collected from different animals like cattle, sheep, goat, pig and poultry contain *L. monocytogenes* but don't exhibit any clinical symptoms of listeriosis (Meng & Doyle 1997; Wesley 1999). *Listeria* species have previously been reported in Odisha from milk samples of cows and from faecal samples of various domestic animals (Sarangi et al. 2009; Sarangi & Panda 2012). In the present study *Listeria* species were isolated from 24 (23.07%) out of 104 faecal samples of healthy zoo animals. *Listeria* infection is mostly food borne

(Ryser 1999) and raw meat, vegetable and fruits used as animal feeds in zoological parks might be the major source of *Listeria* infections.

Listeria species have been reported from a number of wild animals in the past. *L. monocytogenes* have been isolated from the faecal sample of foxes, Roe Deer, badgers, hares and Pine Marten (Weis 1975), African Lion (Haigh et al. 1978), Grey Fox (Black et al. 1996), primates, hippopotamus, fox and bear (Arumugaswamy & Gibson 1999), farm chinchilla (Sabocanec et al. 2000), captive antelopes (Bauwens et al. 2001), wild boars (Hayashidani et al. 2002), Colobus Monkey (Kock et al. 2003), jackal, Indian Fox, Sambar, wolf and Spotted Deer (Kalorey et al. 2006) and from other deer, raccoon and moose (Lyautey et al. 2007). Similarly, *L. ivanovii* has been isolated from Grauer's Gorilla and Red Ruffed Lemur (Bauwens et al. 2003) and from a septicaemic chinchilla (Kimpe et al. 2004). However, we isolated *Listeria* species from tiger, hyena, leopard, zebra, elephant, blue bull, barking deer and porcupine which all have to date not been reported to carry *Listeria*.

Our overall infection rate of 23.07% is comparable to infection rates found by other researchers. Bernagozzi et al. (1999) reported 24.1% of *Listeria* spp. in faeces of wild animals in National Park of Casentine forest (112 samples). Bauwens et al. (2001) isolated *Listeria* spp. from 22.8% of the faecal samples of Wild Animal Park, Planckendael (70 samples). Bauwens et al. (2003) also reported a *Listeria* occurrence of 21% in faecal samples of wild animals in Antwerp Zoo (200 samples). In India, Kalorey et al. (2006) isolated *L. monocytogenes* in 16% of faeces of wild animals (50 samples). A much lower infection rate was reported by Yoshida et al. (2000), who only found an infection rate of 6.1% (623 samples) in all animals tested. However in Japanese macaques the infection rate was also 20%. Recently, Yadav et al. (2011) isolated two *Listeria* species (*L. monocytogenes* and *L. innocua*) out of three positive isolates from 56 faecal samples collected from mammals and birds at Baroda Zoo, Vadodara, Gujarat State, India, which equals to a prevalence of only 5.3%. However as the range of species and animals examined in these studies are entirely different it is not possible to conclusively compare the infection rates found.

In the present study 11 (10.57%) *L. monocytogenes* were isolated which differ from the observation of Arumugaswamy & Gibson (1999) who after examination of 86 animals of Taronga Zoological Garden, New South Wales, Australia, revealed 25.6% were carrier of *Listeria* species and 18.6% were excreting *L. monocytogenes*. This difference may be due to varying environmental condition

between different locations, previous occurrence of the disease and hygienic measures adopted in the zoo.

Listeria spp. have in the past been reported to be sensitive to antibiotics active against gram positive bacteria (Hawkins et al. 1984), but some time later reports of resistance in *Listeria* spp. appeared (Abraham et al. 1998; Walsh et al. 2001). An increase in antibiotic resistance in *Listeria* spp. is in line with a global pattern of an increasing antibiotic resistance, including resistances against multiple antibiotics in many groups of bacteria. The occurrence of antibiotic resistance in non-pathogen forms poses a major risk as there is possibility to transfer the resistance factor from non-pathogens to pathogenic organisms (Walsh et al. 2001).

In our study we found high to moderate sensitivity towards ciprofloxacin, levofloxacin, amoxicillin, azithromycin, enrofloxacin, chloramphenicol and amikacin and resistance towards oxytetracyclin, gentamycin, cephadroxil, penicillin-G, tobramycin, ceftotaxim, cephalixin, ceftriaxone and nalidixic acid. Similar results have been reported by Lyautey et al. (2007) who found resistance against kanamycin, gentamycin, streptomycin and rifampicin. This differs from findings of Butko et al. (1972), who reported the sensitivity of *Listeria* spp. to erythromycin, chlortetracycline, streptomycin, levomycin, neomycin and monomycin and resistance to penicillin. This variation in antibiotic sensitivity might be due the above mentioned global increase in antibiotic resistance in the bacteria over decades.

The present study shows the occurrence of *Listeria* species in captive wild animals of Nandankanan Zoo, Baranga, Odisha, which may act as nidus of infection for other susceptible animals and cause death of animals. A further study investigating the role of *Listeria* in sick wild animals is necessary and emphasis should be given to control the food borne transmission of the diseases.

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