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Prevalence and Seroprevalence of *Listeria monocytogenes* among Slaughtered Food Animals and Human Cancer Patients

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The present study was aimed to study the seroprevalence of *Listeria monocytogenes* amongst the food animals and cancer patients in and around Nagpur Region, Maharashtra, India. A total of 750 samples comprising of 600 samples from food animals and 150 samples from cancer patients were collected from various slaughter houses and Government. Hospital Nagpur, respectively. 50 samples of each meat, blood and serum was collected from 50 slaughtered goat, male cattle, buffaloes and pigs. From 50 cancer patient, each of stool, blood and serum samples were gained. Out of total sample processed 23 were found positive for Listeria spp. biochemical characterization leads 12 isolates turned out as *L. monocytogenes*, 01 *L. seeligiri*, 01 *L. innocua*, 01 *L. grayi*, 05 *L. welshimeri.* Human, buffalo and all blood samples except pig were found nil for the listeria while remaining showed positivity for the same. *L. monocytogenes* was recovered from (two isolates) beef, (four isolates) chevon and (six isolates) pig. In serodiagnosis highest seroprevalence was noted in goats (80%), followed by cattle (44%), pigs (42%) and buffalos (26%) and 26% in human cancer patients. From the research findings study focuses the prevalence of *L. monocytogenes* among food animals in Nagpur region of Maharashtra, India. It can be concluded that high seroprevalence is an indication for the persistence of organism in geographical area.

Key words: Food animal, Human cancer patient, *Listeria monocytogenes*, Prevalence, Seroprevalence.

Meat and meat products are important source of high quality nourishment for ever increasing human population in world. Though meat is good source of nutrient; it may act as potential source of infection. In recent years zoonotic diseases including those of food borne origin have gained more importance and food chain has been recognized as one of the main route of transmission. Listeria is important food borne pathogen between human and animal[1] and various studies confirm the view that Listeria as a frequent contaminant in food products, and India is no exception[2]. Among microorganisms, *Listeria monocytogenes* is one of the most important emerging zoonotic public health hazards causing a food borne disease listeriosis. Listeriosis has increased manifold during past two decades[3], responsible for 30% of food borne deaths from 1996 to 2005 in the United States and had a high fatality rate of 16.9%[2]. Food borne outbreaks of

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listeriosis are also reported from Europe and America[3]. In India, reports of outbreaks of listeriosis in animal populations are reviewed by many workers as Phadke *et al*.[4], Rahman *et al*.[5], Malik *et al*.[6]. *L. monocytogenes* have been isolated from the meat of goats, sheep and buffaloes[7,8] and raw meat has been linked for the listeriosis [9,10].

Listeria is ubiquitous organism occurring in both terrestrial and aquatic habitats. It is a group of Gram-positive, facultatively anaerobic, non-spore-forming bacteria[10]. The genus comprises ten species as *Listeria monocytogenes, Listeria ivanovii, Listeria seeligeri, Listeria innocua, Listeria welshimeri, Listeria grayi, Listeria marthii, Listeria rocourtiae, Listeria fleischmannii,* and *Listeria weihenstephanensis*[11]. Of these, *L. monocytogenes* is an opportunistic pathogen of zoonotic significance responsible for 85% of animal cases and about 98% of human cases with a very high mortality rate of 20 to 30%[12] associated with neural, visceral and reproductive clinical entities leading to septicemia, abortions and stillbirth[13] whereas *L. ivanovii* is considered a pathogenic species for animals[9].

In case of human beings pregnant women, new-born babies, elderly people and immunocompromised individuals are grouped as high risk for listeriosis[14]. In pregnant women and newborn babies organism is considered as the third-most-common cause of meningitis and meningoencephalitis, the prognosis is very poor in immunocompromised patients[15,16].

Virulence factor of *L. monocytogenes* not found in other *Listeria* species called as LLO is stated as an appropriate test antigen and thus has been identified as a candidate antigen for a serological assay[17]. All virulent strains of intracellular pathogenic *L. monocytogenes* elaborate an extra-cellular hemolysin, listeriolysin O (LLO)[18] which is a pore-forming toxin of the cholesterol-dependent cytolysin family shown to be an essential virulence factor[19]. It helps in the escape of the *L. monocytogenes* into the cytoplasm of the host cell during infection. Serodiagnosis is being taken as quick method of detection, ELISA-based formats designed to detect its major virulence factor listeriolysin (LLO), have been developed for diagnosis of listeriosis in animals[20]. Seropositivity against LLO has been suggested to serve as reliable indicator of infection in animals and human beings which can be detected soon after clinical onset of listeriosis and antibodies persist for at least several months[21].

Due to global increase in reported incidence of listeriosis from foods, many countries have established a "zero tolerance" policy towards *L. monocytogenes* in ready to eat foods[22]. Reports of listeriosis from humans in India are scanty, either because of failure to identify the isolate, its rarity, low incidence rate or lack of awareness[23]. In Nagpur region, large mass of consumers prefer chevon, beef, buffalo meat and pork, where goats, cattle, buffalo and pigs are being slaughtered at four major slaughter houses in addition at many door-steps of butchers' houses. Nevertheless, the disease largely remains undiagnosed and under reported, epidemiological data available on listeriosis in India to date is not adequate for assessing the extent of infection in human beings and animals[2]. Therefore study was planned to detect the prevalence and seropositivity of *Listeria monocytogenes* in Nagpur region of Maharashtra, India.

MATERIAL AND METHODS

Biologicals and Laboratory animals

The standard bacterial strains of *Listeria monocytogenes* (MTCC 1143), *Staphylococcus aureus* (MTCC 3160) and *Rhodococcus equi* (MTCC 1135) were obtained from Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India. Chemicals/conjugates required for the study were procured from Sigma Aldrich (US) and Merk/Gene bank, India.

For raising antisera against *L.monocytogenes*, adult healthy rabbits of New Zealand white breed weighing 2.5 to 3 kg were chooses and maintained in strict hygienic conditions with proper supply of *ad-libitum* feed and water throughout the study period.

Sample collection

A total of 750 samples were randomly collected, of which 600 from food animal comprising of meat, blood and serum from 50 each of goat, male cattle, buffaloes and pigs slaughtered at slaughter houses in and around Nagpur region and 150 samples from 50 human cancer patients comprising of blood, stool and serum from each one attending Government Medical College and

Hospital (GMCH), Nagpur. All samples were sealed, packed and transported in an ice box to the laboratory and processed immediately. The locations of samples collected were shown in Table 1 and Table 2.

Isolation of *L. monocytogenes*

The isolation of the *Listeria* species from meat and clotted blood were carried out as per the standard protocol described by USDA[24.] Further identification was carried out by subjecting to gram staining, motility tests, biochemical characterization (Catalase test, Oxidase test, Methyl Red and Voges- Proskauer (MR-VP) tests, Nitrate reduction test) and sugar fermentation tests (D-mannopyranoside, L-rhamnose and D-xylose) as per the method described by Bergey's Manual of Systematic Bacteriology[25].

Purification of the virulence factor Listeriolysin-O (LLO)

Listeriolysin O (LLO), a virulence factor of *L. monocytogenes* was purified from standard strain of *L. monocytogenes* (MTCC 1143) as per the method suggested by Lhopital *et al.*[26] by using diethylaminoethyl (DEAE) cellulose (Sigma, Aldrich, US) chromatography [27]. The pooled fractions (protein) were concentrated using polyethylene glycol (PEG-20,000) at 4^0C. Obtained protein was estimated by using the method suggested by Lowry *et al.* [28]. The characterization of purified protein was done by subjecting to Sodium-dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) for estimation of molecular weight as per Lammeli[29]. The protein was visualized by employing silver staining technique as per method of Rosenberg[30].

Rising of antiserum against purified LLO

Hyper immune serum was raised against purified LLO in adult healthy rabbit as per the method of Barbuddhe [31]. Briefly, 100 µg of purified LLO was dissolved in one ml of Normal saline solution (NSS)and one ml of frieunds complete adjuvant, mixed properly with needle until become viscous and completely blocks the gauge of needle. The mixture injected Intra-cutaneously on back at multiple sites with a distance of 1-2 cm. Rabbit was test bled at 14th and 21st days after first injection for checking the titer. The anti-LLO serum (Hyper immune serum) was separated and stored at -20^0C after addition of thiomersal @ 0.01 per cent as preservative.

Detection of antibodies against Listeriolysin O (ALLO) by Indirect ELISA

The indirect plate ELISA was performed as per the method of Low *et al.*[32] and standardized by Checker board analysis. Briefly, polyvinyl microtitre plates (Nunc, Denmark) were coated with 100µl/ well of coating buffer containing purified LLO as an antigen in a concentration of 1µg and 2µg per well and incubated at 4^0C overnight. The coated plates were washed thrice with phosphate buffered saline (PBS), pH 7.2 to remove unadsorbed antigen. The unsaturated sites of the plates were blocked by adding 5 per cent skimmed milk powder prepared in PBS-T (PBS containing 0.05 per cent Tween-20) @ 200µl /well and incubated at 4^0C overnight. The plates were then washed thrice with PBS-T. The hyperimmune serum as well as healthy (zero day) serum was diluted in range of 1:100 to 1:800 and each dilution was added @100 µl/well and plates were further incubated at 37^0C for 2 h. The plates were then washed thrice with PBST and added with anti-species Horseradish peroxidase (HRPO) conjugate (Merck, India) in the range of 1:1000 to 1:4000 (except for anti-porcine HRPO conjugate from Sigma @ 1:20000) at the rate of 100 µl/well and again incubated at 37^0C for 1 hr. The plates were then washed thrice with PBS-T and finally added with 1 mg/ml solution of O-phenylene-diamine dihydrochloride (OPD) (Sigma Aldrich, US) as substrate @ 100µl per well. The plates were then incubated for 15 min in dark and the colour developed was measured at 492 nm by ELISA reader (Multiskan Go, Thermofisher Scientific, Finland).

RESULTS

Out of 500 samples (400 Animal and 100 Human samples) 23 samples were found positive for Listeria spp. After biochemical characterization 12 isolates turned out to be *L. monocytogenes*, 01 *L. seeligiri*, 01 *L. innocua*, 01 *L. grayi*, 05 *L. welshimeri* (Table 3). Human samples, buffalo samples and blood from all animals were found negative while remaining all other samples showed positivity. Two isolates of *L. monocytogenes* were recovered from beef, four from chevon and six from pork. Three isolates of *L. seeligiri* isolated one from each beef, chevon and pork. Two isolates of *L. innocua* was isolated one each from chevon

and pork whereas, one *L. grayi* and five *L. welshimeri* were isolated from pork and beef respectively.

Purified protein/LLO was characterized with respect to its molecular weight 58.86 kDa by employing SDS-PAGE showed a final purified band of 57.92 kDa. Overloading of the gel with purified LLO did not reveal any contaminating bands in subsequent trials.

The standardization of indirect ELISA could be achieved as antigen (LLO) at concentration of 0.5µg/ well, serum dilution at 1:200 and anti-species HRPO conjugate at 1:2000 (except for anti-porcine HRPO conjugate from Sigma @ 1:20000) for final screening of test sera samples as the combination had Positive to Negative (P/N) ratio of 2.0 when compared with healthy serum. The serum samples with a P/N ratio of 1.5 to 2.0 was taken as suspected for listeriosis.

Seropositivity was revealed among all food animals and human patients. Among the four food animals tested, highest seroprevalence was noted in goats (80%), followed by cattle (44%), pigs (42%) and buffalos (26%). In human cancer patients 26% of seropositivity was obtained (Table 4).

Table 1. Details of samples collected from Slaughterhouses

Food animals	Type and number of sample			Place of collection (Location of slaughter houses)
	Meat	Blood	Serum	
Cattle	50	50	50	Bhandewadi and Kamti, Nagpur
Buffalo	50	50	50	Kamti and Bhandewadi, Nagpur
Goat	50	50	50	Itwari, Gaddigodam and Gittikhadan, Nagpur
Pig	50	50	50	Imamwada, Hazaripahad and Gittikhadan, Nagpur

Grand total = 600

Table 2. Samples collected from Human cancer patients

Risk group	Type and number of samples			Place of Collection
	Stool	Blood	Serum	
Cancer patients Nagpur	50	50	50	Patients attending GMCH,

Grand Total = 150

Table 3. Prevalence of *Listeria* spp. among food animals and Human cancer patients

	Sample (No=50)	*Listeria* Spp.					No. of positive *Listeria* spp.
		Lm	Ls	Lin	Lg	Lw	
Cattle	Beef	02	01	0	0	05	08
	Blood	0	0	0	0	0	0
Buffalo	Carabeef	0	0	0	0	0	0
	Blood	0	0	0	0	0	0
Goat	Chevon	04	01	01	0	0	06
	Blood	0	0	0	0	0	0
Pig	Pork	04	01	01	01	0	07
	Blood	02	0	0	0	0	02
Human	Faecal	0	0	0	0	0	0
	Blood	0	0	0	0	0	0
Total	500	12	01	01	01	05	23

Lm= *Listeria monocytogenes*, Ls= *Listeria seeligiri*, Lin= *Listeria innocua*, Liv= *Listeria ivanovii*, Lg= *Listeria grayi*, Lw= *Listeria welshimeri*

DISCUSSION

Listeria can easily enter in food chain on the basis of its ability to survive in harsh conditions and persistance in environment for long period[33]. Several developed countries have enforced strict regulations over occurrence of *L. monocytogenes* in food[34]. To understand the listeriosis in detail, there is a need of systematic and coordinated studies to estimate the prevalence of *L. monocytogenes* in different habitats, occurrence of listeriosis in humans as well as in animals and their concordance of occurrence and actual disease[2]. Prevalence studies will help to understand the epidemiology of disease that could be used to improve management of Listeriosis.

The present study was aimed at detection of prevalence of *L. monocyotgenes* in and around Nagpur region, Maharashtra, India. In the region goats, male cattle, buffalo and pigs are being slaughtered at large extent with consumer's high predilection so contributing large source of meat. Listeria species were identified by 5.75 % of all samples from food animals, contributing 10.5% of meat and 1% of blood samples. *L. monocytogenes* (5%), *L. ivanovii* (0.5%), *L. innocua* (0.5%), *L. seeligiri* (0.5%), and *L. welshimeri* (2.5%) were isolated from meat samples and *L. monocytogenes* (1%) from blood samples in the study. The *L. monocytogenes* isolates were mainly recovered from cattle, goats and pigs. The findings are in lined with several researchers[35,36,37] who reported prevalence of *L. monocytogenes* as 6.4 , 7.7 and 2.63 % respectively among raw beef samples while none of the buffalo beef samples were found positive. The findings are in accordance with Barbuddhe *et al.*[8] and Hassan *et al.*[38] recorded zero prevalence amongst the buffalo meat samples.

Table 4. Detection of antibodies to Listeriolysin-O (ALLO) among food animals and human cancer patients

Source	No. of serum samples analyzed	No. of positive sera samples (ALLO positivity)
Cattle	50	22(44%)
Buffaloes	50	13(26%)
Goats	50	40(80%)
Pigs	50	21(42%)
Human cancer patients	50	13(26%)

8% prevalence of *L. monocytogenes* from goat meat samples was in harmony with the Barbuddhe *et al.*[7] who reported 6.66% and 7.4% prevalence from the same species. However, Rahimi *et al.*[39] and Hayat Ennaji *et al.*[40] reported higher value of 32.7 and 23.3 % from Iran and *Morocco* respectively.

Isolation from 8% pork samples registered in the present study in lined with Autio *et al.*[41] and Molla *et al.*[42] who reported 9.33% and 7.54 % prevalence in Finland and Addis ababa respectively. Chasseignaux *et al.*[43], Bonardi *et al.*[44] and Peccio *et al.*[45] reported 21, 21.3 and 29.41 % prevalence at France, North East Italy among pork samples respectively. However Kanugati *et al.*[46] stated 50.2 % at Nasco, United States; whereas Wesley *et al.*[47] cited 1.4% at United States and Zhou and Jiao[48] reported 0.95 % prevalence in China among pork samples. The variation among these isolations can be attributed to the geographical variation of location[49], environmental factors , host factor, source of samples as well as combination of the media for the isolation followed[50].

No isolations from blood samples of all food animals except pig (4%) were noted in the present study. However, Chaudhari *et al.*[51] and Shakuntala *et al.*[52] reported 1.6 % and 4.44% isolations of *L. monocytogenes* from buffalo blood samples collected from slaughter house and clinical samples in Uttar Pradesh (UP) respectively. Similarly, Brahmbhatt and Anjaria[53] and Elezebeth *et al.*[49] cited no recovery from goat blood samples collected from Gujarat and slaughter house Bareilly (UP) but BhanuRekha *et al.*[54] reported 1.77% positivity of *L. monocytogenes* from goat blood slaughtered at Uttar Pradesh. The negativity among blood samples can be contributed to the fact that septicemia is relatively uncommon in Listeriosis[55]. The variation can be attributed to the place /source of collection of samples as well as the actual bacteremic condition of the animal. Observations on the isolation of *Listeria* spp. other than *L. monocytogenes* viz. *L. welshimeri*, *L. innocua*, *L. seeligeri* and *L. grayi* confirms prevalence of these within the Nagpur region, Maharashtra, India.

Seroprevalence of 44 % among cattle in present study is on higher side as compared to Dutta and Malik [56] (2.8%), Nigam *et al.*[57] (13.8%).

Kalorey *et al.*[58] reported 5.48% Seroprevalence from Vidarbha region using Listeriolysin-O as an antigen by indirect ELISA. Among buffalo, 26% of seroprevalence was comparable with authors[31,59] who reported 41.6% and 52.5% of seropositivity respectively. Similarily, higher seroprevalence of 80 % was noticed among goats as compared to those reported by several researchers as 41.1% by Barbuddhe *et al.*[7] and 21.69% by Bhanu Rekha *et al.*[54]. In case of 42% prevalence could be compared with 43% positivity for agglutinin in gaming pigs reported by Hislop[60].

The high positivity for ALLO observed in present study might be attributed to variation in the nature and behavior of antigen could not be denied. Perpetuation of the strain in geographical region and possibility of exposure of these animals to low infective doses which has been reported to elicit persistent immune response to LLO equal to those higher infective doses[26].

Amongst the human stool and blood samples examined from cancer patients found nil for the presence of listeriosis, the findings are in agreement of Dhanashree *et al.*[16] and Kaur *et al.*[61] whereas, Safdar *et al*.[62] reported 0.47% of prevalence among cancer patients at New York. Seroprevalence among human cancer patients could be compared with Barbuddhe *et al.*[63] who reported 31.8% and 32.7% of seroprevalence among venereal disease persons and farm workers respectively in north India. Present results could not proved and cited necessity of large number of samples from human cancer patients to revalidate the association among immunocompromised cancer patients with seroprevalance against listeriosis.

CONCLUSION

By considering research findings the present study focuses the prevalence of *L. monocytogenes* among food animals in Nagpur region of Maharashtra, India. High seroprevalence indicate the persistence of organism in geographical area. Pathogenesity of 12 *L. monocytogenes* isolates has already been assessed by Dudhe *et al*.[64] indicating high virulence of these species and thus having epidemic potential. It warns the possibility of transfer of this pathogen from animals to foods of animal origin and thereafter the food chain. Therefore, prevalence and seroprevalence of *L. monocytogenes* need to be studied with wide epidemiological investigations specifically in meat borne implications which will help to prevent this zoonotic significant pathogen.

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