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Isolation, Identification and Antibiotic Susceptibility Profiling of *Listeria* spp. from Raw Chicken Meat in Durg District of Chhattisgarh, India

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ABSTRACT

Present work was conducted to determine the total aerobic plate count of raw chicken meat samples, isolation of the *Listeria* spp. and determining their pathogenicity along with antibiotic susceptibility pattern. The 100 raw chicken meat samples, collected from different retail outlets in and around Durg district of Chhattisgarh, revealed mean APC of 23.67×10^5 cfu/g (6.374 $\log_{10}$ cfu/g). Cultural examination of raw chicken meat samples showed an overall 37% prevalence of *Listeria* spp., comprising of *L. monocytogenes* (16%), *L. grayi* (11%), *L. welshimeri* (5%), *L. ivanovii* (3%) and *L. innocua* (2%). All the *Listeria* isolates exhibited a typical β-heamolysis with narrow zone on sheep blood agar and enhancement of hemolytic zone in CAMP test. The haemolytic Listerial isolates developed kerato conjunctivitis in Anton's test and stunting as well as hemorrhages in liver and heart along with conspicuous thickening of CAM in chicken embryos. Results of antibiotic susceptibility testing of all *Listerial* isolates further revealed that most of isolates were multidrug resistance to antibiotics. The present work revealed that the raw chicken meat may act as an important source of *Listeria* for human being. The presence of multiple drug resistance among *Listeria* spp. isolates provides a evidence of the emergence of multi drug resistant *Listeria* strains, pointing to an increase in the potential threat to human health.

Keywords: *Listeria*, chicken meat, isolation, characterization, Chhattisgarh

Ever since the food borne nature of Listeriosis was established, there is increasing interest in understanding the risk associated with this organism in various foods. The widespread nature of *Listeria* allow easy access to a variety of raw foods including meat, milk, sea foods (Sunil *et al*., 2013) and food products during various phases of production, processing, manufacturing and distribution. *L. monocytogenes* is one of most virulent food borne pathogen with case fatality rate of 20-30% (Ramaswamy *et al*., 2007). As these pathogens are capable of surviving even under refrigerated conditions, posing threat to the food industries and there by the consumers. With the increase in consumption of manufactured ready-to-eat foods, *L. monocytogenes* has been recognized as an important opportunistic human food borne pathogen (Liu, 2006). In humans, listeriosis is a very rare but serious illness that can lead to abortion or serious cases of meningitis or encephalitis, and even death (Ahmed *et al*., 2017).

Many *Listeria* spp. isolated from human samples, milk, meat and environmental sources were found to be resistant to antimicrobials commonly used in human and veterinary medicine. A remarkable multi-drug resistance was observed in all recent studies. The indiscriminate use of antimicrobials has led to the appearance of antimicrobial-

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resistant *Listeria* spp. In addition, antimicrobials used as growth promoters in animal feed have resulted in the dissemination of antimicrobial-resistant *Listeria* spp. into the environment. Therefore despite the use of efficient antibiotic therapy, listeriosis represents a public health problem since it may be fatal up to 30% in most of the outbreaks. This threatening nature of listeriosis also prompted the World Health Organization (WHO) to suggest that various food products must be frequently investigated for the presence of *L. monocytogenes* on a worldwide basis (World Health Organization, 1990). Furthermore, only very few surveys about the presence of *Listeria* spp. in raw meat have been conducted in Asia particularly in India. Hence, the present study was proposed to generate information regarding the prevalence of *Listeria* spp. and their antimicrobial susceptibility profiling in raw chicken meat sold in and around Durg district of Chhattisgarh.

MATERIALS AND METHODS

During the present study, a total of 100 raw chicken meat samples for isolation of *Listeria* spp. and for total aerobic count were collected from different retail outlets in and around Durg district of Chhattisgarh. All the meat samples were collected aseptically in UV sterilized polyethylene sachets and transported immediately to the laboratory under chilled condition, stored at 4°C and processed within 24 hours.

The aerobic plate count (APC) of each chicken meat sample was determined following the method described by International Commission on Microbiological Specifications for Foods (ICMSF, 1978) with minor modifications. Ten-fold serial dilution of each sample was prepared in sterile normal saline solution (NSS) up to 10^{-8} dilution. Inoculum from each dilution has then spread on the surface of the Plate Count Agar medium (Himedia, India) and kept at room temperature for 30 min for adsorption followed by incubation at 37°C for 24 hrs. The plate with colonies between 30-300 was counted and bacterial count was determined by multiplying the number of colonies with reciprocal of dilution factor.

Isolation of *Listeria* spp. from raw chicken meat samples was attempted following the method described by Donnelly and Baigent (1986). Briefly, the 5g sample was triturated under aseptic conditions and inoculated into 45 ml of University of Vermont medium (UVM-I, containing 12 mg of acriflavin hydrochloride) and incubated at 30°C for 18- 24 hrs. Thereafter, 0.1ml enriched inoculum from UVM-I was transferred to 10 ml of UVM–II (containing 25 mg of acriflavin hydrochloride) and incubated again at 30°C for 24-36 hrs. The enriched inoculum from UVM-II was then streaked directly on PALCAM agar, *L. monocytogenes* Differential (LMD) agar and Mac-Bride agar and incubated at 37°C for 48 hrs. The presumptively identified *Listeria* spp. on these media were subjected to staining, morphological characterization and were examined for characteristics tumbling motility at 20-25°C in Brian Heart Infusion (BHI) broth. The *Listeria* isolates were further identified by Catalase, Oxidase, Methyl Red (MR), Voges-Proskauer (VP) and Nitrate reduction tests. All the catalase-positive, oxidase-negative, MR and VP positive and nitrate negative *Listeria* isolates were also tested for mannitol, rhamnose, sucrose, xylose, and α-methyl D-mannopyranoside fermentation. The biochemically characterized *Listeria* isolates were then examined for the type and degree of heamolysis in CAMP test as well as on sheep blood agar (SBA) (Nikas, 2009). The pathogenicity of biochemically confirmed *Listeria* isolates was also assessed by Anton's test and by inoculating in chicken embryo via allantoic cavity route following the method described by Nigam *et al.* (1998).

All 37 *Listeria* isolates recovered from the chicken meat samples were further subjected to *in vitro* antibiotic susceptibility pattern on Mueller-Hinton agar (MHA) (HiMedia, India) by the disc diffusion method (CLSI, 2012). All the isolates were tested for their susceptibility against 17 most commonly used antibiotics (Himedia, India): Amoxycillin (30 µg), Bacitracin (10 units), Chloramphenicol (30 µg), Ciprofloxacin (5µg), Colistin (10 µg), Ceftriaxone (30 µg), Ceftriaxone + Tazobactum (30µg/10), Doxycycline hydrochloride (30 µg), Enrofloxacin (10 µg), Erythromycin (15 µg), Gentamicin (10 µg), Penicillin-G (10 units), Rifampicin (5µg), Norfloxacin (10 µg), Streptomycin (10 µg), Sulphadiazine (100 µg) and Tetracycline (30 µg). The zones of inhibition were measured by antibiotic susceptibility scale (Himedia, India) to the nearest millimeter. The zone diameter for individual antimicrobial agents was then translated into susceptible and resistant categories according to the interpretation table supplied by the Himedia, India.

RESULTS AND DISCUSSION

During the present study, the highest APC value recorded was 35×10^5 cfu/g (6.5004 $\log_{10}$ cfu/g), whereas lowest value was 11×10^5 cfu/g (6.04 log cfu/g), with mean value of 23.67×10^5cfu/g (6.374 $\log_{10}$ cfu/g). These findings are in agreement to the findings of Nair *et al*. (1990) who reported aerobic plate count of 6.372 $\log_{10}$ cfu/g in dressed birds. On contrary, lower count of 4.82 $\log_{10}$ cfu/g in fresh and frozen poultry meat and 5.4 $\log_{10}$ cfu/g bacteria in raw chicken nuggets was reported by Eglezos *et al.* (2008). Comparatively higher APC counts of 7.398 $\log_{10}$ cfu/g and 7.14 $\log_{10}$ cfu/g in poultry meat sampleswere reported by Tompkins *et al*. (2008) and Patyal *et al.* (2012) respectively. Wide variations in the APC values may occur due to differences in sampling methods, sampling sites, handling, and modes of evaluation, climatic conditions, fecal contamination and lack of cleanliness on the retail outlets of meat or slaughter house (Nikas, 2009).

Listeria colonies appeared grayish green surrounded by diffused black zone on PALCAM agar, azurine blue with opacity around it on LMD agar and grayish translucent on Mac-Bride agar. Similar observations have been reported by other investigators (Kalorey, 2006; Yadav, 2008; Nikas, 2009).

Out of 100 raw chicken samples, 37 were found positive for *Listeria* spp. indicated positivity of 37%. Among the isolates, different species of *Listeria* were found as given in Table 1. The findings of present study are similar to the findings of Nikas (2009) who reported 35% prevalence of *Listeria* spp. in chicken samples from Mhow area of Madhya Pradesh. Centinkaya *et al*. (2004) also recorded 31% prevalence of *Listeria* spp. from chicken samples from Busraprovince.

In present study various species of *Listeria* isolated were *L. monocytogenes* (16%), *L. grayi* (11%), *L. welshimeri* (5%), *L. ivanovii* (3%) and *L. innocua* (2%). Predominance of *L. monocytogenes* depends on the type of meat and country. The incidence of *L. monocytogenes* is most predominant in isolated species. Similar finding were reported by several researchers such as 13.5% in raw minced meat in Belgium (Uyttendaele, 1999), 17.6% in raw meat in Spain (Simion de *et al*., 1992) and 12.5% in raw meat samples in New Zealand (Hudson *et al*. 1992). However, higher value of 69% in minced meat samples was recorded by Buchanan *et al*. (1998). Feber and Peterkin (1991) also found 63% prevalence in precooked ready to eat poultry.

All the isolates appeared as cocco-bacilli having positive catalase and negative oxidase activity with characteristic tumbling motility at 25°C. The isolates were positive for MR test and VP test and found negative for nitrate reduction test. They were considered as 'presumptive *Listeria* isolates'. Out of thirty seven isolates, twenty two isolates produces acid from α-methyl D-mannopyranoside, sucrose while these isolates failed to produce acid from mannitol and xylose, confirmed as *L. monocytogenes*. Two isolates produced acid from α-methyl D-mannopyranoside, mannitol, sucrose and xylose but failed to produce acid from rhamnose confirmed as *L. innocua*. Three isolates produced acid from xylose, while these isolates failed to produce acid from mannitol, α-methyl D-mannopyranoside, rhamnose and identified as *L. ivanovii.* Five isolates produced acid from rhamnose, xylose and α-methyl D-mannopyranoside, but failed to produce acid from mannitol, identified as *L. welshimeri*. Eleven isolates produced acid from mannitol, and α-methyl D-mannopyranoside, but failed to produce acid from xylose, rhamnose, confirmed as *L. grayi.*

The findings of present study are in confirmation with the earlier reports (Walse *et al*., 2003; Kalorey *et al*., 2005; Gunjal *et al*., 2006; Yadav; 2008; Nikas, 2009). Biochemical characterization of isolates is a useful tool in classification of genus *Listeria* up to *species* level.

All the biochemically confirmed *Listeria* isolates were streaked on 5% SBA and observed for haemolytic changes. A typical β-haemolysis with narrow zone was exhibited by 15 isolates, while 10 isolates showed weak haemolysis on

Table 1: Prevalence of *Listeria* spp. in raw chicken meat samples collected from retail meat shops of Durg city in Chhattisgarh

Samples	Total No. of samples analyzed	Samples positive for *L. monocytogenes*	Samples positive for *L. ivanovii*	Samples positive for *L. welshimeri*	Samples positive for *L. grayi*	Samples positive for *L. innocua*	Total Prevalence of *Listeria spp.* (%)
Raw Chicken meat	100	16	03	05	11	02	37%

blood agar, 13 isolates were non-haemolytic and 5 isolates were doubtful. A typical β-haemolysis with narrow zone was exhibited by all biochemically confirmed *Listeria* isolates. Haemolysis is an important characteristic, which is directly related to the pathogenicity of *Listeria* and attributed to the production of virulent factor listeriolysin, where as non-hemolytic *Listeria* spp. were practically considered as non-pathogenic. *Listeria* isolates showed characteristic enhancement of haemolytic zone in CAMP test. Kenar *et al*. (2006) also characterized all biochemically confirmed *Listeria* isolates by CAMP test with *S. aureus* and *R. equi* and reported positive reaction. The virulent strains of *L. monocytogenes* are strongly haemolytic against sheep erythrocytes due to extra cellular 58-kDa protein, Listeriolysin O (LLO) secreted by isolates. The characteristic enhancement of the β-heamolytic zone towards *S. aureus* was due to the synergism between β- toxin produced by *S. aureus* and LLO, confirming *L. monocytogenes* (Farber and Peterkin, 1991).

All the biochemically confirmed *Listeria* isolates in chicken embryos showed stunting as well as haemorrhages. The liver, heart and muscle of embryos showed congestion when compared with control. The chorio-allantoic membrane showed conspicuous thickening and inoculated embryos died after 42 hrs post inoculation. Similar lesions in chicken embryos were also reported by Nigam *et al.* (1998). The findings of Anton's test showed development of kerato-conjunctivitis within 24-36 hrs. Conjunctivitis in rabbit due to inoculation of *Listeria* isolate was also reported by Kenar *et al.* (2006).

In present study, most of the *Listeria* isolates were showed multi-drug resistance to majority of the antibiotics tested with the highest sensitivity against Tazobactum/Ceftriaxone. Overall, high per cent of isolates were sensitive against different antibiotics such as Tazobactum/Ceftriaxone (95%), Amoxycillin (69.8%), Chloramphenicol (58%), Tetracyclin (58%) and Ciprofloxacin (51.2%). While moderate percent of isolates were sensitive against Ceftriaxone (34.9%), Enrofloxacin (20.9%) and Penicillin-G (18.6%). Least number of isolates were sensitive against Erythromycin, Streptomycin and Sulphadiazine 1, 6.3% each, Doxycycline hydrochloride (11.6%), Bacitracin (9.3%), Rifampicin (6.9%). Gentamicin and Norfloxacin (4.7% each), Colistin (2.3%). *Listeria* isolates were showing variable resistance to antibiotics. Overall, very high percent of isolates were resistant to Norfloxacin (90.7%), followed by Bacitracin, Rifampicin and Colistin (81.4% each), Doxycycline hydrochloride (72%), Erythromycin and Gentamicin (69.8% each), Penicillin-G and Sulphadiazine (65% each), Enrofloxacin (60.5%), Streptomycin (58%), Ceftriaxone (46.5%), Amoxycillin (18.6%) and Chloramphenicol (11.6%).

The result of present study indicated that Tazobactum / Ceftriaxone were found effective against *Listeria* isolate, which is in accordance with the findings of Bhikane *et al*. (2009). Highest degree of sensitivity was observed against new drug Tazobactum/Ceftriaxone, it may be due to latest beta lactamase inhibitor, which is potent and highly specific irreversible inhibitor. Tazobactum binds with beta lactamase and blocks their destructive hydrolytic activity and Ceftriaxone produces bactericidal effect by inhibition of cell wall synthesis. Addition of the two drugs enhanced efficacy against beta lactamase and extended spectrum. It lowers minimum inhibitory concentration (Bhikane *et al.,* 2009).

In present study sensitivity of isolates towards Amoxycillin was 69.8%. This is similar to the findings of Kumar *et al.* (2005) who reported 85.7% sensitivity, on contrary, Willayat *et al*. (2005) reported that Amoxycillin was not found effective against any of isolates. Sensitivity of isolates against Chloramphenicol was 58%. This is in accordance with the findings of Sharda *et al*. (1991) who reported 66.7% sensitivity, similar findings were also given by several workers (Phadke *et al.,* 1979; Wang *et al*., 1992; Brahmbhatt and Anjaria, 1993; Nigam *et al.,* 1998). However, Willayat *et al.* (2005) reported that none of the isolates were sensitive to Chloramphenicol. Moderate sensitivity of isolates was found to Tetracycline (58%). This is in accordance to the findings of Kuma r*et al.* (2005). On contrary, Rota *et al.* (1996) reported that Tetracycline was not found effective against Listerial isolates. Sensitivity of isolate towards Ciprofloxacin was 51%, this is in agreement with the finding of Kumar *et al.* (2005) who recorded 66.7% sensitivity. Moderate sensitivity of isolate against Ceftriaxone was 34.9%, this is in agreement with the finding of Bhikane *et al*. (2009). Least sensitivity was observed against Enrofloxacin (20.9%), this is in agreement with the finding of Nikas (2009). *Listeria* isolates were found to be variably resistant to the antibiotics. Overall, very high percent of isolates were resistant to Norfloxacin (90.9%), Colistin (81.4%),

Gentamicin (69.8%), Enrofloxacin (60.5%) and the least resistance (18.6%) was observed against the Amoxycillin, this is in agreement with the finding of Yadav (2008) and Nikas (2009). Resistance of isolates to Bacitracin was 81.4%, this is in agreement with the findings of Kumar *et al.* (2005). Resistance of isolates against Penicillin-G was 65%. This is in agreement with the finding of Phadke *et al.* (1979) and Yadav (2008). On contrary, Sharda *et al*. (1991) reported that Penicillin-G was sensitive against isolates. Resistance of isolates to Sulphadiazine was 65%. Result of present study is closely related with findings of Yadav (2008) and Nikas (2009). The results drug sensitivity of *Listeria* spp. varies with place, time, species and even disease to disease in the same animal (Sharda *et al.,* 1991; Kumar *et al.,* 2005; Yadav, 2008; Nikas, 2009). The antimicrobial agents are of a great value for devising *in vitro* antibiogram pattern of *Listeria* spp. Increase in antibiotic resistance among *Listeria* spp. are in line with a general worldwide pattern of an increasing prevalence of antibiotic resistance, including multiple antibiotic resistances among many groups of bacteria. Many pathogens are developing resistance to most currently used antibiotics, and there are increasingly frequent reports of pathogens which are resistant to almost all available antibiotics**.** Antibiotic resistance in bacteria has been linked to over-use of antibiotics in animals and humans (Rao, 1998) since these therapeutic compounds were identified nearly 60 years ago. Such resistance may arise from a mutation in an intrinsic chromosomal gene, or by acquisition of exogenous genetic material carrying single or multiple resistance determinants. Antibiotic resistance in *Listeria* species is due to the acquisition of three type mobile genetic elements: self-transferable and mobilizable plasmids and conjugative transposons (Charpentier *et al*., 1995).

CONCLUSION

The present study indicated that the raw chicken meat is an important source for *Listeria* infection in human being and presence of multiple drug resistance among *Listeria* spp. isolated from chicken meat samples provides a evidence of the emergence of multi drug resistant *Listeria* strains, pointing to an increase in the potential threat to human health posed by this pathogen. Further studies are needed to confirm and explore this relationship. Since listeriosis is transmitted primarily via foods, the presence of antimicrobial-resistant *Listeria* in raw food products has an important public health implication especially in developing countries like India, where antibiotics use is widespread and in uncontrolled manner. Due to the high number of antimicrobial- resistant isolates, we recommend that *in vitro* antimicrobial susceptibility testing of *Listeria* be performed and there after appropriate treatment be instituted especially for cases of food-borne listeriosis with severe or prolonged symptoms or in immune-compromised patients.

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