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Serotyping, RAPD PCR, Antibiotic Susceptibility Profiling of *Listeria* spp. Isolated from Raw Chicken Meat

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ABSTRACT

The present study was aimed at finding the prevalence, serotyping, RAPD and Setyping of *Listeria monocytogenes* in raw chicken meat purchased from different retail outlets and local butcher shops across the Nagpur city. Cultural examination of 100 raw poultry meat samples, revealed the prevalence of *Listeria* spp. (35 %). *L. grayi* was predominant (15 %) among the isolates followed by *L. monocytogenes* (12 %), *L. welshimeri* (5 %), *L. innocua* (2 %) and *L. seeligeri* (1%) respectively. Among the majority of strains of *L. monocytogenes*, virulence marker genes *hly*A and *iap*, all together or in combination were detected. Serotyping PCR study revealed that all *L. monocytogenes* isolates to be serotype 4b, which is the major serotype involved in the human listeriosis. RAPD PCR analysis of *L. monocytogenes* isolates revealed two types of banding patterns. All 35 strains of *Listeria* showed *in-vitro* susceptibility to antibiotics like vancomycin (93.88 %) ceftriaxone + Tazobactum (92.71%) and moderate sensitivity to enrofloxacin (38.78 %), ceftriaxone (34.90%) and ampicillin (34.69 %). Whereas, all the isolates were least sensitive to erythromycine (6.49%) and sulphasomidine (4.08 %). All the *Listeriae* isolates were refractory to penicillin. The present findings indicate the presence of multiple drug resistance among *L. monocytogenes* and other *Listeria* spp. isolated from raw poultry meat samples. Potentially pathogenic *L. monocytogenes* isolates recovered from raw poultry meat samples also signifies the zoonotic potential of listeriosis in the local area of Nagpur. High prevalence of *L. monocytogenes* and other *Listeria* spp. in raw poultry meat and their sensitivity to the antibiotics tested, clearly indicates that vancomycin and ceftriaxone +tazobactam should be used as a drug of choice for treatment of listeriosis.

Key words: Serotyping PCR, RAPD, Antibiotic Sensitivity test, ceftriaxone, sulphasomidine, tazobactam

1. INTRODUCTION

Foodborne listeriosis has three main clinical features, namely, meningitis, septicemia, and abortion. It can cause febrile gastroenteritis in healthy humans, but in susceptible persons it may lead to septicemia and meningitis [1]. Four major serovars of *L. monocytogenes* strains can be categorized into four distinct serogroups, IIa (serovars 1/2a, 1/2c, 3a, and 3c), IIb (1/2b, 3b, 4b,4d, and 4e), IIc (1/2c and 3c), and IVb (4b, 4d, and 4e) by targeting four marker genes with aid of multiplex PCR . Food production environment is commonly contaminated with serotypes 1/2a, 1/2b, 1/2c, and 4b [2]. Serotyping, antibiotic susceptibility testing and automated repetitive sequence-based rep-PCR used to assess the contamination of food processing environments [3, 4].

Serotyping is a universally accepted subtyping method for *Listeria monocytogenes* [5]. *L. monocytogenes* strains are serotyped according to variation

in the somatic (O) and flagellar (H) antigens. More than 14 serotypes of *L. monocytogenes* have been described only three serotypes (1/2a, 1/2b, and 4b) cause the vast majority of clinical cases [6]. *Listeria monocytogenes* is divided into at least 12 serotypes (1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7). The virulence of *Listeria monocytogenes* seems to be serotype dependent with serotypes 1/2a, 1/2c, 1/2b and 4b being involved in 98% of documented human listeriosis cases [7].

Tracking contamination have led to the development of molecular typing techniques with high discriminatory power, among which Random Amplification of Polymorphic DNA (RAPD) PCR is a highyielding and rapid method [8]. Several molecular typing has been used to differentiate Listeria species that include RAPD (Random Amplifica- tion of Polymorphic DNA), AP-PCR (Arbitrarily Primed PCR), ERIC-PCR (Enterobacterial Repetitive Intergenic Consensus PCR), REP-PCR (Repetitive sequence-based PCR) and virulence gene sequencing have been used to characterize strains of *L. monocytogenes*.

A β-lactam antibiotic (e.g., ampicillin or penicillin) combined with an aminoglycoside (e.g., gentamicin) is the reference therapy for human listeriosis, while the second choice of treatment is vancomycin, erythromycin and trimethoprim- sulfamethoxazole combination for pregnant women or patients allergic to β-lactams [9]. Listeria species are generally susceptible to a wide range of antimicrobials, but the first multiresistant *L. monocytogenes* strain has been isolated in 1988, since this year, antibiotic-resistant *L. monocytogenes* isolates have been recovered from food, environment, and human listeriosis cases [3]. *Listeria monocytogenes* causes severe infections (listeriosis) in humans and primarily affects young, old, pregnant, and immunocompromised people [10]. Antimicrobial resistance particularly multidrug resistance in microorganisms has become a major global public health problem in recent decades. The excessive and inappropriate use of antimicrobial agents is responsible for the emergence of resistant bacterial strains [4]. Invasive *Listeria monocytogenes* infections carry a high mortality despite antibiotic treatment. The rareness of the infection makes it difficult to improve antibiotic treatment through randomized clinical trials [11]. Antibiotics have successfully been used to treat human listeriosis for decades. However, antibiotic-resistant strains of *L. monocytogenes* have been emerged [12].

2. MATERIAL AND METHODS

2.1 Collection and processing of Samples

A total of 100 samples comprising muscles of broiler chicken were collected from local poultry market in Nagpur, India and isolation of listeriae was attempted from the collected poultry meat samples of muscles as per method described by Donnelly and Baigent, (1986)[13].

2.2 Molecular characterization

The DNA was extracted from suspected colonies and tissues using Himedia DNA extraction kits. The extracted DNA was subjected to PCR using published primers. Standardization of PCR was done by using standard strain of *L. monocytogenes* 4b (MTCC 1143).

2.3 Serotyping PCR for *Listeria monocytogenes*:

The multiplex PCR assay was standardized for the detection of three major serovars of *L. monocytogenes* namely 1/2a, 1/2b and 4b isolated from raw poultry meat by following the methodology as described by Doumith *et al.* (2004)[14] with suitable modifications.

2.4 Standardization of RAPD (Arbitrarily Primed) PCR for *Listeria monocytogenes:*

For the random amplification of polymorphic DNA of *L. monocytogenes* isolated from raw poultry meat samples, PCR assay was standardized in order to

generate different RAPD profiles so that it allowed the discrimination of the different strains isolated. By using 10-mer primer OPM-01(5' – GTT GGT GGC T – 3') (Lawrence *et al*., 1993)[15]. Random Amplification of Polymorphic DNA (RAPD) PCR is a high yielding and rapid method (8). RAPD-PCR is used as a rapid molecular typing method for typing food-borne pathogens including Listeria in food microbiology [16].

2.5 Antibiotic Sensitivity of the isolates

All the *Listeria* isolates recovered were examined for *in-vitro* antibiotic sensitivity. The test was performed by employing disc diffusion method described by Bauer *et al.* (1966)[17] using 12 different antibiotic discs procured from M/s. Hi Media Lab. Ltd., India . The diameter of zone of inhibition was measured to nearest millimeter.

3 RESULTS

3.1 Isolation, Identification and molecular characterization:

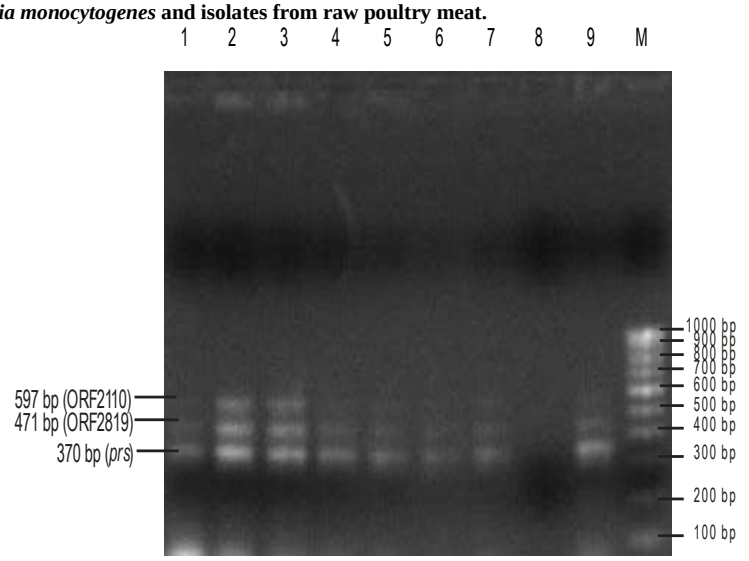
A total of 100 poultry meat samples collected from retail outlets were screened for listeriae. Screening of 100 raw poultry meat samples for listeriae indicated an overall positivity in 35 (35%) samples. Of these, 12 (12 %) isolates were confirmed as *L. monocytogenes*, 15 (15 %) as *L. grayi* and two (2%) as *L. innocua* and five (5 %) as *L. welshimeri.* One (1 %) isolate was confirmed as *L. seeligeri* recovered from the raw poultry muscles

All the biochemically confirmed *Listeria* isolates were subjected to PCR using *hly* an *iap* gene. PCR analysis of all recovered Listeria isolates showed presence of *iap* gene whereas 12 *L. monocytogenes* isolates showed presence of *hly*A and *iap* .

3.2 Serotyping PCR for *Listeria monocytogenes*

All the 12 *L. monocytogenes* isolates from the raw poultry meat were serotyped by employing the multiplex PCR assay. The study revealed all 12 *L. monocytogenes* to be serotype 4b (Figure 1). all *L. monocytogenes* isolates recovered found to be of serotype 4b.

Figure 1: Serotyping PCR Agarose gel electrophoresis of DNA fragments generated by multiplex PCR assay with the serotyping reference strain of *Listeria monocytogenes* and isolates from raw poultry meat.

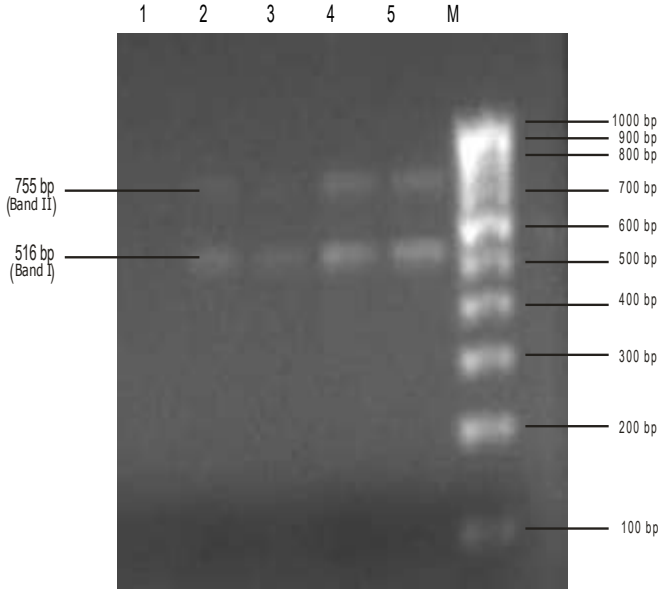


Lane M: 100 bp DNA ladder
Lane 1 - 7: *Listeria monocytogenes* strains isolated from raw poultry meat.
Lane 8: Negative control
Lane 9: *Listeria monocytogenes* reference strain of serovar 4b (MTCC 1143)
Genes corresponding to the amplified fragments are indicated on the right.
Molecular sizes are given at the left (in base pairs)

3.3 RAPD (Arbitrarily Primed) PCR for *Listeria monocytogenes*

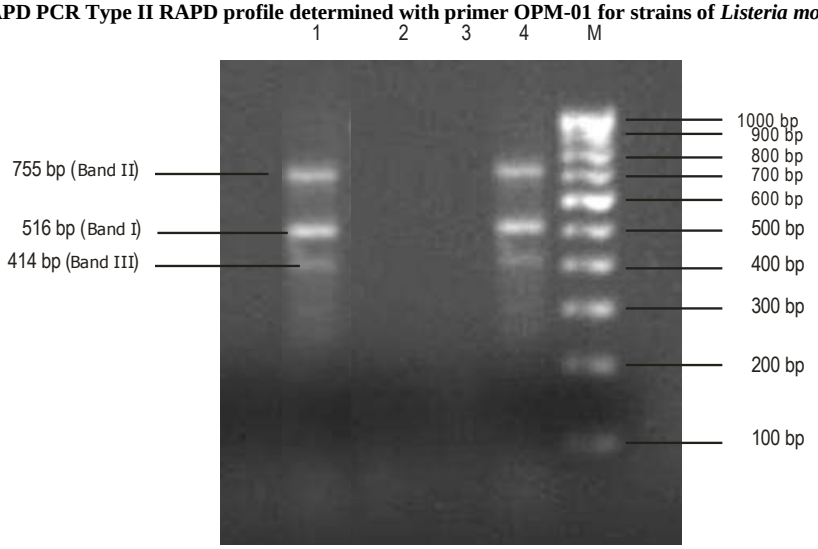
Twelve strains of *L. monocytogenes* from raw poultry meat samples. Two types of RAPD profiles were obtained; type I having 2 bands and type II having three bands within the molecular size range of 100 to 1000 bp. Bands having molecular size range of 755 bp and 516 bp were common to both types of profiles with an additional band having molecular size range of 414 bp in type II profile (Figure 2 and 3).

Figure 2: RAPD PCR Type I RAPD profile determined with primer OPM-01 for stains of *Listeria monocytogenes*.



Lane M: 100 bp DNA ladder
Lane 1: Negative Control
Lane 2 – 5: *Listeria monocytogenes* strains isolated from raw poultry meat.
Bands corresponding to the amplified fragments are indicated on the left.
Molecular sizes are given at the right (in base pairs).

Figure 3: RAPD PCR Type II RAPD profile determined with primer OPM-01 for strains of *Listeria monocytogenes*.



Lane M: 100 bp DNA ladder
Lane 1 and 4: *Listeria monocytogenes* strains isolated from raw poultry meat.
Lane 2 and 3: Negative Control
Bands corresponding to the amplified fragments are indicated on the left.
Molecular sizes are given at the right (in base pairs).

Type I RAPD profile was observed in 10 strains of *L. monocytogenes* isolated from raw poultry meat and type II RAPD profile was observed in 2 strains of *L. monocytogenes*. RAPD analysis revealed that type I RAPD profile was more common (83.33 %) than type II RAPD profile (16.66 %) among the strains of *L. monocytogenes* recovered from raw poultry meat samples, allowing the discrimination within the same serotype. In present study, two types of RAPD profiles (I and II) were found in serogroup 4b and this kind of relationship between RAPD profile within the serotype has also been reported in *B. thuringiensi.*

3.4 Antibiotic Susceptibility profiling

In this study the *in-vitro* antibiotic sensitivity spectrum of 35 isolates of *Listeria* spp. recovered from raw poultry meat samples was studied. Overall, maximum numbers of isolates were sensitive to vancomycin (93.88 %) and ceftriaxone + Tazobactam (92.71%) Lesser sensitivity was recorded against enrofloxacin (38.78 %) and ampicillin (34.69 %). Listeriae isolates were least sensitive to the ceftriaxone (18.37 %), gentamicin, erythromycin and tetracycline (8.16 %, each) followed by chloramphenicol (7.12 %) erythromycin (6.49) and sulphasomidine (4.08 %). All the listeriae isolates were resistant to penicillin.

In the present study, *L. monocytogenes* isolates were variably susceptible to the antibiotics tested. All 12 *L. monocytogenes* isolates showed the highest sensitivity to vancomycin (100 %) and ceftriaxone+tazobactum (95.51%). This was followed by ampicillin and enrofloxacin (58.82 %, each), ceftriaxone (41.18 %). Comparatively, less sensitivity of *L. monocytogenes* isolates was detected to erythromycin and chloramphenicol (17.65 %, each). The highest resistance was offered to the gentamicin, tetracycline, erythromycin and sulphasomidine (88.24 %, each) by *L. monocytogenes* isolates recovered from the raw poultry meat.

One isolate of *L. seeligeri* was resistant to the maximum antibiotics i.e. gentamicin, penicillin, erythromycin, chloramphenicol, ampicillin, ceftriaxone and sulphasomidine and sensitive to enrofloxacin, tetracycline and vancomycin.

Five *L. welshimeri* isolates recovered showed the highest sensitivity to vancomycin (100 %). Comparatively lesser sensitivity was recorded to the ceftriaxone (25 %), followed by gentamicin, enrofloxacin and ampicillin (12.50 %, each) and were refractory towards penicillin, erythromycin, chloramphenicol, tetracycline and sulphasomidine.

L. grayi isolates (15) showed the highest sensitivity to vancomycin (85 %). In contrast, lower sensitivity was detected for ampicillin (25 %) and enrofloxacin (20 %). All isolates showed least sensitivity to gentamicin and tetracycline (5 %, each) and complete resistance to penicillin, erythromycin, chloramphenicol, ceftriaxone and sulphasomidine. The two isolates of *L. innocua* were sensitive to enrofloxacin and vancomycin. Comparatively lesser sensitivity was recorded to the erythromycin and ampicillin (33.34%, each). These isolates were completely resistant to gentamicin, penicillin, chloramphenicol, tetracycline, ceftriaxone and sulphasomidine.

4. DISCUSSION

4.1 Serotyping PCR for *Listeria monocytogenes*

All *L. monocytogenes* isolates recovered from raw poultry meat were found to be of serotype 4b. So the results of the present serotyping PCR study are in accordance with those of the [6,18]. As serotype 4b is the predominant serotype responsible for the *Listeria* associated foodborne outbreaks, it can be of immense importance to consider these 12 *L. monocytogenes* isolates for the further epidemiological investigation to differentiate amongst them for the strain-specificity using advanced techniques like PFGE etc.

Three primer sets were used in conjunction with a previously described Division III primer set in order to classify 122 *L. monocytogenes* strains into five serotype groups [1/2a(3a), 1/2b, 1/2c(3c), 4b(d,e), and 4a/c]. Results of the PCR method agreed with those of the conventional slide agglutination method for 97, 100, 94, and 91% of strains belonging to serotypes 1/2a, 1/2b, 1/2c, and 4b, respectively (5). Among the strains, the serotypes 1 ⁄ 2a, 1 ⁄ 2c, 1 ⁄ 2b, 4b and 3a were identified. The isolates were classified into serogroups I (58.10%), II (22.85%), III (12.38%) and IV (6.67%) [19].

A multiplex-PCR based serotyping assay revealed 88.24% (15/17) of the strains belonging to the serovar group 4b, 4d, 4e and 11.76% (2/17) to the serovar group 1/2b, 3b. Conventional serology indicated that 13 (76.47%) *L. monocytogenes* isolates to be of serotype 4b, 2 (11.76%) serotype 4d, and 2 (11.76%) serotype 1/2b [20]. The serotypes most frequently identified was 1/2a, 4b, 1/2b (in total 92%) [21]. Molecular serotyping of the *L. monocytogenes* isolates by multiplex PCR revealed the predominance of the serogroups 1/2a and 4b from milk and milk samples [22]. Using molecular sub typing techniques, *Listeria monocytogenes* is divided into three major phylogenetic lineages, and a multiplex PCR method can differentiate five *L. monocytogenes* subgroups: 1/2a-3a, 1/2c-3c, 1/2b-3b-7, 4b- 4d-4e and 4a-4c [23].

4.2 RAPD (Arbitrarily Primed) PCR for Listeria monocytogenes

In *L. monocytogenes* the homogenous nature of serogroup 4b strains. Since in the present study there was discrimination among 4b strains isolated, it appears that the primer OPM- 01 and the conditions mentioned above offer a typing method which is more discriminatory and suitable. RAPD using primer OPM- 01 has been found to be a rapid and valuable technique for typing *L. monocytogenes*. It also offers certain advantages over serotyping as RAPD offers greater discrimination of strains. So, when used in combination, serotyping and RAPD offer a higher level of differentiation than either method used alone. In comparison with other molecular techniques for typing *L. monocytogenes*, such as restriction endonuclease pattern analysis study and RFLP, RAPD is more rapid and less labor-intensive by eliminating the need for pure DNA and allows better discrimination within serogroup.

Seventy-five *L monocytogenes* isolates of human listeriosis, the intestinal contents of cows and beef were divided into 5 major clusters, 17 sub-clusters and 28 minor clusters by typing using random amplification of polymorphic DNA (RAPD) [24]. Twenty-one *Listeria monocytogenes* isolates originated from food, animal, and clinical sources were evaluated by RAPD-PCR typing using four random primers (OPM-01, HLWL-74, HLWL-82, and HLWL85), both individually and in combination. The actA gene-typing and PCR-based serotyping were also used for the characterization of the isolates. Data were analyzed and clusters were displayed in dendrogram. Among four primers, OPM-01 showed the highest discriminatory power. The dendrogram for human, food, and livestock isolates showed four different clusters (A-D) and two singletons. Using RAPD-PCR, lineages I, II, and the serogroups were discriminated [16].

4.3 Antibiotic Susceptibility profiling

The overall antibiotic sensitivity pattern of the *Listeria* spp. isolated into consideration, the present findings indicate the presence of multiple drug resistance among *L. monocytogenes* and other *Listeria* spp. isolated from raw poultry meat samples and provides evidence of the emergence of multiresistant listeriae strains, pointing to an increase in the potential threat to human health posed by this pathogen. Earlier antibiotic susceptibility of *L. monocytogenes* isolated from vegetables, environment and water samples [25] have been recorded. The present findings of antibiotic sensitivity of *Listeria* spp. isolated

are in accordance with earlier studies. The susceptibility of *Listeria* spp. and *L. monocytogenes* isolates recovered from Portuguese poultry carcasses samples recorded the high percentages of *Listeria* spp. (84 %) and *L.monocytogenes* (73 %) isolates to be resistant to one or more antimicrobial agents of different groups and noticeable frequency of the resistance of *L. monocytogenes* isolates to enrofloxacin. Least sensitivity was observed against enrofloxacin (20.9%) [26] this is in agreement with the finding of Nikas ,2009 [27]. The findings of the present investigation are in agreement with those of Antunes *et al*. (2002) indicating that poultry could be a potential vehicle of foodborne infections due to strains of *L. monocytogenes* that are resistant to antimicrobial agents.

Resistance to penicillin is a common finding in a number of studies [28]. Comparatively lesser sensitivity was recorded to the ceftriaxone (25 %), followed by gentamicin, enrofloxacin and ampicillin (12.50 %, each) in our study whereas A low number of isolates were resistant to gentamycin [29, 30]. Among the 259 strains studied, 145 strains revealed multidrug-resistance (resistance to≥3 antibiotics) and predominantly belonged to serotype IV (59%) Strains were mainly resistant to daptomycin, tigecycline, tetracycline, ciprofloxacin, ceftriaxone, trimethropim/sulfamethoxazole and gentamicin isolated from food, food-processing plants and human samples in Germany (31) similar finding was recorded from this study.

The 85 Listeria isolates tested, 12 *L. monocytogenes* were identified and tested for their sensitivity to 14 antimicrobial agents. All the 12 isolates (100%) were resistant to nine antimicrobial agents and sensitive to gentamicin. Only one isolate was found to harbour the hylA gene from raw meat and meat products in Zaria, Nigeria [32].

A small fraction of in total 524 Listeria spp. isolates (3.1%) displayed acquired antibiotic resistance mainly to tetracycline (n = 11), but also to clindamycin (n = 4) and trimethoprim (n = 3), which was genotypically confirmed from foodborne, clinical, and environmental Listeria isolates to assess antibiotic resistance [33] in accordance with this study. The occurrence of Listeria species in turkey meats was investigated to check the antimicrobial susceptibility of the isolated strains. The isolates were distributed between *L. monocytogenes* (25.53%), *L. innocua* (34.04%), *L. grayi* (31.91%) and *L. welshimeri* (8.51%). A total of 55.3 % of Listeria spp. isolates were multi-resistant to at least 3 of the antimicrobial agent tested. The level of multi-resistance was higher in *L. monocytogenes* strains (66.7%) than in *L. innocua* (62.5%) and *L. grayi* (53.3%). Listeria spp. isolates were highly resistant to ampicillin, cephalothin, penicillin, meticillin, oxacillin, and trimethoprime-sulfamethoxazole [34] the similar finding was recorded from this study.

Antimicrobial susceptibility screening of the *L. monocytogenes* isolates exhibited high resistance against ceftazidime (96.67%), cloxacillin (90%), cefuroxime (86.67%), augmentin (86.67%), ceftriaxone (80%) and erythromycin (66.67%) from Different Food Sources in Enugu, Nigeria [35]. A high drug resistance rate was observed in antimicrobial susceptibility test of *Listeria monocytogenes* among pregnant women in Tigray region, Ethiopia and recorded succeptibility to penicillin G (66.7%), clindamycin (66.7%), amoxicillin (50%) and vancomycin (50%). However, isolates were relatively sensitive to ciprofloxacin (75%), erythromycin (75%), trimethoprim/ sulphamethaxazole (66.7%) and chloramphenicol (60%) [36].

These antibiotics are generally used to fight infections caused by enteric bacteria. All the isolates were observed to be resistant against penicillin, imipenem and amoxicillin (100%) . The second-highest resistance was recorded against erythromycin, clarithromycin, ampicillin, tigecycline, rifampicin and fusidic acid (91.7%),

followed by cephalothin (83.3%), trimethoprim and tetracycline (75%). Gentamicin and meropenem appeared to be the most effective as all the isolates were found to be susceptible to them [37].

The most frequent serotypes were 4b and 4ab in human and non-human isolates, respectively. The resistance of *L. monocytogenes* isolates to the first-lineantibiotics namely penicillin, ampicillin/amoxicillin, gentamicin, and trimethoprim-sulfamethoxazole has been increased in recent years [38].

5. 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. Since listeriosis is transmitted primarily via foods, the presenceof 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, It is 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. The presence of multidrug resistance isolates which transfer the antibiotic resistance to community. Also, some of the isolates are pathogenic serotypes that play a major role in human listeriosis outbreaks. Subtyping data revealed the heterogeneous nature of the *L. monocytogenes* isolates. RAPD, serotyping have a considerable discriminatory power and antibiotic succeptible testing play important role and are cost effective and less tedious and time consuming.

Declaration by Authors

Ethical Approval: Approved

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