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Multiple Antibiotic Resistance among *Listeria* Strains, Including *Listeria monocytogenes* Isolated from Animals of Gujarat State, India

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A B S T R A C T

There is scarcity of information on multiple drug resistance strains of *Listeria* spp. especially in Indian conditions from animal environment. Present study reports the multiple drug resistance strains of *Listeria* spp isolated from animal and its environment. All the confirmed 48 *Listeria* isolates were characterized by biochemical test and *in vitro* pathogenicity test. For antimicrobial susceptibility / resistance, Bauer-Kirby disc diffusion assay was carried out. Results indicated that a high per cent of isolates were resistant to carbenicillin (93.75 %) followed by colistin and norfloxacin (91.68 % each), cephaloridine (89.59 %), and cephalexin (81.25 %), kanamycin and sulphadiazine (64.58 % each), cloxacillin, streptomycin (60.42 % each), co-trimoxazole (52.08 %), trimethoprim (50.00 %), amikacin (47.92 %), pefloxacin (37.50 %), novobiocin, gentamicin (31.25 % each), oxytetracycline (27.08 %), ampicillin, cephalothin (22.92% each); lesser per cent of isolates were observed resistant for vancomycin (18.75 %), erythromycin (16.67 %), penicillin-G (14.58 %), chloramphenicol (12.50 %), tetracycline (10.42 %), while least number of resistant isolates were observed against amoxycillin (6.25 %), amoxyclav (4.16 %), and enrofloxacin (2.07 %). Taking overall antibiotic sensitivity pattern of the *Listeria* spp. isolated into consideration, the present findings indicate the presence of multiple drug resistance in *Listeria* spp. isolated from animal samples pointing towards an increase in the potential threat to animal and human health posed by this pathogen.

Introduction

Listeriosis has been recognized as an emerging foodborne bacterial infection and a nagging public health hazard (Farber and Peterkin, 1991). It is a serious invasive disease caused by pathogenic strains of genus *Listeria*, affecting animals and man. *Listeria* spp. including *Listeria monocytogenes* are ubiquitous in nature and is commonly found in the intestines of animals and human being without causing illness. But *Listeria monocytogenes* causes disease in a wide variety of animals including sheep, goats, cattle, buffaloes, dogs, horses, chickens, rabbits and also human beings (Katiyar, 1960). Its role in causation of meningo-encephalitis, septicemia, abortion, endometritis, cervicitis, mastitis, kerato-conjunctivitis, local purulent lesions etc. has been established beyond doubt

(Gitter, 1980). The antimicrobial agents are of great value for devising curative measures against bacterial infections. But the selective pressure exerted by indiscriminate use of drugs in clinical settings and heavy use as growth promoters for farm animals have likely accelerated the appearance and dissemination of multiple drug resistance in bacteria (Charpentier and Courvalin, 1999). Bacteria have outstanding ability to develop resistance to almost all antimicrobial agents, so we may expect that even *Listeria* spp., which is considered to be susceptible to antimicrobial agents, will develop multiple drug resistance. Evolution of such resistant bacteria may create problem in treatment of acute infections in man and animals. The appearance of new resistance mechanisms, the development of multi-drug resistance or combinations of resistance, and the facility with which genetic material encoding resistance may, in certain cases, spread horizontally between different species of bacteria, all increases the feeling of vulnerable to diseases that were thought to have been controlled when antibiotics were first developed. The first multi resistant strain of *L. monocytogenes* was isolated in France in 1988 (Poyart-Salmeron *et al.*, 1990).

Emergence of antibiotic resistance in *Listeria* spp. takes place by acquisition of three types of mobile genetic elements: self-transferable and mobilizable plasmids and conjugative transposons (Charpentier and Courvalin, 1999). Use of antimicrobials in livestock production is suspected to significantly add to multiple drug resistance in species of bacteria, which are common to humans and animals (Acar and Rostel, 2001).

Drug susceptibility or resistance pattern of *Listeria* spp. isolated from animal environment under Indian conditions have not yet worked out in detail. This was the major motivator for the present investigation to know the well-characterized 48 *Listeria* strains including 28 *Listeria monocytogenes* isolated from animal environment for sensitivity to 26 different antibiotics.

Materials and Methods

The reference strains

The standard strains of *Listeria monocytogenes* 4b (MTCC 1143), *Staphylococcus aureus* (MTCC 1144), *Staphylococcus aureus* (ATCC 25923), *Str. agalactiae* (NCIM 2401), *Bacillus* spp. (ATCC 6638), *Ps. aeruginosa* (ATCC 27853), *Rhodococcus equi* (MTCC 1135) *Escherichia coli* (ATCC 25922), and *Escherichia coli* (MTCC 723) were obtained from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. *E. coli*, *Klebsiella* spp., *Proteus* spp. and *Shigella* spp. were obtained from Department of Veterinary Microbiology, College of Veterinary Science & A. H., Anand, India. All the strains were maintained by periodically sub-culturing in brain heart infusion (BHI) agar.

All the confirmed 48 *Listeria* isolates, which were recovered from the clinical samples, were subjected for antibiotic sensitivity. The *in vitro* antibiotic sensitivity tests of the isolates were conducted with minor modifications as per the method of Bauer *et al.*, (1966). Briefly, the procedure followed is a loopful of the growth from slant was inoculated in BHI broth and incubated at 37^{o}C for 6 h. A sterile cotton swab was dipped into the broth culture; excess of the bacterial suspension was removed by pressing and rotating the swab against the inner walls of the test tube. Streak the entire agar surface of the plate with the swab three times, turning the plate at 60^{o} angle between each streaking. The surface of pre-incubated and sterile Muller-Hinton agar (Hi Media Ltd., Mumbai) petri plate was kept at room temperature for 30

min. to allow the inoculum to be adsorbed on the surface. Antibiotic sensitivity disc (Hi Media Ltd, Mumbai) were placed with the help of flamed forceps on the plates at equal distance and sufficiently (30 mm) separated from each other. The plates were incubated overnight at 37^0C. Antibiotics used in the present study were ampicillin, amoxycillin, amikacin, amoxyclav, carbenicillin, cephalexin, cephaloridine, cephalothin, colistin, chloramphenicol, ciprofloxacin, cotrimoxazole, cloxacillin, enrofloxacin, erythromycin, gentamicin, kanamycin, novobiocin, norfloxacin, penicillin G, pefloxacin, streptomycin, sulphadiazine, oxytetracycline, tetracycline and vancomycin.

Diameters of zone of inhibition were measured and the interpretation of the results was made in accordance with the instructions supplied by the manufacturer.

Results and Discussion

A total of 2417 samples collected from animals and its surrounding environment were screened for *Listeria*. Those isolates which exhibited catalase-positive, oxidase - negative, methyl red and Voges Proskauer-positive and nitrate-negative reactions along with the characteristics tumbling motility at 25^oC were considered as "presumptive" *Listeria* isolates. The presumptive *Listeria* isolates were further tested to biochemical characterization and differentiation by sugar fermentation pattern, and *in vitro* assay for virulence associated characterization for the identification of "confirmed" *Listeria* isolates upto species level besides adjudging their pathogenic potential.

All the 48 strains were characterized for haemolysis activity, CAMP test (Annonymous, 1994), PIPLC assay (Leclercq, 2004), PCPLC assay (Coffey *et al.*, 1996). All the 28 *L. monocytogenes* strains were found positive for aforementioned characters, while all the 20 *L. innocua* strains were found negative for aforementioned characters.

All the 28 strains were serotyped as *L. monocytogenes* 4b using multiplex PCR assay following the methodology as described by Doumith *et al.*, (2004) with suitable modifications like annealing temperature was increased from 45^oC to 47^oC and instead of 2.0% agarose gel 1.5% was used.

All the 48 isolates of *Listeria* were tested for *in vitro* sensitivity towards 26 antibacterial agents.

In the present study, *Listeria* isolates were found variably resistant to the antibiotics tested. Overall, very high per cent of isolates were resistant to carbenicillin (93.75 %) followed by colistin and norfloxacin (91.68 % each), cephaloridine (89.59 %), and cephalexin (81.25 %). Higher per cent of isolates were resistant to kanamycin and sulphadiazine (64.58 % each), cloxacillin and streptomycin (60.42 % each). While moderately high per cent of isolates were resistant to co-trimoxazole (52.08 %), trimethoprim (50.00 %), amikacin (47.92 %), pefloxacin (37.50 %), novobiocin and gentamicin (31.25 % each), oxytetracycline (27.08 %), ampicillin and cephalothin (22.92% each); lesser per cent of isolates were observed resistant for vancomycin (18.75 %), erythromycin (16.67 %), penicillin-G (14.58 %), chloramphenicol (12.50 %), tetracycline (10.42 %), while least number of resistant isolates were observed against amoxycillin (6.25 %), amoxyclav (4.16 %), and enrofloxacin (2.07 %).

Three isolates of *Listeria* were resistant to the 21 antibiotics, 2 isolates were resistant to 17 antibiotics, 3 isolates were resistant to 16 antibiotics, 5 each isolates were resistant to 12 and 15 antibiotics, 4 isolates were resistant to

14 antibiotics, 1 each isolate was resistant to 11 and 13 antibiotics, 8 isolates were resistant to 10 antibiotics, two each isolates were resistant for 3, 6, 7, 8 and 9 antibiotics, three each isolates were for 4 and 5 antibiotics.

About 26 different antibiotic resistance patterns were observed in *L. monocytogenes* isolates, whereas all the isolates of *L. innocua* gave different antibiotic resistance pattern (Table 1–3). Resistance to antibiotics varied from 1 to 17 for *L. monocytogenes* and 12 to 21 for *L. innocua*. Resistance of *Listeria* isolates to individual as well as different classes of antibiotics was presented in Table 4.

With great expansion of livestock industry in India, *Listeria* spp. is emerging as a problem of economic concern to all phases of industry from production to marketing to consumer health significance, to the clinicians due to emergence of multiple drug resistant bacterial strains, and to veterinarians due to reservoirs of infection.

It was evident that antibiotic resistance was becoming more and more extensively reported in all bacteria, not only in pathogens, but commensals was also not left out. The occurrence of antibiotic resistance in non-pathogens poses key risk, if less direct, to human health. Despite the increasing number of clinical isolation of *Listeria* spp. exhibiting resistance to multiple antibiotics, plasmid mediated multiple resistance was not yet reported extensively. While many antibiotic-resistant bacteria in foods were currently saprophytic or commensals in habit, their resistance genes can be transferred to other food-borne bacteria, including pathogenic species within the gastrointestinal tract. This process may have undesirable clinical implications for the host, and for the wider population coming into contact with derived antibiotic resistant pathogens. Thus, supplementary information was urgently required on the patterns of dispersion and transmission of antibiotic resistance among the wider prokaryotic kingdom.

Table.1 Overall multiple drug resistance of *Listeria* isolates

Sr. No.	No. of Drug	No. of Resistant Isolates	% of Resistant Isolates (N = 48)
1	3	02	04.16
2	4	03	06.25
3	5	03	06.25
4	6	02	04.16
5	7	02	04.16
6	8	02	04.16
7	9	02	04.16
8	10	08	16.67
9	11	01	02.07
10	12	05	10.42
11	13	01	02.07
12	14	04	08.33
13	15	05	10.42
14	16	03	06.25
15	17	02	04.16
16	21	3	06.25

Table.2 Multiple drug resistance pattern of *Listeria monocytogenes*

No. of Drugs	Resistance Pattern	No. Of Isolates
1	Cb	1
2	Ak, K	1
3	Cp, Cx, K	1
4	Cb, Cl, Cr, Nx	1
4	Cb, Nv, Nx, T	1
5	Cb, Cl, Cr, Nx, Sz	1
5	Cb, Cl, Cp, Cr, Nx	1
6	Cb, Cl, Cp, Cr, E, Nx	1
6	Cb, Cl, Cr, Nv, Nx, Tr	1
6	Am, C, Cb, Cl, Nv, Sz	1
7	Cb, Cl, Cp, Cr, Nx, O, Sz	1
7	A, Cb, Cl, Cr, K, Nx, Tr	1
8	Cb, Cl, Cp, Cr, Nx, O, Sz, Tr	1
8	Cb, Cl, Cp, Cr, Nv, Nx, O, Sz,	1
8	Cb, Cl, Co, Cr, K, Nx, S, Tr	1
9	A, Cb, Cl, Cp, Cr, Cx, K, Nx, O	1
9	Cb, Cl, Cp, Cr, Nv, Nx, O, Sz, T	1
10	Ak, Cb, Cl, Cp, Cr, Cx, K, Nx, S, Sz	3
10	A, Cb, Cl, Co, Cp, Cr, Cx, K, Nx, Tr	1
10	Cb, Cl, Co, Cp, Cr, K, Nx, Pf, S, Tr	1
10	Ak, A, Cb, Cl, Cp, Cr, Cx, Nx, Pf, Tr	1
11	Ak, A, Cb, Cl, Co, Cp, Cr, Cx, G, Nx, Tr	1
11	Cb, Cl, Co, Cp, Cr, Cx, K, Nx, Pf, S, Tr	1
12	Cb, Cl, Co, Cp, Cr, Cx, K, Nx, P, S, Sz, Tr	1
12	Ak, Cb, Ch, Cl, Cp, Cr, G, K, Nx, S, Sz, Va	1
17	Ak, Cb, Ch, Cl, Co, Cp, Cr, Cx, E, G, K, Nv, Nx, O, Pf, S, Sz,	1

Note: Amikacin (Ak), Amoxycillin (Am), Amoxyclav (Ac), Ampicillin (A), Carbenicillin (Cb), Cephalexin (Cp), Cephaloridine (Cr), Cephalothin (Ch), Chloramphenicol (C), Cloxacillin (Cx), Colistin (Cl), Co-trimoxazole (Co), Enrofloxacin(Ex), Erythromycin (E), Gentamicin (G), Kanamycin (K), Oxytetracycline (O), Pefloxacin (Pf), Penicillin-G (P), Norfloxacin (Nx), Novobiocin (Nv), Streptomycin (S), Sulphadiazine (Sz), Tetracycline (T), Trimethoprim (Tr), Vancomycin (Va)

Table.3 Multiple drug resistance pattern of *Listeria monocytogenes*

No. of Drugs	Resistance Pattern	No. Of Isolates
12	Cb, Cl, Co, Cp, Cr, Cx, K, Nx, Pf, S, Sz, Tr,	1
12	A, Cb, Cl, Co, Cp, Cr, Cx, Nx, O, S, Sz, Tr	1
13	Ak, Cb, Cl, Co, Cp, Cr, Cx, K, Nx, Pf, S, Sz, Tr,	1
13	Ak, Cb, Ch, Cl, Co, Cp, Cr, Cx, G, K, Nv, Nx, Tr	1
13	C, Cb, Cl, Co, Cp, Cr, Cx, G, K, Nx, O, S, Tr	1
14	Ak, Cb, Ch, Cl, Co, Cp, Cr, Cx, G, K, Nx, P, S, Sz,	1
14	A, Am, Cb, Cl, Co, Cp, Cr, E, K, Nx, Pf, S, Sz, Tr	1
14	Ak, Cb, Cl, Co, Cp, Cr, Cx, G, K, Nx, Pf, S, Sz, Tr	1
14	Cb, Cl, Co, Cp, Cr, Cx, K, Nx, O, P, Pf, S, Sz, Tr	1
15	Ak, Cb, Ch, Cl, Cp, Cr, K, Nv, Nx, O, Pf, S, Sz, T	1
15	Ak, Cb, Ch, Cl, Co, Cp, Cr, Cx, K, Nx, Pf, S, Sz, Tr	1
15	Ak, A, Cb, Ch, Cl, Co, Cp, Cr, Cx, G, K, Nv, Nx, S, Sz,	1
15	A, Cb, Ch, Cl, Co, Cp, Cr, Cx, G, Nx, O, S, Sz, T, Tr	1
16	Ak, Cb, Ch, Cl, Co, Cp, Cr, Cx, K, Nv, Nx, Pf, S, Sz, Tr, Va	1
16	Ak, Cb, Ch, Cl, Cp, Cr, Cx, G, K, Nv, Nx, P, Pf, S, Sz, Va	1
16	Ak, A, Cb, Cl, Co, Cr, Cx, G, K, Nv, Nx, P, S, Sz, Tr, Va	1
17	Ak, A, Cb, Ch, Cl, Co, Cp, Cr, Cx, E, K, Nx, O, Pf, S, Sz, Tr	1
20	Ak, Ac C, Cb, Ch, Cl, Co, Cp, Cr, Cx, E, Ex, G, K, Nv, Nx, P, S, Sz, Va	1
21	Ak, A, Am, C, Cb, Ch, Cl, Co, Cp, Cr, Cx, E, G, K, Nv, Nx, O, Pf, S, Sz, Tr	1
21	**Ak, Ac C, Cb, Cl, Co, Cp, Cr, Cx, E, G, K, Nv, Nx, P, Pf, S, Sz, T, Tr, Va**	**1**

In present study, *Listeria* isolate were resistant to ampicillin and cephalothin (22.92 % each), erythromycin (16.67 %), gentamicin (31.25 %), chloramphenicol (12.5 %) and tetracycline (10.42 %). The present findings were in partial correlation with that of Rota *et al.*, (1996) who reported resistance to ampicillin, cephalothin, erythromycin, gentamicin chloramphenicol and tetracycline to be 15.3, 20.8, 41.0, 27.8, 6.7 and 8.9 per cent, respectively,

Phadke *et al.*, (1979) reported all the isolates were sensitive to terramycin, chloramphenicol, streptomycin and resistant to erythromycin and penicillin. Shah and Dholakia (1983) reported all the *L. monocytogenes* strains were sensitive to penicillin, ampicillin and neomycin, while 66.67 per cent strains were sensitive to streptomycin, tetracycline and gentamicin. Sharda *et al.*, (1991) reported 100.0 per cent isolates were sensitive to oxytetracycline, followed by Chloramphenicol (66.67 %), penicillin, gentamicin (33.33 % each) and 100.0 per cent resistance to ampicillin. Brahmbhatt and Anjaria (1993) observed 100.0 per cent sensitivity against chloramphenicol, neomycin and gentamicin and 87.5 per cent against co-trimoxazole and tetracycline and 74.0 per cent against ampicillin. Walsh *et al.*, (2001) reported *Listeria* isolates were resistant to tetracycline (6.7 %) and penicillin (3.7%). Willayat *et al.*, (2005) reported *L. monocytogenes* isolates was highly sensitive to gentamicin but resistant to tetracycline, ampicillin, amoxycillin and chloramphenicol. The findings of the various authors (Phadke *et al.*, 1979; Shah and Dolakia, 1983; Sharda *et al.*,

1991; Walsh *et al.,* 2001; Safdar and Armstrong, 2003) were in partial agreement with the present investigation.

Kumar *et al.,* (2005) reported multidrug resistant *Listeria* from meats and fish. Antibiotic sensitivity of 14 isolates revealed that maximum resistance was recorded against cloxacillin (100.0 %), followed by vancomycin (92.85 %), amoxycillin, cephalothin and amoxyclav (85.71 % each), erythromycin (78.57 %), clindamycin and co-trimoxazole (70.0 % each). The maximum sensitivity was observed with ciprofloxacin and tetracycline (66.66 % each). Most of the isolates were resistant to 7-14 antibiotics. Three *L. monocytogenes* isolates showed resistance to most of the antibiotics tested, while one was resistant to 13 antibiotics and another to 12 antibiotics. Our results agreed to the above report.

Resistance of *L. monocytogenes* and *L. innocua* showed variations to different classes of antibiotics like aminoglycosides, cephalosporins, penicillin and quinolones, which needs further investigation with role of different types of enzymes involved in resistance pattern, both for *L. monocytogenes* and *L. innocua*.

Walsh *et al.,* (2000) reported that incidence of antibiotic resistance was low but the range of antibiotics to which resistance has been acquired is wide. But in present investigation there were three strains of *Listeria* spp. that were resistant to 21 antibiotics. So it is of great concern that this expanding range of antibiotics now includes those drugs that is used for treatment of human and animal listeriosis. The high number of multiple drug resistant strains of *Listeria* found in this study seems to suggest that mobile genetic elements encoding resistance to a wide range of antibiotics in this genus have appeared and are spreading.

Antibiotic resistance pattern varied from *L. monocytogenes* and *L. innocua*, where most of the *L. monocytogenes* isolates were sensitive to different antibiotics while *L. innocua* isolates were resistant upto 21 antibiotics. Multiple resistance of later may be due its non-pathogenic nature were less number of genes were required as compared to former where multiple virulence associated genes are maintained. Multiple resistance of the *L. monocytogenes* isolates was linked to the presence of a self-transferable plasmid that was proposed to originate in *Enterococcus* and *Streptococcus* (Poyart-Salmeron *et al.,* 1990).

Most of the isolates were recovered from feces or environmental sources, resistance to multiple antibiotics may be due to *Enterococcus* and *Streptococcus* harbouring conjugative plasmids and transposons which are present at very high numbers (Charpentier and Courvalin, 1999). For *Listeria* spp. intestinal tract represents an ecosystem most favorable for direct exchange of genetic information between *Enterococcus* and *Streptococcus*.

Taking 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 animal samples and provides evidence of the emergence of multidrug resistant *Listeria* strains, pointing to an increase in the potential threat to human health posed by this pathogen.

The results of this study confirm that major changes in the nature and incidence of antibiotic susceptibility amongst *Listeria* spp. had occurred within the last two decades. It can be postulated from the study that resistance observed may be due to widespread use of these antibiotics in therapies, or as a

supplement in animal feeds, with subsequent dissemination through known multiple *Listeria* spp. In other cases, the factors underlying the acquisition and persistence of antibiotic resistance are much less clear. It was of concern that expanding range of antibiotic resistance now includes a number of antibiotics used to treat listeriosis, e.g. penicillin, ampicillin, tetracycline and gentamicin.

As *L. monocytogenes* is an emerging food borne pathogen, it also presents the potential threat to human health through the consumption of contaminated food like milk and milk products, meat and meat products. General consent is that ampicillin or penicillin alone or in combination with gentamicin was the treatment of choice for listeriosis. More comprehensive and continuous monitoring of the course and nature of the acquisition and dissemination of antibiotic resistance by this *Listeria* pathogen and other members of the genus is obligatory.

Impacts

Resistance for antibiotics was observed for penicillin, ampicillin, tetracycline and gentamicin which are used for treatment of listeriosis in both animals and human beings and thus may be concern for Veterinarians and Human Physicians in treating the listeriosis.

Listeria spp. including *L. monocytogenes* isolated from animal and its environment showed multiple drug resistance pointing towards an increase in the potential threat to animal and human health posed by this pathogen.

Key modificatioin in the nature and incidence of antibiotic susceptibility amongst *Listeria* spp. had occurred within the last two decades which may be due to widespread use of these antibiotics in therapies, or as a supplement in animal feeds, with subsequent dissemination through known multiple *Listeria* spp.

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