International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
www.chemijournal.com
IJCS 2020; 8(1): 2825-2829

Received: 07-11-2019
Accepted: 09-12-2019

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Detection of antimicrobial drug resistance in *Listeria monocytogenes* of cattle origin

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DOI: https://doi.org/10.22271/chemi.2020.v8.i1aq.8696

Abstract
The objective of this work was to investigate the antimicrobial drug susceptibility of *Listeria monocytogenes* strains detected from 200 samples of cattle origin by conventional bacteriology and species specific PCR. A total of 13 (6.50%) *L. monocytogenes* strains were detected from faeces (2.00%), offals/ internal organs (6.00%), raw meat (4.00%) and farm water (14.00%). The *L. monocytogenes* strains were found to be susceptible to to most of the antimicrobial drugs tested. Highest resistance to Penicillin (23.08%) followed by Ciprofloxacin (15.39%) and Nalidixic acid and Ampicillin (7.07%) was detected in *L. monocytogenes* strains. However, no multidrug resistant *L. monocytogenes* was detected in the present study. The detection of antimicrobial resistant *L. monocytogenes* in food animals and their environment indicates that these strains may be transferred to consumers via the food chain leading to compromise in the effective treatment of human listeriosis.

Keywords: *Listeria monocytogenes*, antimicrobial drug, cattle, Aizawl, Mizoram

Introduction
Food borne infections and intoxications remain as one of the major global concern in the field of food security and food safety. Animal origin foods are considered to be the most common source of zoonotic diseases. *Listeria monocytogenes* is a major cause of zoonotic listeriosis and food borne infection. Infections with this bacterium are currently associated with the highest fatality rate of approximately 17% among foodborne pathogens (EFSA, 2012; Wu *et al.*, 2015) [7]. The global outbreaks in steady rates of increase after 1980s led the World Health Organization (WHO) to conclude that *L. monocytogenes* is an environmental contaminant mainly transmitted to humans through food (Granier *et al.*, 2011) [10]. The organism is hardy in nature which can grow in wide range of temperature, pH and osmotic pressure allowing its survival for longer duration in the adverse environmental conditions (Adzitey and Huda, 2010) [1]. It is also important to note that *Listeria* is a widely distributed bacterium in nature and commonly found in soil, sewage, dust, water and causes listeriosis in humans and animals (Norton *et al.*, 2001) [17]. Outbreak and sporadic cases of human listeriosis have been associated with contamination of various food items milk, meat and meat products (Sur *et al.*, 2012) [24] and pork (Goulet *et al.*, 2012) [9]. However, outbreaks of food borne infections are not well documented in developing countries including India. A poor hygienic practice in local food production and processing chain favours the entry and transmission of different food borne pathogens. In India, *L. monocytogenes* should be absent in 25 gram of frozen and canned meat and fish products as per food safety and standard regulation, 2011. The organism has been isolated from reproductive disorders and mastitis of cattle, buffalo, sheep and goat (Kaur *et al.*, 2007; Rawool *et al.*, 2007; Shakuntala *et al.*, 2006) [13, 19, 20] from different parts of India. Little is known about *L. monocytogenes* antimicrobial resistance, especially for non-human-origin isolates from India more particularly from the North Eastern region. The food production sector of animal origin in North Eastern region of India is still un-organized and there is ample scope of contamination of food with bacterial pathogens. The present work was undertaken to study the antimicrobial drug susceptibility of *L. monocytogenes* strains isolated from different samples of cattle origin and their environment from Aizawl district of Mizoram.

Materials and Methods

The study was conducted for a period of one year from July, 2017 to June, 2018 in Aizawl district of Mizoram, India. A total 200 number of samples of cattle sources including faeces, offals/ internal organs and raw meat from slaughtered animals and farm water (50 each) were collected randomly following aseptic measures at periodic intervals for detection of *L. monocytogenes.*

The isolation and identification of *L. monocytogenes* from faeces, internal organs/ offals, raw meat and water samples were done by using the standard methodologies as per USDA FSIS (2002) [25] and Food and Drug Administration (2015) [8] with slight modification. The methods involved two steps enrichment in UVM broth and selective plating on PALCAM, McBride and TSYEA agar. The organism was phenotypically identified based on cultural characteristics, Gram staining reaction and biochemical characteristics (Catalase, Oxidase, Motility, Indole, Methyl Red, Voges-Proskauer, Citrate utilization, fermentation patterns of sugars like L-Rhamnose, D-Xylose and Mannitol) (Quinn *et al*. 1994) [18]. The presumptively identified *L. monocytogenes* strains were subjected to in vitro pathogenicity test by using beta haemolysis on 5% sheep blood agar and Christie, Atkins, Munch- Petersen **(**CAMP) test as per ISO (1996) [11]. The phenotypically confirmed bacterial strains were further confirmed by PCR detection of species specific *16S-rRNA* gene of *L. monocytogenes* by using published primers *(*Weidmann *et al.*, 1993) [26].

A panel of antimicrobial drugs were selected for antimicrobial susceptibility test considering their use in animal and human and effective against Gram-positive bacteria. All the *L. monocytogenes* strains were subjected to *in vitro* antimicrobial drug sensitivity test by disc diffusion method (Bauer *et al*.,1966) [3] against 14 antibiotics namely Penicillin G (10units), Amoxicillin/clavulanic acid (30µg), Ampicillin (10µg), Oxacillin (1µg), Streptomycin (10 µg), Erythromycin (15 µg), Cephotaxim/ Clavulanic acid (30/10µg), Ceftriaxone (30 µg), Chloramphenicol (30 µg), Nalidixic acid (30 µg), Ciprofloxacin (5 µg), Gentamicin (10 µg), Tetracycline (30 µg) and Trimethoprim (5 µg). The pure cultured bacterial strains were inoculated into Brain Heart Infusion (BHI) broth and incubated for 24 hours at 37 °C. A broth culture of 200 µl was taken on Muller Hinton Agar plates and spread eventually with the help of sterile L-shaped spreader. On drying of the culture, antibiotic discs were placed on media aseptically with the help of sterile forceps and incubated at 37 °C for 24-48 hours. The diameter of zone of inhibition was compared with the standard known value against each specific antimicrobial agent from interpretation guide line (Hi-Media).

Results and Discussion

From the 200 different samples of cattle sources (faeces, offals/ internal organs, raw meat and farm water) from Aizawl district, a total 18(9.00%) samples were found to be presumptively positive for *L. monocytogenes* based on phenotypic characteristics in which isolates turned into black colour in UVM broth and showed specific colony characteristics on different agars like green colonies with black holoes in PALCAM agar, dense white to iridescent white appearing as crushed glass in McBride agar and clean glass like colonies in TSYEA agar after 24-48 hours of incubation at 37 ˚C. The *L. monocytogenes* strains were phenotypically confirmed based on the positive Gram staining reaction, tumbling motility, different biochemical reactions like catalase positivity, oxidase negativity, indole negativity, methyl red positivity, Voges-Proskauer positivity, citrate negativity and fermentation reaction of sugars namely L-rhamnose (positive) D- Mannitol (negative) and D- Xylose (negative) and *in vitro* pathogenic reactions of weak haemolysis on 5% SBA and positive CAMP test against *Staphylococcus aureus.* On PCR of *16S-rRNA* gene, 13(6.50%) numbers of *L. monocytogenes* were detected with highest numbers in farm water (7.00%) followed by internalorgans/ offals (6.00%), raw meat (4.00%) and lowest in faeces (2.00%) (Table-1).

Higher occurrence rate of *L. monocytogenes* from faecal samples of ruminants were recorded by Lawan *et al.* (2003) [15] (10.00%) and Kalorey *et al.* (2006) [12] (16.00%) from Nigria and Nagpur, India, respectively. The occurrence of haemolytic *L. monocytogenes* isolates from various meat samples was reported by Khan *et al* (2013) [14] like in raw chicken (6.0%), fish meat (4.0%) and beef (2.5%). Sur *et al* (2012) [24] also reported that high prevalence of *L. monocytogenes* in chicken meat ranging from 7.14 to 42.03% is of high risk in hyper and wet markets of Malaysia. Healthy cattle are capable of serving as reservoirs for *L. monocytogenes*. The food products like milk, meat and processed milk and meat products may be contaminated through accidental contact of raw products with faeces, water and silage. The incidence of *L. monocytogenes* in cattle faeces, internal organs/ offals and farm water under unorganised sector of cattle production provided information about the probable carrier status of the organism in cattle and contamination status of their products. The contamination of beef and offals or organs is mainly due to the improper food handling practices carried out in wet markets, use of unhygienic containers, improper food handling, cross-contamination from other contaminated foods and from infected field workers at the market (Chai *et al.*, 2007) [6]. It is well established that food product contamination is associated with food-processing environments harbouring *L. monocytogenes* (Olsen *et al*, 2005) [16].

Table 1: Phenotypic and molecular detection of *L. monocytogenes* from different samples of cattle origin from Aizawl district, Mizoram

S. No	Type of Sample	Sample tested	Phenotypically positive *L. monocytogenes*	Genotypically positive *L. monocytogenes*
1	Faeces	50	2 (4.00)	1 (2.00)
	Internal organs/ offals	50	3 (6.00)	3(6.00)
	Raw meat	50	3 (6.00)	2(4.00)
3	Farm water	50	10(20.00)	7(14.00)
	Total	200	18 (9.00)	13 (6.50)

Antimicrobial resistance is emerging as a silent pandemic in both developed and developing countries threatening the global health, food security and safety (Sharma *et al*, 2017; Chauhan *et al*, 2018) [22, 5] and its prevalence among food borne pathogens has increased during recent decades (Akbar and Anal, 2014) [2]. The resistant strains of bacterial

pathogens percolate through the environment and may colonize the human population via food chain of animal origin, contact through occupational exposure or waste run off from animal production facilities. Resistant bacteria may be readily transferred from food animals to human beings as the similar kind of antimicrobial agents are used in human practice also.

Listeria monocytogenes is naturally susceptible to a range of antibiotics that act on Gram-positive bacteria including penicillin, ampicillin, amoxicillin, gentamicin, erythromycin, tetracycline, rifampicin, co-trimoxazole, vancomycin and imipenem. However, most strains of *L. monocytogenes* show natural resistance to current fluoroquinolones and cephalosporin (Byrne *et al.*, 2016) [4]. Additionally, reduced sensitivity or resistance to beta-lactams has been encountered.

Out of the 13 strains of *L. monocytogenes*, the single strain isolated from cattle faeces was susceptible to the panel of antimicrobials tested (Table 2). Further analysis of antimicrobial susceptibility test results showed the highest resistance to Penicillin (23.08%) followed by Ciprofloxacin (15.39%), Nalidixic acid (7.07%) and Ampicillin (7.07%) in *L. monocytogenes* strains. Out of the 13 isolates, 4 (30. 77%) isolates were resistant to at least one or more antimicrobials namely Penicillin G (2), Ampicillin (1), Ciprofloxacin (2) and Nalidixic acid (1). Although the number of resistant strains is low, out of the thirteen strains, 42.86% (3/7) strains were from the water sources of cattle farm environment were resistant to the antimicrobial drugs of Penicillin and Quinolone groups namely Penicillin (2), Ampicillin (1), Nalidixic acid (1) and Ciprofloxacin (2). However, highest 23. 08% (3/13) *L. monocytogenes* strains identified from farm water (2) and internal organs/ offals (1) were resistant to Penicillin G. One (14.29%) (3/7) strain from farm water was resistant to Nalidixic acid while the strains from other samples were sensitive to the antimicrobial agent (Table 2). One isolate from farm water was found to be resistant to three antibiotics namely Penicillin G, Ampicillin and Ciprofloxacin while one isolate from offal was found to be resistant to Penicillin and Ampicillin. However, no multidrug resistance pattern of the bacterial pathogen was detected in the present study as an organism has to be resistant to atleast three different classes of antimicrobial drugs. Resistance of the *L. monocytogenes* strains isolated from farm water detected in the present study against the quinolone group of antibiotics namely Nalidixic acid and Ciprofloxacin were similar with studies conducted in different countries. Soni *et al* (2014) [23] and Wu *et al* (2015) [27] reported *L. monocytogenes* isolates resistant to tetracycline, ampicillin, streptomycin and ciprofloxacin from raw food including vegetables and meat. Sharma *et al.* (2012) [21] detected 80-90 percent resistance of *L. monocytogenes* strains from raw milk in Meerut and Babugarh Cantt, Hapur (India) to Nalidixic acid whereas some strains were also resistant to the Ampicillin and Ciprofloxacin.

In Mizoram, cattle farming are gaining a momentum in recent years and the farmers rely on use of antimicrobial drugs for ensuring health, to reduce morbidity and mortality and to enhance production performance of the dairy cattle. Penicillin is one of the most frequently prescribed drugs for most of infectious diseases in veterinary medicine due to its relatively cheaper price and easy availability in the market which might be one of the reasons for the development of higher resistance profile to the agent. On the other hand, clinicians usually treat listeriosis with aminopenicillins in combination with an aminoglycoside such as gentamicin.

Table 2: Antimicrobial susceptibility of *L. monocytogenes* strains isolated from cattle sources in Aizawl district, Mizoram

Antimicrobials	Type of sample			
	Faeces (1)	Internal organs/Offals (3)	Raw meat (2)	Farm water (7)
	Sensitive (S)/ Resistant (R)	Sensitive (S)/ Resistant (R)	Sensitive (S)/ Resistant (R)	Sensitive (S)/ Resistant (R)
Penicillin G	S	S (66.66%) R (33.34%)	S	S (57.14%) R (42.86%)
Ampicillin	S	S (66.66%) R (33.34%)	S S	S
Amoxycillin clavulanc acid	S	S	S	S
Oxacillin	S	S	S	S
Streptomycin	S	S	S	S
Erythromycin	S	S	S	S
Cephalexin	S	S	S	S
Ceftriaxone	S	S	S	S
Chloramphenicol	S	S	S	S
Ciprofloxacin	S	S R	S S	S (71.42%) R (28.58%)
Gentamicin	S	S	S	S
Tetracycline	S	S	S	S
Trimethoprim/Sulphamethoxazole	S	S	S	S
Nalidixic acid	S	S R	S	S (85.71%) R (14.29%)

Conclusion

The detection of antimicrobial resistant *L. monocytogenes*, although in low numbers from different samples of cattle sources namely internal organs/ offals and farm water in Aizawl district of Mizoram indicated the public health significance of this pathogen. The presence of antimicrobial resistant *L. monocytogenes* in food animals and their environment has an important public health implication especially in developing countries where there is widespread and uncontrolled use of antibiotics along with high contamination status of raw food of animal origin as the animals are slaughtered unhygienically under unorganized sector of meat production. Based on the evidence of recent increase of human listeriosis, regular surveillance of *L. monocytogenes* should be maintained for early detection of any shift in the antimicrobial resistance of food or

environmental isolates. In the present scenario of cattle farming, dairying and slaughtering of animals in Mizoram, treatment based on *in-vitro* antimicrobial susceptibility tests should be practised. Health education about the risk of consumption of raw or undercooked foodstuffs should be implemented.

Acknowledgements
The authors duly acknowledge to the Dean, College of Veterinary Sciences & Animal Husbandry, Central Agricultural University, Imphal for providing the funds with necessary facilities to conduct this study under the Department of Veterinary Public Health and Epidemiology.

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