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Molecular characterization of drug resistant *Listeria monocytogenes* from food and water samples

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ABSTRACT

Aims: Present research is focused on the molecular level characterization of drug-resistant *L. monocytogenes* identified from food and water samples from Tamil Nadu, India.
Methodology and results: A total of 39 food and water samples were collected from local markets and retail shops in Tamil Nadu, India and processed for the isolation and identification of bacteria. Morphology of the bacteria was analysed under a fluorescent microscope. Isolated bacteria were serotyped and screened for the presence of virulence-associated genes haemolysin (*hly*A) and invasive associated protein (*iap*A) by Real-time polymerase chain reaction. The qPCR positive isolates were also typed by random amplified polymorphic DNA–PCR for epidemiological study. Antibiotic resistance test was done with 16 commercial antibiotics by disc diffusion method. A total of 8 (20.51%) *L. monocytogenes* were identified belonging to the serotype group 1/2a, 1/2b, 1/2c and 4b. PCR assays revealed the presence of *hly*A (456 bp) and *iap*A (131 bp) genes. In RAPD, OPA-10 primer was found to generate the distinct polymorphic fragment among the isolates. All the isolates were 100% resistant to rifampicin, co-methoxazole, linezolid and oxacillin and 100% sensitive to tetracycline and chloramphenicol. Tetracycline and chloramphenicol are suggested to be a very effective antibiotic against the tested *L. monocytogenes* isolates.
Conclusion, significance and impact of study: The *hly*A and *iap*A based quantitative PCR technique could be a rapid molecular technique for the detection of *L. monocytogenes* used in this study. Serotyping along with RAPD-PCR was able to discriminate between the isolates and therefore could serve as a robust and sensitive tool for typing antibiotic-resistant strains of *L. monocytogenes*.

Keywords: L. monocytogenes, Antibiotic resistant, PCR, RAPD, Serotyping

INTRODUCTION

Listeria monocytogenes is a disease-causing, opportunistic, Gram-positive bacteria. This facultative anaerobe is a very virulent foodborne pathogen and causes Listeriosis, a disease that is associated with high rates of hospitalisation and fatality. Listeriosis arises from food contamination and is associated with encephalitis, miscarriage, septicaemia and sometimes even results in death (Martinez *et al*., 2017). It commonly occurs more in pregnant women, immune-compromised individuals, HIV affected people and ageing adults (Kureljuši *et al*., 2017). This disease is one of the noted causes for deaths resulting from foodborne infections, with about 260 cases per year. Among the 13 serotypes known, 1/2a, 1/2b and 4b are the serogroups that are known to most frequently cause contamination in foods (Toomas *et al*., 2013).

Contamination of *L. monocytogenes* is in general found in raw milk, meat and fishery products. In uncooked poultry meat products, as much as 50% contamination has been documented in certain regions. Nearly 3% to 6.5% raw milk samples tested positive for contamination and this is owing to the inclination of *Listeria* to grow in moist environments over a range of temperatures (Ibba *et al*., 2013). It remains viable in acidic pH (4.4), increased salt concentration (14%), and low water activity and can, therefore, thrive in food processing equipment. *L. monocytogenes* may infect many processed foods and is thus also able to contaminate meat products (Adeshina *et al*., 2017). This ubiquity of *L. monocytogenes* is one of the foremost concerns in the food industry because it is very tedious to completely remove the organisms from all small niches and crevices.

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Listeria monocytogenes are capable of forming biofilms on many abiotic surfaces after short contact times of 20 min (Zadernowska *et al*., 2017). This formation of biofilms on surfaces like glass and stainless steel gives it the ability to be resistant to harsh chemicals such as detergents and antibiotics; this is because the microbes in the film are embedded in an Extracellular Polymeric Substance (EPS) (Escolar *et al*., 2017). Drug resistance also arises as a consequence of the misuse of antibiotics and incorrect dosage. Such Multi-Drug Resistant Organisms (MDRO) not only render the previous generation of antibiotics futile but also put an additional strain on infection. This is a major cause for concern since it prevents the effective medication of Listeriosis in human beings. Research suggests that this bacterium is by nature resistant to intermediate and current generation sulphamethoxazole and cephalosporins. There is also the possibility that it can resist third generation antibiotics of rifampin, oxacillin, gentamicin and such (Morobe *et al*., 2009).

Currently, these bacteria can be detected by conventional methods such as extensive biochemical tests, which include plating in selective media and CAMP test. ALOA Agar and Palcam Agar are various selective media available for culturing isolated colonies. The CAMP test involves plating the *L. monocytogenes* colonies on 5% sheep blood agar, and recording zones of haemolysis after 24-48 h. These methods generally take a few days to complete and require the bacteria to reach an appreciable initial concentration. DNA fingerprinting by RAPD markers may serve as a possible typing technique, based on the fact that for any oligonucleotide sequence, the genome of the organism is expected to have sequences with an extent of homology to the primer (Altuntas *et al*., 2012). Since the distribution of these sequences is extremely randomized, the result is a set of RAPD sequences and the pattern retrieved will carry the identity profile of the organism. The profile of the bands, size and the core patterns are analysed for the selected strains. This aids in the discrimination between the strains and within the serogroups. In addition, real-time PCR can be performed using the non-specific dye SYBR green. The specificity could be tested by carrying out a melting-curve analysis, where the temperature of the peak may be compared with the melting temperature of the specific amplified product (Guilbaud *et al*., 2005).

A significant step towards management of listeriosis outbreak is to devise a rapid screening technique for the pathogen. In addition, the acquired resistance of *L. monocytogenes* is escalating rapidly, which calls for the identification of new drug targets. Therefore, this study aims to characterise *L. monocytogenes* to develop a faster and more efficient way to detect it in food samples.

MATERIALS AND METHODS

Collection of food samples

In total, 39 food samples consisting of pork (14), chicken (7), beef (5), chevon (3), milk (4) and water (6) were collected for investigation. These samples were randomly procured from local markets and retail shops in Tamil Nadu and were stored in 4 °C under refrigeration during both transport and storage. They were promptly transported to Molecular Diagnostics and Bacterial Pathogenomics Research Laboratory for various biochemical and microbiological analysis.

Isolation and identification of *L. monocytogenes*

Samples of 1 g each were homogenised in 9 ml of Listeria Enrichment broth (Himedia, Mumbai) for 2 min. 1 mL from each of the LE broth suspension tubes was consequently transferred to 9 mL of LE broth, which was then incubated at 37 °C for 24 h. Resulting samples were then streaked on Listeria Selective Agar (PALCAM) along with the further addition of Selective Supplement and incubated for 24 h at 37 °C. Those colonies that showed the characteristic morphology of *L. monocytogenes* underwent various biochemical tests like CAMP Test (Khan *et al*., 2012), catalase test, indole test, oxidase test, and Voges-Proskauer test.

Fluorescent staining of *L. monocytogenes*

Listeria monocytogenes cultures were grown overnight in BHI broth and washed with 1× PBS (pH 7.4). The washed cell of 10 µL was smeared on glass slides and air dried. 200 µL of Propidium Iodide (20 µg/mL) was added and evenly spread to stain all the cells. The stained cells were incubated in dark for 30-45 min and observed under fluorescent microscope (Nikon Eclipse 80i) using 535/617 nm detector (FL-2 detector).

Template DNA preparation for Real-Time PCR

The template DNA for real-time PCR was prepared by extracting the genomic DNA from the isolated *L. monocytogenes* using QIAamp® genomic DNA kits (Qiagen, USA) following its technical handbook instructions with little modifications.3 mL of overnight grown bacterial cultures from BHI broth were taken in 1.5 mL of Eppendorf tube and centrifuged at 13,000 rpm for the 30 sec at 4 °C to discard the supernatant. Prior to centrifugation at the same speed, 1× PBS was used to wash the pellet containing the cells, twice. The supernatant was again discarded, and the cells of *L. monocytogenes* were thoroughly suspended in 20 µL of proteinase K. To the cell suspension, 10 µL of lysozyme (10 mg/mL) was added and incubated at 60 °C until the tissue was completely lysed and was centrifuged at the same speed to discard the supernatant. To the pellet, 4 µL of RNase A (100 mg/mL) was added and mixed for 15 sec by pulse-vortexing and incubation was carried out at 15-25 °C for 2 min. Further, 200 µL of buffer AL was introduced and mixed for 15 sec by pulse-vortexing and incubated at 70 °C for 10 min. The suspension was briefly centrifuged at the same speed for the 30 sec and 200 µL of ethanol was introduced to the mix and again centrifuged. The mixture was taken in a QIAamp Mini spin

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column and to discard the filtrate, centrifugation was performed for 1 min at 8000 rpm. To the 2 mL collection tube which includes the QIAamp Mini spin column, 500 µL of buffer AWL was added and centrifuged for 3 min at 14,000 rpm to discard the filtrate. 200 µL of buffer AE was taken in a column and incubated for 1 min at room temperature followed by centrifugation for 1 min at 8000 rpm and this step was done again. The template DNA thus extracted from the bacteria was stored at –20 °C.

The concentration of the extracted DNA was analyzed using NanoDrop™ (2000/2000c spectrophotometer) (Thermo Scientific™, USA) following its nucleic acid measurement support information. Absorbance was measured at 260 nm and the generated spectral image was reviewed to evaluate the quality of the nucleic acid and its concentration (ng/µL) was calculated using the modified Beer-Lambert equation.

Real-Time PCR for detection of virulence genes

Isolated bacteria were screened for virulent gene detection like haemolysis (*hly*A) (456 bp) and invasive associated protein (*iap*A) (131 bp) by real-time PCR (Guilbaud *et al.,* 2005). Specific reverse and forward primers for *iap*A genes were 5'-tgacagcgtgtgtagtagca-3' and 5'-acaagctgcacctgttgcag-3' and *hly*A were 5'-gcaacgtatcctccagagtgatcg-3' and 5'-gcagttgcaagcgcttggagtgaa-3' were synthesized commercially (Eurofins Genomics Pvt. Ltd, Bangalore). *Escherichia coli* (MTCC 1143) was used as negative control and *L. monocytogenes* (MTCC 723) was used as positive control in qPCR. An amplification mixture of 15 µl containing 7.5 µl of 2× Rotor-Gene SYBR Green PCR Master Mix (Qiagen) containing HotStar Taq® *Plus* DNA Polymerase, Rotor-Gene SYBR Green PCR Buffer and dNTP mix containing dATP, dCTP, dGTP and dTTP, 0.5 µM of forward and reverse primer and 1.5 µL of template DNA was made up by adding 5.85 µL of RNase-Free water for the detection of the food-borne pathogen *L. monocytogenes.* qPCR amplification was performed in a thermal cycler (Rotor-gene Q, Qiagen). After initial denaturation at 95 °C for 10 min, the real-time PCR cycle (45×) had denaturation and annealing at 95 °C and 60 °C for 10 sec and 20 sec respectively. A standard curve representing the detection of *L. monocytogenes* genome copies was generated by Rotor-gene Q series software version 2.3.1.49 (Qiagen, Germany). The specificity was tested by melting curve analysis during which, the temperature was increased every 5 sec by 0.1 °C. The high-resolution melt curve was analyzed for peaks at a melting temperature that corresponds to the temperature at which the product of amplification melts.

Serotyping

The serotypes of *Listeria monocytogenes* were differentiated using Multiplex-PCR as described by Doumith *et al.* (2004). The templates DNA for PCR were extracted as described above. Multiplex-PCR was carried out in 25 µL reaction volume in a thermal cycler, Mastercycler® EP gradient (Eppendorf, Germany) and 2.5 µL of the supernatant was used in every amplification. Ultra-pure water was used as negative control and the serotypes of *L. monocytogenes* namely, 1/2a (35Lis12), 1/2b (22Lis12), 1/2c (TP16) and 4b (33Lis12) laboratory isolates procured from Division of A.H, ICAR, India was used as positive controls.

DNA fingerprinting by RAPD markers

Isolates of *L. monocytogenes* were screened using a set of 20 numbers, 10-mer oligos primer Kit A (A1-A20) commercially available from Operon Technologies (USA). Primer OPA-10 (5'-gtgatcgcag-3') resulted in descriptive fingerprints and was evaluated with the remaining isolates. The end concentration of template DNA for RAPD analysis was maintained in a range of 186 - 189 (ng/µl) by diluting the genomic DNA in the desired volume of sterilized Milli-Q water. The amplification reaction for RAPD was performed in an end volume of 50 µl containing 25 µL of 2× PCR master mix (4 mM $MgCl_2$; 0.4 mM of each dNTPs [dATP, dCTP, dGTP, dTTP]); 0.5 units/µ l of *Taq* polymerase; 150 mM Tris-HCl PCR-RAPD buffer (pH 8.5), 1µM of primer and 5 µl of template DNA. PCR amplification was carried out in Rotor-Gene Q, a thermal cycler (Qiagen) with cycling parameters of initial 5 min denaturation at 94 °C followed by 45 cycles of denaturation followed by annealing and chain extension at 94 °C, 36 °C and 72 °C for 1 min, 1 min and 2 min respectively. An extension was performed in the end for 10 min at 72 °C followed by holding at 4 °C. Gene Ruler 100bp plus DNA ladder (0.1 µg/µL; Thermo Scientific, USA) was used as a marker. The PCR amplicons (5 µL) electrophoresed in 1.5% agarose (Promega, USA) and the ethidium bromide (0.4 µg/mL) stained gel was captured using Gel doc system with software (Bio-Rad, USA). The diversity and distribution of PCR-RAPD fingerprints of selected representative cultures of *L. monocytogenes* were analysed using BioNumerics, version 7.6 software (Applied Maths, USA).

Antibiotic susceptibility

The susceptiveness of *L. monocytogenes* to a panel of 16 antibiotics namely ampicillin (10 mcg), erythromycin (15 mcg), novobiocin (5 mcg), penicillin G (10 units), methicillin (30 mcg), kanamycin (30 mcg), tetracycline (30 mcg),chloramphenicol (10 mcg), rifampicin (5 mcg), vancomycin (5 mcg), co-trimoxazole (25 mcg), oxacillin (1 mcg), amoxicillin (10 mcg), ciprofloxacin (1 mcg), linezolid (10 mcg) and streptomycin (25 mcg), was tested using standard disc diffusion method according to 2017 European Committee of Antimicrobial Susceptibility Testing (EUCAST) guidelines (Kahlmeter *et al*., 2006). The organisms were streaked using a swab on Nutrient Agar plates, followed by positioning discs containing antibiotics on the plates. Following incubation for 24 h at 37 °C, the diameter of the resulting clear zone of inhibition was recorded. The resistant breakpoint was analysed using National Committee for Clinical Laboratory

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Standards (NCCLS) (Villanova, 1990) with control for Gram-positive bacteria as *Listeria monocytogenes* (MTCC 1143) and *Staphylococcus aureus* (NCIM 2079).

RESULTS AND DISCUSSION

Isolation and identification of *L. monocytogenes*

Of the 39 food samples, 8 (20.5%) isolates were identified as *L. monocytogenes* based on the following bacteriological examinations. All isolates produced typical grey-green colonies on Listeria Selective Agar which are ringed by a black zone. Isolates were also found to be positive for the indole test, catalase test, Voges-Proskauer test and CAMP assay, and negative for oxidase test. Among the 8 isolates, 3 (37.5%) were from pork and 1(12.5%) each from water, beef, milk, chevon and chicken (Figure 1). The highest percentage of isolation of 37.5% was from pork meat. In this study, 3 (21.4%) isolates were obtained from 14 pork samples and 1 (33.33%) isolate was obtained from 3 chevon samples. This result is found to be greater than those obtained by other research studies for pork (Trimoulinard *et al.,* 2017; Ryu *et al.*, 2013), for chevon (Islam *et al.,* 2016). In our study, 1 (20%) out of 5 beef samples and 1 (14.3%) out of 7 chicken samples were analysed to be positive for *L. monocytogenes*. Those results obtained for meats such as beef and chicken were similar to the results those obtained by (18%) Islam *et al.* (2016) and (16.66%) Abay *et al.* (2017) respectively. Prevalence of this microbe in milk (25%) and water (16.67%) is marginally higher than the results obtained by other researchers for water and milk respectively (Lyautey *et al.,* 2007; Ibba *et al.,* 2013).

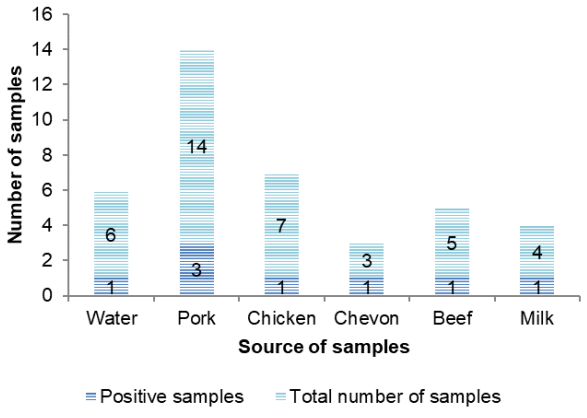

Figure 1: Distribution of *L. monocytogenes* among various food samples

Fluorescent staining of *L. monocytogenes*

The stained cells when observed under a microscope after 45 min of incubation fluoresced bright orange-red and appeared to be rod-shaped (Figure 2). Use of dyes such as Nisin-EGFP complex is useful in identifying *L. monocytogenes* specifically, as demonstrated by Tan *et al.* (2017). Hazeleger *et al.* (2006) showed that double staining procedures utilising dyes such as DAPI (4', 6-Diamidino-2-phenylindole) and FM 4-64 are more beneficial to visualise DNA-rich spots and membrane spots.

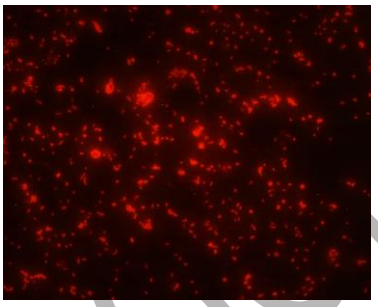

Figure 2: Ultrastructure study of *L. monocytogenes* by fluorescence microscopy under 40×.

Real-Time PCR for detection of virulence genes

The concentration of the extracted genomic DNA from *L. monocytogenes* was recorded to be between 186-189 ng/µL at 260 nm absorbance. qPCR assay was performed for 39 food and water samples and a relationship between the Ct (cycle threshold) value and the number of amplicons present was obtained for two genes, *iap*A (131 bp) and *hly*A (456 bp) (Das *et al.,* 2015) after 45 cycles of amplification as shown in Figure 4 and Figure 6. The Ct values were ranging from 1-8 and qPCR results were found to be 100% and 87.5% positive for *hly*A and *iap*A respectively. Similar work was carried out using the listeriolysin O gene (*hly*) by Guilbaud *et al.* (2005). Linearity for both the genes was observed for 20 cycles and the fluorescent signal emitted by the dye bound to the amplicons doubled every cycle of amplification during the exponential phase. After 36 cycles, the emission of fluorescence began to level out and this indicated the attainment of saturation. Linear regression analysis produced a correlation coefficient of 1 for both the genes and the slope was –3.185 for *hly*A and –3.073 for *iap*A. Real-time PCR analysis of *L. monocytogenes* revealed a detection limit of 1214 and 418 copies of genomes for *hly*A and *iap*A respectively. High-resolution melt curve analysis performed by raising the temperature by 0.1˚C every 5 sec, indicated the specificity of qPCR assay by revealing similar peaks at 84.2 °C for *hly*A and between 82.0 °C - 82.3 °C for *iap*A as shown in Figure 3 and Figure 5. This was performed because of the non-specific binding nature of SYBR Green I dye, and thus co-amplified nonspecific PCR products could be differentiated from the specific product of amplification. The peaks generated indicated the amount of amplicons produced and this range was in proximity for amplicons from a specific target gene.

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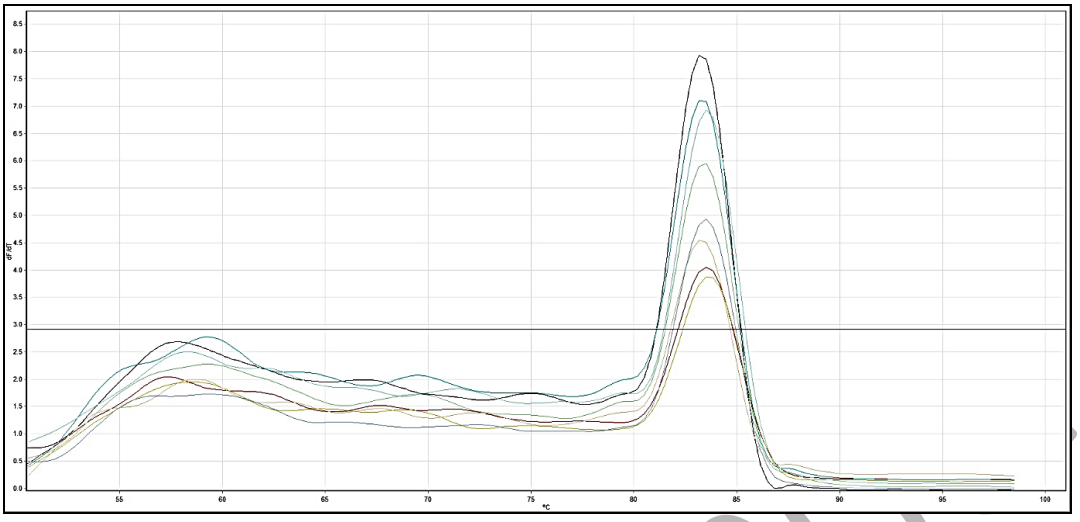

Figure 3: Melt curves indicate the melting peaks of the amplified part of the *hly*A gene among eight isolates of *L. monocytogenes*. The plot dF/dt (X-axis) vs. Temperature (°C) (Y-axis) with a threshold of 2.912 shows that the melting temperature of the amplified PCR products significantly has the same value (84.2 °C), ensuring that desired gene was amplified.

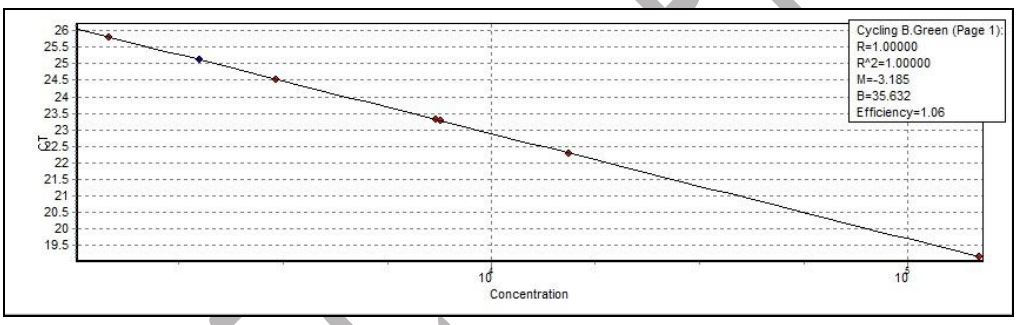

Figure 4: Standard plot of Ct value vs concentration of DNA (copies/µL) representing the different quantities of a *hly*A gene from *L. monocytogenes* isolates. The detection limit was as low as nearly 1214 copies in this run.

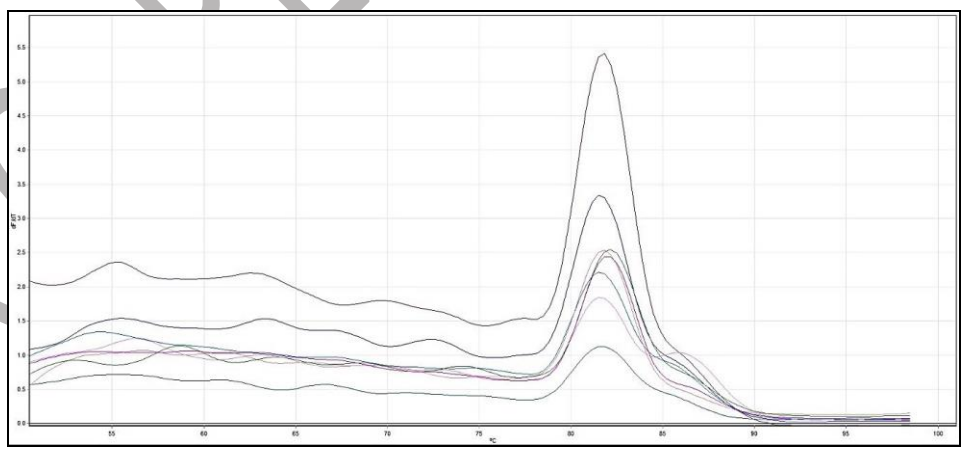

Figure 5: Melt curves indicate the melting peaks of the amplified part of *iap*A gene among eight isolates of *L. monocytogenes*. The plot dF/dt (X-axis) vs Temperature (°C) (Y-axis) shows that the melting temperature of the amplified PCR products significantly has the same value (82.0 °C - 82.3 °C), ensuring that the desired gene was amplified.

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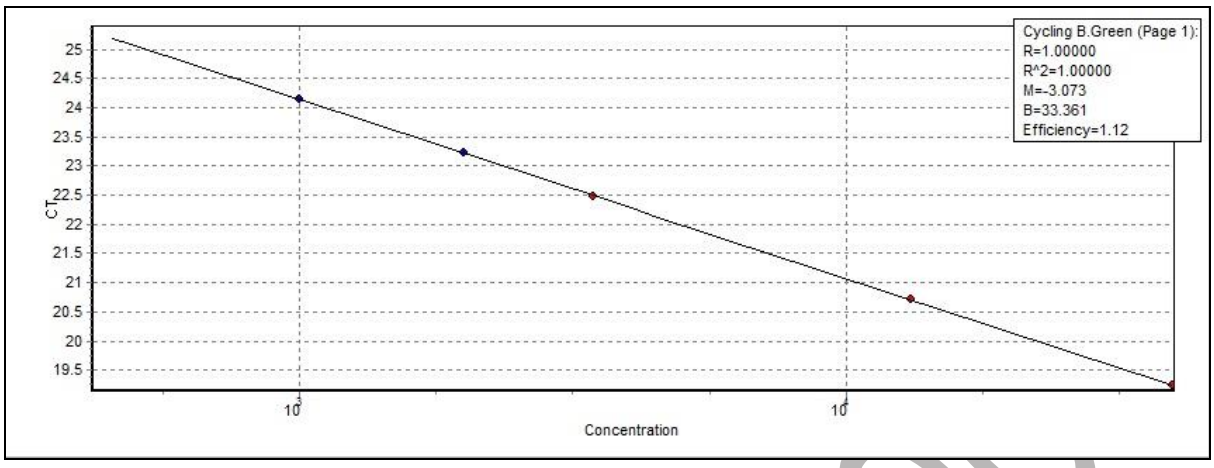

Figure 6: Standard plot of Ct value vs Concentration of DNA (copies/µL) representing the different quantities of *iap*A gene from *L. monocytogenes* isolates. The detection limit was as low as nearly 418 copies in this run.

Table 2. PCR RAPD fingerprint of *L. monocytogenes* generated with primer OPA-10**.**

Fingerprint type	OPA-10 (A)	OPA-10 (B)	OPA-10 (C)	OPA-10 (D)	OPA-10 (E)	OPA-10 (F)	OPA-10 (G)
Template DNA Conc. (ng/µl)	186	186	187	189	188	189	189
Source	Pork	Pork	Chicken	Milk	Beef	Water	Chevon
Isolates	LMM15	LMM16 LMM12	LMM13	LMM17	LMM14	LMM18	LMM27
Total No (%) of isolates	1 (12.5%)	2 (25%)	1 (12.5%)	1 (12.5%)	1 (12.5%)	1 (12.5%)	1 (12.5%)

Serotyping

Serotyping by multiplex PCR indicated the prevalence of four common serogroups of *L. monocytogenes* namely 1/2a, 1/2b, 1/2c and 4b involved in the contamination of commercial foods (Table 1). Similar work was reported by Vasconcelos *et al.* (2008). Serogroup 1/2a showed the highest prevalence while 1/2c was less prevalent.

Table 1: Serogrouping and virulence determination of *L. monocytogenes* isolated from various sources

Isolate number	Source	Place	Sero group	Virulence gene profile	
				*hly*A	*iap*A
LMM12	Pork	Tamil Nadu	1/2a	+	+
LMM16			1/2c	+	+
LMM15			4b	+	+
LMM14	Beef		1/2a	+	+
LMM18	Water		4b	+	+
LMM13	Chicken		1/2a	+	-
LMM17	Milk		1/2b	+	+
LMM27	Chevon		1/2b	+	+

DNA fingerprinting by RAPD markers

RAPD fingerprinting with the primer OPA-10 resulted in 7 distinct fingerprint patterns [OPA-10 (A) through OPA-10 (G)] (Table 2). The amplicon sizes were found in molecular weight ranging from 335 bp to 1500 bp with a common 335 bp fragment in 6 of the 7 fingerprint patterns (Figure 7). A similar analysis was done by Lawerence *et al.* (1993). We have also found that the final and optimum concentration of template DNA at 186-189 ng/µL was sufficient to generate good RAPD fingerprinting profiles with all the primer evaluated with OPA series. Since OPA-10 generated a good reproducible fingerprinting patterns among the 8 *L. monocytogenes* isolates studied, the software analysis was done with this primer only. Out of the 7 patterns, 4 (A, B, E, F) had the same bands at around 1000 bp. At around 500 bp, B and F patterns have the same bands, while at 1500 bp, only pattern C is found to produce a band. The fingerprinting patterns prove the existence of genetic variance among the isolates of *L. monocytogenes*. Such diversity is independent of source and geographical landmark. Czajka, J and Batt, C. A. (1994) reported that isolates obtained from clinical samples were different from food samples by analyzing the patterns produced for 19 primers. Thus, the resulting unique pattern facilitates the discrimination between organisms from different sources irrespective of its serogroup.

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Table 3: Antibiotic susceptibility tests for *L. monocytogenes* to a panel of antibiotics

Antibiotics (Concentration)	Isolate number and zone of inhibition (mm) for antibiotic							
	LMM12	LMM14	LMM18	LMM13	LMM17	LMM27	LMM15	LMM16
Ampicillin (10 mcg)	S (39)	S (36)	R (7)	R (25)	S (30)	S (29)	S (31)	R (2)
Methicillin (30 mcg)	R (8)	S (30)	S (25)	R (5)	R (4)	S (34)	S (30)	R (1)
Co-methoxazole (25 mcg)	R (0)	R (9)	R (0)	R (8)	R (4)	R (0)	R (0)	R (9)
Chloramphenicol (10 mcg)	S (21)	S (20)	S (20)	S (18)	S (16)	S (18)	S (20)	S (22)
Tetracycline (30 mcg)	S (35)	S (37)	S (37)	S (29)	S (29)	S (31)	S (42)	S (30)
Amoxicillin (10 mcg)	R (4)	R (2)	R (5)	R (4)	S (18)	R (4)	R (4)	R (6)
Penicillin G (10 units)	R (5)	R (2)	R (3)	R (4)	R (7)	S (33)	S (29)	R (5)
Rifampicin (5 mcg)	R (2)	R (0)	R (0)	R (0)	R (0)	R (2)	R (0)	R (0)
Novobiocin (5 mcg)	S (31)	S (28)	S (30)	R (3)	R (5)	S (33)	R (2)	R (10)
Kanamycin (30 mcg)	S (27)	S (30)	S (28)	R (4)	R (9)	R (3)	S (25)	S (25)
Oxacillin (1 mcg)	R (2)	R (0)	R (3)	R (2)	R (2)	R (4)	R (2)	R (7)
Erythromycin (15 mcg)	S (41)	S (38)	S (34)	S (30)	S (26)	R (5)	R (6)	R (8)
Ciproflaxin (1 mcg)	R (5)	R (4)	R (4)	R (6)	R (4)	S (18)	R (8)	R (6)
Linezolid (10 mcg)	R (6)	R (8)	R (2)	R (0)	R (0)	R (2)	R (1)	R (4)
Streptomycin (25 mcg)	S (29)	R (2)	R (4)	R (4)	R (10)	R (2)	R (2)	R (1)
Vancomycin (5 mcg)	R (7)	R (2)	R (2)	S (18)	R (0)	R (0)	R (0)	R (0)

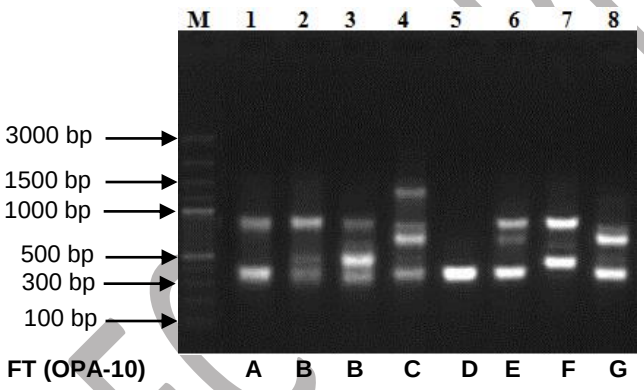

Figure 7: RAPD fingerprint of *L. monocytogenes* isolates with primer OPA-10. Lanes 1-8: Isolate numbers LMM15, LMM16, LMM12, LMM13, LMM17, LMM14, LMM18, and LMM27, respectively. M: Gene Ruler 100 bp plus DNA ladder (Thermo Scientific). FT: fingerprinting types with OPA-10.

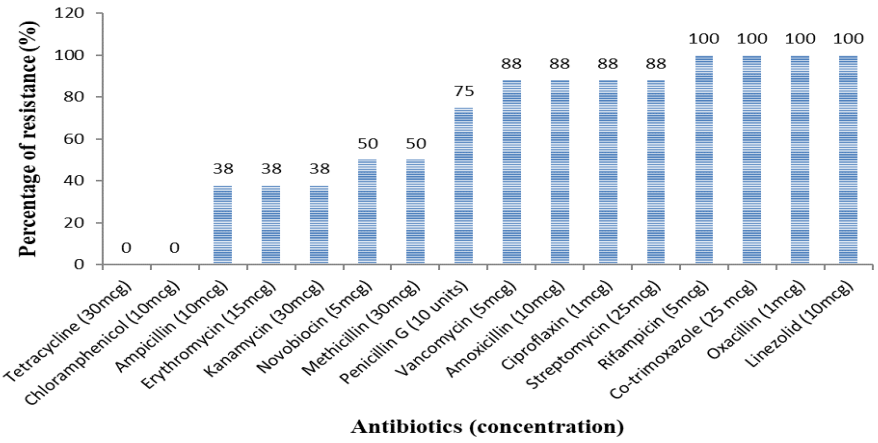

Figure 8: Percentage resistance of *L. monocytogenes* to a panel of antibiotics

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Antibiotic susceptibility

The sensitivity of the isolates to antibiotics was tested using the disc diffusion method and zone of inhibition was recorded. The diameter of this zone was compared against standard charts and the bacteria were classified as sensitive or resistant to that antibiotic (Table 3). The tested organism, *L. monocytogenes* showed high resistance (100%) to rifampicin, trimethoprim / sulphamethoxazole, linezolid and oxacillin and low resistance (0%) to tetracycline and chloramphenicol (Figure 8). While studies showed that this bacterium has high resistance to rifampicin and oxacillin (Escolar *et al*., 2017; Kuan *et al.,* 2017), results regarding chloramphenicol vary with other studies. Surprisingly, resistance to ciprofloxacin (87.5%), streptomycin (87.5%) and penicillin G (75%) was found to be high, whereas studies show sensitivity towards these antibiotics (Morvan *et al.,* 2010; Kuan *et al.,* 2017).

This study shows that *L. monocytogenes* is resistant to amoxicillin (87.5%), vancomycin (87.5%) and co-methoxazole (100%), while a study done in 2014 (Hasegawa *et al.,* 2014) shows that the bacteria are susceptible to them. This may be due to the fact that resistance to these antibiotics was developed in recent years. Use of antibiotics such as ampicillin, erythromycin and kanamycin has shown 37.5% resistance, indicating that the bacteria are somewhat susceptible to them. In contrast, certain studies (Kuan *et al.,* 2017) have shown that *L. monocytogenes* is 0% resistant to ampicillin and 98.3% intermediately resistant to erythromycin. Results regarding tetracycline seem to differ; in the current study, there is 0% resistance to tetracycline, while other research (Yang *et al.*, 2008) has reported 4.07% resistance to the same.

CONCLUSION

This investigation revealed the characteristics of *L. monocytogenes obtained* from various sources such as pork, chicken, chevon, milk and water. Isolation was made efficient using specific medium and the pure colonies thus obtained were subjected to discrete biochemical tests including indole test, catalase test, Voges-Proskauer test, CAMP assay and oxidase test. These tests indicated that the isolated bacteria were *L. monocytogenes* and positive results were obtained for all except the oxidase test. The structure of the bacteria was visualized under fluorescent microscope using Propidium Iodide. Serotyping revealed the prevalence of serogroups 1/2a,1/2b, 1/2c and 4b among the 8 isolates. Presence of virulence-associated genes like *hly*A and *iap*A was rapidly detected using the qPCR technique as it provides better amplification. RAPD analysis produced distinct fingerprint patterns for different isolates and thus turned out to be a reproducible, rapid and powerful typing technique for this bacterium. Antibiotic susceptibility test highlighted the drug resistance *L. monocytogenes* as it plays an important role in the treatment and transfer of this emerging zoonotic food-borne infection. Hence, this work proved to be a potential method to rapidly detect and investigate the outbreak of listeriosis, thus ensuring decreased disease burden on the society.

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