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RESEARCH ARTICLE

Phenotypic and genotypic characterization of antibiotic resistance of *Listeria monocytogenes* isolated from raw milk samples collected from Polonnaruwa, Sri Lanka

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ABSTRACT

Listeria monocytogenes is a food-borne pathogen that can cause severe invasive infection called 'listerosis' in humans. Development of antibiotic resistance is a major setback in the management of conditions caused by *Listeria* in both human and veterinary medicine. In this study, antibiotic resistance of fifty *L. monocytogenes* strains isolated from raw milk samples collected from farms in Polonnaruwa district, Sri Lanka was determined for four commonly used antibiotics; penicillin, ampicillin, streptomycin and tetracycline. The strains were also tested for the presence of selected antibiotic resistant genes (*pen*A, *amp*C, *str*A, *str*B, *tet*A and *tet*B). *L monocytogenes* isolates showed resistance to ampicillin (60%), penicillin (40%) streptomycin (16%) and tetracycline (8%) in diffusion assays. Phenotypic multidrug resistance was exhibited by twenty isolates. The tetracycline resistant gene (*tet*A) was detected in seven isolates, while *tet*B was not detected in any. Presence of streptomycin resistant genes (*str*A or *str*B) was confirmed in seven isolates. Ampicillin (*amp*C) and penicillin (*pen*A) resistant genes were not detected in any of the tested isolates. Except from the samples collected from Sungavila area, isolates from other sampling areas showed resistance to at least one of the antibiotics tested, suggesting that raw milk samples are prone to be contaminated with *L. monocytogenes* strains with different antibiotic resistant profiles. Therefore, necessary hygienic precautions are recommended to avoid any potential public health threats and to safeguard the health of raw milk consumers.

Keywords: Antibiotics; Genotype; *Listeria monocytogenes*; Phenotype; Raw milk

INTRODUCTION

Antibiotic resistance is one of the major obstacles in infectious disease management and patient care. Indiscriminate and excessive use of antibiotics have greatly contributed in developing antibiotic resistance in humans and animals (Imperial and Ibana, 2016; Agyare et al., 2018). Emergence of antibiotic resistance of *L. monocytogenes* isolates from different dairy products, were stated in several studies (Yakubu et al., 2012; Olaimat et al., 2018). Except the emergence of drug-resistant pathogenic bacteria, antibiotic resistance can also have a selective pressure on normal commensal microbiota. The rapid dissemination of antibiotic resistance from commensal microorganisms to the pathogenic microbiota can mainly occur through horizontal gene transfer (Aminov et al., 2001).

Listeria monocytogenes is a foodborne pathogen, which causes a serious foodborne illness called 'listeriosis' in both animals and humans. Invasive listeriosis can lead to serious clinical manifestations such as meningitis or septicaemia in immune compromised hosts including infants, organ transplant recipients, cancer and AIDS patients and in pregnant women with the fatality rates of 20 – 30% (Vázquez-Boland et al., 2001; Hilliard et al., 2018; Şanlıbaba and Tezel, 2018).

Further, listeriosis can cause abortions and central nervous system diseases in economically important animals such as cattle and sheep in livestock industry (Borucki et al., 2005). The abortions and other serious health problems can lead to reduction of milk production and eventual death of these animals. The skin and intestinal tract in livestock animals are habitats for many foodborne pathogens and opportunistic bacteria that can contaminate meat and milk products during slaughter and milking. Additionally, these bacterial pathogens can enter into various other natural environments and can contaminate fresh harvest through

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fertilizers made using animal excrements (Srinivasan et al., 2005). *Listeria* spp. can be a potential contaminant of milk and milk products during udder infections of buffaloes and cows (Osman et al., 2016).

Although *L. monocytogenes* is generally susceptible to various antibiotics targeting Gram-positive bacteria, evidence suggests that the use of antimicrobials in animal production may lead to the development of antimicrobial resistance (Manyi-Loh et al., 2018; Conter et al., 2009).

Polonnaruwa district, the main area of this study, is well known for the farming and dairy industry in Sri Lanka. The overuse, as well as the improper use of antibiotics in cattle farming within the study area could act as a driving force in the development of antibiotic resistance among *Listeria* spp. These resistant strains may harbor genes for antibiotic resistance and these genes can be transmitted to microbiota in human through dairy products such as raw milk (Santorum et al., 2012). Ultimately, the antibiotics may not be successful in the treatments for the diseases caused by not only *Listeria*, but also other problematic human pathogens as well. Recently, many investigators have found that commensal bacteria may also act as reservoirs of antibiotic resistance genes comparable to those found in human pathogens (Hilliard et al., 2018).

Phenotyping and genotyping methods can contribute in providing valuable inputs on strains, which may be responsible for an outbreak or to understand the relationship between isolates implicated in an outbreak, and also may help in determining a source of transmission during an outbreak (Mazen et al., 2018). In this study, we have used both phenotyping and genotyping methods to determine the antibiotic resistance patterns of *Listeria* isolates against four widely used antibiotics (penicillin G, ampicillin, tetracycline, streptomycin) in cattle farming in Polonnaruwa district, Sri Lanka.

MATERIALS AND METHODS

Bacterial strains used

Listeria monocytogenes (ATCC 51776) and *L. innocua* (ATCC 33090) were used as the positive and negative control strains respectively, for the determination of cultural characteristics of the isolates, as well as for PCR assays for the detection of *L. monocytogenes*. A *Salmonella typhi* strain, obtained from the Bacteriology Laboratory, Medical Research Institute, Colombo 08, was used as the positive control for the detection of genotypic resistance of isolates. This strain was previously confirmed for its' multidrug resistance for penicillin G, ampicillin, tetracycline, and streptomycin. The multidrug resistance of this *Salmonella typhi* strain was re-confirmed using both phenotypic and genotypic characterization.

Fifty isolates of *L. monocytogenes* from the previous study conducted by Ramesh et al, 2019 were used to determine the antibiotic resistant profiles in this study. These *Listeria* strains were isolated from raw milk samples collected from Madirigiriya, Sangabohigama, Aluthwewa, Pansalgodella, Kaduruwela, Bakamoona, Sungavila, Gallella and Jayanthipura milk collecting centers located in Polonnaruwa district, Sri Lanka. Isolates were confirmed using their typical colony characteristics on *Listeria* Selective Agar (OXOID) prior to their molecular confirmation.

Identification of *L. monocytogenes*

DNA extraction

Total genomic DNA extraction of bacterial isolates was carried out using QIAGEN bacterial DNA extraction kit (QIAamp DNA Mini Kit) according to the manufacturer's instructions. The purity and the integrity of the extracted DNA samples were determined by spectrophotometer (Eppendorf Bio Spectrometer® basic).

Molecular identification of L. monocytogenes

PCR primers targeting the 'Listeriolysin O' gene were used for the confirmation of *L. monocytogenes* (Herman et al.,1995). To obtain very sensitive detection of *L. monocytogenes*, two-step nested PCR amplification was used in this procedure. In the first PCR of the Nested-PCR assay, the primers LM1 (F) and LM2 (R) were used and, LL5 (F) and LL6(R) were used as nested primers for the second step (Table 1). The first PCR reaction was done under the following thermal cycling conditions using the Bio-Rad PCR System (MYCyclerTM thermal Cycler): 95 °C for 2 min, 96.5 °C for 30 s, 57 °C for 30 s and 72 °C for 60 s for a total of 30 cycles and final extension was carried out at 72 °C for 5 min.

PCR was performed in a final volume of 50 µl containing 10 µl of 5×PCR buffer, 5 µl of $MgCl_2$ (25 mM), 1 µl of nucleoside triphosphate (dNTP 10 mM) mix, 0.25 µl of 5 U/µl of Taq polymerase (GoTaq Flexi DNA polymerase; Promega Corp.), and 50 pmol of each LM1 (F) and LM2 (R) primers. For the first amplification, 10 µl of the sample DNA was added. For the second PCR reaction, 10 µl of the diluted product from the first amplification (10^{-1}) was used as the template with other reactants as described above. The procedure was carried out for the DNA extracted from both enriched milk samples and cultures. The thermal cycle of the second PCR reaction included; 95 °C for 2 min, 95°C for 30 s, 55 °C for 30 s, 72 °C for 60 s for a 35 cycles and final extension was carried out at 72 °C for 5 min. Ten microliters of the final product were electrophoresed on a standard agarose (2%) gel (Cambrex

Table 1: Primers used for the amplification of 'Listeriolysin-O' gene for the confirmation of the *Listeria* spp.

Primer name	Primer sequence (5'-3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
LM1	CCTAAGACGCCAATCGAA	57	701	Border et al., 1990
LM2	AAGCGCTTGCAACTGCTC			
LL5 (nested)	AACCTATCCAGGTGCTC	55	267	Thomas et al., 1991
LL6 (nested)	CTGTAAGCCATTTCGTC			

Bio Science, Rockland, ME) with Trisborate-EDTA buffer system. Amplicons were visualized under UV light after staining with ethidium bromide for 5-10 min (0.5 μg/ml in the running buffer). The expected size of the final PCR product was 267 bp.

Determination of antimicrobial susceptibility of L. monocytogenes

Fifty purified bacterial isolates were tested by the standard disk diffusion or well diffusion method to determine their susceptibility for four different antibiotics [penicillins – ampicillin (10μg) and penicillin-G (10IU); tetracyclines – tetracycline (30μg); aminoglycosides – streptomycin (10 μg)]. Clinical and Laboratory Standards Institute (CLSI) guidelines – 2017 were followed in this methodology. Due to the unavailability of breakpoints for *Listeria* spp. in CLSI guidelines, break points for *Staphylococcus* spp, were used in this study (Chen et al., 2018; Faridi et al., 2021).

A swab was taken from each bacterial suspension (~1×10^7 CFU/ml) and stroked trolley on Mueller-Hinton agar (Merck, Germany), and then the antibiotic disks (PadtanTeb, Iran) to be tested were placed on the agar plates containing the organism (Maktabi et al., 2015). Where standard antibiotic disks were not available, the test was carried out using a modified method of standard well diffusion assay (Holder and Boyce, 1994). The diameter of inhibition zone was measured after the incubation at 35 °C for 24 h, and the results were interpreted as resistant, intermediate or sensitive based on the presence or the absence of clear zones. The test was triplicated in order to minimize errors. Multidrug resistance was reported as the bacterial resistance to more than one antibiotic.

Statistical analysis was performed using SPSS.13 statistical software and Microsoft Excel 2016. Pearson Chi-Square test was used to determine significance of data (α level=0.05). The percentage results of diffusion assays were compared using ANOVA test to determine the statistical significance.

Amplification of antimicrobial resistant genes

PCR detection of genes involved in conferring resistance to selected antibiotics was performed using a method described in previous study (Srinivasan et al., 2005). Oligonucleotide sequences and predicted sizes of the resulting amplicons are listed in Table 2. Presence of antimicrobial resistant genes in *L. monocytogenes* encoding for a) penicillin binding protein gene (*pen*A); b) beta lactamase–ampicillin resistant gene (*amp*C); c) tetracycline efflux pump (*tet*A and *tet*B) and d) streptomycin phosphotransferases (*str*A and *str*B) was determined. Primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Amplification of target gene fragments for all bacteria was performed using a DNA thermal cycler (MYCyclerTM -BioRad) and the Taq-polymerase kit (Promega). The PCR was performed in a final volume of 50 μl containing 30 μl of sterile water, 5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl), 2 μl of 15 mM $MgCl_2$, 2 μl of deoxyribonucleoside triphosphates (2.5 mM each dATP, dTTP, dGTP and dCTP), 1.0 μl of each appropriate primer, 1–10 μl of template, and 0.5 μl (5U/μl) of Taq-DNA polymerase. Initial denaturation was done at 94°C for 4 min. Thirty PCR cycles were performed under following conditions: denaturation at 94 °C for 45 s, primer annealing at optimum temperature for 45 s, and primer extension at 72 °C for 45 s in each cycle. The final extension was done at 72 °C for 7 min and then the reaction condition was maintained at 4 °C until further analysis. The annealing temperatures were optimized with all primer sets. Genomic DNA from multi-drug resistant *S. typhi* was used as the positive control in all PCR reactions. In the reagent control, sterile molecular-grade water was used instead of template DNA in the reaction mixture. Ten microliters from each PCR product were analyzed using standard agarose (2%) gel electrophoresis (Cambrex Bio Science) with Trisborate-EDTA buffer system. Resulting gels were visualized under UV light after staining with ethidium bromide for 5-10 min (0.5 μg/ml in the running buffer).

RESULTS

Phenotypic characterization and molecular confirmation of *L. monocytogenes* isolates

Eighty *L. monocytogenes* isolated from field-collected raw milk samples, which were previously inoculated into Mueller Hinton Broth (OXOID) and stored at-20°C, were sub cultured into *Listeria* Selective Agar (OXOID) plates by streak plate method and incubated at 36±1 °C for 24 hours. Eighty isolates from this were cultured on *Listeria* Selective medium. Out of these eighty, total of sixty-two isolates showed characteristic intense black-colored colony

appearance after the incubation at 36±1 °C for 24 hours and after the enrichment.

Sixty-two culture positive isolates were confirmed by PCR amplification targeting the region of 'listeriolysin O' gene. The expected PCR amplicon (~267 bp) was observed in all (n=62) isolates after analyzing on a 2% agarose gel (Fig. 1).

Fifty bacterial isolates, which were positively identified as *L. monocytogenes* using both culture and PCR methods, were selected for further analysis.

Determination of antibiotic resistance

Antibiotic resistant profiles were obtained for fifty *Listeria monocytogenes* isolates for the selected antibiotics. Different antibiotic resistant patterns were recorded for different isolates based on the results obtained for penicillin, ampicillin and tetracycline from standard disk diffusion assays, as well as for streptomycin from well diffusion assay (Fig. 2).

The highest percentile resistance was observed for ampicillin (60%), whereas lowest resistance was observed against tetracycline (8%) among the tested isolates. Intermediate resistance was observed only for streptomycin (18%) and tetracycline (6%). According to the interpretative criteria in CLSI standards, intermediate resistance range was not stated for penicillin and ampicillin. The highest percentile susceptibility was obtained for tetracycline (86%) among the tested *L. monocytogenes* strains, while the lowest susceptibility was observed for ampicillin (40%). Majority of isolates (68%) were resistant to the antibiotics tested, some showed intermediate resistance and susceptibility for the tested concentrations (Table 3). According to the statistical analysis, there was a significant difference in the percentage antibiotic resistance in each antibiotic tested ($p<0.05$, αlevel $=0.05$).

According to the results of phenotypic characterization of this study, out of the thirty-four resistant isolates, fourteen (41.2%) isolates were resistant for only one antibiotic (Pen-G-4, Amp-9, Str-1 and Tet-0). And also, another fourteen isolates were

Table 2: Annealing temperature and melting temperatures of primers of antibiotic resistant genes

Gene	Primer name	Primer sequence (5'-3')	Melting T(°C)	Annealing T(°C)	Amplicon size (bp)
*pen*A	PenA	F-ATCGAACAGGCGACGATGTC	57.4	47.6	500
		R- GATTAAGACGGTGTTTTACGG	51.4		
*amp*C	AmpC	F -TTCTATCAAMACTGGCARCC	49.2	50.0	550
		R-CCYTTTTATGTACCCAYGA	51.4		
*str*A	StrA	F- CTTGGTGATAACGGCAATTC	51.8	46.9	548
		R- CCAATCGCAGATAGAAGGC	53.3		
*str*B	StrB	F- ATCGTCAAGGGATTGAAACC	52.7	46.9	509
		R- GGATCGTAGAACATATTGGC	50.6		
tet A	TetA	F –GGCCTCAATTTCCTGACG	53.6	50.4	372
		R-AAGCAGGATGTAGCCTGTGC	57.7		
tet B	TetB	F –GAGACGCAATCGAATTCGG	53.9	49.8	228
		R-TTTAGTGGCTATTCTTCCTGCC	54.8		

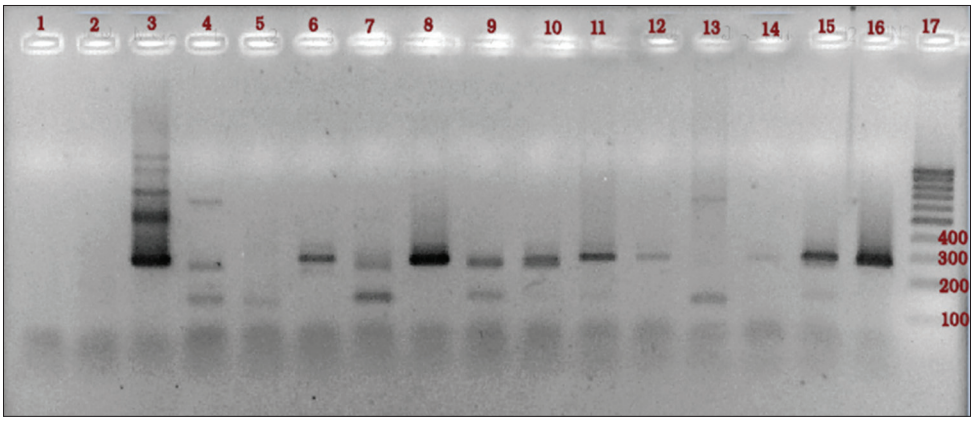

Fig 1. PCR amplification of 'Listeriolysin-O' gene for the confirmation of *L. monocytogenes*. Lane 1 contains the negative PCR reaction with MiliQ water. Lane 2 and 3 contain products of the PCR with genomic DNA of *L. innocua* ATCC 33090 (negative control) and *L. monocytogenes* ATCC 51776 (positive control) respectively. Lane 17 contains 100 bp marker. Amplified fragments of the 'Listeriolysin O' of isolates '17' (Lane 4), '22' (Lane 6), '27' (Lane 7), '29' (Lane 8), '33' (Lane 9), '47B3' (Lane 10), '47B4' (Lane 11), '53B15' (Lane 12), '53B9' (Lane 14), '57B13' (Lane 15), '59B15' (Lane 16) were approximately 267 bp in size. The expected band was not observed in isolates '18' (Lane 5) and '63B16' (Lane 13). The PCR products were electrophoresed in 2% agarose gel at 100V for 30 mins.

Table 3: Percentage resistance/susceptibility of tested *Listeria monocytogenes* isolates

Antibiotic	Percentage resistance/ susceptibility			CLSI Breakpoints (for *Staphylococcus* spp.) (Zone diameter in mm)		
	R (%)	I (%)	S (%)	R	I	S
Penicillin (10IU)	40	-	60	≤28	-	≥29
Ampicillin (10µg)	60	-	40	≤28	-	≥29
Streptomycin (10µg)	16	18	66	≤11	12-14	≥15
Tetracycline (30µg)	8	6	86	≤14	15-18	≥19

R-Resistance
I-Intermediate
S-Susceptibility

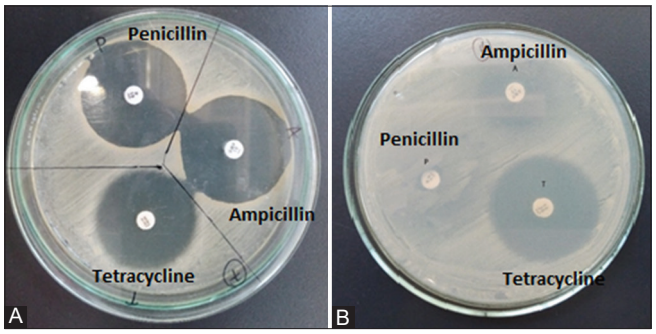

Fig 2. *L. monocytogenes* on Mueller Hinton agar with antibiotic discs. Penicillin-G (10 units), ampicillin (10µg/µl) and tetracycline (30µg/µl) discs were placed on bacterial lawns and incubated at 36°C for 24 hours. (A) After incubation, inhibition zones were observed around all three antibiotic discs for isolate 63B-19. (B) A clear zone was observed only around tetracycline disc for isolate M-48 and there were no identifiable clear zones observed around ampicillin and penicillin discs.

resistant to at least two antibiotics (Pen, Amp - 10; Amp, Tet - 03; Amp, Str - 01) tested, while other six (Pen,Amp,Str- 05; Pen, Amp,Tet- 01) isolates showed resistance to at least three antibiotics. However, none of the isolates exhibited resistance for all the four antibiotics tested. These findings reveal the presence of multidrug resistant isolates in these areas.

According to the phenotypic characterization of the antibiotic resistance of the *L. monocytogenes* strains isolated from raw milk samples collected from nine areas in this study, isolates from Madirigiriya area were resistant to all the antibiotics tested (Fig. 3). However, there was no resistance observed in isolates from Sungavila area for the antibiotics tested. Resistance to penicillin was observed in all the areas except Sungavila, while ampicillin resistance isolates were observed in Madirigiriya (30%) Pansalgodalla (50%), Bakamoona (16.7%) and Gallella (16.7%) areas. Streptomycin resistance was found in many areas such as Madirigiriya (10%), Aluthwewa (33.3%), Bakamoona (33.3%), Gallella (16.6%) and Jayanthipura (20%). However, tetracycline resistance was found only in isolates from Madirigiriya (20%) and Pansalgodella (50%) areas. Intermediate resistance in *L. monocytogenes* was widely found for streptomycin than that of tetracycline.

Molecular detection of the antibiotic resistant genes in *L. monocytogenes* isolates

The theoretical annealing temperatures of primers were obtained by using melting temperatures (T_m) provided by the primer product description. Annealing temperatures were optimized by performing a temperature gradient PCR, starting at 5°C below the lowest T_m of the primer pair. Resulted annealing temperatures were shown in the Table 2.

*tet*A and *tet*B

The *tet*A and *tet*B genes were detected using TetA and TetB primers. Multiplex PCR was performed for this detection as these two primers sets had very close annealing temperatures of 50.4°C and 48.9°C respectively. Totally, fifty strains were tested for the presence of *tet*A and *tet*B genes and seven isolates gave the expected band size for *tet*A after the PCR products were electrophoresed in 2% agarose gel at 100 v for 30 mins.

*str*A and *str*B

Detection of *str*A and *str*B genes was done by using StrA and StrB primers. Multiplex PCR was performed as the two primers pairs had the same annealing temperature. Totally, fifty isolates were tested for the presence of *str*A and *str*B genes, however seven isolates gave the expected band for *str*A or *str*B genes. The expected 548 bp and 509 bp gene products could not be resolved and identified separately due to the lower resolving power of the agarose gel used.

*amp*C and *pen*A

The expected bands of 550 bp and 500 bp for the detection of the presence of *amp*C and *pen*A genes respectively, were not observed with any of the fifty *L. monocytogenes* isolates tested with the annealing temperatures (50.0°C, 47.6°C) used. And also, the expected band for the *pen*A gene was not observed with the positive control.

*str*A/*str*B genes were confirmed in 7 isolates (14%), whereas another 7 isolates (14%) had the targeted band for *tet*A gene amplification. The presence of streptomycin resistance genes and tetracycline resistant genes were considerably higher than that of penicillin and ampicillin in isolates. Thirteen isolates showed genotypic antibiotic resistance for streptomycin or tetracycline, whereas only one showed genotypic multidrug resistance for streptomycin and tetracycline. All the genotypic and phenotypic findings of this study were summarized in Table 4.

DISCUSSION

Since the first report of antibiotic resistance in *L. monocytogenes* (Graves et al., 1994; Poyart-Salmeron

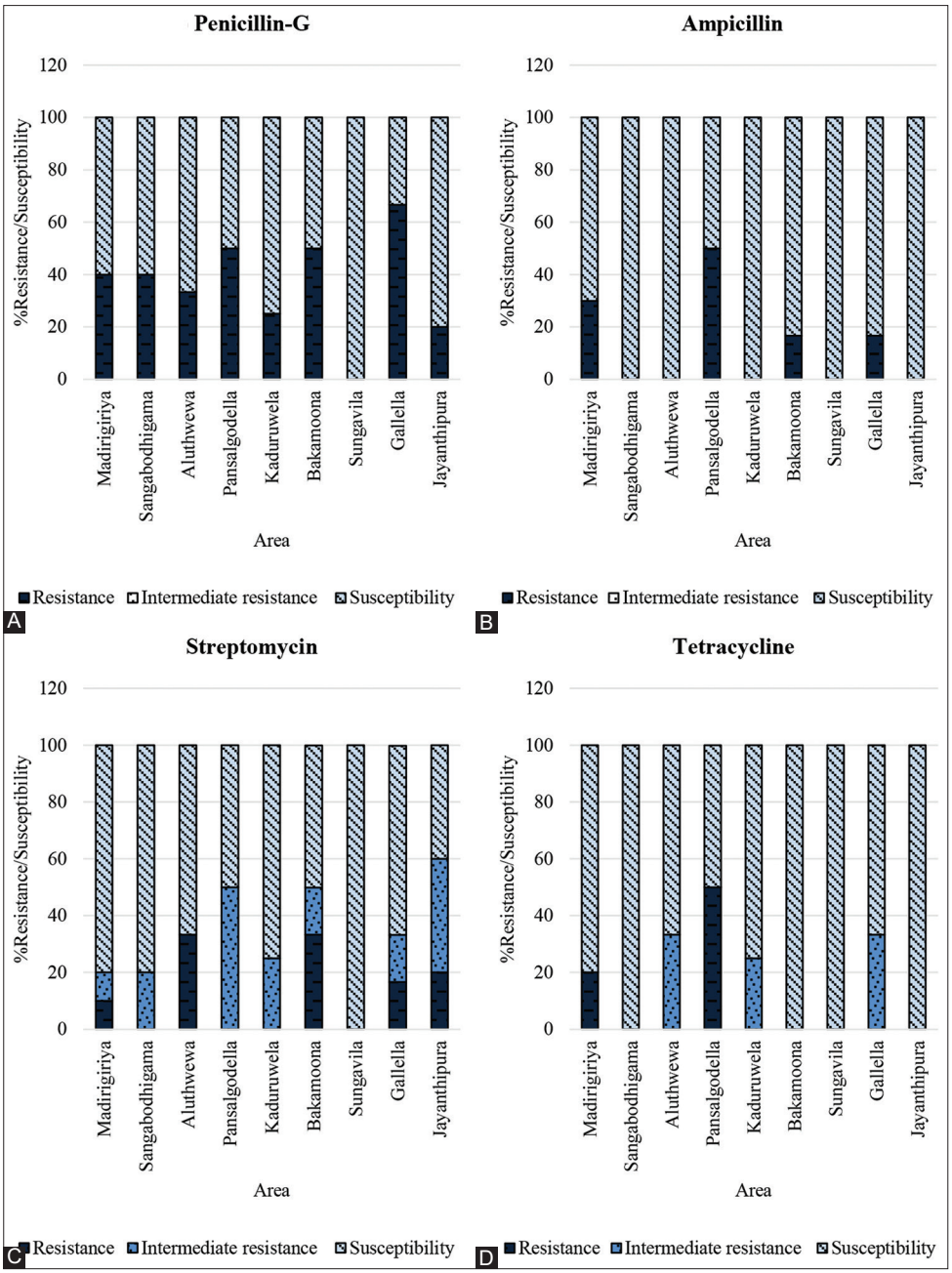

Fig 3. Percentage distribution of antibiotic resistant isolates. Resistance intermediate resistance and susceptibility patterns of *L. monocytogenes* isolates from different areas for the antibiotics of (A) Penicillin-G (B) Ampicillin (C) Streptomycin (D) Tetracycline.

et al., 1990), there has been an increase in the emergence of antimicrobial resistance of *L. monocytogenes* isolated from a variety of sources (Charpentier et al., 1995). The prevalence of antimicrobial resistance in *L. monocytogenes* appears to fluctuate considerably based on data published so far. According to previous studies, antimicrobial susceptibility of *L. monocytogenes* has been assayed using clinical isolates of human, animal and food in most studies (Charpentier and Courvalin, 1999). Although *L. monocytogenes* can be transmitted through various kinds of food sources, contamination of milk and milk products with *L. monocytogenes* is a serious problem to the world, especially in the developing countries like Sri Lanka. But only few reports are available on studies that have been conducted to determine the presence of *L. monocytogenes* in our dairy products in Sri Lanka (Withana and Mirando, 1967; Wijesundera et al., 1992; Jayamanne and Samarajeewa, 2001; Wijendra et al., 2014). According to them, the percentage of *L. monocytogenes* contaminated milk samples in Sri Lanka is much higher, compared to that of developed countries. This is the first study that specifically evaluated the presence of antimicrobial resistance of *L. monocytogenes* isolates from raw milk samples in Polonnaruwa district in Sri Lanka, using both phenotypic and genotypic characteristics.

Table 4: Phenotypic and genotypic characterization of *L. monocytogenes* isolates

Antibiotic	Resistance/ intermediate resistance observed phenotypically[a]	Presence of the targeted gene[b]	Number of isolates
Penicillin	+	+	00
	+	-	20
	-	+	00
	-	-	30
Ampicillin	+	+	00
	+	-	30
	-	+	00
	-	-	20
Streptomycin	+	+	05
	+	-	12
	-	+	02
	-	-	31
Tetracycline	+	+	05
	+	-	01
	-	+	01
	-	-	43

[a]Both phenotypically 'Intermediate resistant' and 'Resistant' isolates were taken into account

[b]The genes coding for the tetracycline efflux pump (*tet*A and *tet*B), streptomycin phosphotransferases (*str*A and *str*B), penicillin binding protein (*pen*A), and beta lactamase–ampicillin resistance (*amp*C) were targeted for tetracycline, streptomycin, penicillin and ampicillin respectively.

Results of this study demonstrated that many of *L. monocytogenes* (68%)strains isolated from nine milk collecting centers of Polonnaruwa district were resistant to at least one of the tested antimicrobials. Furthermore, thirteen *L. monocytogenes* isolates tested in this study carried one or more antimicrobial resistant genes and these isolates may have the potential to function as an antimicrobial resistance gene pool for other commensal and pathogenic bacteria in the dairy farm environment.

In line with previous studies (Srinivasan et al., 2005; Skowron et al., 2019), the present study revealed the presence of phenotypic resistance to ampicillin, penicillin-G, tetracycline and streptomycin in *L. monocytogenes*. In this study 68% of isolates were phenotypically resistant to at least one antibiotic and much higher phenotypic resistance was exhibited by ampicillin (60%) and penicillin (40%). Parallelly, much higher frequencies were observed in oxacillin (93%) followed by penicillin (90%) and ampicillin (60%) in *L. monocytogenes* dairy product isolates in Iran (Harakeh et al., 2009). Also, it was reported that all five bovine raw milk isolates of *L. monocytogenes* were resistant to the majority of antibiotics tested (Sharma et al., 2017). In contrast, previous studies reported high susceptibility in *L. monocytogenes* isolated from cheese to ampicillin and penicillin (Marco et al., 2000; Margolles et al., 2001; Faridi et al., 2021).

In present study, the lowest resistance was observed for tetracycline (8%). Similarly, high tetracycline susceptibility was found in *L. monocytogenes* isolates from pork and slaughterhouses in Brazil (Moreno et al., 2014). Intermediate resistance is also an important factor to be considered because these isolates also have antibiotic resistance genes and the expression of it may less under these experimental conditions. In this study, for the comparison of genotypic resistance and the phenotypic resistance, both resistant and intermediate resistant strains were taken into account as shown in Table 4. However, there was no intermediate range specified in the interpretative criteria for penicillin and ampicillin in CLSI (2017) guidelines.

According to the results obtained from PCR amplifications, positive results for the amplification of the respective targeted antibiotic resistant genes were not observed for some of the phenotypically resistant strains. For example, 8 (16%) *L. monocytogenes* isolates were phenotypically resistant to streptomycin, while only 7 (14%) isolates (only some showed both resistance) produced amplicons of the expected size confirming the presence of *str*A or *str*B genes. Even though, phenotypically *L. monocytogenes* strains were resistant to ampicillin and penicillin respectively, none of these isolates have shown the presence of *amp*C and *pen*A genes. These findings were comparable with previous studies (Srinivasan et al., 2005; Skowron et al., 2019). According to that, some of the *L. monocytogenes* isolates displayed phenotypic resistance to multiple antimicrobials, but did not contain antimicrobial resistant genes as evaluated by PCR.

Only few antibiotic resistant genes were selected in this study due to time and budget limitations, though many genes were responsible for the resistance of tested antibiotics. Tetracycline resistance is mediated by genes such as *tet*A, *tet*B, *tet*C, *tet*D, *tet*S and *tet*M (Srinivasan et al., 2005). But only the presence of *tet*A and *tet*B were tested in the current study. Therefore, the absence of tested genes indicates only that the antibiotic resistance exhibited, as a phenotypic trait of these isolates may not conferred by the tested genes. Also, it is possible that the other genes and genetic determinants may mediate the observed resistance. Thus, the presence of different antimicrobial resistant genes did not always correlate with antimicrobial resistance exhibited phenotypically by foodborne pathogens. Various other mechanisms, such as decreased outer membrane permeability (Farmer and Hancock, 1992), activation of efflux pump (Charvalos et al., 1995) or a mutation in a ribosomal protein gene (Yan and Taylor,1991) are known to have significant contributions in defining such phenotypes. Further studies are needed to conclude on this hypothesis.

In contrast, there were some strains, which were confirmed for the presence of resistant genes. However, these strains did not show the relevant phenotypic resistance. For

example, four (8%) isolates of *L. monocytogenes* tested were phenotypically resistant to tetracycline, while the *tet*A gene was found in seven (14%) isolates. That may be because of the presence of the antibiotic resistant genes is not the only factor responsible for phenotypic antimicrobial resistance. Also, there should be suitable environmental conditions such as limitation of nutrients, change in temperature, presence of antibiotics to express these antimicrobial genes.

In the present study twenty *L. monocytogenes* isolates, displayed phenotypic resistance to multiple antibiotics. However, none of the isolates showed resistance to all four antibiotics tested. Other studies have reported multidrug resistance in *L. monocytogenes* isolated from food and animals to chloramphenicol, erythromycin, gentamicin, kanamycin, streptomycin and rifampin (Charpentier et al., 1995; Walsh et al., 2001). According to Srinivasan et al., (2005), fifty percent of *L. monocytogenes* strains isolated from the dairy farm environment were multidrug resistant and carried different combinations of antimicrobial resistant genes, while our findings has indicated that the observed multidrug resistance percentages were around 40% and 2% for the tested isolates based on the phenotypic and genotypic characterizations respectively. Finding of these multiple resistant strains will be useful in clinical disease management.

Genetic methods may confirm the presence of specific genes conferring antimicrobial resistance, however, the presence of antimicrobial resistance genes alone does not necessarily imply that bacteria are resistant, as it is possible that resistance genes may not be expressed (Michalova et al., 2004). In clinical setting actually phenotypical resistance is more useful than genotypic resistance. Because the clinical treatments are based on the expressed resistance of the strains.

The fifty isolates studied in this work have been characterized and confirmed to *L. monocytogenes* by the PCR amplification of 'listeriolysin O' gene. Therefore, it can be confirmed that some cattle in the farms, where these raw milk samples were collected, were already infected with *L. monocytogenes*. Majority of these isolates exhibited phenotypic resistance against one or more antibiotics tested, suggesting that they may possess a possible resistance mechanism against tested antibiotics. Therefore, there is a risk of spreading this antibiotic resistance within the microbial populations present in the particular environment, including among the other commensal and pathogenic microorganisms. Furthermore, unpasteurized or poorly pasteurized milk can act as a medium in transferring these resistance microorganisms from cattle to human. Moreover, the critical issue of spreading of antibiotic resistome is that it may contribute to the failure of available antibiotics in treating serious illnesses.

In this study, the resistant isolates for all the antibiotics tested were found in Madirigiriya area. However, None of the *Listeria* strains isolated from Sungavila, exhibited resistant characteristics against any of the antibiotic tested. Isolates form all the other areas, were found to have phenotypic resistance for one or more antibiotics tested. Exposure to different antimicrobials in different geographical regions during different time periods may be a reason behind the wide variation of antimicrobial resistance patterns among *L. monocytogenes* isolated from dairy farms (Srinivasan et al., 2005). However, statistical analysis revealed that the antibiotic resistance results were not depend on the collected areas in this study. To make conclusions, there should be extended researches with larger samples and more data related to the usage of antibiotics in these areas.

In the dairy industry, antimicrobial resistance of *L. monocytogenes* could be a huge problem in future. Pregnant women should pay special attention to avoid contact with potential sources of *Listeria* (Osman et al., 2016). Not only that, but also farmers who are immunocompromised, should take the same precautions. According to our study, raw milk samples were contaminated with *L. monocytogenes*. Therefore, there may be a potential risk of transfer of *L. monocytogenes* in cows to humans by raw milk or poorly pasteurized milk. The maintenance of good hygienic practices and pasteurization of milk before consumption is a must to reduce the risks of human infection with *L. monocytogenes*.

CONCLUSION

In conclusion, this study has revealed the presence of antibiotic resistant strains of *L. monocytogenes* isolated from raw milk samples collected from dairy farms in Polonnaruwa District, Sri Lanka. Several isolates exhibited phenotypic resistance against penicillin-G, ampicillin, streptomycin and tetracycline in the disk diffusion assay. Majority of the tested antibiotic resistance observed as a phenotypic trait in the isolates could not be confirmed through genotypic characterization. Therefore, based on the results of this study, no relationship could be found in the antibiotic resistance traits of *L. monocytogenes* isolates through phenotypic and genotypic characterization. Further studies with other possible genes conferring the resistance to the tested antibiotics are needed to understand the genetic determinants of the antibiotics resistant traits expressed phenotypically. Based on the results of this study, it can be suggested that the raw milk samples are potentially prone to be contaminated by *L. monocytogenes* strains with different antibiotic resistant profiles. These strains may contribute in expanding the global microbial

resistome causing a significant threat to public health. Therefore, the need to improve the safety of milk and dairy products is crucial to avoid health risks, as well as the emergence of antibiotic resistance. During the entire process, from the collection and processing of milk and dairy products, to their final consumption, it is vital to take necessary hygienic precautions to safeguard the health and safety of the consumers.

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Author contribution

H. B. C. Harshani: Conceptualization (Supporting); Investigation (lead); Methodology (lead); Writing-original draft preparation (lead); Formal analysis and data curation (lead). R. Ramesh: Conceptualization (supporting); Resources (equal); Writing – review and editing (equal); Project supervision (equal). A. P. Halmillawewa: Project supervision (equal); Data curation (equal); Writing – review and editing (lead); Resources (equal); W. A. S. Wijendra: Conceptualization (lead); Project supervision (equal); Methodology (supporting); Resources (equal); Review and editing (equal).

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