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Detection of Phenotypic and Genotypic Antibiotic Resistance among *Listeria monocytogenes* Isolated from Different Food Samples in Yola

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Abstract

Listeria monocytogenes is an opportunistic bacterial pathogen implicated in several lethal illness outbreaks. Future outbreaks may be more complicated to manage because of the surfacing of antibiotic resistance among L. monocytogenes strains in food products. Accordingly, this study aimed to determine the phenotypic and genotypic antibiotic resistance of L. monocytogenes previously isolated from different food samples in Yola. Antibiotic resistance of L. monocytogenes was determined via disc diffusion and polymerase chain reaction (PCR). Exactly 72.72% of the isolates were multi-drug resistant (MDR) with high resistance to Beta-Lactams and sulfonamides. Percentage resistance to ceftriaxone, ampicillin, augmentin, cefuroxime, and sulfamethoxazole/trimethoprim were 100%, 72.7%, 64.0%, 64.0%, and 54.5% respectively. The MDR isolates were subjected to PCR, and antibiotic resistance genes bla_{CTX-M}, *7(100%),* bla_{TEM}, *7(100%), and sul1 7(100%) were detected in (72.72%) i.e. all the MDR isolates. L. monocytogenes isolates from food samples in this study area exhibited phenotypic and genotypic resistance to multiple antibiotics. This implies that future outbreaks of L. monocytogenes in the study area may be complicated to manage using the commonly used antibiotics tested in this study, representing a major public health concern.*

Keywords: Antibiotic, Food-borne, Listeria, Phenotypic, Resistant-genes.

INTRODUCTION

Listeria monocytogenes is an important, ubiquitous, food-borne microbe that can contaminate food products during or after processing. It poses a significant risk to the food industry, particularly producers of ready-to-eat (RTE) foods, due to its ability to proliferate over a vast range of adverse environmental conditions encompassing low temperature, low pH, and high salt. *L. monocytogenes* represents a major public health concern because it may cause severe human illness with serious consequences. Septicemia, meningitis, meningoencephalitis in immunocompromised individuals, invasive infections in the newborn and elderly, and serious complications during pregnancy (abortion and stillbirth), with a fatality rate that can reach up to 20% to 30% (Scallan *et al.*, 2011; Swaminathan & Gerner-Smidt, 2007). Therefore, antibiotic treatment is usually needed to control the infection caused by this bacterium.

L. monocytogenes, in general, is considered vulnerable to a wide range of antibiotics, which have bactericidal effects against Gram-positive bacteria, including tetracyclines, erythromycin, ampicillin, and gentamicin (Teuber, 1999). However, most strains of *L. monocytogenes* display native resistance to cefotaxime, cefepime, fosfomycin, oxacillin, and licosamides (Lecuit & Leclercq, 2009).

The prevalence of antimicrobial drug resistance among foodborne pathogens has increased due to its use in human therapy and animal farming for therapeutic and prophylactic purposes. Consequently, multidrug resistance among these foodborne pathogens, including *L. monocytogenes*, has been observed (Wong *et al.*, 2012). Increasing the prevalence of multi-drug resistant *L. monocytogenes*, particularly resistance to ampicillin, penicillins, aminoglycosides, and sulphonamides, is an emerging problem worldwide (Morobe *et al.*, 2009). Recently, antibiotic resistance among *L. monocytogenes* isolated from foods and the environment has increased, particularly for those antibiotics commonly used to treat listeriosis. Monitoring changes in the antibiotic resistance of *L. monocytogenes* due to the continuing emergence of resistant strains is needed. Therefore, this study aimed to determine the phenotypic and genotypic antibiotic resistance of *L. monocytogenes* previously isolated from different food samples in Yola.

MATERIALS AND METHODS

Source of Organisms

Listeria monocytogenes were previously isolated from food samples (i.e., cabbage, fresh fish, raw meat, yogurt, and frozen chicken) obtained in Yola, the Capital of Adamawa state. The isolation and morphological/biochemical identification of the organism was done in the Microbiology Department laboratory Modibbo Adama University Yola, while the molecular identification was done in Chevron forensic laboratory also of Modibbo Adama University Yola.

Antibiotic Susceptibility Test

The antibiotic susceptibility of the *Listeria monocytogenes* isolates was determined by the disc diffusion method on Mueller Hinton agar, as described by Osman *et al.* (2016). The antibiotic discs used were ampicillin (10µg), amoxicillin clavulanic acid (augmentin) (30µg), cefuroxime (20µg), sulfamethoxazole-trimethoprim (25µg), gentamycin (10µg), erythromycin (15µg), ciprofloxacin (5µg) and ceftriaxone (30µg).

Exactly 0.1ml of the standard inoculum of *L. monocytogenes* was transferred onto the surface of the Mueller Hinton agar plate and spread onto the entire media surface with a sterile glass spreader. The inoculated plates were allowed to dry for about 15 minutes, and the antibiotic discs were placed on the agar plates using sterile forceps, making sure they made immediate and complete contact with the agar surface and incubated at 37^0C for24 hours as described by Oyelami *et al.*, (2018). The diameter of the zone of clearance (including the diameter of the disc) was measured with a ruler to the nearest millimeter. Zones of inhibition were recorded and interpreted as susceptible and resistant based on the interpretive guidelines of the Clinical Laboratory Standard Institute (CLSI 2021) Harshani *et al.*, (2022).

DNA extraction

Freshly grown *L. monocytogenes* colonies collected from 24-hour nutrient agar culture plate surfaces were used for DNA extraction (Maria *et al.*, 2018). Qiagen QIAamp DNA mini kit was used and the DNA extraction procedure was according to the manufacturer's instructions.

DNA Quantification

Nanodrop One (Thermo Scientific) was used to determine the quantity of the extracted *L. monocytogenes* DNA in ng/µL. Its purity was also determined by its absorbance reading at the A260/A280nm wavelength.

PCR Amplification and Detection of Antibiotic Resistance Genes

Antibiotic resistance genes, which comprised Temoneira (bla_{TEM}), Cefotaximase-Munich (CTX-M) (bla_{CTX-M}), and Sulphadryl 1 variable (*sulI*), were amplified in isolates that showed phenotypic resistance Beta Lactams and sulfonamides antibiotic. The primers used were bla_{TEM} F: 5'-ATT TCC GTG TCG CCC TTA TTC-3': R: 5'CGT TCA TCC ATA GTT GCC TGA C-3' to amplify 800bp, bla_{CTX-M} F: 5'-AAC RCR CAG ACG CTC TAC-3' R: 5'-TCG AGC CGG AAS GTG TYA T-3' to amplify 650bp and *sul I* F: 5'-TTC GGC ATT CTG AAT CTC AC-3' R: 5'-ATG ATC TAA CCC TCG GTC TC-3' to amplify 822bp as previously reported by Mpondo *et al.*, (2021) and Ntshanka *et al.*, (2022). Inqaba Biotech synthesized the primers. The PCR reaction mixture (25 µL) included 10 µL of 50 ng DNA, 2.5 µL of 10X PCR buffer, 1µL MgCl2, 1-µL of dNTPs, 1 µL of each primer (50 pmol/mL), and 0.5 µL of Taq DNA polymerase (Thermo Fisher Scientific). PCR amplification started with an initial denaturation step at 94 ˚C for 5 min, followed by 35 cycles (denaturation at 95 ˚C for 50 sec, annealing at primer-specific temperatures of (60 ºC, 55.5 ºC, and 60.5 ºC, respectively) for 30 sec, and extension at 72 ˚C for 30 sec) and a final extension for 10 min at 72 ˚C. All amplicons were electrophoresed in agarose gel (1.5% agarose in 0.5 X TBE buffer), stained with ethidium bromide, and visualized under a UV light transilluminator as reported by Mpondo *et al.* (2021). The sizes of the PCR products were compared with a standard 100bp DNA marker. A reaction mixture with no DNA template was incorporated as a negative control.

RESULTS

Antibiotic Susceptibility Pattern of *L. monocytogenes* Isolates

Results of the antibiotic susceptible test showed that all the isolates were resistant to ceftriaxone (100%), followed by ampicillin (72.7%), augmentin (64.5), cefuroxime (64.5%), and sulfamethoxazole/trimethoprim (54.5), and however, showed 100% susceptibility to ciprofloxacin. The *L. monocytogenes* also showed high susceptibility to erythromycin (90.9%) and gentamycin (82.7%). Exactly 2.5% of the isolates (Lm2C and Lm11Y) were resistant to up to 75% of the antibiotics tested with a Multiple Antibiotic Resistance Index (MARI) of 7.5, while 3.2% (Lm1C, 5M, and 6Fc) were resistant to only one antibiotic with MARI of 1.25 as presented in **Tables 1**. Multi-drug resistance was, therefore, observed in 8/11(72.72%) of the isolates. The susceptibility test plates were presented in **Plate 1**.

Quantity and Purity of the Extracted DNA

Nanodrop spectrophotometric qualification of the extracted *L. monocytogenes*' whole genome DNA revealed the quantity and quality of the DNA, which range from 124-131ng/µl and absorbance from 1.85-1.92 at A260/A280nm wavelength. **Plate 2** shows the gel picture of the extracted DNA.

Prevalence of Antibiotic Resistance Genes among Antibiotic Resistant *L. monocytogenes* Isolates

PCR amplification of antibiotic resistance genes (ARGs) revealed that all of the six (6) sulfamethoxazole/trimethoprim resistant isolates harbored *sulI*; Lm3C, which is susceptible to sulfamethoxazole/trimethoprim but resistant to the entire beta-lactams tested also harbored the *sulI* gene (Plate 3 (a)). Prevalence of sulI gene among the isolates tested was 7/7(100%). Similarly, the phenotypically resistant isolates (Lm2C, Lm3C, Lm4C, Lm 8Fc, Lm9Fc, Lm10Fc, and Lm11Y) against all the beta-lactams (ceftriaxone, ampicillin, augmentin, and cefuroxime) possessed both bla_{TEM}, 7/7(100%) and $\mathit{bla}_{CTX\text{-}M}$ 7/7(100%) genes as shown in (Plate 3(b)) and (Plate 3(c)).

Table 1: Susceptibility Profile of *Listeria monocytogenes* Isolated from Food Samples

S/n	Isolates	Antibiotics tested								
	Code	AMP	AUG	CXC	SXT	GN	ERY	CIP	CFX	MARI
1	Lm 1C	S	S	S	S	S	S	S	R	1.25
2	Lm 2C	R	R	R	R	R	S	S	R	7.50
3	Lm 3C	R	R	R	S	S	R	S	R	6.25
4	Lm 4C	R	R	R	R	S	S	S	R	6.25
5	Lm 5M	S	S	S	S	S	S	S	R	1.25
6	Lm 6Fc	S	S	S	S	S	S	S	R	1.25
7	Lm 7Fc	R	S	S	S	S	S	S	R	2.50
8	Lm 8Fc	R	R	R	S	R	S	S	R	6.25
9	Lm 9Fc	R	R	R	R	S	S	S	R	6.25
10	Lm 10Fc	R	R	R	R	S	S	S	R	6.25
11	Lm 11 Y	R	R	R	R	R	S	S	R	7.50
	PRPA (%)	72.7	64.0	64.0	54.5	18.3	9.1	0.00	100	

AMP= Ampicillin (10µg) AUG=Augmentin (30µg) CXC= Cefuroxime (20µg), SXT= Sulphamethoxazole/Trimethoprim (25µg) GN= Gentamicin (10 µg), ERY =Erythromycin (15µg), CIP= Ciprofloxacin (5µg), CFX= Ceftriaxone (30µg). PRPA= percentage Resistance per Antibiotic. MARI= Multiple Antibiotic Resistance Index (0.2 as threshold value)

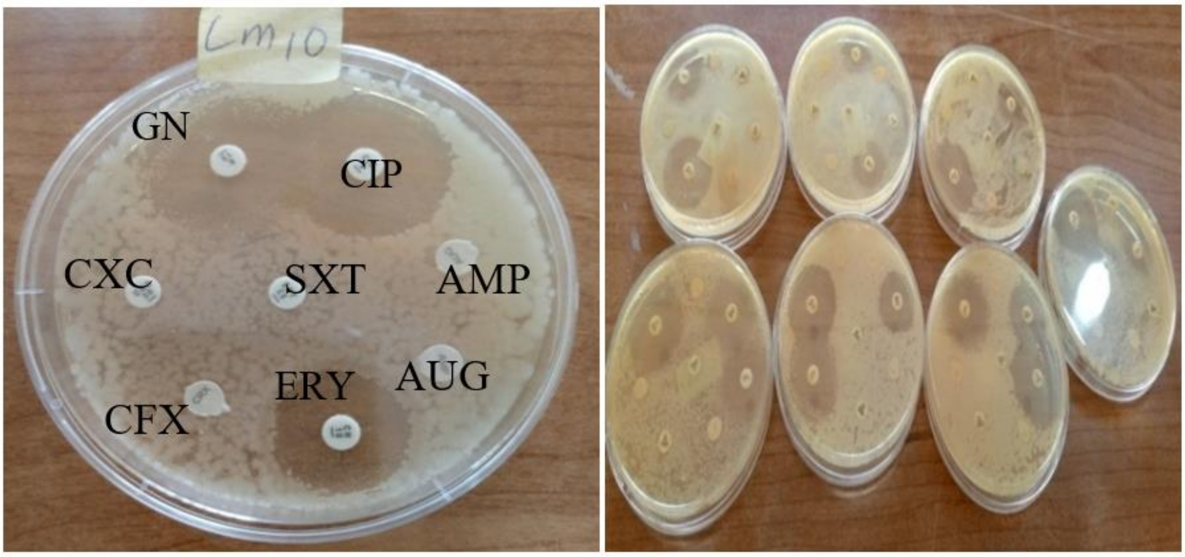

Plate 1: Antibiotic Susceptibility Test Plates of *Listeria monocytogenes* Isolates with MARI > 2.5. (Lm2C, Lm3C, Lm4C, Lm8Fc, Lm9Fc, Lm10Fc and Lm11 Y) Susceptible to Ciprofloxacin, Erythromycin, and Gentamycin Key AMP= Ampicillin (10µg) AUG=Augmentin (30µg) CXC= Cefuroxime (20µg), SXT= Sulphamethoxazole/Trimethoprim (25µg) GN= Gentamicin (10 µg), ERY =Erythromycin (15µg), CIP= Ciprofloxacin (5µg), CFX= Ceftriaxone (30µg)

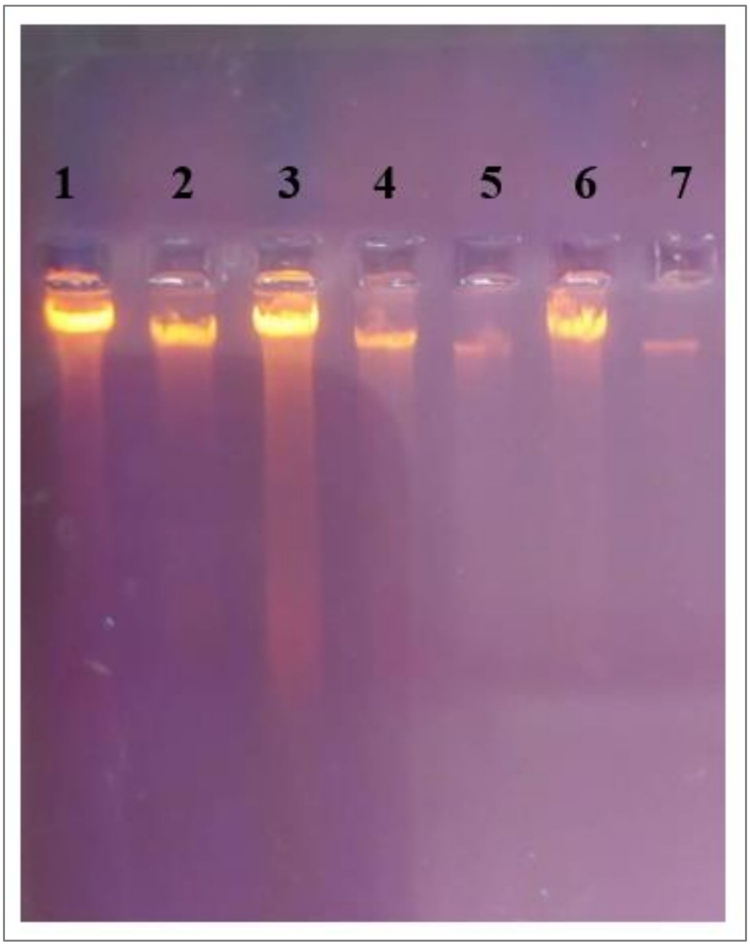

Plate 2: Extracted DNA of *Listeria monocytogenes* isolates that showed phenotypic resistance against Beta Lactams and sulfonamides. Lanes 1-7 for the isolates Codes Lm2C, Lm3C, Lm4C, Lm8Fc, Lm9Fc, Lm10Fc, and Lm11Y respectively.

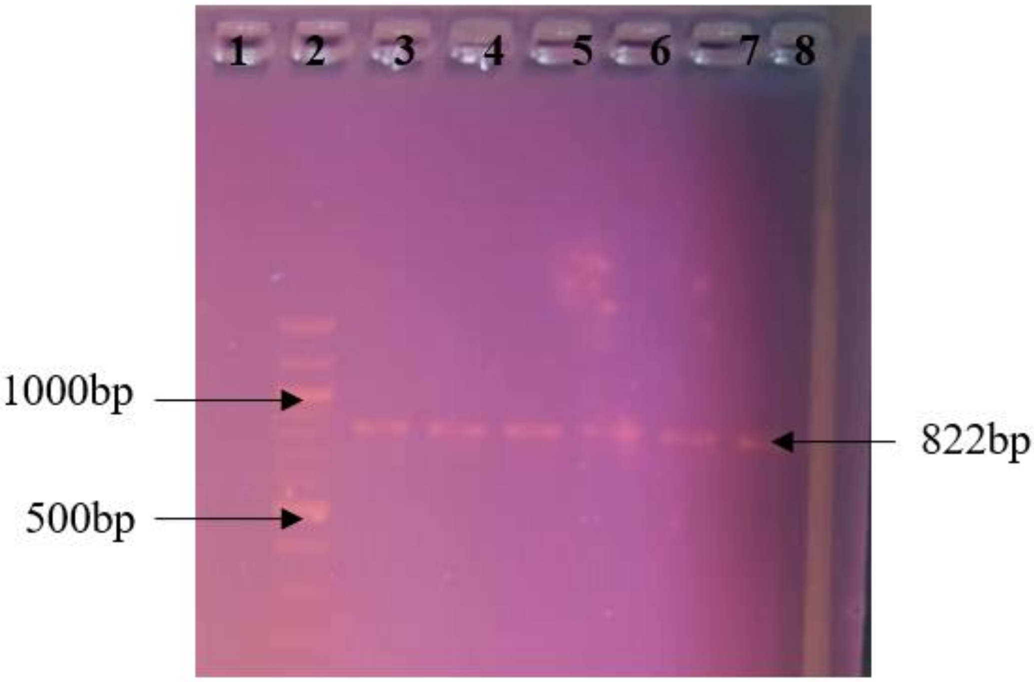

Plate 3(a): Gel Picture Showing *SulI* Resistance Gene (822 Bp). Lane 1: Negative Control; Lane 2: Molecular Weight Marker (100 bp); Lanes 3-8: Positive Isolates, Lm3C, Lm4C, Lm8Fc, Lm9Fc, Lm10Fc and Lm11Y.

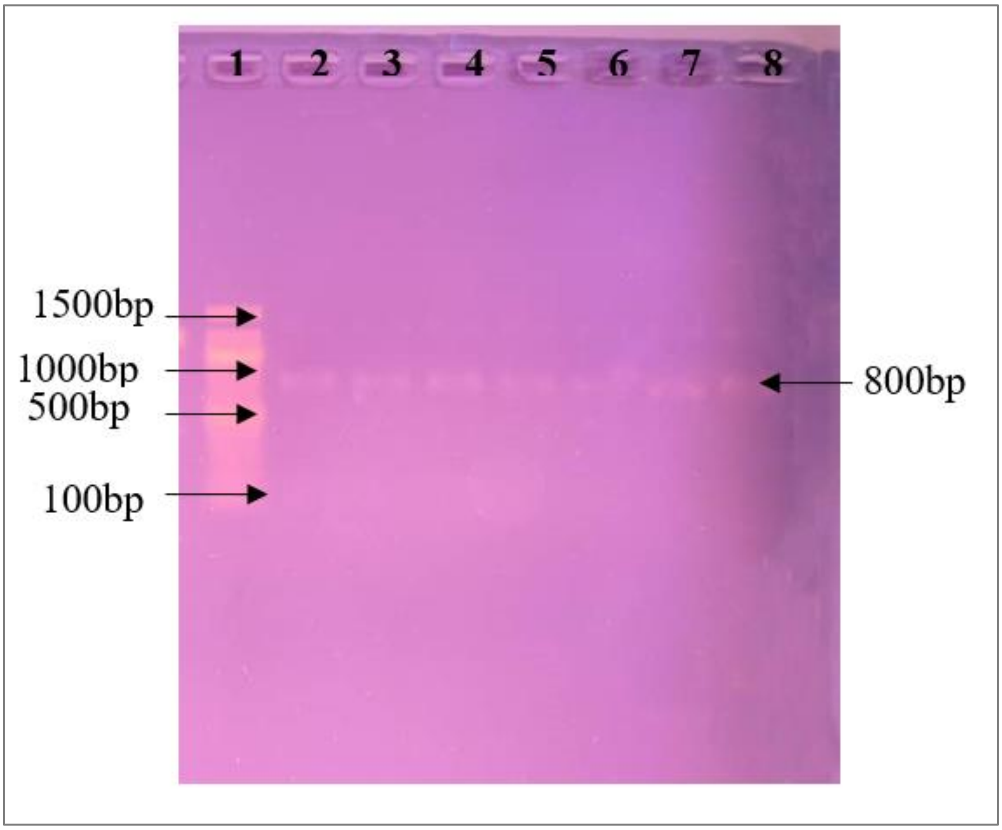

Plate 3(b): Gel Picture Showing *Bla*$_{TEM}$ Resistance Gene (800 Bp). Lane 1: Molecular Weight Marker (100 Bp); Lanes 2-8: Positive Isolates; Lm2C, Lm3C, Lm4C, Lm8Fc, Lm9Fc, Lm10Fc and Lm11 Y. Lane 9: Negative Control.

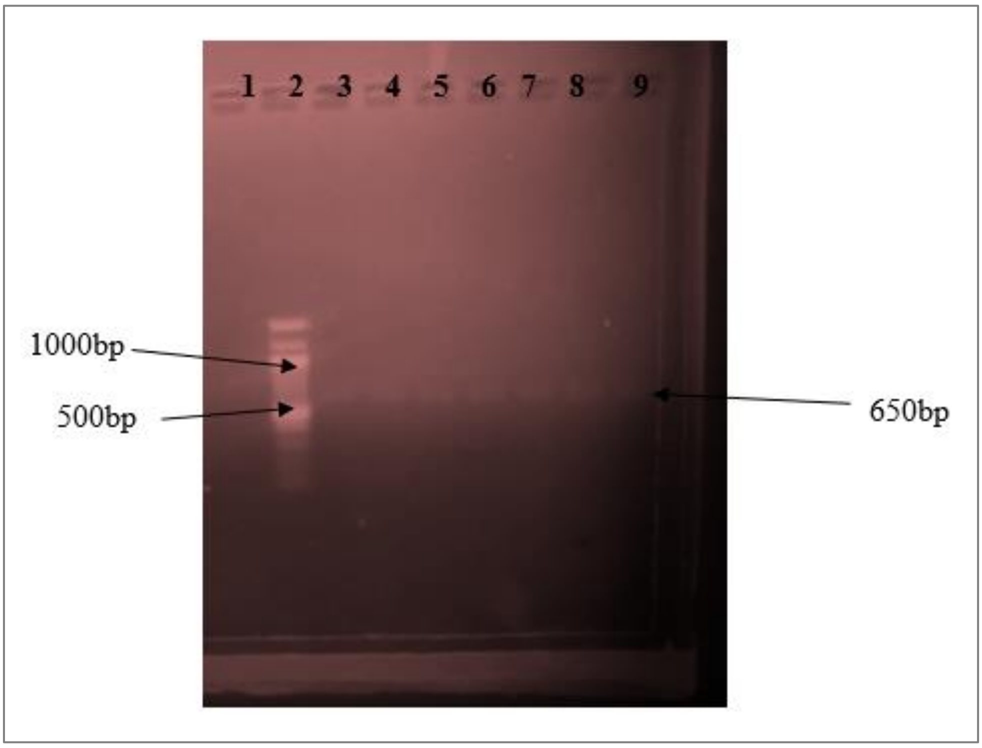

Plate 3(c): Gel picture showing *bla*$_{CTX-M}$ resistance gene (650 bp). Lane 1: Negative control; Lane 2: Molecular weight Marker (100 bp); Lanes 3-9: positive isolates. Lm2C, Lm3C, Lm4C, Lm8Fc, Lm9Fc, Lm10Fc and Lm11Y

DISCUSSION

The antibiotic susceptibility pattern observed in this study indicates that ciprofloxacin, gentamycin, and erythromycin are the most effective antibiotics against *L. monocytogenes* and can, therefore, serve as the most appropriate drugs to be prescribed for empirical treatment of listeriosis in the study area. This finding is similar to Khan *et al.* (2014), where ciprofloxacin and gentamycin were observed as the most effective antibiotics. Ntshanka *et al.* (2022) also reported susceptibility to ciprofloxacin (50%) and gentamycin (95%). Amajoud *et al.* (2018) also reported *L. monocytogenes*' susceptibility to ciprofloxacin, erythromycin, and gentamicin.

Antibiotic resistance of *L. monocytogenes* isolates in this study indicated that the overall incidence of antibiotic resistance is relatively high (72.7%), contrary to the low (23%) resistance reported by Oyelami *et al.* (2018). This, therefore, shows that resistance among food-borne pathogens has increased, as Matthias et al. proposed (2018). The differences between the two results might be due to differences in attitudes towards the use of antibiotics in the study areas, which might expose the organisms to antibiotics or other factors that can make them acquire resistance to antibiotics. High resistance to ceftriaxone (100%), ampicillin (72.7%), augmentin (64.5), cefuroxime (64.5%), and sulfamethoxazole/trimethoprim (54.5) was observed. In contrast, however, (Ntshanka *et al.*, 2022) reported high susceptibility to augmentin (65%) and resistance against erythromycin (0.5%). This may be because some food-borne pathogens like *L. monocytogenes* are intrinsically resistant to certain antibiotics and are related to their general physiology. Resistant strains may also transfer the resistance to other microorganisms, whereas other pathogens develop antibiotic resistance by mutation or genetic alteration. In addition, during their adaptation to environmental stresses, pathogens can become more resistant to antibiotics (Amin *et al.*, 2018).

The high multidrug resistance observed in this study supports that resistance among food-borne pathogens may increase due to the heavy use of growth promoters and antibiotics in livestock farming (Matthias *et al.*, 2018). It has been reported that many drugs, antibiotics, and hormones are applied in the livestock industry to benefit from their meat and produce more milk (Wong *et al.*, 2012). Also, disinfectants in the food industry may bring about biocide tolerance, resulting in bacterial cross-resistance to antibiotic drugs, which has already been reviewed for *L. monocytogenes* (Arslan and Fuzdemir 2008). Antibiotic resistance is considered one of the major threats to global public health, food security, and food development because it makes disease harder to treat as antibiotics become ineffective, which may increase the morbidity and mortality rate, as well as medical costs (WHO, 2018). It is also evident that *L. monocytogenes* strains from food products resist several antibiotics, including those frequently prescribed to treat human listeriosis, such as ampicillin, penicillin, and Gentamycin (Amin *et al.,* 2018).

All of the *L. monocytogenes* isolates that showed phenotypic resistance to β-lactams and Sulfonamides antibiotics in this study exhibited the presence of all three antibiotic-resistance genes investigated. The three (3) antibiotic resistance genes (bla_{CTX-M}, bla_{TEM}, and *sul1*) confer resistance against the two different antibiotic classes (β-lactams and Sulfonamides), which most of the isolates resisted phenotypically. This indicates conformity between the phenotypic resistance and the presence of resistance genes in most isolates. However, some isolates possessed the ARGs but were phenotypically susceptible. This is possible because the detection of different genes of resistance to antibiotics does not always correlate with the phenotypic antibiotic resistance of foodborne pathogens (Srinivasan *et al.*, 2005). Previously, Mpondo *et al.* (2021) reported the detected *sul*1 and bla_{TEM} among other ARGs in different strains of *L. monocytogenes*. Ntshanka *et al.* (2022) also detected ARGs, including *Sul*1 (100%) and bla_{TEM}, in a study that showed a high occurrence of multidrug-resistant *L. monocytogenes* and clinical ARGs in fresh vegetables. Chepkemei *et al.* (2022) also reported the detection of the *Sul*1 gene and similar ESBL genes, where the predominant ESBL genes detected were bla_{TEM} and bla_{CTX-M}.

L. monocytogenes isolates might have acquired genes for antibiotic resistance through antibiotic selection pressure or gene transfer mechanisms from other bacteria in the farm area, such as cabbage isolates. This gene transfer may also occur during the refrigeration of frozen chicken and yogurt-borne isolates. Studies have shown conjugative transfer of antibiotic resistance, i.e., the acquisition of enterococcal and streptococcal plasmids into the genus Listeria and subsequent transfer of these plasmids within the genus, including transmission to *L. monocytogenes* (Srinivasan *et al.*, 2005). It is known that *bla* genes encoding antibiotic resistance may be placed on transferable elements such as plasmids or transposons. This localization of antibiotic-resistance genes can facilitate a horizontal spreading of antibiotic resistance among (food-borne) bacterial strains (Zeynudin *et al.,* 2018) and pose a major therapeutic challenge in clinical settings.

CONCLUSION

L. monocytogenes isolates from food samples in this study area exhibited phenotypic and genotypic resistance to multiple antibiotics. This implies that future outbreaks of L. monocytogenes in the study area may be complicated to manage using the commonly used antibiotics tested in this study, representing a major public health concern.

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