South Asian Journal of Research in Microbiology

Volume 18, Issue 9, Page 10-20, 2024; Article no.SAJRM.121041
ISSN: 2582-1989

Screening for Resistant Genes and Plasmid Curing Profile Reagent of *Salmonella enterica* and *Listeria monocytogenes* from Seafood Sold in Rivers State, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author BPN designed the study, performed the statistical analysis, wrote the protocol and first draft of the manuscript. Authors ST and ANP supervised and managed the analyses. Authors ANP and ONAC managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: https://doi.org/10.9734/sajrm/2024/v18i9384

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/121041

Original Research Article

Received: 12/06/2024
Accepted: 14/08/2024
Published: 24/08/2024

ABSTRACT

Preamble: Plasmid curing has been a successful technique in reducing resistant bacteria and controlling antibiotic resistance which promote pathogenicity of an organism.
Aim: This study is focused on screening resistant genes and plasmid curing profile reagent of

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Cite as: *P.N., Barika, Sampson, T., Akani, N.P., Ogbuleka, N.A.C., and Chuku, W. 2024. "Screening for Resistant Genes and Plasmid Curing Profile Reagent of Salmonella Enterica and Listeria Monocytogenes from Seafood Sold in Rivers State, Nigeria". South Asian Journal of Research in Microbiology 18 (9):10-20. https://doi.org/10.9734/sajrm/2024/v18i9384.*

Salmonella enterica and *Listeria monocytogenes* from seafood sold in Rivers State, Nigeria.
Study Design: A completely randomized design was used for the study and the work run from June 2023 to December 2023.
Methodology: A total of one hundred and twenty-six (126) raw and parboiled samples of *Crassostrea gasar* (Oyster) (42), *Buccinum undatum* (Whelks) (42) and *Panaeus monodon* (Prawn) (42) were purchased from three (3) Local Government Area markets and subjected to standard conventional methods such as culturing, isolation and identification using PALCAM agar (for *Listeria monocytogenes*) and Triple sugar iron test (for *Salmonella enterica*), molecular screening using polymerase chain reaction and plasmid curing using an intercalating agent (acridine orange).
Results: The bacterial isolates were identified using molecular technique as: *Salmonella enterica*-AM04528*, Salmonella bongori*-NCTC12419*, Salmonella enterica*-KKP3882, *Listeria ivanovii*-PM-44*, Listeria monocytogenes*-HN1*, Listeria monocytogenes*-F6540*, Salmonella enterica*-19_85*, Salmonella enterica*-KKP1761*, Listeria monocytogenes*-NITRR/R1*, Salmonella enterica*-777SA01*, Listeria monocytogenes*-HR27 and *Listeria monocytogenes*-FC3. The resistant genes were present as bla_{TEM} (58.33%) and bla_{SHV} (91.67%) in *Salmonella enterica* and *L. monocytogenes* isolates. Plasmid curing with Acridine orange (An intercalating agent) reversed the resistant trait (100%) in the twelve (12) most resistant *Salmonella enterica* and *L. monocytogenes* isolates after susceptibility testing.
Conclusion: This study provides valuable insight into the genetic mechanism of antibiotic resistance of the two pathogens and highlights the potential of plasmid curing as a therapeutic approach and proper management of seafood.

Keywords: Screening; resistant genes; plasmid curing; Salmonella enterica; Listeria monocytogenes and seafood.

1. INTRODUCTION

Human pathogenic bacteria associated with sea foods such as fishes, oysters and prawns are known for their causation of human illnesses when these foods are consumed raw or undercooked. Pathogenic bacteria are naturally found in the environment inhabited by seafood. “Anthropogenic contamination is responsible for introducing diarrheagenic bacterial pathogens such as *Escherichia coli*, *Listeria monocytogenes* and *Salmonella enterica*, some of which could be highly resistant to multiple drugs due to their human and animal origin. The use of antimicrobials in fish farms is also responsible for developing antimicrobial resistance in fish-borne human pathogens. The incidence of drug-resistant pathogenic bacteria in seafood offers a formidable challenge in ensuring food safety and preventing the global spread of antibiotic-resistant bacteria via seafood” [1].

The bla_{TEM} and bla_{SHV} are resistance gene for beta lactam drugs such as Penicillins, Monobactams, Cephalosporins and Carbapenems used for the treatment of Gram positive and Gram-negative bacterial infection. These antibiotics contain a nucleus of 6-aminopenicillanic acid (lactam plus thiazolidine) ring and other ringside chains giving them their unique nature. “Plasmid profile analysis is one of the oldest molecular techniques used for epidemiological investigation. In this technique, plasmid DNAs are extracted from bacteria and the DNA is separated on agarose gel electrophoresis. It is easy to perform this technique and to interpret the results except that plasmids are mobile extrachromosomal elements that can spontaneously be lost or readily acquired by bacteria and thus isolates that are related epidemiologically can easily display different plasmid profiles” [2]. “The same researchers also reported that plasmids have transposons which may contain resistant determinants that can readily be lost or acquired, quickly changing the composition of plasmid DNA. Plasmids exist in a variety of spatial conformations (linear, nicked and supercoiled) which result in different migration velocities when submitted to agarose gel electrophoresis and this affects the reproducibility of this technique” [2].

“Resistance plasmids (R plasmids) have been reported to be the most frequent cause of antibiotic resistance in most bacteria including the *Salmonella* and *Listeria* species which have exhibited resistance to different antibiotics such as beta-lactam antibiotics, aminoglycosides and fluoroquinolone” [3,4]. “They allow the movement of genetic materials including antimicrobial resistant genes between bacterial species and

genera through gene exchange processes thereby causing a rapid spread of antibiotic resistance" [3,5]. "The plasmid-associated resistance can be gotten rid of by plasmid curing, which occurs spontaneously during bacterial cell division or by treating the bacteria with some physical or chemical reagents such as acridine orange or ethidium bromide" [6]. "The reagents can cause a single nick in the plasmid, thereby, initiating its relaxation and subsequently affecting its replication. Application of the curing assay may help to mitigate the spread of antibiotic resistance encoded by R-plasmid and consequently stops the spread of the antibiotic resistance" (Burussow et al., 2004) [7]. Hence, this research is focused on screening for resistant genes and plasmid curing (acridine orange) profile of *Salmonella enterica* and *Listeria monocytogenes* from seafood sold in Rivers State, Nigeria.

2. MATERIALS AND METHODS

2.1 The Study Area

Three markets in Rivers State, Kaa market in Khana Local Government Area, Creek Road market in Port Harcourt Local Government Area (PHALGA), and Bakana Market in Degema Local Government Area, were used for the study.

2.2 Sample Collection

One hundred and twenty-six (126) samples of raw and parboiled seafoods, including Whelks (*Buccinum undatum*), Oysters (*Crassostrea gasar*), and Prawns (*Penaeus monodon*), were purchased from three markets, placed in sterile polythene bags, then put in ice chests, and aseptically transported to the Department of Microbiology laboratory at Rivers State University, Port Harcourt, for bacteriological analysis after identification by Prof. G.C. Akani in the Department of Animal and Environmental Biology, Rivers State University.

2.3 Microbiological Analysis

2.3.1 Bacterial isolation and identification

Salmonella and *Listeria* were isolated by picking representative or discreet colonies based on their size, margin, surface, elevation, texture, transparency and coloration (blackish and Grey-green) on *Salmonella-Shigella* agar and a selective medium Polymyxin Acriflavin Lithium-chloride Ceftazidime Esculin Mannitol (PALCAM) agar supplemented with Listeria Selective Supplement II (FD063) respectively. Identification of the organism was further conducted through biochemical such as citrate utilization test, methyl

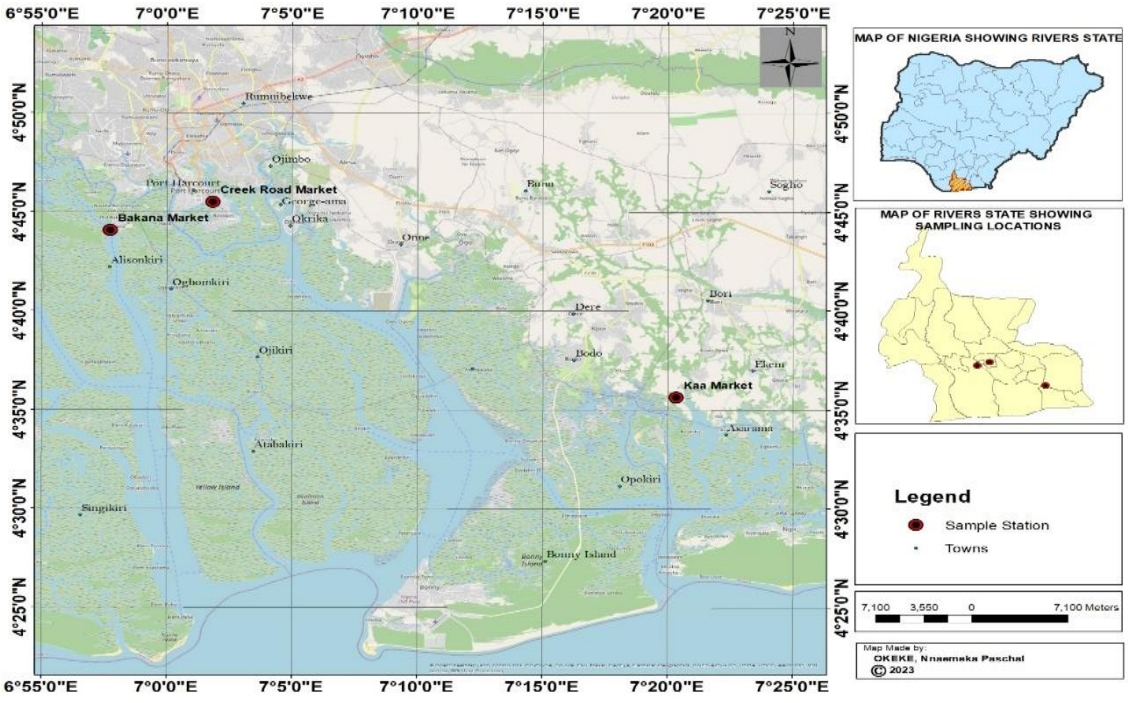

Fig. 1. Map of the study area

red, Catalase, Coagulase, indole test, Voges Proskaeur test, Urease sugar fermentation test, triple sugar iron test and hydrogen sulphide production to confirm *Salmonella enterica* [8].

2.4 Molecular Studies

2.4.1 DNA extraction and quantification

Boiling method was used for the extraction process as described by Bell et al. [9]. Pure culture of the *Salmonella* and *Listeria* isolate was put in Luria-Bertani (LB) Broth and incubated at 37^{o}C. Zero point five milliliter (0.5ml) of the broth culture of the *Salmonella* and *Listeria* in Luria Bertani (LB) were dispensed into properly labeled Eppendorf tubes and filled to mark with normal saline and were centrifuged at 14000rpm for 3 minutes and the supernatant was decanted leaving the DNA at the base. This process was repeated 3 times. The cells were re-suspended in 500ul of normal saline and heated at 95^{o}C for 20 min. The heated bacterial suspension was cooled on ice (About 10minutes) and spun for 3 min at 14000rpm. The supernatant containing the DNA was transferred to a 1.5ml micro-centrifuge tube and stored at -20^{o}C for other down-stream reactions [9]. The extracted DNA was quantified by using the Nanodrop 1000 Spectrophotometer as described by Olsen and Marrow [10].

2.4.2 Amplification of 16S rRNA

"The 16srRNA Amplification was carried out using an ABI 9700 Applied Biosystems, thermal Cycler, as described by Srinivasan et al. [11]. The 16s rRNA region of the rRNA gene of the bacterial isolates was amplified using the forward primer; 27F: 5'- AGAGTTTGATCMTGGCTCAG-3' and Reverse primer; 1492R: 5'-CGGTTACCTTGTTACGACTT-3' and to a final volume of 50 µL for 35 cycles. The PCR mix includes: (Taq polymerase, DNTPs, $MgCl_2$), the primers at a concentration of 0.5uM and the extracted DNA as template, Buffer 1X and water. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light trans-illuminator for a 1500bpamplicons" [11].

2.4.3 Amplification of bla_{TEM} and bla_{SHV} genes

"The bla_{TEM} and bla_{SHV} genes from the *Salmonella enterica* and *Listeria monocytogenes* isolates were detected using the TEM-F: 5'-ATAAAATTCTTGAAGACGAAA-3' and TEM-R: 5'-GACAGTTACCAATGCTTAATCA-3' and SHVF: 5'-GGGTTATTCTTATTTGTCGC-3' and SHVR: 5'-TTAGCGTTGCCAGTGCTC-3' primers on ABI 9700 Applied Biosystems thermal cycler at a final volume of 50 micro-litres for 35 cycles. The PCR mix included: The X2 Dream Taq Master Mix supplied by Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M and 50ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 58ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extension, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV trans-illuminator for a 500bp and 928bp product size" [9].

2.4.4 DNA sequencing

The Big-Dye Terminator kit on a 3510 ABI sequencer was used for sequencing of the amplified products. The sequencing was done at a final volume of 10ul, the components included 0.25ul BigDye® terminator v1.1/v3.1, 2.25ul of 5 x Big-Dye sequencing buffer, 10uM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition were as follows; 32 cycles of 96°C for 10s, 55°C for 5s and 60°C for 4minutes [11].

2.4.5 Phylogenetic analysis

Similar sequences were downloaded from the National Center for Biotechnology Information (NCBI) database using BLASTN prior to the edition of the obtained sequences using the bioinformatics algorithm Trace edit. MAFFT was used to align these sequences. The evolutionary history was inferred using the Neighbor Joining method in MEGA 6.0 [12]. The evolutionary distances were computed using the Jukes-Cantor method [13].

2.5 Plasmid Curing using Acridine Orange as Curing Agent

"The curing assay was carried out by aseptically mixing 0.5ml of acridine orange solution with 1ml of Mueller Hinton broth, each in a sterilized test tube. A loopful of the pure colony of each resistant isolate was inoculated into the test tube containing the mixture and then agitated carefully and incubated overnight at 37°C. From each of

the plasmid curing aliquots and was streaked aseptically, onto a freshly prepared Brain Heart Infusion agar plate and incubated overnight at 37°C" [14,15].

2.6 Molecular Screening of the Isolates for Plasmid after Curing Assay

"A colony was picked from the Brain Heart Infusion cultures of the isolates and inoculated into the 200µl solution containing 100mM glucose, 50mM Tris hydrochloride (pH 8), 10mM EDTA. The mixture was incubated at 37°C. The mixture was then treated with 50µl lysozyme, 400 µl of 1% sodium dodecyl sulphate in 0.2N NaOH. Lastly, 300µl of 30% potassium acetate was added and incubated in ice for 5min and then centrifuged at 5000rpm for 5min" [16]. "The supernatant was pipetted out and mixed with an equal volume of isopropanol and allowed for 5min and then 100µL was pipetted carefully from the upper part of the supernatant into another Eppendorf tube and used as the plasmid. The extracted plasmid (10µl) was casted into 1% agarose gel, containing acridine orange an intercalating dye and allowed to run for 30min under electrophoresis. The gel was examined under UV light documentation system (BioDoc-It™) for bands (above 500bp)" [17].

2.7 Determination of Isolates for Antibiotics Susceptibility Pattern after Plasmid Curing

"A loopful of the suspension after curing was streaked aseptically onto a freshly prepared nutrient agar plates and incubated overnight at 37°C. Subsequently, from the culture plates suspensions were prepared in sterilized normal saline and standardized to 0.5 McFarland scale. Then, 0.1ml of the standardized inocula were spread each onto Mueller Hinton agar plate and the antibiotics resisted (Ciprofloxacin, Cefixime, Ceftazidime, Cefuroxime, Augmentin, Cefotaxime, Impenem/Cilastatin and Ampiclox) previously by the isolates were placed onto the agar plates and then incubated at 37°C for 24hours. The zone of inhibition around the antibiotic discs were recorded and interpreted sensitive, intermediate and resistance according to the Clinical Laboratory Standard Institute" (CLSI)manual [18].

3. RESULTS

A total of twelve (12) (6 *Salmonella enterica* and 6 *Listeria monocytogenes* of the most resistant isolates after susceptibility testing in Barika *et al*., 2023 were identified using molecular technique. The Agarose gel electrophoresis of the amplified 16SrRNA gene of bacterial isolates before sequencing showed that Lanes 1 – 12 represent the 16SrRNA gene bands (1500bp) while lane L represents the 100bp molecular ladder (Fig. 2). The evolutionary distance between the bacterial isolates from this study and the accession numbers and their closest relatives on the phylogenetic tree is revealed on Fig. 3.

Agarose gel electrophoresis image of the amplified *TEM* gene of the twelve (12) most resistant isolates to antibiotics shows that Lane 2, 3, 4, 6, 9, 11 and 12 were the *TEM* gene band at 500bp while Lane L represents the 100bp molecular ladder. This shows that seven (7) out of the twelve (12) isolates screened for *TEM* gene had the gene present in their genetic material as shown on Fig. 3. The gel electrophoresis image shows the amplified *SHV* gene of the twelve (12) isolates shows that Lane 1, 2, 3, 4, 5, 6, 7, 9, 10, 11 and 12 showing the *SHV* gene band at 928bp while Lane L represents the 100bp molecular ladder. This indicates that eleven (11) out of the twelve (12) isolates screened for *SHV* gene had the gene present in their genome as shown on Fig. 4.

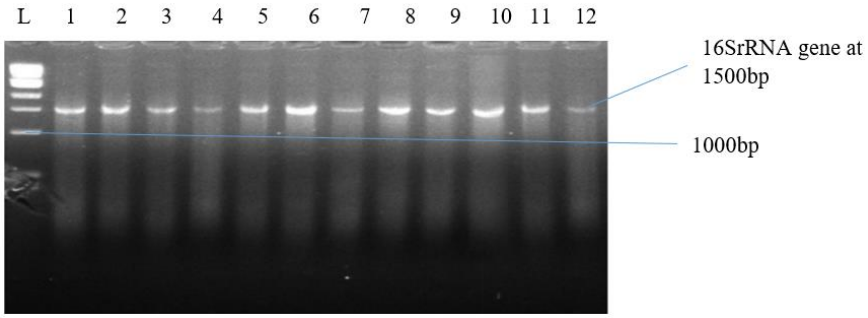

Fig. 2. Amplified 16S rRNA gene of the bacteria isolates at 1500bp

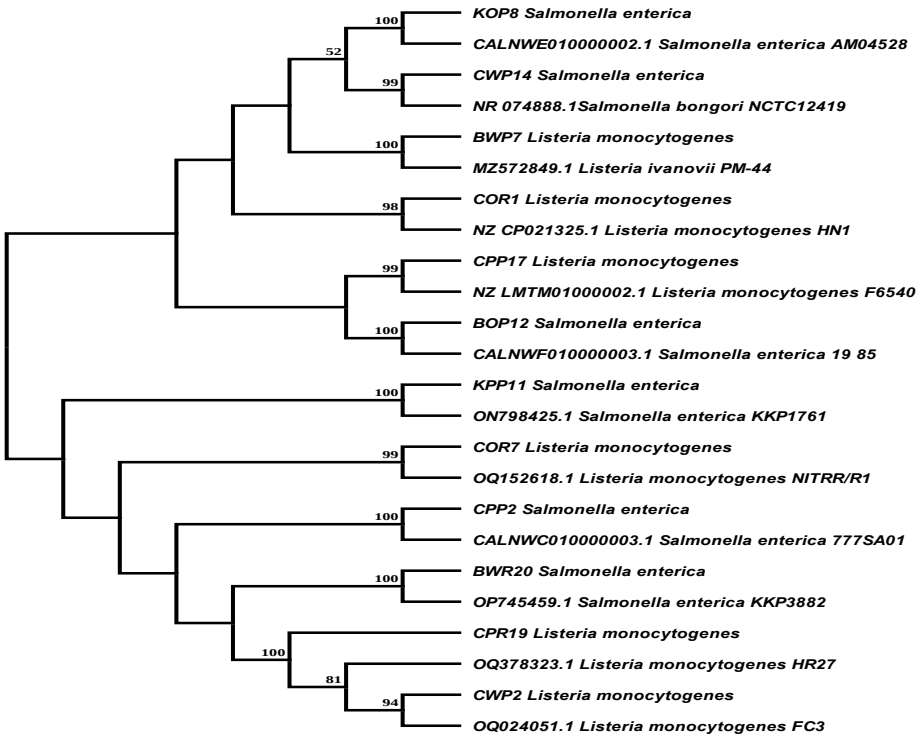

Fig. 3. Phylogenetic tree showing evolutionary distance between bacterial isolates

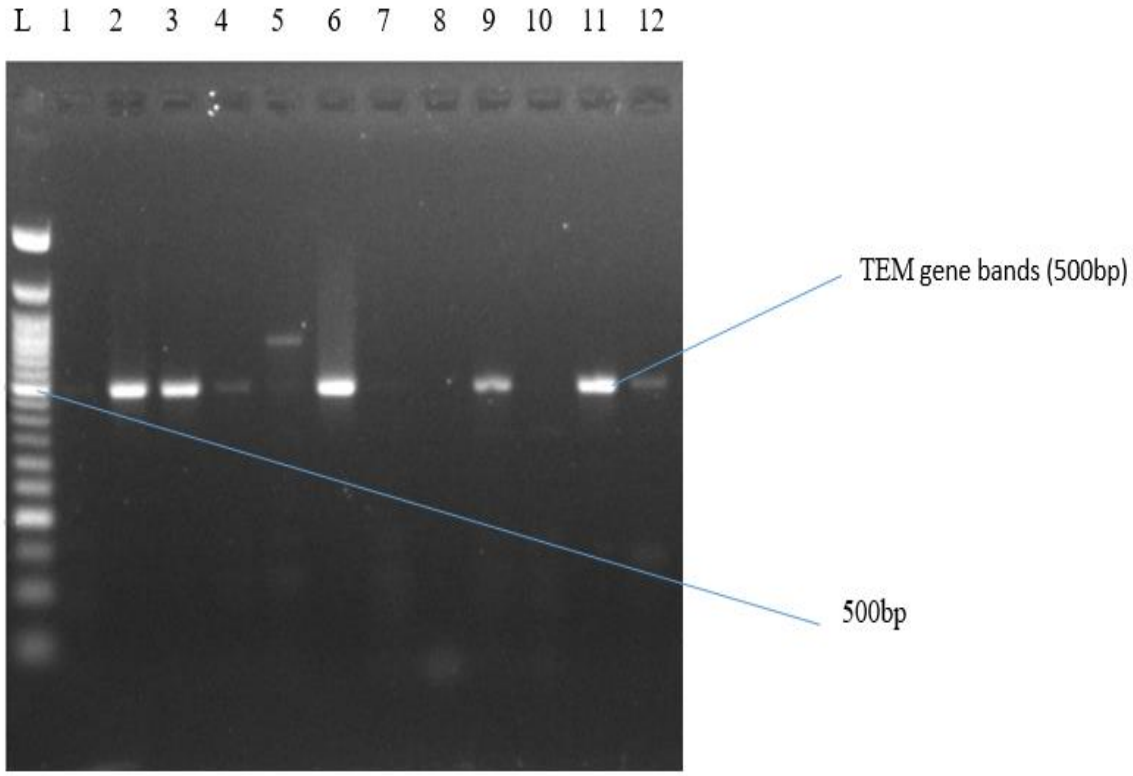

Fig. 4. Agarose gel electrophoresis of the amplified TEM gene bands of the bacterial isolates at 500bp with a 50bp molecular ladder

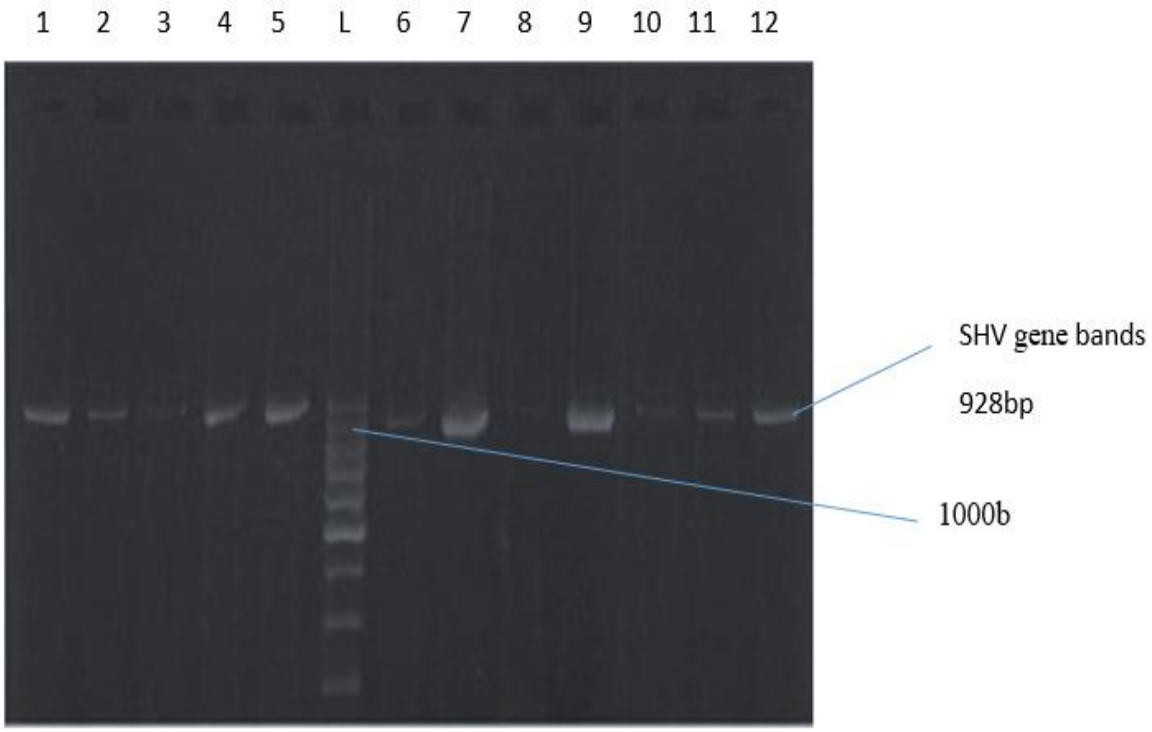

Fig. 5. Agarose Gel electrophoresis of the amplified SHV gene bands of the bacterial isolates at 928bp with lane L representing a 100bp molecular ladder

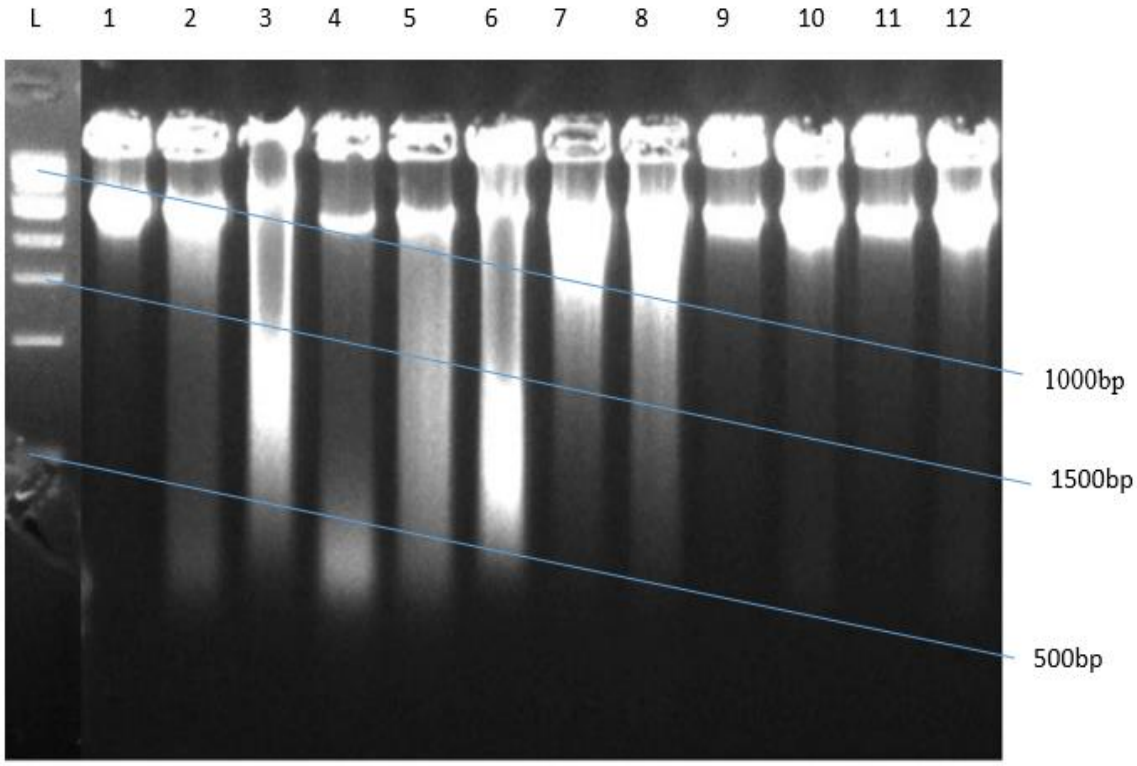

Fig. 6. Plasmid DNA of the bacterial isolates before curing

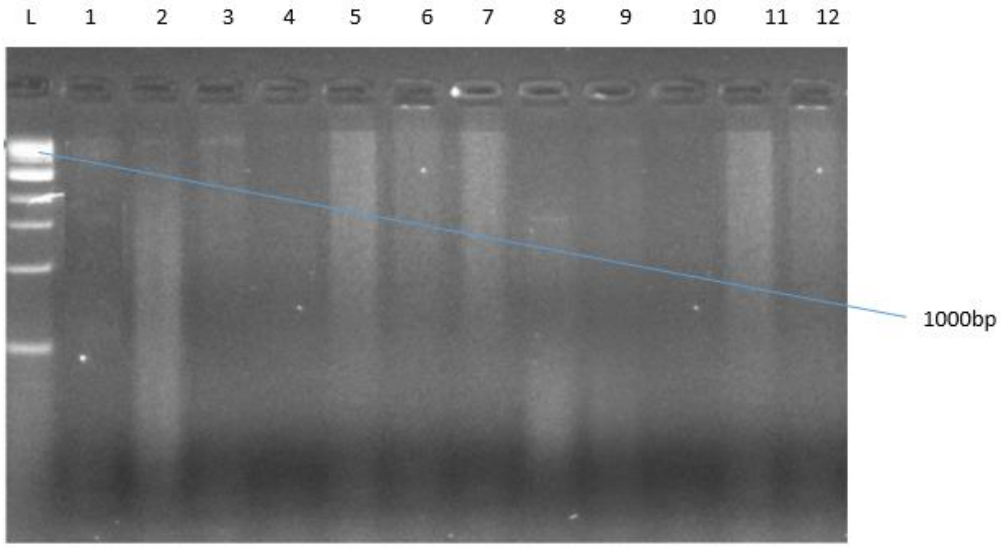

Fig. 7. Cured plasmid DNA of the bacterial isolates

Table 1. Percentage resistant patterns of test isolates before and after plasmid curing with curing agents (acridine orange)

S/N	Isolate code	NCBI BLAST/Strain	Resistance n(%)	Curing with AO (Susceptibility) N(%)
1	KOP8	*Salmonella enterica – AM04528*	12(100)	12(100)
2	CWP14	*Salmonella bongori-NCTC12419*	12(100)	12(100)
3	BWP7	*Listeria ivanovii*-PM-44	6(75)	8(100)
4	COR1	*Listeria monocytogenes*–HN1	7(87.5)	8(100)
5	CPP17	*Listeria monocytogenes* –F6540	6(75)	8(100)
6	BOP12	*Salmonella enterica*-19_85	12(100)	12(100)
7	KPP11	*Salmonella enterica*-KKP1761	12(100)	12(100)
8	COR7	*Listeria monocytogenes* –NITRR/R1	5(62.5)	8(100)
9	CPP2	*Salmonella enterica* –777SA01	12(100)	12(100)
10	BWR20	*Salmonella enterica* –KKP3882	12(100)	12(100)
11	CPR19	*Listeria monocytogenes* –HR27	7(87.5)	8(100)
12	CWP2	*Listeria monocytogenes* –FC3	5(62.5)	8(100)

n=Number of Antibiotic they are resistant to; N=number of antibiotics Susceptible to; AO-Acridine Orange

The agarose gel electrophoresis visualizing the presence of the DNA fragments of the twelve (12) resistant bacterial isolates showing that all the isolates with plasmid gene before curing as shown below in Fig. 5. Agarose gel electrophoresis revealing the presence of the DNA fragments of the twelve (12) resistance bacterial isolates showed that all the isolates did not carry plasmid gene after curing with acridine orange as shown below as shown in Fig. 6.

4. DISCUSSION

The extract from the obtained 16S rRNA sequence of the isolates produced during the Basic Local Alignment Search Tool (BLASTN) in the National Centre for Biotechnology Information (NCBI) database were highly similar to the sequence from the NCBI non – redundant nucleotide (nr/nt) data base and the 16S rRNA classification of the isolates KOP8, CW14, COR7, BWP7, COR1, CPP17, BOP12, KPP11, CPP2, BWR20, CPR19 and CWP2 which identified bacterial isolates as *Salmonella enterica* -AM04528, *Salmonella bongori*-NCTC 12419, *Listeria ivanovii*-PM-44, *Listeria monocytogenes*-HN1, *Listeria monocytogenes*-F6540, *Salmonella enterica*-19_85, *Salmonella enterica*-KKP1761, *Listeria monocytogenes*-NITRR/R1, *Salmonella enterica*-777SA01,

Salmonella enterica-KKP3882 *Listeria monocytogenes* -HR27*, Listeria monocytogenes*-FC3 with accession numbers CALNWE010000002.1, NR_074888.1, MZ572849.1,NZ_CP021325.1,NZ_LMTM01000002.1, CALNWF010000003.1, ON798425.1, OQ152618.1, CALNWC010000003.1, OP745459.1, OQ378323.1 and OQ024051.1 and relatedness of 100, 99, 100, 98, 99, 100, 100, 99, 100, 100, 81, 84(%). The phylogenic trees showing the evolutionary distance or relationship tree of the twelve (12) bacterial isolates as inferred from their nucleotide sequences using the Neighbour-Joining method.

The twelve (12) bacterial isolates of *Salmonella* and *Listeria* were screened for the presence of resistance genes which includes bla_{TEM} and bla_{SHV} during the amplification of the rDNA of the isolates. The bla_{TEM} genes were present in *Salmonella bongori*-NCTC12419, *Salmonella enterica*-19_85, *Salmonella enterica*-KKP1761, *Salmonella enterica*-KKP3882, *Listeria monocytogenes*-F6540, *Listeria monocytogenes*-HR27*, Listeria monocytogenes*-FC3 genome while *Salmonella enterica*-AM04528*, Salmonella bongori*-NCTC12419, *Salmonella enterica*-19_85*, Salmonella enterica*-KKP1761, *Salmonella enterica*-777SA01*, Salmonella enterica*-KKP3882, *Listeria ivanovii*-PM-44, *Listeria monocytogenes*-F6540, *Listeria monocytogenes*-NITRR/R1, *Listeria monocytogenes*-HR27 and *Listeria monocytogenes*-FC3 possess the bla_{SHV} gene in their genomes.

This extended spectrum βeta Lactamase gene (bla_{TEM} and bla_{SHV}) has been widely known to be the cause of resistance of the bacterial isolates to first and second generation cephalosporin and penicillin antibiotics used in this study and the excessive production of can increase the ability of isolates to resist these antibiotics completely [19,20]. The bla_{TEM} and bla_{SHV} genes codes for the production of bla_{TEM} and bla_{SHV} beta-lactamase enzyme which destroy or inactivate the beta-lactam ring of antibiotics thereby inhibiting the activity of the antibiotics especially in Gram-negative bacteria [21].

However, bacterial resistance to beta-lactams drugs is also expressed through the production of beta-lactamases as discussed earlier but could also be mediated by the decreased penetration to the target site. The resistance to beta-lactam antibiotics observed in this study could be attributed to the continuous usage and exposure of these antibiotics to the bacterial isolates [22,19]. Parvathi *et al*. [23] made an observation from their research that the increasing use of antibiotics without prescription in developing countries especially Nigeria where there are no regulatory policies to effectively prohibit this action, has rendered the commonly used antibiotics completely ineffective in the treatment of infections. Acridine orange (An intercalating agent) have considerable potentials for treating plasmid-mediated antibiotic resistance in bacteria. “The resistant genes recorded against all the isolates used in the study, highlighted the terrible future awaiting the antibiotic era, unless some scientific approaches are put in place to curtail such public health hazard. However, the plasmid curing assay employed in this study may be one of such scientific approaches that may help to reduce the spread of antibiotic resistance in bacteria. This is because after the curing, the drugresistant bacteria was completely corrected and drastically reduced in the studied isolates. This is in agreement with the previous findings on impact of plasmid curing on multi-drugresistant *E. coli”* (Oriomah and Akpe, 2019;) [24] *Pseudomons aeruginosa* [6] *Vibrio* and *Salmonella* species [14]. “The role of the curing on reverting the antibiotic multidrug resistance depends on whether the resistance is acquired or induced, The complete elimination of resistance against antibiotics used in this study by curing, suggests that the resistance is acquired, confirming further that the efficacy of antibiotics administered by injection is easily conserved” [25-28].

5. CONCLUSION AND RECOMMENDATIONS

This study has demonstrated the presence of resistant genes (Bla_{TEM} and Bla_{SHV}) and plasmid curing potential of Acridine orange as a remedy to antibiotic resistance in *Salmonella enterica* and *Listeria monocytogenes*. These finding emphasize the need for continued surveillance and proper management of seafood to prevent spread of these pathogens and potential outbreak of foodborne illnesses.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscripts.

ACKNOWLEDGEMENT

I want to appreciate Professor N.P. Akani, Dr. T. Sampson, Dr. Ogbuleka and W. Chuku for their inputs in making this research a success.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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