

Original Research Article

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

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 detecting resistant genes and plasmid curing profile of *Salmonella enterica* and *Listeria monocytogenes* from seafood sold in Rivers State, Nigeria.

Study design: 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, molecular screening and plasmid curing.

Results: The bacterial isolates were identified molecularly 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

INTRODUCTION

Human pathogenic bacteria associated with sea foods such as fishes, oysters and prawns are known for their causation of human illnesses when 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 multidrug-resistant human pathogenic bacteria in wild-caught and farmed shrimp offers a formidable challenge in ensuring food safety and preventing the global spread of antibiotic resistant bacteria via seafood (Espejo *et al.*, 2017). 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 (Trinidad *et al.*, 2003). 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 (Trinidad *et al.*, 2003).

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 (Carattoli, 2013; Zhang *et al.*, 2019). 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 the antibiotic resistance (Carattoli, 2013; Daneman *et al.*, 2013). 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 (Elias *et al.*, 2013). The reagents can cause a single nick in the plasmid, thereby, initiating its relaxation and subsequently affects its replication. Application of the curing assay may help to mitigate the spread of the antibiotic resistance encoded by R-plasmid (Letchumanan *et al.*, 2015) consequently stops the spread of the antibiotic resistance (Burussow *et al.*, 2004). Hence, this research is focused on detecting resistant genes

and plasmid curing profile of *Salmonella enterica* and *Listeria monocytogenes* from seafood sold in Rivers State, Nigeria

MATERIALS AND METHODS

Description of 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

Sample Collection

One hundred and twenty-six (126) samples of raw and parboiled seafoods, including Whelks (*Buccinumundatum*), 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

Microbiological Analysis

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 red, Catalase, Coagulase, indole test, Voges Proskauer test, sugar fermentation test and specifically hydrogen sulphide production to confirm *Salmonella enterica* (Cheesbrough, 2005).

Molecular Studies

DNA Extraction and Quantification

Boiling method was used for the extraction process as described by Bell *et al.* (1998). Pure culture of the *Salmonella* and *Listeria* isolate was put in Luria-Bertani (LB) Broth and incubated at 37°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 filling to mark with normal saline and was 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°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°C for other down-stream reactions (Bell *et al.*, 1998). The extracted DNA was quantified by using the Nanodrop 1000 Spectrophotometer as described by Olsen and Marrow (2012).

Amplification 16S rRNA

The 16srRNA Amplification was carried out using an ABI 9700 Applied Biosystems, thermal Cycler, as described by Srinivasan *et al.* (2015). The 16s rRNA region of the rRNA gene of the bacterial isolates were 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, MgCl₂), 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, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; annealing, 52°C for 30 seconds; extension, 72°C for 30 seconds for 35 cycles and final extension, 72°C 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 1500bp amplicons (Srinivasan *et al.*, 2015).

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

The bla_{TEM} and bla_{SHV} genes from the *Salmonella enterica* and *Listeria monocytogenes* isolates were amplified 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, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; annealing, 58°C for 30 seconds; extension, 72°C for 30 seconds for 35 cycles and final extension, 72°C 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 (Bell *et al.*, 1998).

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 (Srinivasan *et al.*, 2015).

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 were used to align these sequences. The evolutionary history was inferred using the Neighbor Joining method in MEGA 6.0 (Saitou and Nei, 1987). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969).

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 (Zhang, *et al.*, 2012; Vengadesh *et al.*, 2015).

Molecular Screening of the Isolates for Plasmid after the Curing Assay

A colony was picked from the Brain Heart Infusion cultures of the isolates and inoculated into the a 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 were incubated in ice for 5min and then centrifuged at 5000RPM for 5min. the supernatant was pipetted out and mixed with equal volume of isopropanol and allowed for 5min. 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) (Olukoya and Oni, 1990).

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 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 (CLSI, 2017).

RESULTS

A total of six (6) each *Salmonella enterica* and *Listeria monocytogenes* of the most resistant isolates were identified molecularly. 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 (Plate 1). 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 Figure 1.

The agarose gel electrophoresis image showing the amplified *TEM* gene of the twelve (12) most resistant isolates to antibiotics shows that Lane 2, 3, 4, 6, 9, 11 and 12 showing 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 Plate 2. The gel electrophoresis image showing the amplified *SHV* gene of the twelve (12) most resistant isolates to antibiotics 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 Plate 3.

The agarose gel electrophoresis visualizing the presence of the DNA fragments of the twelve (12) resistance bacterial isolates showing that all the isolates with plasmid gene before curing as shown below as shown in Plate 4. 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 Plate 5.

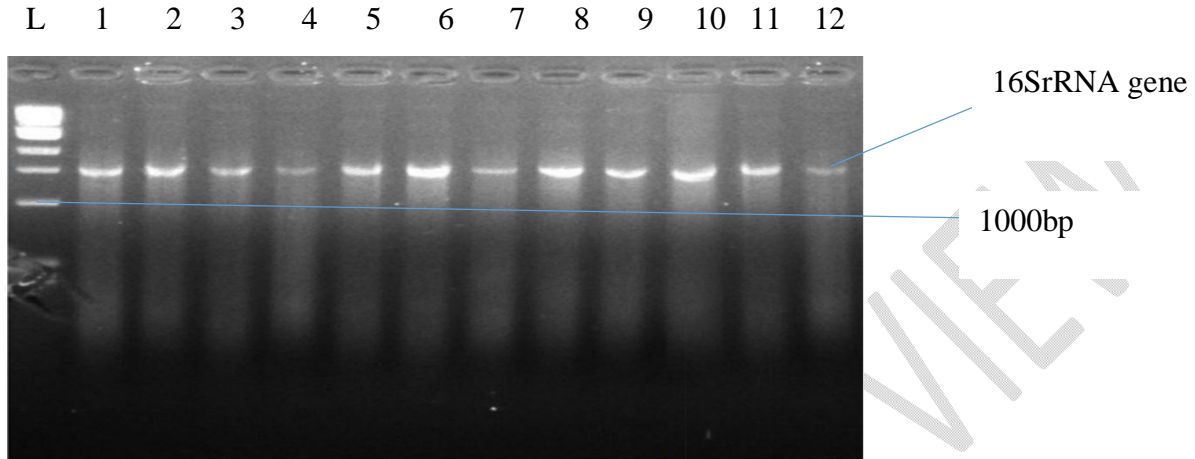


Plate 1: Amplified 16S rRNA Gene of the bacteria Isolates at 1500bp

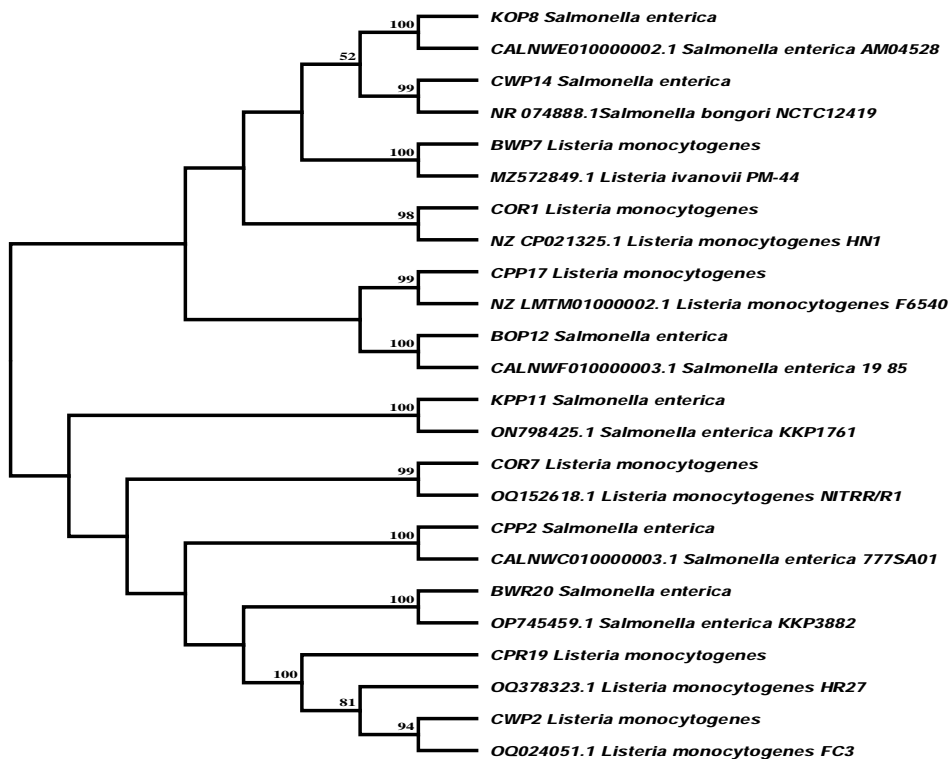
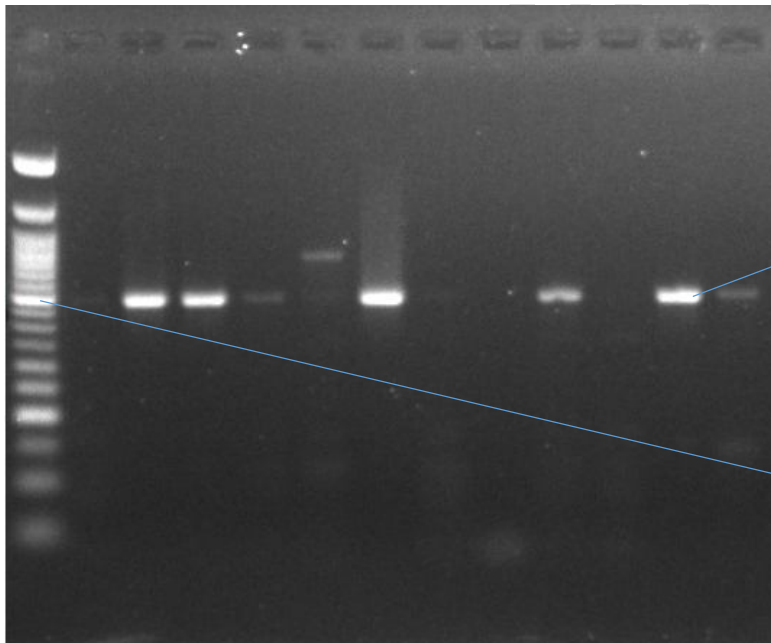


Figure 1: Phylogenetic Tree showing evolutionary distance between bacterial Isolates

L 1 2 3 4 5 6 7 8 9 10 11 12



TEM gene bands (500bp)

500bp

Plate 2: Agarose Gel Electrophoresis of the Amplified *TEM* Gene Bands of the Bacterial isolates at 500bp with a 50bp molecular ladder

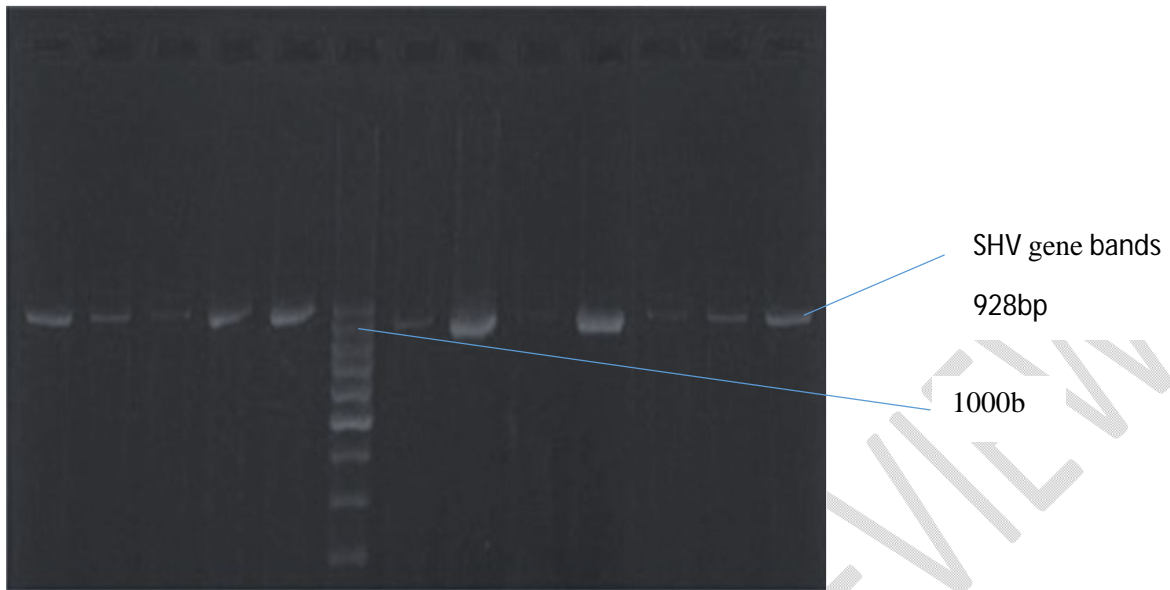


Plate 3: Agarose Gel Electrophoresis of the Amplified *SHV* Gene Bands of the bacterial isolates at 928bp with Lane L representing a 100bp molecular ladder

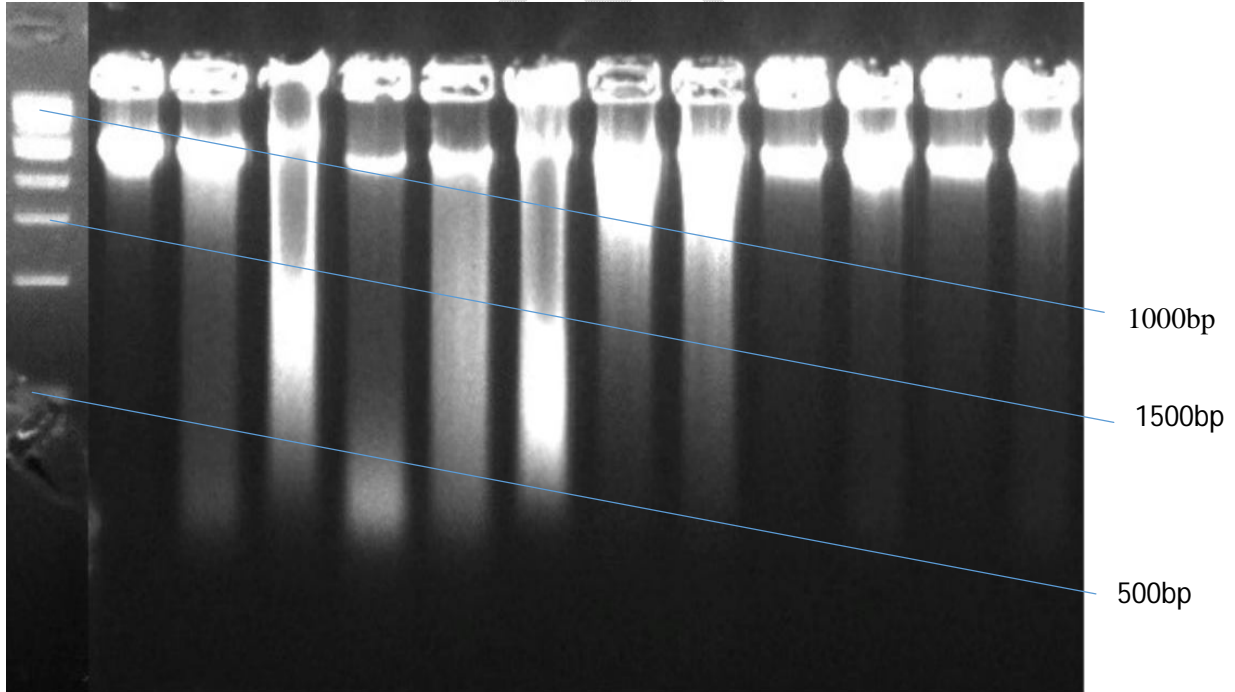


Plate 4: Plasmid DNA of the Bacterial Isolates before curing

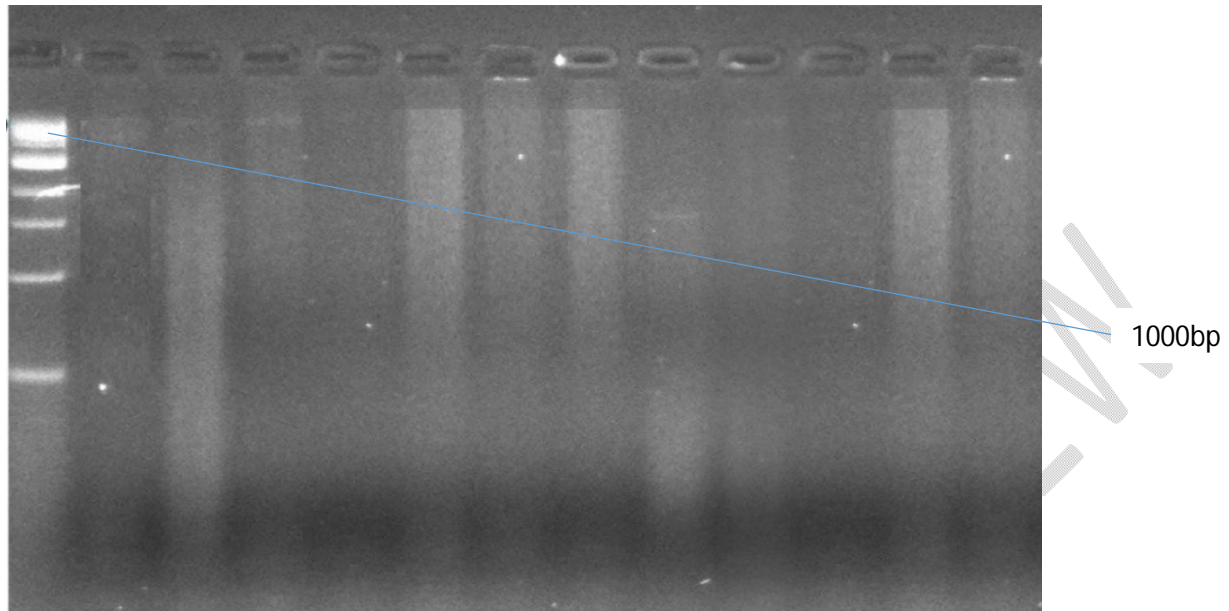


Plate 5: Cured Plasmid DNA of the Bacterial Isolates

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 | <i>Salmonella enterica</i> AM04528 | – 12(100) | 12(100) |
| 2 | CWP14 | <i>Salmonella bongori</i> -NCTC12419 | 12(100) | 12(100) |
| 3 | BWP7 | <i>Listeria ivanovii</i> -PM-44 | 6(75) | 8(100) |
| 4 | COR1 | <i>Listeria monocytogenes</i> -HN1 | 7(87.5) | 8(100) |
| 5 | CPP17 | <i>Listeria monocytogenes</i> F6540 | – 6(75) | 8(100) |
| 6 | BOP12 | <i>Salmonella enterica</i> -19_85 | 12(100) | 12(100) |
| 7 | KPP11 | <i>Salmonella enterica</i> -KKP1761 | 12(100) | 12(100) |
| 8 | COR7 | <i>Listeria monocytogenes</i> NITRR/R1 | – 5(62.5) | 8(100) |
| 9 | CPP2 | <i>Salmonella enterica</i> -777SA01 | 12(100) | 12(100) |
| 10 | BWR20 | <i>Salmonella enterica</i> -KKP3882 | 12(100) | 12(100) |
| 11 | CPR19 | <i>Listeria monocytogenes</i> -HR27 | 7(87.5) | 8(100) |
| 12 | CWP2 | <i>Listeria monocytogenes</i> -FC3 | 5(62.5) | 8(100) |

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

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*-NCTC12419, *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.

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-animopenicillanic acid (lactam plus thiazolidine) ring and other ringside chains giving them their unique nature. This extended spectrum β 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 (Park *et al.*, 2013). 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 (Ford *et al.*, 2015).

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 (Sauvage *et al.*, 2008; Barika *et al.*, 2021). Parvathi *et al.* (2004) 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 significant multidrug resistance index 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 multidrug resistance index 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-drug resistant *E. coli* (Oriomah and Akpe, 2019; Zaman *et al.*, 2010), *Pseudomons aeruginosa* (Elias *et al.*, 2013), *Vibrio* and

Salmonella species (Zhang, *et al.*, 2012). The role of the curing on reverting the antibiotic multidrug resistance depends on whether the resistance is acquired or induced (Dueduet *et al.*, 2017), 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 (Guillermo *et al.*, 2012).

CONCLUSION AND RECOMMENDATIONS

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

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UNDER PEER REVIEW