Molecular Characterisation and Detection of Antibiotic Resistant Genes in *Escherichia coli* isolated from *Senilia senilis* (Cockle) in Rivers State, Nigeria

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Abstract: In controlling *E. coli* infections antibiotics that were once effective are now ineffective due to the bacteria acquired resistance to these antibiotics. This research is carried out to characterize and identify *Escherichia coli* isolated from edible cockle (*Senilia senilis*) by molecular methods and screened for the presence of bla_{TEM} gene that confer resistance to Extended spectrum β-Lactam antibiotics. The study was carried out from January to June 2020 at the Department of Microbiology, Rivers State University, Port Harcourt, Nigeria and the *E. coli* isolates were subjected to multiple antibiotic susceptibility test using Kirby-Bauer disc diffusion method and resistant isolates were screened for the presence of the resistance gene bla_{TEM}). This screening was carried out via the process of DNA extraction, quantification, amplification of the gene using appropriate primer and agarose gel electrophoresis which showed the DNA extracts that had bla_{TEM} genes when amplified. Forty (40) *E. coli* were isolated and identified culturally and molecularly from *Senilia senilis*. Results showed the presence of bla_{TEM} gene in 5 out of the eight (8) isolates screened for bla_{TEM} gene. Results also revealed that 52.5% of isolates had MAR index greater than 0.2 indicating high source of contamination where antibiotics are often used. Molecular characterization via sequencing of the 16S rRNA gene of the Seven (7) most resistant isolates confirmed that the isolates were strains of *Escherichia coli*. This study demonstrated the resistance ability of *E. coli* and the main factor behind its resistance. Further investigation into antimicrobial resistance is recommended for the administration of drugs most especially for the treatment of food-mediated *E. coli* infections.
Keyword: bla_{TEM}, *Escherichia coli*, Molecular Characterisation, Resistant genes and *Senilia senilis*

INTRODUCTION

In controlling *E. coli* infections antibiotics that were once effective are now ineffective due to the bacteria acquired resistance to these antibiotics. The microbe's resistance to these antibiotics may function as a potential source in the transportation of antimicrobial resistance to humans (Schroeder *et al*., 2002). The disease threat from antibiotic resistance strains has increased in recent years and the occurrence of multiple antibiotic resistance among the enteric species could be a problem associated with transfer of resistance to human beings (Immaculate *et al*., 2012).

Multiple antibiotic resistance indexes are used in differentiating the source of pollution in this seafood. Transmission of resistant clones and resistant plasmid of *E. coli* from cockles to humans commonly occurs (Van de Boogard and Stobberingh 2000). However, after the spread of antibiotic therapy, a number of enteric organisms have developed resistance to antibiotic and this antimicrobial resistance can spread through horizontal transfer of resistant genes from one type of bacteria to another. Enzyme production is the major mechanism of acquiring resistance of *E. coli* to β-lactam antibiotics. β-lactamases rupture the amide bond of the β-lactam ring leaving obtained products that lack antimicrobial activity (Fred, 2006). *E. coli* is naturally susceptible to carboxypenicillins, ceftazidimes and aztreonams; however, it can acquire resistance to third generation cephalosporins. This happens readily through the constitutive excessive production of *BlaTEM* β-lactamase (Fred, 2006). The *BlaTEM* β-lactamase enzyme belonging to molecular class C is naturally produced in low quantities by *E. coli* and determines resistance to aminopenicillins and some of the early cephalosporins. However, chromosomal cephalosporinase production may increase from 100 to 1000 times in the presence of inducing β-lactams (including imipenem) (Kumar, 2017). *E. coli* is highly resistant to cephalexin, cephalosporin, ampicillin and amoxicillin/Clavulanic acid (Kumar, 2017). The presence of resistance, together with the acquisition of virulence genes can lead to clonal expansion and spread of disease-causing agents (Henrick *et al*., 2000).

Hence it is expedient to characterise and detect antibiotic resistant genes in *Escherichia coli* isolated from *Senilia senilis* in Rivers State, Nigeria.

MATERIALS AND METHODS

Study Area

The study was carried out in three different locations in Rivers State; Creek Road Market in Port Harcourt Local Government Area (4.7583^{o} N, 7.0209^{o} E), Kaa Market in Khana Local Government Area (4^{o} 40' 34.64" N 7^{o} 21' 54.68" E) and Buguma Main Market in Asari-toru Local Government Area (4^{o} 44' 10.10" N 6^{o} 51' 44.50" E).

Sample Collection/Preparation

A total of 42 raw and shucked *Senilia senilis* were collected randomly from the three different locations (Creek Road Market, Kaa Market and Buguma Main Market) in River State, Nigeria. The samples were labelled properly, put in ice-chest and transported aseptically to the Department of Microbiology Laboratory for bacteriological analysis in Rivers State University. Preparation of the stock analytical unit was done by weighing 10gof*Seniliasenilis* (shell is removed) samples and homogenized (Using mortar and pestle) in 90ml of the diluent.

Isolation and Identification of *E. coli*

Escherichia coli was isolated by picking representative or discreet colonies based on its greenish metallic sheen colouration on Eosin Methylene Blue agar, Identification of the organism was further conducted through biochemical such as citrate utilization test, methyl red, indole test, Voges Proskaeur test, sugar fermentation test to confirm *E. coli* (Cheesbrough, 2005; Aditi*et al*., 2017)

Antibiotic Susceptibility Test

The antimicrobial susceptibility profiles of the *E. coli* isolates to conventional antibiotics were determined using the Kirby Bauer disk diffusion method on sterile Mueller Hinton agar. Standardization of the *E. coli* isolates was carried out by adjusting to 0.5 McFarland turbidity standard. The swab is dipped into the bacterial suspension and streaked over the surface of the agar plates and the procedure was repeated several times, rotating the agar plate 60° C each time to ensure even distribution of the inoculum. The plates were left to dry for 3–5 min. Ten conventional antibiotics disk impregnated with (Cephalexin (CEP)–10 µg, Ofloxacin (OFX)-10 µg, Nalidixic acid (NA) – 30 µg, Pefloxacin (PEF)-10 µg, Gentamicin (CN)-10 µg, Augmentin (AU)-30 µg, Ciprofloxacin (CPX)-10 µg, Trimethoprim (SXT) – 30 µg, Streptomycin (S)-30 µg and Ampicillin (PN)-30 µg) were aseptically placed on the surface of the inoculated agar plate with sterile forceps. Each disk was pressed down to ensure full contact with the surface of the agar. The plates were then incubated 24 hours at 33 to 35°C in an inverted position. The zones of inhibition were measured in millimetre (mm) using a meter rule and compared to (CLSI, 2017).

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 *E. coli* isolate was put in Luria-Bertani (LB) Broth and incubated at 37^{o}C.Zero point five millilitre (0.5ml) of the broth culture of the *E. coli* in Luria Bertani (LB) was put into properly labeledEppendorf 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 resuspended 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 (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 and TEM Gene'

The 16srRNA Amplification was carried out using an ABI 9700 Applied Biosystems

Thermal Cycler, as described by Srinivasan The 16s rRNA region of the rRNA gene of the *E. coli* isolates were amplified using the forward primer; 27F: 5'-AGAGTTTGATCMTGGCTCAG-3' and Reverse primer; 1492R: 5'-CGGTTACCTTGTTACGACTT-3' and TEM genes were amplified using TEMForward: 5'-ATGAGTATTCAACATTTCCGTG-3' and TEMReverse: 5'-TTACCAATGCTTAATCAGTGAG-3' primers on ABI 9700 Applied Bio-systems thermal cycler at a final volume of 40 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 (Srinivasan *et al*., 2015)

DNA Sequencing

Sequencing of the amplified product was carried out using the Big-Dye Terminator kit on a 3510 ABI sequencer. The sequencing was done at a final volume of 10ul, the components included 0.25 ulBig Dye® terminator v1.1/v3.1, 2.25ul of 5 x BigDye 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 *et al.* (2015). 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).

Determination of Multiple Antibiotic Resistance Index (MAR)

Multiple antibiotic resistance was done as described by Lambert, 2003 and Osunduya*et al.,* 2013) Multiple antibiotic resistance (MAR) index was ascertained by using the formula MAR = a/b, where a stands for the number of antibiotic to which the test isolate depicted resistance and b stands for the total number of antibiotics to which the test isolate has been evaluated for susceptibility (Krumperman, 1985).

RESULTS

The forty (40) *E. coli* isolates were isolated and identified as showed in Table 1. The result of the susceptibility pattern shows that the *E. coli* isolates were more susceptible to Nalidixic acid (85%), streptomycin (77.5%), Augmentin (75%) and resistant to Pefloxacin (42.5%) and cephalexin (40%) (Table 2). The agarose gel electrophoresis of the amplified 16S rRNA gene of the most resistant *E. coli* isolates before sequencing showed that Lanes B1-B4 represent the 16SrRNA gene bands (1500bp) while lane L represents the 100bp molecular ladder (Figure 1). The evolutionary distance between the *E. coli* isolates from this study and the accession numbers and their closest relatives on the phylogenetic tree is revealed in Figure 2.

The agarose gel electrophoresis shows the amplified *TEM* gene of the eight (8) most resistant *E. coli* isolates to antibiotics shows that Lane 1, 4, and 6-8 showing the *TEM* gene band at 400bp while Lane L represents the 100bp molecular ladder.

Table 1: Isolation and Identification of the *E. coli* Isolates from S. *senilis*(Raw and Shucked)

	Colony Characteristics						Gram Stain		Biochemical and Sugar Fermentation														
S/N & Isolate Code	Form/ Shape	Elevation	Surface	Margin	Colour on EMB	Opacity	Reaction	Shape	Catalase	Oxidase	Citrate test	Indole	Methyl Red	VP	Motility test	Coagulase	Glucose	Lactose	Mannitol	Urease	Growth on EMB	Suspected Organism
1. FAS1	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
2. FC1	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
3. CF12	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
4. FKH1	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
5. FKH2	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
6. FKH3	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
7. PKH1	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
8. PAS1	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
9. FC2	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
10. FAS2	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
11. PKH2	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
12. PAS2	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
13.PAS3	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
14.CF13	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
15.FAS3	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
16.CP1	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
17.FKH4	Circular	Raised	smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
18.CP4	Circular	Raised	Smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
19.CF3	Circular	Raised	Smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
20.PKH3	Circular	Raised	Smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
21.CF4	Circular	Raised	Smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
22.CF5	Circular	Raised	Smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
23.CP2	Circular	Raised	Smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
24.CF6	Circular	Raised	Smooth	entire	GMS	translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
25. FKH5	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
26. FKH6	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
27. CF7	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
28. CP8	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
29. FAS4	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
30. FAS5	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
31. CP3	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
32. FKH7	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
33. FAS6	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
34. CP9	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
35. CF9	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
36.FKH8	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
37.CF10	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
38.CF11	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
39.FAS7	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*
40.CP10	Circular	Raised	smooth	entire	GMS	Translucent	-	rod	+	-	-	+	+	-	+	-	+	+	+	-	+	*EC*

KEY: Green Metallic Sheen (GMS), Eosin Methylene Blue Agar (EMB), Positive (+) and Negative (-), *EC = Escherichia coli*

This shows that 5(62.5%) out of the 8 *E. coli* isolates screened for *TEM* gene had the gene present in their genetic material as shown on Fig: 3. The result from Table3, showed that 19 (47.5%) of the *E. Coli* isolates have MAR index of 0.1, 9(22.5) of the isolates have a MAR index of 0.2 while 1(2.5%) of the *E. coli* isolate have a MAR index of 0.3.

Table 2: Susceptibility Pattern of *Escherichia coli* isolated from Cockles (*Senilia senilis*) in Rivers State

Antibiotics	Conc. (µg)	Resistant n (%)	Intermediate n (%)	Susceptible n (%)
CEP	(10)	16(40.0)	8(20.0)	16(40.0)
OFX	(10)	11(27.5)	8(20.0)	21(52.5)
NA	(30)	6(15.0)	0(0.00)	34(85.0)
PEF	(10)	17(42.5)	22(55.0)	1(2.5)
CN	(10)	8(20)	4(10)	28(70.0)
AU	(30)	2(5)	8(20.0)	30(75.0)
CPX	(10)	15(37.5)	10(25.0)	15(37.5)
SXT	(30)	4(10.0)	8(20.0)	34(70.0)
S	(30)	9(22.5)	0(0.00)	31(77.5)
PN	(30)	13(32.5)	13(32.5)	14(35.0)

Key: CN (Gentamicin), CPX (Ciprofloxacin), CEF(Cephalexin), OFX (Ofloxacin), AU (Augumentin), PEF (Pefloxacin), NA (Nalidixic acid), S (Streptomycin), SXT (Trimethoprim-Sulfamethoxazole), PN(Ampicillin)

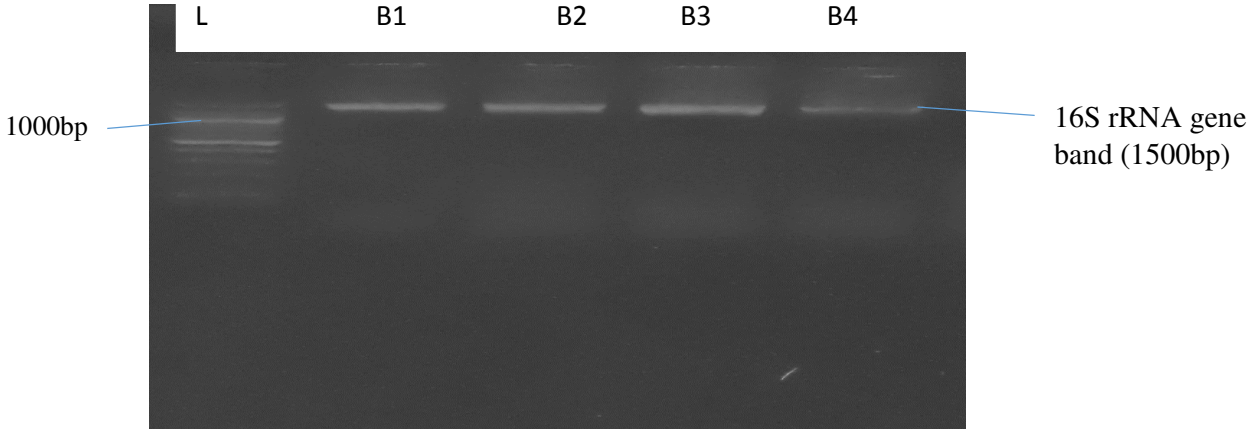
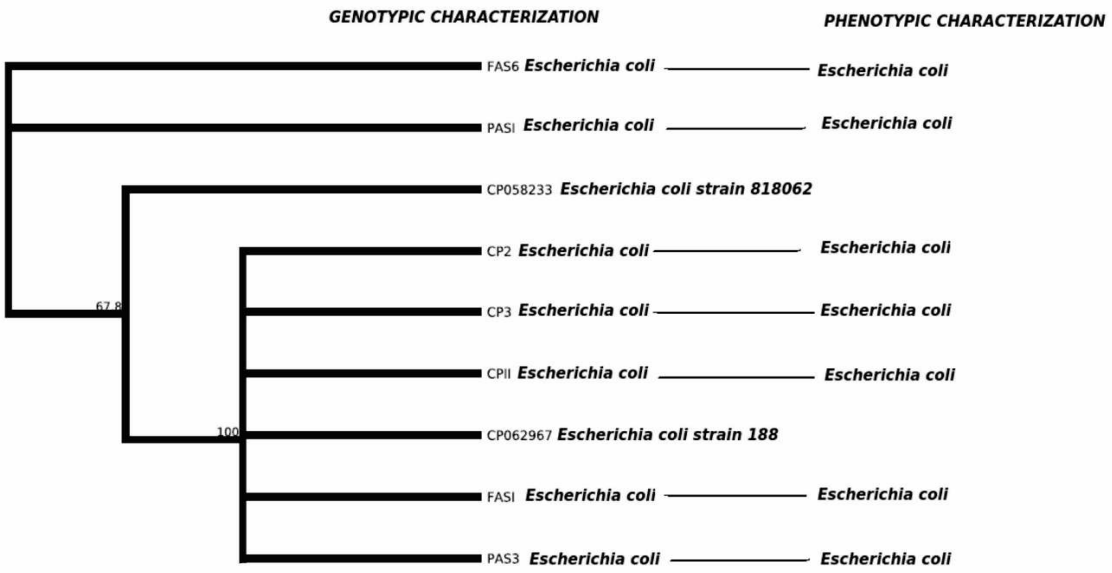


Figure 1. Agarose Gel Electrophoresis showing the Amplified 16S rRNA Gene of Selected *E. coli* isolates.



Key: PAS-Parboiled Asari-Toru LGA, FAS-Fresh Asari-Toru LGA, CP-Creek Road Parboiled, CP062967 and CP058233 are Accension numbers

Figure 2. Phylogenetic tree showing the evolutionary distance between the bacterial isolates

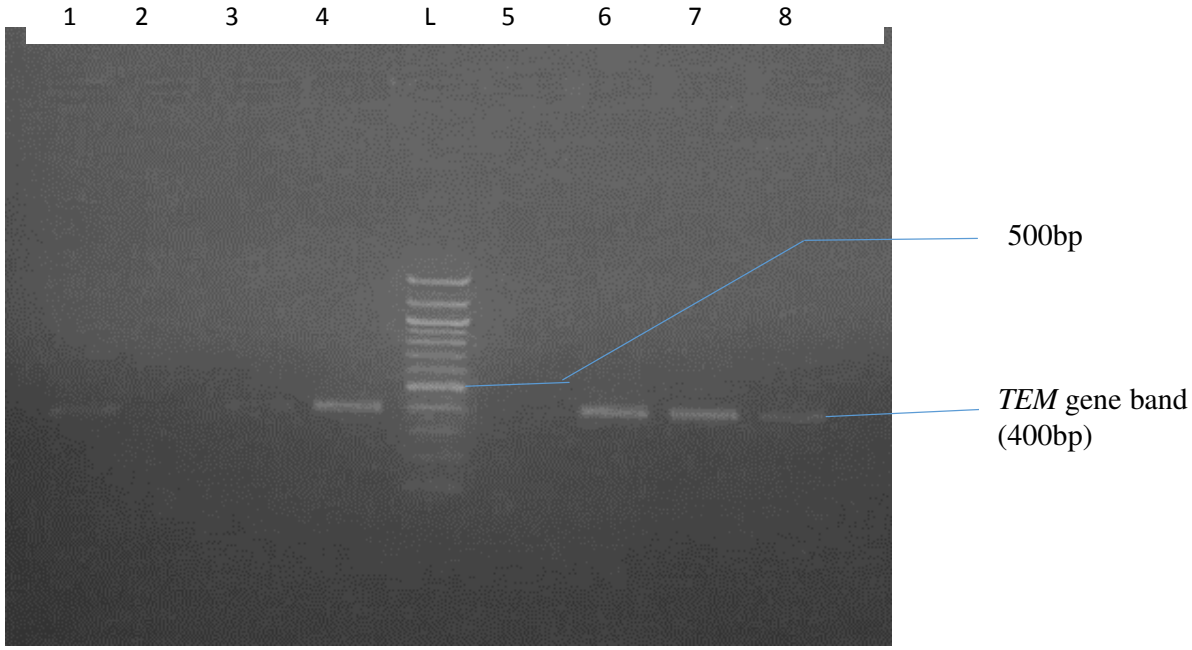


Figure 3. Agarose Gel Electrophoresis Showing the Amplified *TEM* Gene Bands of the *E. coli* isolates

Table 3: Multiple Antibiotic Resistance Index of *Escherichia coli* (N=40)

MAR Index	Number (%)
0.1	19(47.5)
0.2	9(22.5)
0.3	1(2.5)
0.4	3(7.5)
0.5	2(5.0)
0.6	3(7.5)
0.7	3(7.5)

KEY: Multiple Antibiotic Resistance (MAR)

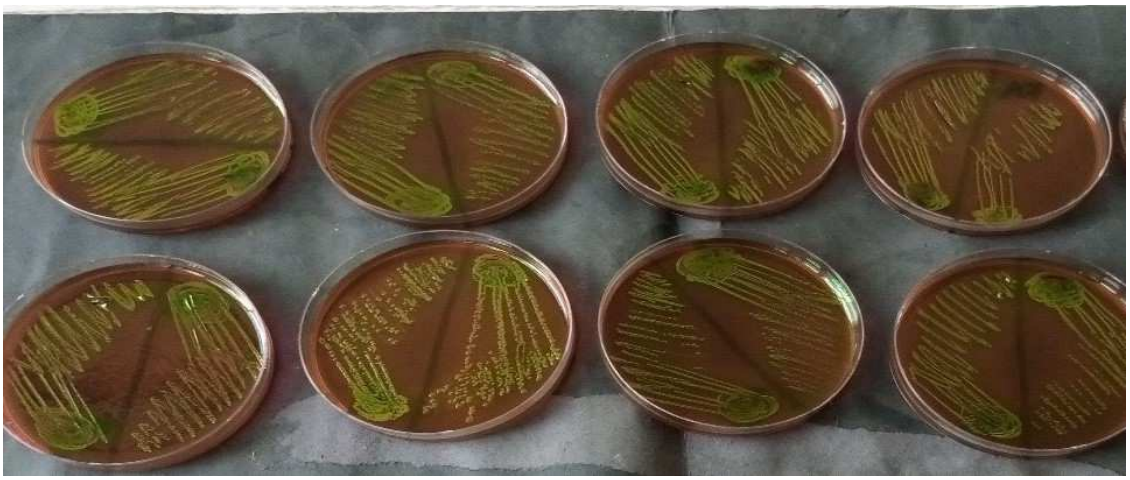


Plate 1: Pure Cultures of *E. coli* Isolates

DISCUSSION

The epidemiology and distribution of *E. coli* infections is greatly altered or influenced by the methods used in characterization and identification of most *E. coli* isolates, as many microbiologists employ cultural and morphological means in identifying bacterial isolates (Sampson *et al*., 2020). Molecular techniques have been employed recently, to facilitate the reliable identification of the etiological agent of these infections. Amplification of sequences that is specific for an organism can be done through polymerase chain reaction. This research is aimed at molecular characterisation of *E. coli* and screened for genes that confer resistance such as TEM genes from *E. coli* isolated from *Senilia senilis* (Edible cockle). The results showed an exact match from the obtained 16SrRNA sequence of the isolate produced during the mega blast search were highly similar to the sequences from the NCBI non-redundant nucleotide (nr/nt) database as in Fig 1. The evolutionary distances computed using the Jukes-Cantor method were in agreement with the phylogenetic placement of the 16SrRNA of the isolates FAS6, PAS1, CP2, CP3, CP11, FAS1 and PAS3 within the *Escherichia* spp revealed a closely relatedness to *Escherichia coli.*

The increase in antibiotic resistance and multidrug resistance in *Escherichia coli* is a great concern to the environment and public health as reported previously in Sampson *et al*. (2020), the presence of bla_{TEM} gene which was screened through molecular technique in this study has been widely known to be responsible for the resistance of *E. coli* isolates to cephalosporin, pefloxacin and penicillin antibiotics used. The eight (8) most resistant *E. coli* isolates subjected to molecular technique to screen for bla_{TEM} genes showed that 5 (62.5%) out of the eight were positive for bla_{TEM} gene (Fig. 2) present in their genome which confer resistance to most first generation cephalosporin and its overproduction can increase the ability of isolates to resist these antibiotics completely (Schultsz and Geerlings, 2012). The bla_{TEM} gene codes for the production of bla_{TEM} beta-lactamase enzyme which destroy the beta-lactam ring of antibiotics thereby inhibiting the activity of the antibiotics as reported by Sauvage *et al*. (2008). They were sensitive to nalidixic acid that target nucleic acid synthesis, because the drug inhibits DNA gyrase during DNA replication (Schultsz *et al*., 2012)

There is little information on the molecular characterization and detection of resistant genes from *E. coli* isolated from seafood carried out in Nigeria, however the work of Muhammad *et al.* 2009 reported the presence of bla_{SHV} and bla_{TEM} in *E. coli* from environmental sources.

Multiple Antibiotic Resistance (MAR) index of *E. coli* isolated in this study revealed that the percentage of isolates with MAR index ≥ 0.2 was 52.5% (Table 1). It is of high importance to know that MAR index values greater than 0.2 indicates high risk source of contamination where antibiotics are often used (Davis *et al*., 2016; Krumperman, 1985). This finding is very critical as it indicates that these antibacterial agents may not be potent in the management/treatment of infections caused by *Escherichia coli* (Sampson *et al*., 2020). The presence of resistant strains of *E. coli* in *Senilia senilis* (Cockle) which may have risen from the market environment due to poor hygienic practices poses a problem for public health as it can cause a wide range of infections such as gastrointestinal infections, endocarditis and septicaemia especially in immunocompromised individuals (Rossolini and Thaller, 2010). The understanding of bacterial genomics through this research can inform biologist on the evolution of resistant strains of organisms as well as resistant genes (Rossolini and Thaller, 2010).

CONCLUSION

This study has showed that the resistance of *E. coli* to antibiotics is mainly due to the presence of bla_{TEM} resistant genes among other factors not highlighted in this study. The molecular identification of *Escherichia coli* was needed to produce its exact identity of the organism due to their public health importance. The risk of resistance of *E. coli* to antibiotic is high considering the MAR index obtained in this study. From this study it can be inferred that *Senilia senilis* (Cockle) accumulate *Escherichia coli* that can cause serious foodborne disease as well as multiple antibiotic resistant traits and this is connected with microbiological quality of its marine environments and serve as a microcosm for antibiotic resistant microbial population. Enhanced sanitary conditions are strongly recommended for the market and the retailing environments.

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