Molecular Characterization and Plasmid Curing of Multidrug Resistant Enterohemorrhagic *Escherichia coli* Isolated from Some Fecal and Water Samples in Port Harcourt Metropolis

S. A. Wemedo [a], N. P. Akani [a] and A. A. Akintola [a*]

[a] Department of Microbiology, Faculty of Science, Rivers State University, Nkpolu-Oroworukwo, P.M.B. 5080, Port Harcourt, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Public health is at risk due to the fact that enterohemorrhagic *E. coli* (EHEC) is becoming resistant to most antibiotics that demonstrates widespread misuse of antibiotics. The study was aimed at molecularly identify and perform plasmid curing with extracts of *Morinda citrifolia* (MC) and *Xylopia aethiopica* (XA) on multidrug resistant EHEC isolates that are obtained from some fecal and water samples in Port Harcourt, Rivers State. After isolation of *E. coli*, eleven (11) *E. coli* isolates were identified as EHEC. The Molecular Identification of seven (7) most resistant isolates using PCR confirmed 100% EHEC isolates. Of the 7 resistant EHEC isolates, the *Amp*C Gene and *bla*TEM Gene were detected in 71.4% and 42.9% respectively. Results of plasmid curing test revealed that the extracts of *Xylopia aethiopica*, *Morinda citrifolia* and Acridine Orange used as the control reduced the total percentage of multiple antibiotic resistant EHEC isolates from 35.61% to 10.61%,

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Corresponding author: Email: alexisakins58@gmail.com;

18.18% and 16.67%, respectively. The extracts had the ability to cure plasmids, with *X. aethiopica* extract being the most successful against resistant EHEC isolates. In conclusion, this investigation revealed the genetic makeup of EHEC strains, detected their resistance genes and demonstrated the curing of the plasmids of resistant EHEC isolates found in fecal and water samples from the study area, both of which constitute a serious risk to public health.

1. INTRODUCTION

The Enterohemorrhagic *E. coli* (EHEC) or Shiga toxin-producing *E. coli* (STEC) family of *E. coli* is one of the *E. coli* strains that can cause serious intestinal infections in both people and domestic animals [1]. They are among the most prevalent strains that lead to serious food-related illnesses in people. Its production of the powerful toxin called shiga toxin sets it apart from other strains of *E. coli* [1]. Excessive antibiotic use is thought to be the primary cause of antibiotic resistance. It's possible for this antibiotic resistance to develop due to gene mutations or horizontal gene transfer [2]. Multidrug resistant (MDR) bacteria may have a number of drug resistance genes and bacterial enzymes. Human morbidity and death rates have increased significantly as a result of the multidrug resistant *E. coli* strains that have emerged quickly [3]. Beta-lactamases are bacterial enzymes that give *E. coli* resistance to beta-lactam antibiotics like penicillin and cephalosporins by hydrolyzing the beta-lactam ring. Extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamases are two of the new varieties of beta-lactamase enzymes that have evolved (Babic et al., 2006); [4,5]. The following resistance genes are the most often discovered beta-lactamases in Gram-negative bacteria: TEM, SHV, OXA, CMY, and CTX-M beta-lactamases [5]. The majority of ESBLs and AmpCs are found on mobile genetic elements (plasmids, transporons or intergrons). Conjugation, transformation, and transduction have been found to contain mobile genetic components that facilitate horizontal gene transfer techniques used to spread bacterial cells to other organisms (Livermore et al., 2006). There is an increasing concern for public health over multi-drug resistance enterohemorrhagic *E. coli,* which develops resistant genes and bacterial enzymes [1]. An evergreen, fragrant tree belonging to the Annonaceae family*, Xylopia aethiolipica* which can reach a height of 20 meters. It is indigenous to Africa's savanna zones, lowland rainforests, and moist fringe forests. Common names for the plant include "spice tree," "Africa pepper," "Ethiopian pepper," and "Guinea pepper." The fruits are both highly nutritious and therapeutic [6]. Both as a spice and an herbal remedy, the dried fruits and leaves are employed. African food, traditional medicine, and construction all make substantial use of *X. aethiolipica* [6]. An infusion of the plant's bark or fruit has been effective in treating bronchitis, dysentery, and toothaches by acting as a mouthwash. Additionally, it has been used as a treatment for fever and biliousness. The bark is used to treat rheumatism, stomachaches, and asthma when steeped in palm wine [7]. The fruits are added to other remedies as a stimulant and carminative, as well as a cough remedy. The root's powder is used as a dressing and a local cancer treatment. It has been discovered that the fruit of the plant *X. aethiolipica* contains diterpenes known as kauranes, including xylopic acid and kauremoic acid [8,9]. The fruit-bearing tree *Morinda citrifolia,* sometimes known as the "Noni plant," is a member of the Rubiaceae family of coffee plants. The pungent, vomit-like scent of fresh fruit has turned it into a famine food in most areas, although it is still a common diet in some cultures and has been employed in traditional medicine [10]. Additionally, it has been made available as a supplement in a variety of forms, including juices, skin care products, and pills. Fruits, seeds, barks, leaves, and flowers from *M. citrifolia* are used separately for their specific nutritional and medicinal benefits. *M. citrifolia* is becoming more and more well-liked around the world as a dietary supplement, a food functional ingredient, or a natural health booster [11]. However, *M. citrifolia* is thought to have the highest concentration of chemical compounds and phytochemicals that have immune-stimulating, analgesic, hypotensive, anti-inflammatory, antiviral, antifungal, and anticancer properties [12]. As a result, this investigation revealed genetic makeup of EHEC strains and curing of resistant EHEC found in some fecal and water samples from the study area, both of which constitute a serious risk to the public health.

2. MATERIALS AND METHODS

2.1 Collection and Transport of Samples

A total of 84 samples, including 21 samples of human squat, 21 samples of cow feces, 21 samples of chicken droppings, and 21 samples of water were collected from four (4) distinct sources. All samples were collected in sterile universal sample vials and transported aseptically to the bacteriological analysis facility at Rivers State University as soon as possible.

2.2 *E. coli* Plate Counting and Isolation

After the collection of samples, each sample was diluted appropriately, and an aliquot (0.1 ml) of each was placed in duplicate on sterile MacConkey and Eosin Methylene Blue (EMB) agar plates and incubated at appropriate temperatures. After incubation, the plates were counted and isolates of *E. coli* were sub cultured and preserved for further identifications of enterohemorrhagic strain of *E. coli* (Karch et al., 1996); [13].

2.3 *E. coli* Enterohemorrhagic Strain Identification (*E. coli* O157:H7)

For some of the assays used to identify *E. coli* O157:H7, sorbitol MacConkey agar and the sorbitol fermentation test were used. After identification, eleven (11) isolates were identified as EHEC [14-16].

2.4 Agar Disk Diffusion Method (Kirby Bauer's Method)

This method was done according to Kibret et al. [17]. The 11 EHEC isolates were subjected into sterile normal saline tubes whose turbidity was equivalent to 0.5 m McFarland Turbidity Standard. To ensure that the organism was distributed evenly, swab ticks were used to swab the surface of the petri dish, which was filled with Mueller Hinton agar that had already been prepared. The agar was left for three to five minutes to dry. The impregnated antimicrobial discs were equally distributed on the surface of the inoculation plate using sterile forceps, 15 mm from the plate's edge. Each disc was pressed down slightly to establish contact with the agar using the forceps' head. The plates were incubated in an inverted position aerobically at 35°C for 24 hours after the discs were applied. The test plates were checked for growth after incubation. Each plate's zone of inhibition was measured and reported in terms of its diameter (mm) [18].

2.5 Molecular Characterization of EHEC Isolates

2.5.1 Extraction of Bacterial Genomic DNA

The seven (7) most resistant EHEC isolates were cultured in Luria Bertani (LB) broth overnight and five milliliters (5 mils) of LB broth was spun at 14000 revolution per minutes (rpm) for three minutes. The bacteria were then heated for 20 minutes at a temperature of roughly 95°C before being re-suspended in five hundred microliters (500 µls) of normal saline. The heated bacterial suspension was spun for three minutes at 14000 rpm after cooling on ice. After that, the DNA-containing supernatant was transferred to a 1.5 mils microcentrifuge tube and kept at -20°C for further reactions.

2.5.2 Quantification of DNA

A Nanodrop 1000 spectrophotometer was used to measure the genomic DNA of the isolated bacterial isolates. By clicking twice on the Nanodrop icon, the equipment software was launched. The apparatus was initialized with around 2µls of sterile distilled water, and blanked with normal saline. The isolated bacterial DNA in a volume of two microliters was placed on the lower pedestal. The isolated bacterial DNA on the lower pedestal was brought into contact with the higher pedestal. The concentration of the DNA was measured by clicking the "measure" button that was visible on the computer screen.

2.5.3 Amplification of 16S rRNA

Using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3 and 1492R: 5'-CGGTTACCTTGTTACGACTT-3 primers on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microliters for 35 cycles, the 16s RRNA region of the rRNA genes of the isolates was amplified. The X2 Dream Taq Master mix from Inqaba, South Africa (Taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4M, and the extracted DNA as template were all ingredients in the PCR mixture. The following were the PCR conditions: Initial denaturation took place at 95°C for 5 minutes, followed by subsequent denaturations at 95°C for 30 seconds, 52°C for 30 seconds, extension for 35 cycles, and 72°C for 5 minutes of final extension. The result was visualized on a UV transilluminator after being resolved on a 1% agarose gel at 120V for 15 minutes.

2.5.4 DNA Sequencing

Inqaba Biotechnological, Pretoria, South Africa, performed the sequencing using a 3510 ABI sequencer and the BigDye Terminator kit. The BigDye® terminator v1.1/v3.1, 0.25 µl of the 10µM Primer PCR primer, 2.25 µl of the 5 x BigDye sequencing buffer, and 2 to 10 nanogram of PCR template per 100 bp were used in the sequencing, which was done at a final volume of 10 µl. The following were the sequencing requirements: There are 32 cycles of 96°C for 10s, 55°C for 5s, and 60°C for 4 minutes.

2.5.5 Phylogenetic analysis

The bioinformatics algorithm Trace edit was used to alter the obtained sequences, and BLASTN was used to retrieve related sequences from the National Center for Biotechnology Information (NCBI) database. Utilizing ClustalX, these sequences were aligned. The Neighbor-Joining approach in MEGA 6.0 was used to infer the evolutionary history [19]. The evolutionary history of the organisms under study is assumed to be represented by the bootstrap consensus tree generated from 500 replicates [20]. The Jukes-Cantor technique was used to compute the evolutionary distances [21].

2.5.6 Amplification of AmpC and *bla*$_{TEM}$ genes

The AmpC and bla$_{TEM}$ genes were amplified using the methods described by Bell et al. [22].

2.5.7 Use of acridine orange as curing agent

EHEC isolates were inoculated into a Mueller Hinton Broth (MHB) overnight using pure cultures. The culture broth was then given a dose of the chosen curing agent (Acridine Orange) that ranged from 0.1 to 0.5 mg/ml. The cultures were then kept constantly moving throughout an overnight incubation at 37°C for 24 hours. Antibiotic resistance was discovered through testing for antibiotic susceptibility after the treatment. This was done to treat the bacterium and preserve its resistance to antibiotics that had previously been used (Chigor et al., 2010); [23].

2.5.8 Collection of plant leaves

At the Department of Botany at Rivers State University, leaves of *Xylopia aethiopica* and *Morinda citrifolia* were identified and authenticated as belonging to those species. The leaves were collected in the morning from mature trees that were in the flowering stage. In the laboratory, freshly collected leaves of the *X. aethiopica* and *M. citrifolia* species were cleaned, let to air dry for 14 days, and then sieved to produce fine powder samples.

2.5.9 Preparation of plant extracts

Ade-Ademilua's [24] approach was used to create aqueous extracts of the plant leaves. The plant leaves were extracted aqueously by suspending twenty grams (20 g) of finely ground leaf samples in two hundred milliliters (200 mils) of distilled water at a time for 48 hours. The aqueous extract was made in a water bath at 75°C for 1.5 hours after a preliminary O-test revealed that it had no effect on the test organisms while it was at 25°C. The extracts were decanted and filtered through Whatman filter paper No 10 after the aqueous extraction was carried out at room temperature. The membrane filter was used to sterilize the extracted fluids, and they were then dried at 40°C. Reconstituted residues were kept in the refrigerator after being reconstituted in distilled water at stock concentrations of 1.0 to 0.25 grams per milliliter (g/ml) [25].

2.5.10 Determination of plant extracts concentrations

The concentrations of the plant extracts were calculated using stock solutions of the aqueous extracts. To obtain the succeeding concentrations of 1g/ml, 0.75 g/ml, 0.5 g/ml, and 0.25 g/ml, add about 0.1 mil of the extracts to 10 ml, 7.5 ml, 5 ml, and 2.5 ml of distilled water.

2.5.11 Use of plant extracts as curing agents

For this investigation, aqueous extracts of the leaves of *Morinda citrifolia* and *Xylopia aethiopica* were employed. This was done using the techniques outlined by Iheukwumere et al. [25]. The size of the inoculum was determined to be one milliliter of suspended broth solution (1.0×10^8 mil) of each resistant bacterial culture, which was then inoculated into Muller Hinton broth (MHB) and incubated for 24 hours at 37°C. To measure concentrations the agents, the culture was added to a set of test tubes containing, respectively, 1 g/ml, 0.75 g/ml, 0.5 g/ml, and 0.25 g/ml of plant extracts. The test tubes were then incubated at 37°C for 24 hours. Colonies were chosen and inoculated into freshly made Muller Hinton agar plates after 0.1-millimeter (0.1mil) aliquots from each test tube were put into nutrient agar plate and incubated. The same antibiotic discs were then aseptically

inserted into the plate, making sure that they made proper contact with the agar surface. The plates were incubated for 24 hours at 37°C after which plates were examined for cured resistant to previous antibiotic discs [25,26].

3. RESULTS AND DISCUSSION

3.1 Results

The results of the molecular characterization of the seven (7) most resistant EHEC isolates as shown in Plate 1 revealed the 16S rRNA of the following isolates, including HSRSU4, HSRSU14, PDBC6, PDBC13, WSR6, WSR20, and CDM5, exhibited 100% similarity to other species. In accordance with the phylogenetic placement of the isolates' 16S rRNA within the *Escherichia* spp., the evolutionary distances computed using the Jukes-Cantor technique revealed a close relatedness to *Escherichia coli* strains 026:H11, 091:H21, 0157:H7, 0111:H8, and 0157:Non-Motile (NM), as shown in Fig. 1.

Results of detection of resistant genes as shown in Plates 2 and 3 revealed that the 7 resistant EHEC isolates that were subjected to analysis had 5 (71.4%) of AmpC gene and 3 (42.9%) of bla$_{TEM}$ gene.

Results shown in Table 1 revealed that Amp*C* gene was detected in high numbers in water samples and human squat, but it was absent from chicken droppings while bla$_{TEM}$ Gene was detected in cow feces, chicken droppings, water samples, but was not found in human squat.

Results of susceptibility patterns of EHEC isolates were shown in Tables 2 and 3 where Table 2 revealed a greater proportion of EHEC isolates were resistant to cefuroxime (90.91%), meropenem (90.91%), cefotaxime (72.73%), and ceftazidime (54.55%), while susceptibility to the following drugs decreased in that order: gentamicin (100%), chloramphenicol (100%) > tetracycline (63.64%), cotrimoxazole (63.64%) > vancomycin (54.55%) while Table 3 revealed the percentage of the susceptibility patterns of each EHEC isolates and number of antibiotics used.

Results of percentage resistant patterns of test EHEC isolates as shown in Table 4 revealed that aqueous extracts of *Xylopia aethiopica* and *Morinda citrifolia* and chemical agent (acridine orange) exhibited a decrease in the total percentage of multiple antibiotics resistance EHEC from 35.61% to 10.61%, 18.18%, and 16.67%, respectively.
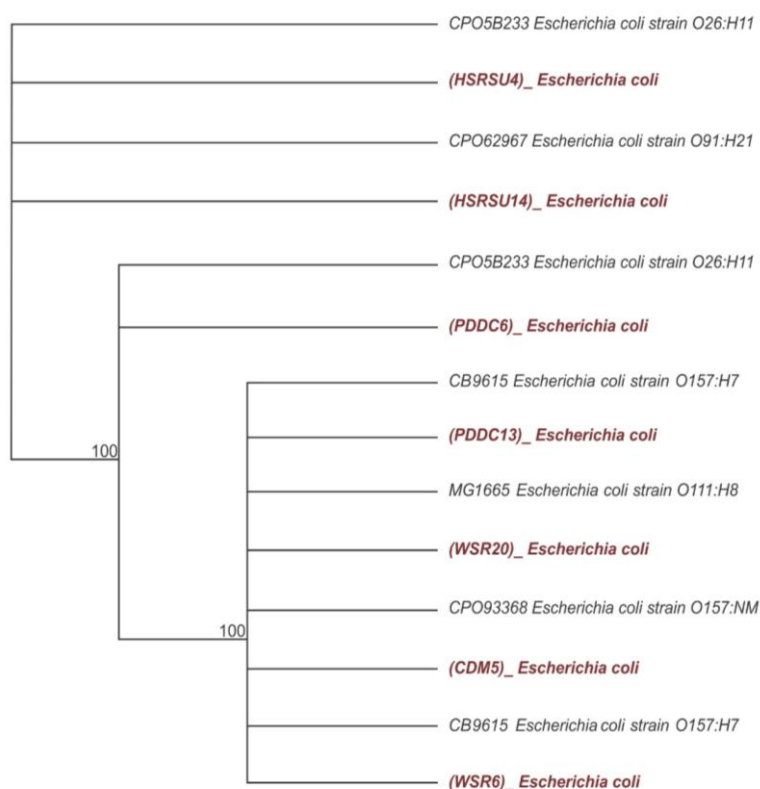

Fig. 1. Phylogenetic Tree displaying the evolutionary distance between the EHEC Isolates
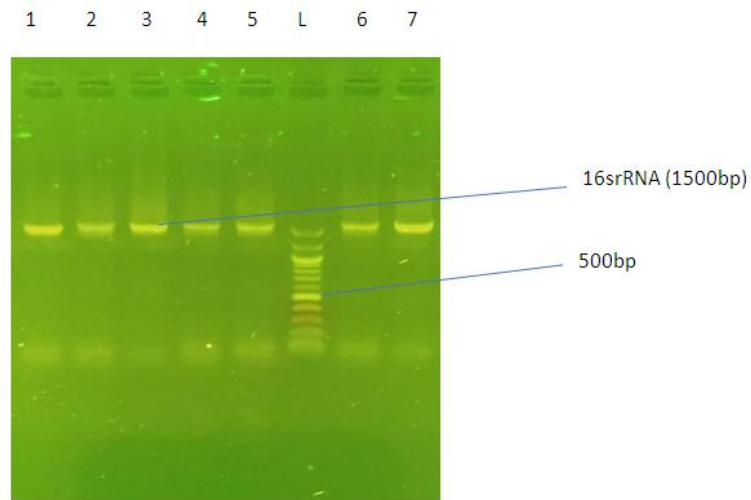
Plate 1. Agarose Gel Electrophoresis displaying the 16SrRNA bands. Lane 1-7 showing the 16SrRNA bands at 1500bp while lane L represents the 100bp molecular ladder
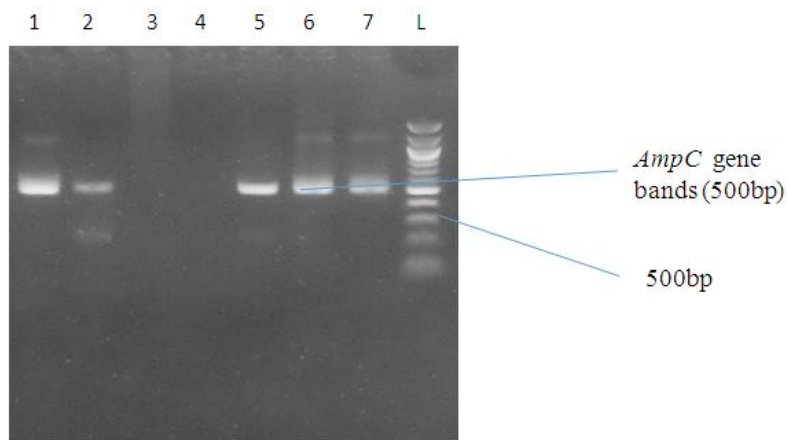

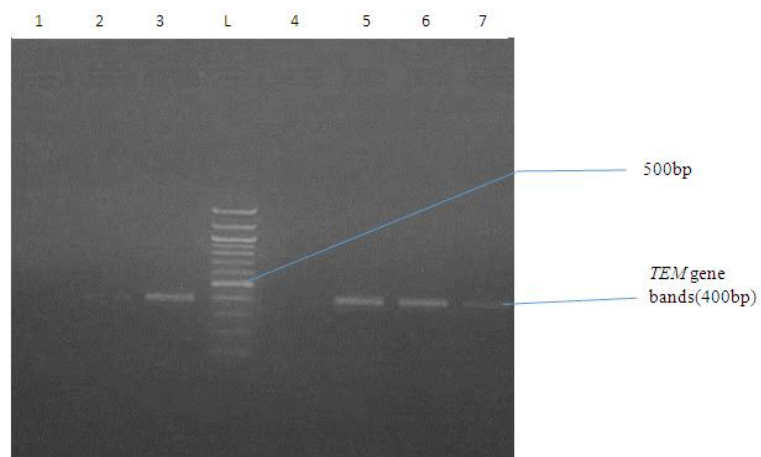**Plate 2. Agarose gel electrophoresis displaying the amplified *AmpC* gene of the 7 EHEC strains**

Plate 3. Agarose gel electrophoresis displaying the amplified *bla*TEM Gene of the 7 EHEC strains

Table 1. Detection of resistant genes in EHEC Isolates from all the samples

(N=7)

Samples/Specimen	Resistant Genes	
	*Amp*C Gene n (%)	*bla*TEM Gene n (%)
Cow Feces	1(20.00)	1(33.33)
Chicken Droppings	0(0.00)	1(33.33)
Water Samples	2(40.00)	1(33.33)
Human Squat	2(40.00)	0(0.00)
Total	5(71.43)	3(42.86)

KEY: N (Number of resistant EHEC Isolates), n (number of isolates present), % (percentage)

Table 2. Susceptibility pattern of enterohemorrhagic *Escherichia coli* (EHEC) from all samples analyzed

(N=11)

Antibiotics Conc. (µg)	Resistantn (%)	Intermediaten (%)	Susceptiblen (%)
Tetracycline (10)	0(0.00)	4(36.36)	7(63.64)
Cotrimoxazole (25)	1(9.09)	3(27.27)	7(63.64)
Gentamycin (10)	0(0.00)	0(0.00)	11(100)
Cefuroxime (30)	10(90.91)	0(0.00)	1(9.09)
Chloramphenicol (10)	0(0.00)	0(0.00)	11(100)
Ceftriaxone (30)	3(27.27)	4(36.36)	4(36.36)
Cefotaxime (30)	8(72.73)	3(27.27)	0(0.00)
Ciprofloxacin (5)	4(36.36)	7(63.64)	0(0.00)
Amikacin (30)	0(0.00)	10(90.91)	1(9.09)
Vancomycin (30)	5(45.45)	0(0.00)	6(54.55)
Ceftazidime (30)	6(54.55)	3(27.27)	2(18.18)
Meropenem (10)	10(90.91)	0(0.00)	1(9.09)

Table 3. Susceptibility pattern of EHEC isolates

S/N	Isolate Code	Total Number of Antibiotics N (%)	Susceptiblen (%)	Resistantn (%)
1	**HSRSU1**	12 (100)	4 (33.33)	4 (33.33)
2	**HSRSU4**	12 (100)	5 (41.67)	5 (41.67)
3	**CDM1**	12 (100)	8 (66.67)	0 (0.00)
4	**CDM5**	12 (100)	6 (50.00)	4 (33.33)
5	**PDBC6**	12 (100)	5 (41.67)	4 (33.33)
6	**WSR6**	12 (100)	3 (25.00)	6 (50.00)
7	**PDBC13**	12 (100)	3 (25.00)	5 (41.67)
8	**HSRSU14**	12 (100)	4 (33.33)	7 (58.33)
9	**PDBC21**	12 (100)	5 (41.67)	4 (33.33)
10	**WSR20**	12 (100)	3 (25.00)	5 (41.67)
11	**WSR21**	12 (100)	5 (41.67)	3 (25.00)
	Total	132 (100)	49 (37.12)	47 (35.61)

KEY: N (Total number of antibiotics), % (Percentage)

Table 4. Percentage resistant patterns of test isolates after plasmid curing with curing agents

S/N	Isolate Code	Resistant	*Xylopia aethiopica*	Curing agents used *Morinda citrifolia*	Acridine orange
		n (%)	n (%)	n (%)	n (%)
1	**HSRSU1**	**4 (33.33)**	2 (16.67)	2 (16.67)	2 (16.67)
2	**HSRSU4**	**5 (41.67)**	2 (16.67)	4 (33.33)	2 (16.67)
3	**CDM1**	**0 (0.00)**	0 (0.00)	0 (0.00)	0 (0.00)
4	**CDM5**	**4 (33.33)**	0 (0.00)	2 (16.67)	1 (8.33)
5	**PDBC6**	**4 (33.33)**	0 (0.00)	2 (16.67)	3 (25.00)
6	**WSR6**	**6 (50.00)**	3 (25.00)	4 (33.33)	4 (33.33)

S/N	Isolate Code	Resistant	*Xylopia aethiopica*	Curing agents used *Morinda citrifolia*	Acridine orange
		n (%)	n (%)	n (%)	n (%)
7	PDBC13	5 (41.67)	1 (8.33)	3 (25.00)	2 (16.67)
8	HSRSU14	7 (58.33)	3 (25.00)	2 (16.67)	3 (25.00)
9	PDBC21	4 (33.33)	0 (0.00)	0 (0.00)	1 (8.33)
10	WSR20	5 (41.67)	3 (25.00)	4 (33.33)	2 (16.67)
11	WSR21	3 (25.00)	0 (0.00)	1 (8.33)	2 (16.67)
	Total	47 (35.61)	14 (10.61)	24 (18.18)	22 (16.67)

KEY: HSRSU (Human Squat Rivers State University), PDBC (Poultry Dropping Bori Camp), CDM (Cow Dung Mgbuoshimini), WSR (Water Sample Rumuokoro), n (number of resistant to antibiotics)

3.2 Discussion

EHEC isolates were 100% validated by the molecular analysis of the seven (7) most resistant isolates in this investigation using the PCR technique. The phylogenetic placement of the isolates within the *Escherichia* spp. in this investigation revealed a close relatedness to *Escherichia coli* strains 026:H11, 091:H21, 0157:H7, 0111:H8, and 0157: NM. The evolutionary distances computed using the Jukes-Cantor technique were in agreement with this investigation. The AmpC gene and bla_{TEM} gene, which are the most common genes in *E. coli* that confer resistance, were analyzed for the presence of beta-lactamase genes in the resistant EHEC isolates. The results showed that the AmpC gene had 71.4% and the bla_{TEM} gene had 42.9% of these genes present in their genomes. AmpC, bla_{CTX-M}, bla_{SHV}, and bla_{TEM} are only a few of the beta-lactamase genes that have been identified in hospital and municipal wastewater effluents (Korzeniewska et al., 2013). The amplified AmpC gene of the seven EHEC isolates with the highest antibiotic resistance was shown by Agarose gel electrophoresis in this investigation. Similar to this, the amplified bla_{TEM} gene of the seven EHEC isolates with the highest antibiotic resistance was also visible on Agarose gel electrophoresis. It is well known that the extended spectrum beta Lactamase genes (AmpC and bla_{TEM}) are to responsible for the EHEC isolates' resistance to the penicillin and cephalosporin antibiotics utilized in the study, and their overproduction can make isolates entirely resistant to these drugs [27]. The bla_{TEM} gene produces the bla_{TEM} beta-lactamase enzyme, which breaks down the beta-lactam ring of antibiotics and inhibits their activity, while the ampC gene produces the ampC beta-lactamase enzyme, which confers resistance to penicillins, second and third generation cephalosporins, and cephamycins [28].

According to this study's analysis of the inhibitory effects of chemical agent and plant extracts on resistant EHEC isolates, the proportion of multiple antibiotic-resistance bacteria was reduced by aqueous extracts of *Xylopia aethiopica* and *Morinda citrifolia*. The extracts of *M. citrifolia* had the least impact on the resistant EHEC isolates. *X. aethiopica* and acridine orange, which served as the control, had the highest potential for plasmid cure on the isolates. It was shown that the extract of *X. aethiopica* had the strongest curative effects on the isolates CDM5, PDBC6, PDBC21, and WSR21, reducing the prevalence of multiple antibiotic resistance to zero percentage. Similar outcomes were seen when PDBC21 isolate was tested against *M. citrifolia* extract. The total percentage of multiple antibiotic resistance in EHEC strains was lowered by the extracts of *X. aethiopica*, *M. citrifolia*, and Acridine Orange used as the control from 35.61% to 10.61%, 18.18%, and 16.67%, respectively, according to the overall findings. According to studies, *X. aethiopica* exhibits a high level of antibacterial activity against isolates of *Escherichia coli* and *Staphylococcus aureus* from patients with gastroenteritis [29].

4. CONCLUSIONS

The discovery of EHEC in water and fecal samples revealed the presence of coliforms and suggested that food poisoning is a likely result, which is a significant reason for public health action. In this investigation, it was discovered that the presence of the AmpC and bla_{TEM} genes in some of the isolates may contribute to EHEC's antibiotic resistance. This study has demonstrated that the extracts of *Xylopia aethiopica, Morinda citrifolia*, and acridine orange all have considerable potentials for treating plasmid-mediated antibiotic resistance in EHEC strains, with *X. aethiopica* having the most potential.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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