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# Determination of Virulence Genes in Extended Spectrum Beta Lactamase-Producing *Escherichia coli* Isolated from Fresh Leafy Vegetables Sold in Rivers State, Nigeria

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

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## ABSTRACT

Extended-spectrum β-lactamases (ESBL'S) are a rapidly growing group of β-lactamases which have the ability to hydrolyze third generation antibiotics such as Cephalosporins and Aztreonam. The aim of this study is the determination of virulence genes in ESBL- producing *Escherichia. coli* isolated from cabbage, spinach and water leaf. but are inhibited by Clavulanic Acid. The randomised experimental design was used to collect cabbage (*Brassica oleracea*), water leaf (*Hydrophyllum macrophyllum*) and spinach (*Spinacia oleracea*) samples from retailer sellers in two different markets (Choba and Rumuosi daily markets) of Obio/Akpor Local Government Area, Rivers State.

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Water leaf samples had a mean count of $2.9\times10^3$, spinach sample mean count was $2.8\times10^4$ Cfu/g and the highest, while the lowest mean count was obtained from cabbage samples; $1.6\times10^3$ Cfu/g. The study has established the presence of pathogenic *E. coli* (Enterohaemorrhagic) which are Shiga toxin producers that are Highly virulent with low infections doses.

*Keywords: Virulence genes; shiga toxin; bacterial pathogens; antibiotics.*

## 1. INTRODUCTION

"Bacterial pathogens of public health importance such as *Escherichia coli 0157:H7*, *Salmonella, Shigella*, *Staphylococcus aureus* and *Clostridium spp*. have been isolated from fresh-cut ready to eat fruits, vegetables and ready-to-eat foods sold on the streets, markets, schools, major cities and fast-food joints/restaurants in Nigeria" [1]. Marnizume et al*.* [2] and Sugiyama et al*.* [3] described « *Escherichia coli* as a facultative anaerobic gram-negative rod-shaped bacterium that can causes infection in both humans and animals". Doyle et al. [4] in an earlier work reported that "majority of the bacterial disease transmission occurs through eating of undercooked contaminated ground meat, consumption of raw milk, cooked and raw vegetables, fruits contaminated by water, cheese, curd and also through consumption of sprouts, lettuce and juice".

"Extended-spectrum β-lactamases (ESBL'S) are a rapidly growing group of β-lactamases which have the ability to hydrolyze third generation antibiotics such as Cephalosporins and Aztreonam, but are inhibited by Clavulanic Acid. They represent the first example in which β-lactamase–mediated resistance to β-lactam antibiotics resulted from fundamental changes in the substrate spectra of the enzymes" [5]. "At present, β-lactam drugs are a key factor in the treatment of bacterial infections worldwide and account for almost 65% of antibiotic usage" (Patel, 2018). "They have been classified into six main groups based on the chemical structure of the β-lactam ring which includes Penicillins, Cephalosporins, Cephamycins, Carbapenems, Monobactams, and β-lactamase inhibitors. These drugs block cell wall synthesis by preventing accurate working of the Penicillin-binding protein (PBP), which has a principal role in the synthesis of the bacterial cell wall, and finally leads to cellular death" (Patel, 2018). "Nevertheless, it is unfortunate that, in recent years, resistance to this important class of antibiotics is also increasing globally. ESBLs are mostly produced by gram-negative bacilli, especially *Enterobacteriaceae* family. Enterobacteriaceae, especially *Escherichia coli, Salmonella spp., Shigella spp*. and *Vibrio cholerae"* [6]. "ESBLs are often encoded by genes located on large plasmids, and these also carry genes for resistance to other antimicrobial agents such as aminoglycosides, trimethoprim, sulphonamides, tetracyclines and chloramphenicol" [7].

After the widespread use of the expanded-spectrum cephalosporins, strains of *Klebsiella pneumoniae* that were resistant to these new drugs began to appear [8], resistance spread to some strains of *Escherichia coli* and, in rare instances, to other gram-negative organisms as well. Investigation into the mechanisms responsible for this resistance revealed the presence of new forms of the older plasmid-mediated TEM (plasmid encoded with β lactamase in gram negative bacteria) and SHV (sulf-hydryl variable active site) β-lactamases. "Mutations within the structural genes encoding the older enzymes had occurred, giving rise to derivatives that possessed an extended substrate profile compared with that of the parental enzymes. Thus, these new enzymes were given the name Extended-Spectrum β-Lactamases (ESBLs) to reflect the fact that they were derivatives of older enzymes and had a new capability to hydrolyze a broader spectrum of b-lactam drugs. The development of resistance to antimicrobial is known to occur through stable genetic change heritable from generation to generation through specific mechanisms including mutation, transduction, transformation and or conjugation" [9].

"*Escherichia coli*, an important gastrointestinal flora known to be capable of accepting and transferring plasmids and which under stress readily transfers those plasmids to other species, is therefore considered an important reservoir of transferable antibiotic resistance genes" (Rahman, 2018). Investigation by CDC indicates that 10.8% of a zero type of this bacterial (e.g *E coli* 0157:H7) tested in 2006 have become resistant to one or more antibiotics

(CDC,2006). Several outbreaks of *E. coli* O157, 0157:H7 and other sero type of this bacterial

have been epidemiologically attributed to consumption of contaminated fresh and cooked vegetables and meat products [9]. The aim of this study is the determination of virulence genes in ESBL- producing *Escherichia. coli* isolated from cabbage, spinach and water leaf.

## 2. METHODOLOGY

### 2.1 Research Design

The randomised experimental design was used to collect cabbage (*Brassica oleracea*) , broad leaf water leaf (*Hydrophyllum macrophyllum*) and spinach (*Spinacia oleracea*) samples from retail sellers in two different markets (Choba and Rumuosi daily markets) of Obio/Akpor Local Government Area, Rivers State. All samples analyzed were within a period of two weeks, the samples were held at 4$^{o}$c for not more than 30min before analysis

### 2.2 Collection of Samples

A total of 30 samples comprising 10 for each of fresh cabbage, spinach and water leaves were randomly bought from two retail markets (Choba and Rumuosi) located in Port-Harcourt, Rivers State. The vegetable samples were bought from the table of the retailers and kept in a sterile polythene bags. It was immediately transferred into the Laboratory for analysis during the morning hours of the day for five days.

### 2.3 Methods of Samples Analysis

#### 2.3.1 Pre enrichment

The vegetable samples were rinsed with sterile distilled water then twenty-five grams (25gms) of each of three leaf sample was agitated differently in a conical flask containing 225mL of 0.1% of peptone water in (Oxoid UK) for 2 min.The homogenate was incubated at 35$^{0}$C for 6 hours.

#### 2.3.2 Isolation of *Escherichia coli*

Tenfold serial dilutions of the pre-enriched leaf samples using sterile 0.1% peptone water as diluent, was made after 6 hours of pre-enrichment. Aseptically 1ml of the homogenized sample was collected using a sterile pipette into 9mL of the sterile diluent which resulted into dilution $10^{-1}$, from the dilution $10^{-1}$, 1mL was taken into another 9mL diluent to make dilution $10^{-2}$ this process continued until dilution $10^{-6}$ was obtained. 0.1mL from each dilution was plated on MacConkey agar and Eosin methylene blue agar respectively and incubated at 35$^{o}$C for 24 hours. After the incubation period, the green metallic sheen colonies on Eosin Methylene Blue Agar presumptively identified as *E. coli* were collected and sub cultured for purification and stored on nutrient Agar slants for preservation. Identification of the isolates was by Grams Staining, and Biochemical Tests.

### 2.4 Identification of Isolates

#### 2.4.1 Gram staining grams staining

A loopful of eighteen-hour old broth culture of the test isolate was obtained with a sterile wire loop and placed on a clean glass slide to make a smear. The smear was heat fixed by passing it through the bursen burner flame intermittently for 5secs., the smear was stained with crystal violet for 60 seconds, rinsed with water and covered with grams iodine for 60second, the smear was decolorized with 70% ethanol and washed off then counter stained with safranin for 30 seconds and washed off. It was blotted dried and viewed at x100 power (oil immersion) of the microscope.

#### 2.4.2 Biochemical test

*2.4.2.1 Citrate utilization test*

This test determines the ability of an organism to utilize Citrate as sole carbon and energy source for growth and ammonium salt as the sole source of nitrogen. Simon citrate agar slant was inoculated with the 18 hours old culture of the test organism, the slant was streaked with a sterile wire loop while the butt of the medium was stab with a sterile inoculating needle and incubated at 37$^{o}$C for 48hrs. Change in color of the medium from green to blue indicates a positive result. Cheesbrough, 1994).

#### 2.4.3 Indole production

The test determines the ability of certain bacteria to decompose the amino acid tryptophan to indole which accumulates in the medium. Sterile peptone water medium was inoculated with 24 hours old culture of the test isolate and incubated at 37$^{o}$C for 48hrs. After incubation, 0.5mL of Kovacs reagent (p-dimethyl-amino benzaldehyde) was added and shaken gently. A red ring formation at the surface of the medium indicates a positive result (Cheesbrough, 1994).

#### 2.4.4 Methyl red –voges proskauer test

The test determines the end products of glucose metabolism in bacteria. Methyl Red positive organisms produce acid as their end- products

and VP positive organisms produces 2,3 – butanediol or acetyl methyl carbinol from fermentation of pyruvic acid. The 24 hours old culture of the test organism was inoculated into 5mL of sterile glucose phosphate medium using a sterile wire loop. The broth was incubated at 37$^{o}$C for 72 hours, after the incubation period the broth culture was divided into two parts, 5drops of methyl red indicator was added to a part for methyl red test, and red colour formation indicated a positive result.

For the VP test, 6 drops of 5% Alpha napthtol and 3 drops of 40% potassium hydroxide was added to the other half of the broth culture it was gently shaken and left to stand for about 30mins. Positive organisms changed the color of the medium to brick red (Cheesbrough, 1994).

#### 2.4.5 Motility test

This test was performed to determine the presence or absence of locomotive organelle characteristics such as flagella in bacteria. Tube method was used, with a sterile inoculating needle 18hour old culture of the test organism was stabbed at the middle of the Sterile semi solid medium, it was incubated at 37$^{o}$C for 48 hours, after the incubation period the tube was observed for growth, diffusion of growth away from the stabbed line indicates a positive test (Cheesbrough, 1994).

#### 2.4.6 Triple sugar iron agar

This test was used to indicate hydrogen sulfide $H_2S$ production in addition to glucose and lactose fermentation with or without gas production. A sterile tube of triple sugar iron agar was inoculated with the test organism by streaking the slant and stabbing the butt and incubated at 37$^{o}$C for 48 hours. Hydrogen sulphide production was indicated by blackening of the medium. Acid production was indicated by yellow color development while gas production was shown by a crack or a split within the medium due to the tension produced by the gas. Acid production within the slope region signifies lactose utilization while at the butt signifies glucose (Cheesbrough, 1994).

### 2.5 Antibiotic Sensitivity Screening of Positive Isolates of *E. coli*

Antibiotic sensitivity screening of test isolates was done using standard disk diffusion test method according to CLSI M100(2020).

#### 2.5.1 Inoculum preparation by direct colony suspension method

Five milliliters (5mls) of sterile 0.85% normal saline was prepared; a loopful of the test isolate (18 hour) was collected and inoculated into the saline to make a suspension. The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard (suspension contains approximately $1\times10^{8}$ CFU/mL), this was performed visually by using adequate light to visually compare the inoculum tube and the 0.5 McFarland standard against a card with a white background and contrasting black line.

#### 2.5.2 Disk diffusion test

Fifteen minutes after adjusting the turbidity of the inoculum suspension, a sterile swab stick was dipped into the adjusted suspension, the dry surface of the Mueller-Hinton agar was inoculated by streaking the swab over the entire sterile agar surface this was repeated twice to ensure even distribution of the inoculum. It was left for 15 minutes before applying the disc containing the different antibiotic and incubated at 37$^{o}$C for 18 hours. After 18 hours the plates were examined for inhibition and diameter of zone of inhibition measured. The measurements were translated as susceptible, intermediate, and resistant categories according to the zone interpretation table (CLSI). The presence of a growth inhibition zone larger than or equal to the established breakpoint diameter is an indication of susceptibility to that antimicrobial agent.

### 2.6 Phenotypic Detection of Extended Spectrum Beta Lactamase

#### 2.6.1 Kirby-bauer disk diffusion method (CLSI M100, 2020)

Detection of ESBL-producing organisms was performed by Double Disc Synergy Test (DDST) method following CLSI recommendations. In this method, first, a suspension of each of the pure bacterial isolate was prepared according to the 0.5 McFarland turbidity standard and cultured on Mueller–Hinton agar. Fifteen minutes after bacterial cultures, pairs of antibiotic disks containing Cefazidime (30 µg) with Cefazidime/Clavulanic acid (30/10 µg), and Cefotaxime (30 µg) with Cefotaxime/Clavulanic acid (30/10 µg) were placed on Mueller–Hinton agar medium center to center, at a distance of 20 mm apart from each other. The plates were incubated for 24 hours at 37°C. Thereafter, the

diameter of inhibition zone was measured. According to CLSI, (2020) guidelines, an increase of ≥5 mm in the zone diameter around the clavulanic acid combination disks versus the same disks alone confirmed the presence of ESBL producer strains.

## 2.7 Determination of *E. coli* Virulence Genes

### 2.7.1 DNA extraction

The extraction of DNA from the isolated *E. coli* was done using the boiling method. This was done by taking a loopful of the bacteria and suspended in 150mL of distilled water, the suspension was boiled at 100$^{o}$C for 10mins in a water bath and was placed on ice for 5mins, and centrifuged for 5minutes at 10,000g to obtain the supernatant. The supernatant was stored in an Eppendorf tube. The DNA was subjected to cocktail mix and condition for PCR. The PCR cycling for *E coli* strains consisted of 95$^{0}$C for 5min which was followed by 40 cycles of denaturing at 950c for 30s, annealing at 600c for 30s and elongation at 720c for 30s.

### 2.7.2 Gel electrophoresis

The presence or absence of band were assessed using gel electrophoresis with a potable gel hood built in blue LED (470nm) by Royal Biotech/Biolympics,1.5% agarose gel at a constant voltage, IX TBE for approximately 1 hour. They were visualized by ethidium bromide staining and photographed under ultraviolet light. the ladder used 100 base pair ladders from Thermofisher. All data obtained were analysed using Microsoft Excel.

## 3. RESULTS AND DISCUSSION

The various results obtained from the study are presented in the tables and figures listed below:

**Table 1. Total green metallic sheen colony counts of the three samples from the different locations**

| Sample | CFU/g | LOG CFU/g |
|---|---|---|
| Water leaf | $2.9\times10^{3}$ | 3.46 |
| Spinach | $2.8\times10^{4}$ | 4.51 |
| Cabbage | $1.6\times10^{3}$ | 3.20 |

*Keys;- CFU/g-Colony forming unit per gram*

**Table 2. Comparative distribution of biochemically and phenotypically confirmed *E. coli* isolated from the water leaf samples**

| Source of samples | No. of sample | No. with *E. coli* (%) | No. without *E. coli* (%) |
|---|---|---|---|
| CHOBA | 5 | 2(40%) | 3(60%) |
| RUMUOSI | 5 | 3(60%) | 2(40%) |
| Total | 10 | 5(50%) | 5(50%) |

**Table 3. A comparative distribution of biochemically and phenotypically confirmed *E. coli* isolated from the spinach samples**

| Source of samples | No. of sample | No. with *E. coli* (%) | No. without *E. coli* (%) |
|---|---|---|---|
| CHOBA | 5 | 4(80%) | 1(20%) |
| RUMUOSI | 5 | 3(60%) | 2(40%) |
| Total | 10 | 7(70%) | 3(30%) |

**Table 4. A comparative distribution of biochemically and phenotypically confirmed *E. coli* isolated from the cabbage samples**

| Source of samples | No. of sample | No. with *E. coli* (%) | No. without *E. coli* (%) |
|---|---|---|---|
| CHOBA | 5 | 2(40%) | 3(60%) |
| RUMUOSI | 5 | 0(0%) | 5(100%) |
| Total | 10 | 2(20%) | 8(80%) |

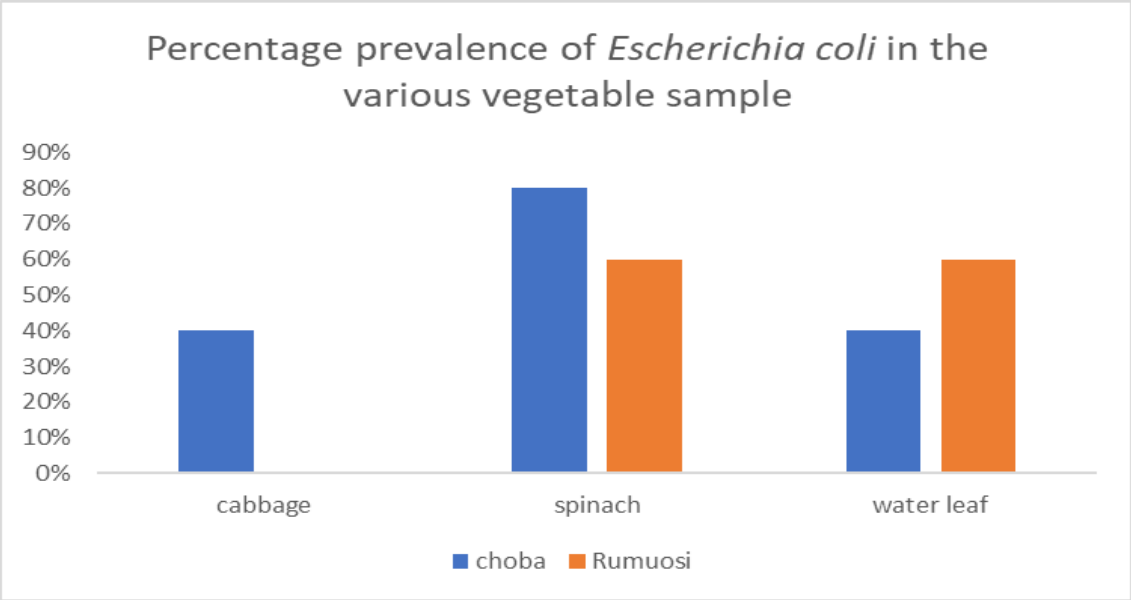


**Fig. 1. Percentage prevalence of *E. coli* in the various vegetable samples obtained from Choba and Rumuosi markets**

**Table 5. Antibiotics susceptibility pattern of *E. coli* isolated from the various vegetable samples n=14**

| Antibiotics | Resistant | | Sensitive | |
|---|---|---|---|---|
| | % of Positive | Inhibition zone (mm) | % of positive | inhibition zone (mm) |
| Tetracycline (30µg) | 9(64.3) | <14 | 5(35.7) | >18 |
| Ciprofloxacin (10µg) | 2 (14.3) | <24 | 12 (85.7) | >28 |
| Ampicillin (10µg) | 11(78.6) | <14 | 3 (21.4) | >16 |
| Contrimozazole (30µg) | 5(35.7) | <15 | 9(64.3) | >16 |
| Chloramphenicol (30µg) | 5(35.7) | <12 | 9(64.3) | >18 |
| Amikacin (30µg) | 6(42.9) | <14 | 8(57.1) | >17 |
| Ceftriazone (10µg) | 12(85.7) | <15 | 2(14.3) | >20 |
| Gentamicin (30µg) | 2(14.3) | <12 | 12(85.7) | >15 |
| Streptomycin (10µg) | 14 (100.0) | < 11 | 0 (0.0) | >15 |
| Nalidixic acid(30µg) | 6 (42.9) | <13 | 8 (57.1) | >16 |

**Table 6. Antibiotics susceptibility pattern of strains of *E. coli* isolated from spinach samples n=7**

| Antibiotics | Resistant | | Sensitive | |
|---|---|---|---|---|
| | % of Positive | Inhibition zone (mm) | % of positive | inhibition zone (mm) |
| Tetracycline (30µg) | 5(71.4) | <14 | 2 (28.6) | >18 |
| Ciprofloxacin (10µg) | 2(28.6) | <24 | 5 (71.4) | >28 |
| Ampicillin (10µg) | 4 (57.1) | <14 | 3 (42.9) | >16 |
| Contrimozazole (30µg) | 2 (28.6) | <15 | 5(71.4) | >16 |
| Chloramphenicol (30µg) | 0 (0) | <12 | 7(100) | >18 |
| Amikacin (30µg) | 2(28.6) | <14 | 5(71.4) | >17 |
| Ceftriazone (10µg) | 6( 85.7) | <15 | 1(14.3) | >20 |
| Gentamicin (30µg) | 2(28.6) | <12 | 5(71.4) | >15 |
| Streptomycin (10µg) | 0(100) | < 11 | 0 (0.0) | >15 |
| Nalidixic acid(30µg) | 3(42.9) | <13 | 4(55.7) | >16 |

**Plate 1. Shows different zone diameter of inhibition of different antibiotics the right side of the plate shows different diameter of inhibition**

**Table 7. Antibiotics susceptibility pattern of *E. coli* isolated from the cabbage samples n=2**

| Antibiotics | Resistant | | Sensitive | |
|---|---|---|---|---|
| | % of Positive | Inhibition zone (mm) | % of positive | inhibition zone (mm) |
| Tetracycline (30µg) | 1(50) | <14 | 1(50) | >18 |
| Ciprofloxacin (10µg) | 0(0) | <24 | 2(100) | >28 |
| Ampicillin (10µg) | 2(100) | <14 | 0( 0) | >16 |
| Contrimozazole (30µg) | 1(50.0) | <15 | 1(50) | >16 |
| Chloramphenicol (30µg) | 2(100) | <12 | 0(0) | >18 |
| Amikacin (30µg) | 1(50) | <14 | 1(50) | >17 |
| Ceftriazone (10µg) | 2(100) | <15 | 0(0) | >20 |
| Gentamicin (30µg) | 0(0) | <12 | 2(100) | >15 |
| Streptomycin (10µg) | 2(100) | < 11 | 0 (0) | >15 |
| Nalidixic acid(30µg) | 1( 50) | <13 | 1 (50) | >16 |

**Table 8. Antibiotics susceptibility pattern of strains of *E. coli* isolated from water leaf samples n=5**

| Antibiotics | Resistant | | Sensitive | |
|---|---|---|---|---|
| | % of Positive | Inhibition zone (mm) | % of positive | inhibition zone (mm) |
| Tetracycline (30µg) | 4(80.0) | <14 | 1(20.0) | >18 |
| Ciprofloxacin (10µg) | 1(20.0) | <24 | 4(80.0) | >28 |
| Ampicillin (10µg) | 3(60.0) | <14 | 2(40.0) | >16 |
| Contrimozazole (30µg) | 1(20.0) | <15 | 4(80.0) | >16 |
| Chloramphenicol (30µg) | 0(0) | <12 | 5(100.0) | >18 |
| Amikacin (30µg) | 1(20.0) | <14 | 4(80.0) | >17 |
| Ceftriazone (10µg) | 4(80.0) | <15 | 1(20.0) | >20 |
| Gentamicin (30µg) | 1(20.0) | <12 | 4(80.0) | >15 |
| Streptomycin (10µg) | 5(100.0) | < 11 | 0(0.0) | >15 |
| Nalidixic acid(30µg) | 2(40.0) | <13 | 3(60.0) | >16 |

*Extended beta lactamase production pattern of E. coli isolated from the various sample sources*

**Table 9. Numbers of *E. coli* isolates positive for ESBLs production**

| Location/Source of isolates | No (%) with Extended Spectrum βlactam |
|---|---|
| **CHOBA** | |
| Spinach | 3 (75%) |
| Cabbage | 1(50%) |
| Water leaf | 1(50%) |
| **RUMUOSI** | |
| Spinach | 1(33.3%) |
| Cabbage | 0(0%) |
| Water leaf | 1(33.3%) |

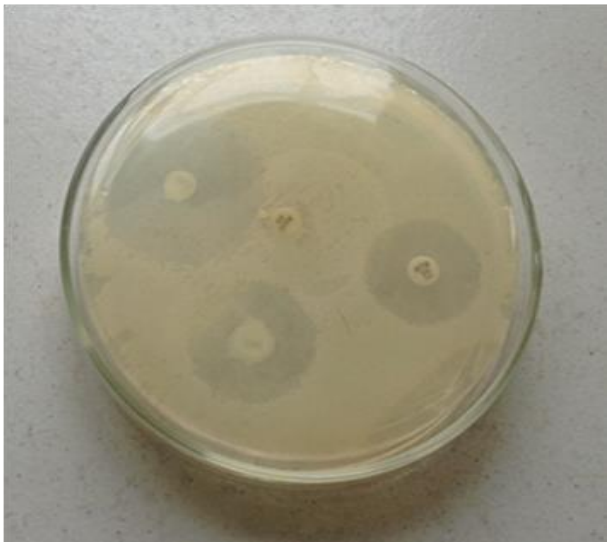

**Plate 2. Shows a negative result obtained by the disk diffusion test, absent of enhance zone of inhibition between one of the beta lactam disks and disk contains clavulanic acid (the center disk)**

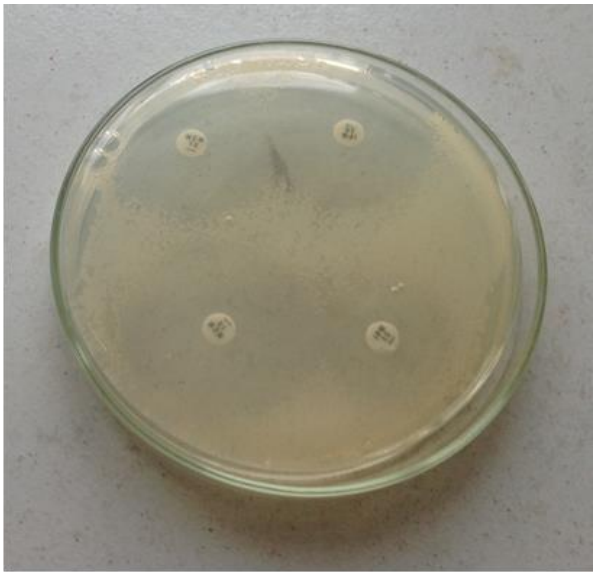

**Plate 3. Shows a positive result obtained by the 2-disk test, presence of enhance zone of inhibition between one of the beta lactam disks and disk contains clavulanic acid**

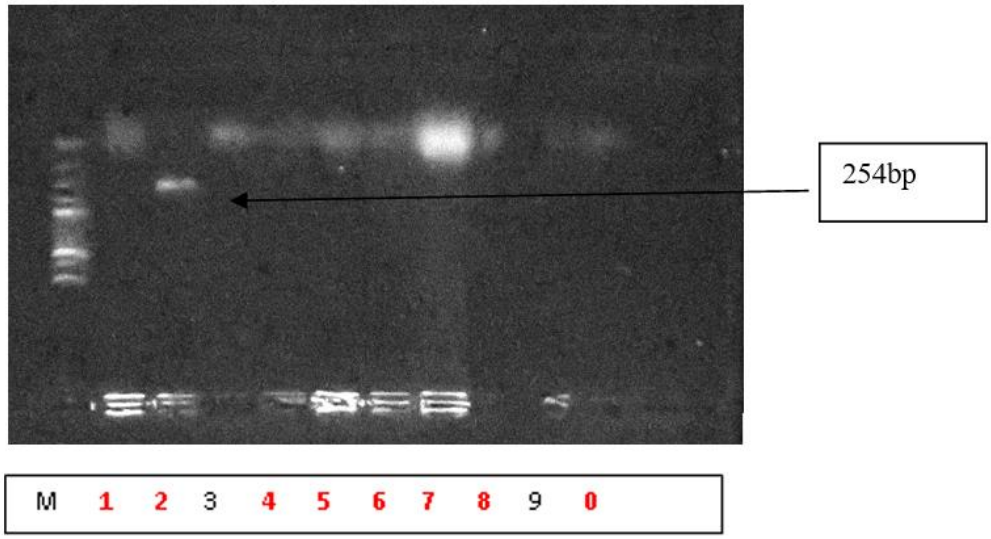


**Plate 4. Gel picture of east virulence gene for *E. coli* showing positive and negative isolates. M is 100bp DNA ladder. Isolates highlighted red are positive for east virulence gene while black are negative**

**Table 10. Primer sequences for *E. coli* virulence genes**

| Sub group | Target gene | Primer Nucleotide Sequence (5'- 3') | Amplicon size (bp) |
|---|---|---|---|
| EAEC | AggRk | F 5' GTATACACAAAAGAAGGAAGC 3'<br>R 5' ACAGAATCGTCAGCATCAGC 3' | 254bp |

The result of green metallic sheen colony on eosin methylene blue agar obtained from the three leaf samples is represented on Table1. Water leaf samples had a mean count of $2.9\times10^3$, spinach sample mean count was $2.8\times10^4$ CFU/g and the highest, while the lowest mean count was obtained from cabbage samples; $1.6\times10^3$ CFU/g. The Percentage prevalence of *E coli* in the various vegetable samples obtained from Choba and Rumuosi markets are presented in Fig. 1. Water leaf and cabbage samples obtained from Choba market had the same prevalence of 40%, while none was isolated from cabbage samples bought from Rumuosi market. Water leaf obtained from Rumuosi had 60% prevalence while Spinach samples obtained from Choba had prevalence of 80% which was the highest obtained from the two markets and the three leafy vegetables. Spinach samples was found to be most contaminated of all the three leafy vegetable samples with a mean count of $2.8\times10^4$CFU/g (Table 1) and highest prevalence rate of 80% (Table .4) while Cabbage samples was contaminated with a count of 1.6 $\times10^3$cfu/g (Table 1) and 40% prevalence (Table 4) which is the lowest. This might be due to the facts that the vegetable creeps on the soil. This result is consistence with the work of Atnafie et al. [10] whose study revealed that several strains of *E. coli* can be isolated from the surface of raw salad vegetables which includes spinach and cabbage. The low prevalence rate of *E. coli* in cabbage samples 2 (20%) out of 10 samples analyzed (Table 4) agreed with Seow et al. [11] who reported that *E. coli* was found cabbage and prevalence was less. The high mean count may indicate the poor handling practice during storage and point of selling. Several studies reported that the major source of microbial contamination was an anthropogenic disturbance in human, animals and irrigation water [12].

Table .5 shows the antibiotic resistance and susceptibility patterns of 14 isolates of *E. coli* isolated from the various sample sources. Resistance was highest for Streptomycin 14 (100%) tetracycline had 9 (64.2%) and sensitivity was highest with ciprofloxacin 12 (85.7%), and gentamicin 12 (85.7%). This agrees with the studies carried out by Musa et al. [13] who reported that all the isolates of *E.coli* from cow were susceptible to ciprofloxacin. Aibinu et al. [14] also reported that 12 (66.6%) out of 18 isolates from animal and human sources are susceptible to ciprofloxacin. Comparison of the

antibiotic resistant pattern of isolates from each sample type was shown in table 6-9. All the *E coli* isolated from spinach samples, exhibited 7 (100%) resistance to streptomycin. Isolates from cabbage also shows 2 (100%) resistance to ampicilin. Multi drug resistance was common amongst the isolates. Fifty percent of the all the isolates were resistant to five antibiotics. The *E. coli* isolated from this study displayed resistance to one or more antimicrobial agents including streptomycin, tetracycline, ampicillin, amikacin, and chloramphenicol. Resistance was highest for streptomycin 14 (100%) followed by ceftriaxone (88.5%) while susceptibility was highest for gentamycin (84%) and lowest for ceftriaxone (Table 4-8). The development of resistance to antimicrobial is known to occur through stable genetic change heritable from generation to generation through specific mechanisms including mutation, transduction, transformation and or conjugation [8]. Comparison of the antibiotic resistant pattern of isolates from each sample type was shown in Table 4-8. Out of the 2 isolated from cabbage samples, 2 (100%) showed resistant to tetracycline while all had 2 (100%) susceptibility to ciprofloxacin. Multi drug resistance was common amongst the isolates. Fifty percent of all the isolates were resistant to five antibiotics and also fifty percent were resistant to more than two antibiotics.

Table 9 reveals the extended beta lactamase pattern of *E*. *coli* isolated from the various sample sources. Spinach samples obtained from Choba market had the highest percentage of ESBL positive *Escherichia coli* 4(80%) while cabbage and water leaf had the same percentage of ESBL positive *E coli* 1(50%). *E. coli* isolated from spinach and water leaf samples obtained from Rumuosi had the same percentage of ESBL producing *Escherichia coli* 1(33.3%) samples, A number of studies have assessed the occurrence of ESBLs among the family of Enterobacteriaeceae (Singhal et al., 2004; Rahal et al., 2002). In this study 7(50%) out 14 *E. coli*, produced ESBLs. This could be as a result of mutations within the structural genes encoding the older enzymes, giving rise to derivatives that possessed an extended substrate profile compared with that of the parental enzymes [8].

The Gel picture of EAST (enterohaemorrhagic and enteropathogenic) Virulence gene for *E. coli*. (Plate 4) shows that out of the ten isolates subjected to the test, eight were positive for Enteroheamorrhagic. Paddock et al., (2013) reported that *E. coli* isolated from vegetables, cattle and human sources were positive for ast, A gene which is frequently associated with diarrhea. Seven (50%) of the total isolates produced Extended β-lactamase. A number of studies have assessed the occurrences of ESBLs among members of the family Enterobacteriaeeae, [15,16,17]. About 31.7% *E .coli* isolated from vegetable farms in Imo State, Nigeria produced ESBLS [18]. A large number of outbreaks of infection due to ESBL-producing organisms have been described on every continent of the globe [19,20]. Clinical outcomes data indicate that ESBLs are clinically significant and, when detected, indicate the need for the use of appropriate antibacterial agents [21].

The Gel picture of EAST (Enterohaemorrhagic and Enteropathogenic) virulence gene for *E. coli.* is shown in Plate 4. Out of the ten isolates subjected to the test, eight were positive for Enteroheamorrhagic and Enteropathogenic virulence gene while the remaining two were negative. Ateba and moses, [22] also detected *Escherichia coli* 0157:H7 virulence genes in isolates from beef, pork, water, human and animal species in Northwest province of South Africa [23-26].

## 4. CONCLUSION

The study has established the presence of pathogenic *E. coli* (Enterohaemorrhagic) which are Shiga toxin producers that are Highly virulent with low infections doses. Spinach samples was found to be most contaminated of all the three leafy vegetable samples with a mean count of $2.8\times10^4$CFU/g and highest prevalence rate of 80% while Cabbage samples was contaminated with a count of $1.6\times10^3$cfu/g (Table .1) and 40% prevalence which was the lowest.

Out of the ten isolates subjected to detection of virulence gene, eight isolates were positive for the virulence genes namely Enteropathogenic (EPEC) and Enterohemorrhagic (EHEC) genes which makes this organism a dangerous pathogen

The presence of this pathogen indicates that consumers of these vegetables in some parts of Rivers State are at risk of acquiring this pathogen which could be transferred from person to person, and animal to animals.

The poor sanitary condition of practices observed in the various markets could be responsible for spread of the pathogen.

## 5. RECOMMENDATION

When patients present with food poisoning symptoms the causative agents should be properly identified and sources of food be identified for epidemiological information and data base for referencing.

Consumers should avoid indiscriminate eating of foods sold in open places and cook their food properly.

The indiscriminate use of antibiotics should also be discouraged since it is implicated as one of the reasons for multiple resistance to antibiotics by pathogens.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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