

Phenotypic and Genotypic Methods used for Detection of Extended Spectrum β -Lactamase Production in Sorbitol and Non-Sorbitol Fermenting *E. coli* Isolates Jointly Resistant to Cefotaxime and Ceftazidime from Vegetables Purchased from Keffi Main Market

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Abstract: Investigations were carried out on phenotypic and genotypic methods used for detection of extended spectrum β -lactamase production sorbitol and non-sorbitol fermenting *E. coli* isolates jointly resistant to cefotaxime and ceftazidime. Vegetable samples were purchased from Keffi main market making a total of hundred (100). Twenty (20) each from pumpkin leaves (*Telfaria occidentalis*), lettuce (*Lactuca sativa*), spinach (*Amaranthus hybridus*), cabbage (*Brassica oleracea*) and carrot (*Daucus carota*). Samples were packaged in sterile separate polythene bags and were immediately transported to Nasarawa state University Microbiology Laboratory for analyses. Sorbitol and non-sorbitol fermenting *E. coli* were isolated and identified from the samples using standard microbiological and biochemical methods. Antibiotic susceptibility test using disc diffusion methods as described by Clinical and Laboratory Standards Institute (CLSI) and the confirmatory test for extended spectrum β -lactamase (ESBL) production in NSF and SFE isolates jointly resistance to both cefotaxime and ceftazidime from vegetables were carried out using double disks synergy method. The molecular detection of ESBL genes such as TEM, SHV and CTX-M in NSF and SFE isolates jointly resistant to both cefotaxime and ceftazidime were carried out using polymerase chain reaction (PCR) method. The detection of ESBL producing NSF and SFE isolates jointly resistant to both cefotaxime and ceftazidime was 100.0%. The order of occurrence of ESBL genes in NSF were; SHV (100.0%), TEM (12.5%) and CTX-M (37.5%). Sorbitol fermenting *E. coli* percentage occurrence of ESBL genes were; SHV (91.7%), TEM (33.3%) and CTX-M (33.3%) respectively. The detection rate of *E. coli* was high in vegetables and most of the isolates were ESBL producers and the most common ESBL gene was SHV gene. Treatment of vegetables for eradication of pathogenic organisms before consumption is vital, so as to prevent infections arising from ingestion of pathogenic organisms.

Keywords: Phenotypic, Genotypic, Cefotaxim, Ceftazidime, Sorbitol, Non-sorbitol, *E. coli*

1. Introduction

Escherichia coli are commensals in the intestines of most animals including man (Lesley-Ann *et al.*, 2014). Sorbitol fermenting (SF) and non-sorbitol fermenting (NSF) are usually the two types of *E. coli* encountered (Ngwaiet *et al.*, 2014). *Escherichia coli* O157: H7 is a non-sorbitol fermenting *E. coli* (Ngwaiet *et al.*, 2014) which causes diarrhea and hemolytic uremic syndrome especially in children (Olorunshola *et al.*, 2000; Annet and Ole, 2009). Infections such as sepsis, meningitis and urinary tract infections have been reported to be caused by NSF *E. coli* (Johnson *et al.*, 2005; Croxnet *et al.*, 2010). Antimicrobial resistance among enteropathogens has been a public health issue in recent years especially in developing countries where enteropathogens are frequently encountered and cause life threatening infections (Johnson *et al.*, 2005). Resistance to β -lactam antibiotics has become widespread throughout the world, and numerous types of extended spectrum β -lactamases (ESBLs) have been detected in various Enterobacteriaceae especially from animal origins (Jensen *et al.*, 2006). Systemic infections with ESBL producing enterobacteriaceae are associated with severe adverse clinical outcomes (Nkneet *et al.*, 2016). It is thus essential for

a diagnostic microbiology laboratory to updated methods for detection of ESBL-producing strains.

2. Materials and Methods

Sample collection

One hundred (100) samples of vegetables such as Cabbage (*Brassica oleracea*), Spinach (*Amaranthus hybridus*), Lettuce (*Lactuca sativa*), Pumpkin leaves (*Telfaria occidentalis*) and Carrot (*Daucus carota*) were randomly purchased directly from vegetable sellers in Keffi main market, Nasarawa State, Nigeria. These vegetable samples were collected separately using sterile polythene bags and were transported to Microbiology Laboratory, Nasarawa State University Keffi, Nigeria in an ice pack for analysis.

Preparation of vegetable samples

The vegetable samples were prepared by modification of the method described by Ngwaiet *et al.* (2014). The vegetables were sliced each using sterile blade and exactly 10.0 g of each sliced vegetable samples were dispensed into 90 ml of sterile distilled water in 250 ml sterile conical flask, it was vigorously shaken and allowed to stand for 4 hours before inoculation

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Isolation of Sorbitol and Non-Sorbitol Fermenting *Escherichia coli*

The Sorbitol and Non-sorbitol fermenting *E. coli* were isolated from vegetable samples that were obtained from Keffi main market, Nasarawa State, Nigeria by modification of the method earlier described by Ngwaiet *et al.* (2014). Exactly, 1ml each prepared vegetable samples such as cabbage, spinach, lettuce, pumpkin leaves and carrot were inoculated into 9 ml of Tryptone Soy broth (TSB) and incubated at 37 °C for 24 h. A loopful of the inoculum in TSB was streaked on Sorbitol MacConkey agar and were incubated for 24 h at 37°C. Colourless (non-sorbitol fermenting) and pinkish (sorbitol fermenting) colonies were sub-cultured on EMB agar plates and incubated at 37 °C for 24 h. Greenish metallic sheen colonies that were grown on EMB agar plates after 24 h incubation were selected as suspected *E. coli*.

Preparation of media and inoculation for isolation of *Escherichia coli*

Preparation of Culture media:

Nutrient Agar, Mueller-Hinton Agar, Simmons Citrate Agar, Peptone water, Eosin Methylene Blue (EMB), MacConkey agar, Tryptone soya broth, Sorbitol-MacConkey agar were all prepared according to manufacturer's standards

Identification of *Escherichia coli*

Standard biochemical tests such as Gram-staining, Indole Test, Methyl Red Test, Voges-Proskauer Test, Citrate Test were carried out adopting methods of Cheesebrough (2006)

Antibiotic Susceptibility Test

The antibiotic susceptibility testing was carried out using Kirby-Bauer disc diffusion method modified by CLSI (2014). Four (4) variants colonies of SF and NSF *E.coli* isolates were inoculated into 5 ml of sterile normal saline in a test-tube, and the turbidity of the bacteria suspension was adjusted equivalents to turbidity of 0.5 McFarland's standard. The McFarland's standard was prepared as follows: 0.5 ml of 1.172 % (w/v) BaCl₂·2H₂O was added to 99.5ml of 1% (v/v) H₂SO₄. A sterile cotton swab stick was soaked in the adjusted SF and NSF *E.coli* suspension and was streaked on Mueller Hinton agar (MHA) plates. The antibiotic discs were placed aseptically on Mueller-Hinton agar plates containing the *E.coli* isolates. The plates were allowed to stand for 1 h for pre-diffusion at room temperature (25°C) before they were incubated at 37°C for 24 h. The diameter zone of inhibition (in mm) was determined; using meter rule and the result was interpreted in accordance with CLSI (2014). Antibiotic disks from Oxoid Ltd (England) that were used include: gentamicin (10µg), cefuroxime (30 µg), Amoxicillin-Clavulanate (30µg), Perfloxacin (10µg), Streptomycin (30 µg), Ampicillin (30µg), Ciprofloxacin (5µg), Chloramphenicol (30µg) and Sulphonamide/sulphomethoxazole (30 µg), Ceftazidime (30 µg), Cefotaxime (30 µg) and Cefoxitin (30 µg).

Confirmatory Test for Extended Spectrum β-Lactamase Production

The confirmatory test for ESBL production by the isolates jointly resistant to both cefotaxime and ceftazidime antibiotics were carried out on SF and NSF *E. coli* isolates

whose diameter zone of inhibition were ≥24mm and ≥22mm using Double Antibiotic Synergy Test (DAST). Swab stick was soaked in a standardized suspension (10⁵Cfu/ml) of SF and NSF *E. coli* jointly resistance to both cefotaxime and ceftazidime antibiotics were streaked on MHA plates and 30µg of Amoxicillin-Clavulanate disks were placed at the center; and 30µg ceftazidime and 30µg cefotaxime disks 20 mm away from the Amoxicillin-Clavulanate disks. The plates were allowed to stand for 1 h for pre-diffusion at room temperature before they were incubated at 37°C for 24 h. Isolates with increase in zone of inhibition of both ceftazidime and cefotaxime towards Amoxicillin-Clavulanate were confirmed as ESBL producers (Nkeneetal., 2016).

Detection of ESBL Genes

Isolates that were suspected positive for ESBL were screened to know the types of beta-lactamase (*bla*) genes they carried. They were subjected to a simpleplex polymerase chain reaction to detect three (3) ESBL genes: *bla*_{SHV}, *bla*_{TEM}, *bla*_{CTX-M}. A total of twenty samples were screened for the presence of *bla* genes. The protocol by Bradford *et al.* (2012) was employed. This was carried out at the Pharmaceutical Microbiology Laboratory of the Niger Delta University, Ammasoma, Bayelsa State.

Preparation and Purification of DNA/ DNA Extraction by boiling method

The DNA extraction was done by boiling as described by Zhao *et al.* (2001). *Escherichia coli* isolates were sub-cultured on MacConkey agar overnight at 37°C to obtain pure cultures. Three distinct colonies of the organism were scooped from MacConkey using a sterile inoculating loop and transferred into 2ml Luria Bertani broth (LB) and incubated at 37°C for 24 hours.

The bacterium cells were harvested by centrifugation at 3200rpm in a micro centrifuge for 2 minutes at room temperature (25⁰) and the supernatant was discarded. The pelleted cells were re-suspended in 1ml of sterile normal saline and the micro-centrifuge tubes were placed in the vortex for five seconds. Centrifugation was carried out at 3200rpm for 1 minute and the supernatant was discarded.

Exactly 0.5ml of sterile normal saline was added to the pellets and the tubes were vortexed for 5 seconds after which they were heated in the block heater at 90°C for 20 minutes. Immediately after heating, rapid cooling was done by transferring the tubes into the freezer for 10 minutes. Cell debris was removed after centrifugation was done at 3200rpm for 1 minute and 300µl of the supernatant was transferred into a sterile 2ml Eppendoff tubes as DNA and stored at -10°C until use.

Estimation of the concentration, purity and yield of the DNA sample was accessed using absorbance method (measurement of absorbance) with the spectrophotometer (Nano drop 1000). For DNA concentration, absorbance readings were performed at 260nm (A₂₆₀) and the readings were observed to be within the instrument's linear range (0.1 – 1.0). DNA purity was estimated by calculating the A₂₆₀/A₂₈₀ ratio and this was done by the spectrophotometer's

computer software (where A_{260}/A_{280} ratio ranges from 1.7 – 1.9).

The isolated DNA was run on agarose gel electrophoresis to confirm the presence of genomic DNA (Zhao *et al.*, 2001).

Polymerase Chain Reaction (PCR) procedure

Singleplex (PCR) was performed in order to identify the types of ESBLs present in the vegetable isolates. The presence of *bla*_{CTX-M}, *bla*_{SHV} and *bla*_{TEM} genes were tested for using previously published primer sets and conditions. The primer sequences and expected amplicon size for each gene are listed as:

Primer	Sequence (5'-3')	bp
<i>bla</i> _{TEM-1/F}	ATAAAATTCTTGAAGACGAAA	890
<i>bla</i> _{TEM-1/R}	GACAGTTACCAATGCTTAATCA	
<i>bla</i> _{SHV-1/F}	ATGCGTTATATTCGCCTGTG	283
<i>bla</i> _{SHV-1/R}	TTAGCGTTGCCAGTGCTC	
<i>bla</i> _{CTX-M1}	GTTACAATGTGTGAGAAGCAG	520
<i>bla</i> _{CTX-M2}	CCGTTTCCGCTATTACAAAC	

The reactions were carried out in 20µl reaction volume which was made up of 10µl of Mastermix (Qiagen), 0.32µl of primers (0.16µl each of forward and reverse primers), 3µl of DNA and 6.68µl of nuclease free water. The primer concentration stood at 0.2M. The reaction tubes were placed in the holes of the thermal cyclor and the door of the machine was closed.

PCR Conditions

Conditions during the reactions were set as: 3 minutes of initial denaturation at 95°C, followed by 35 amplification cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 40 seconds, initial extension at 72°C for 50 seconds, final extension at 72°C for 3 minutes and a hold at 4°C infinitely.

Preparation of 1.5% Agarose Gel

A 1.5% Agarose gel was prepared by dissolving 1.5g of Agarose gel powder in 100ml of Tris-Borate-EDTA (TBE) buffer and heating the mixture for 10 minutes in the microwave oven. The mixture was then cooled and 10µl of ethidium bromide was added to the molten gel and swirled to mix completely. A leveler was used to balance the gel

box, and combs were fixed. The mixture was poured carefully and allowed to polymerize at room temperature for 1 hour. After it had polymerized, the comb was removed from the solidified gel (Zhao *et al.*, 2001).

Running Gel Electrophoresis

Enough TBE buffer was applied in the electrophoretic tank, and the solidified gel was placed on the base of the tank, making it completely immersed in the TBE buffer. The DNA was transferred into the wells of the gel by stabbing the wells and dispensing using a micropipette and this was done carefully to ensure that each well had only one sample. Each gel had one well which contained a DNA ladder (1000bp, Thermo Scientific) in order to estimate the size of the DNA amplicons. Electrophoresis was run at 120 volts for 20 minutes, after which the gels were viewed using ultra-violet trans-illuminator machine (Zhao *et al.*, 2001)

3.2.9 Statistical Analysis

Data obtained in this study on frequency of occurrence of sorbitol fermenting and non-sorbitol fermenting *Escherichia coli* isolates were subjected to Chi-square test using Statistical Package for Social Sciences (SPSS) version 21.0 and the significance was determined at 5% probability.

Table 1: Phenotypic and Genotypic Detection of Extended Spectrum beta-Lactamase Production in Sorbitol Fermenting *Escherichia coli* and Non- Sorbitol Fermenting *Escherichia coli* isolates jointly Resistant to both Cefotaxime and Ceftazidime from vegetables sold in Keffi Main Market, Nigeria

Isolates	No. of resistant isolates	No. (%) ESBL producers	No. (%) ESBL genes		
			SHV	TEM	CTX-M
NSFE	8	8 (100.0)	8 (100.0)	1 (12.5)	3 (37.5)
SFE	12	12(100.0)	11 (91.7)	4 (33.3)	4 (33.3)
Total	20	20 (100.0)	19 (90.5)	5 (25.0)	7 (35.0)

Key: ESBL (Extended Spectrum beta-Lactamase), SFE (Sorbitol Fermenting *Escherichia coli*), NSFE (Non-Sorbitol Fermenting *Escherichia coli*), SHV (Sulphahydinyl Variance enzymes), TEM (Temonera enzymes), CTX-M (Cefotaximase enzymes)

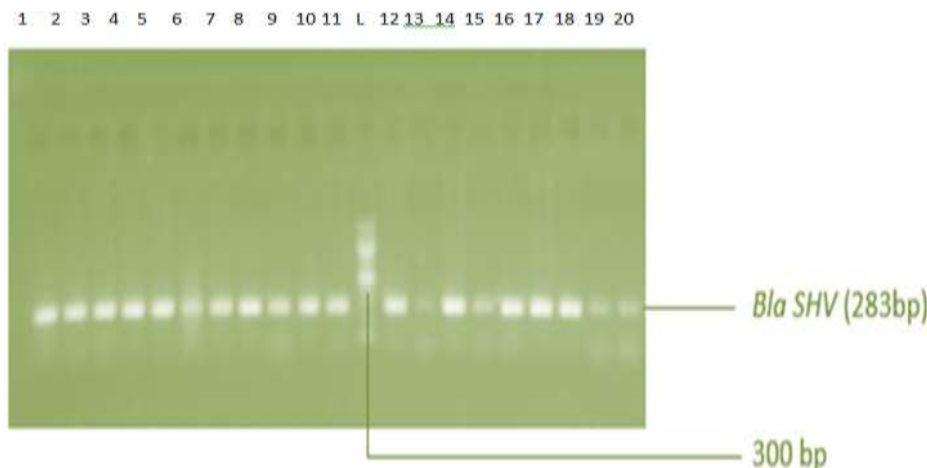


Figure 1: Agarose gel electrophoresis of *bla*_{SHV} gene detected from *Escherichia coli* isolates

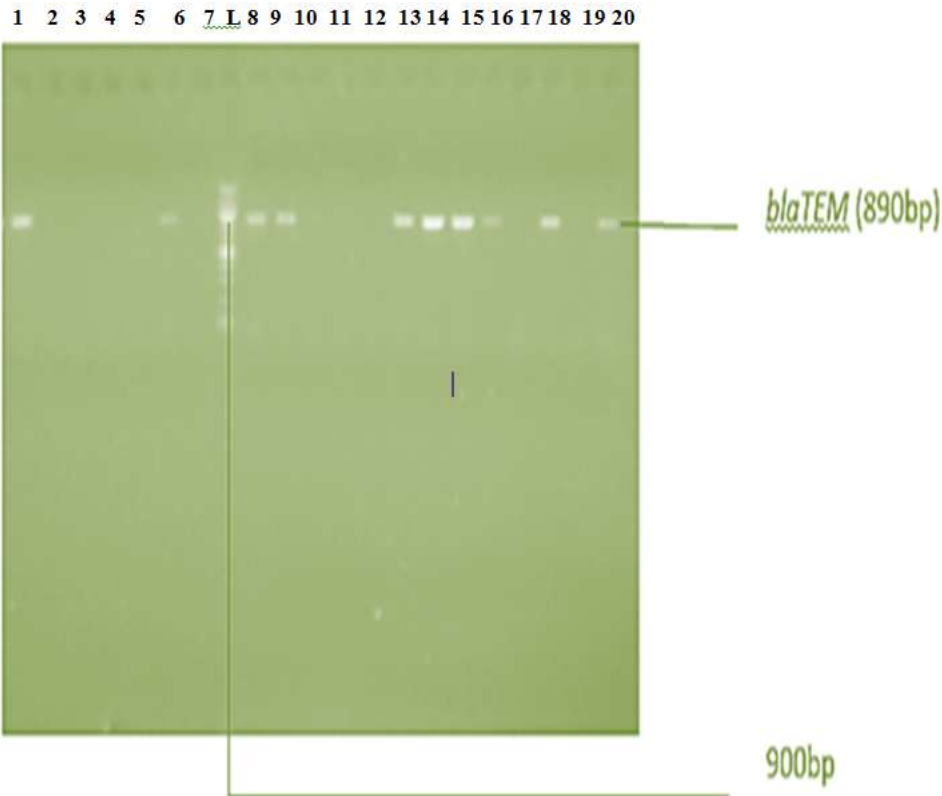


Figure 2: Agarose gel electrophoresis of *blaTEM* gene detected from *Escherichia coli* isolates

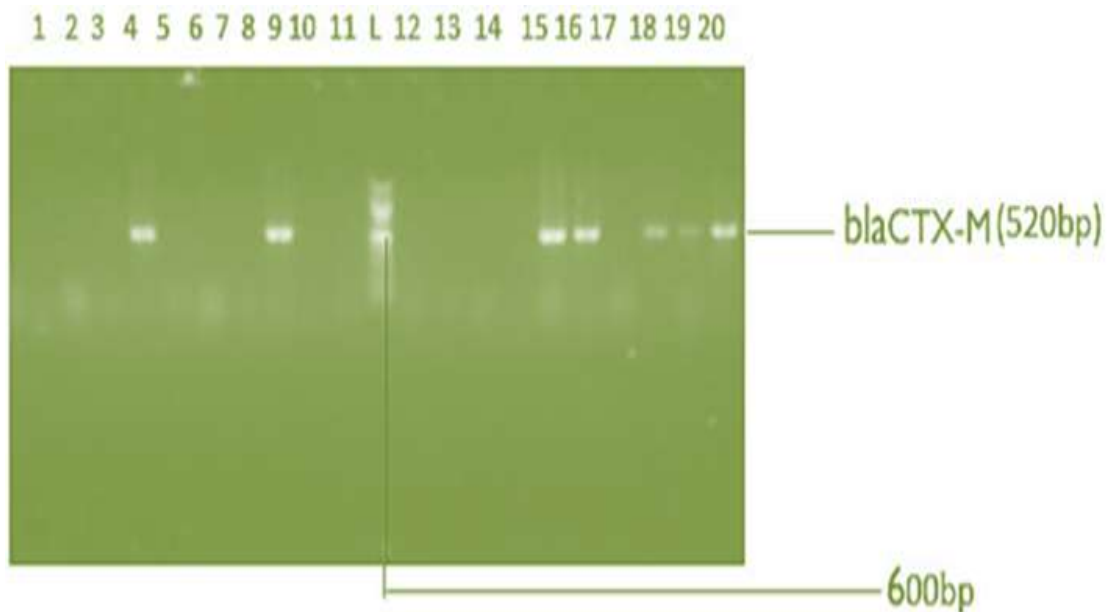


Figure 3: Agarose gel electrophoresis of *blaCTX-M* gene from *Escherichia coli* isolates

3. Discussion

Vegetables are important sources of vitamins, other nutrients and fibre (Annet and Ole, 2009) therefore, consumption of vegetables has increased over two decades. Series of outbreaks have been linked to pathogenic organisms contaminating raw vegetables (CDC, 2013). The occurrence of NSFE in vegetables observed in this study have public health implication because this group of *E. coli* are shown to be *E. coli* 0157:H7 (Ngwaiet *al.*, 2014) that have been reported to cause diarrheal diseases and haemolytic uremic syndrome most especially in children of under-age.

It was observed that all the NSFE and SFE jointly resistant to both Cefotaxime and Ceftazidime were phenotypically confirmed ESBL producers but this however seems to disagree with the study earlier report by Bradford (2012) and Nkene *et al.* (2016). The high detection of ESBL producing *E. coli* in vegetable samples observed in this study is in agreement with the study earlier reported by Anette and Ole (2009). Though, the detection rate of SHV genes was higher than CTX-M and TEM in both SFE and NSFE. The presence of the ESBL gene mentioned above as observed in this study may be responsible for the production of ESBL enzymes that inactivates Cefotaxime and Ceftazidime. Although the mechanism of resistance of both Cefotaxime

and Ceftazidime by NSF and SFE isolates were not evaluated in this study but Jacoby and Meideiros (1991) reported that resistance to Cefotaxime and Ceftazidime is due to the production of ESBL enzymes that inactivates the third generation cephalosporin mentioned above.

4. Conclusion

Sorbitol and non-sorbitol fermenting *E.coli* isolated from vegetables sold in Keffi main market, Nigeria were high. It was also established from this study that all the *E. coli* isolates jointly resistant to both Cefotaxime and Ceftazidime were ESBL producers and the occurrence of SHV genes were higher than CTX-M and TEM gene. Therefore raw vegetables should be properly treated to avoid infections arising from consumption of contaminated vegetables.

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