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 (Telfariaoccidentalis), lettuce (Lactuca sativa), spinach (Amaranthushybridus), cabbage (Brassica oleracea) and carrot (Daucuscarota). 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 NSFE and SFE isolates jointly resistant to both cefotaxime and ceftazidime were carried out using double disks synergy method. The molecular detection of ESBL genes such as TEM, SHV and CTX-M in NSFE and SFE isolates jointly resistant to both cefotaxime and ceftazidime were carried out using polymerase chain reaction (PCR) method. The detection of ESBL producing NSFE and SFE isolates jointly resistant to both cefotaxime and ceftazidime was 100.0%. The order of 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.colicountered (Ngwaiet al., 2014). Escherichia coli O157: H7 is non-sorbitol fermenting E. coli(Ngwaiet 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; Croxenet 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 enteroabacteriaceae are associated with severe adverse clinical outcomes (Nkene 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 (Amaranthushybridus), Lettuce (Lactuca sativa), Pumpkin leaves (Telfariaoccidentalis) and Carrot (Daucuscarota) were randomly purchased directly from vegetable sellers in Keffi main market, Nasarawa State, Nigeria. These vegetables 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 vegetables samples were prepared by modification of the method described by Ngwai 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 Ngwaei 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·H₂O was added to 99.5ml of 1% (v/v) H₂SO₄, A sterile cotton swab stick was soaked in 30μg of Amoxicillin, 30μg of Cefotaxime, 30μg of Ceftazidime, 30μg of Sulphmethoxazole and 30μg of Cefotaxime disks. 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), Perflxacin (10μg), Streptomycin (30 μg), Ampicillin (30μg), Ciprofloxacın (5μg), Chloramphenicol (30μg) and Sulphonamide/ sulphmethoxazole (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 (10°Cfu/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 simplex polymerase chain reaction to detect three (3) ESBL genes: blaTEM, blaSHV, blaCTX. 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 (25°C) 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 Eppendorff 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 *blaCTX-M*, *blaSHV* and *blaTEM* genes were tested for using previously published primer sets and conditions. The primer sequences and expected amplicon size for each gene are listed as:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>blaTEM-F</td>
<td>ATAAAAATTCTGAAAGACGAAA</td>
<td>890</td>
</tr>
<tr>
<td>blaTEM-R</td>
<td>GAGCAATGATCATGCTATCA</td>
<td>283</td>
</tr>
<tr>
<td>blaSHV-R</td>
<td>TTACGTGTTGCAATGC3T</td>
<td>520</td>
</tr>
</tbody>
</table>

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 cycler 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 1000ml 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 ampiclons. Electrophoresis was run at 120 volts for 20 minutes, after which the gels were viewed using ultraviolet 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 β-Lactamase Production in Sorbitol Fermenting *Escherichia coli* isolates jointly Resistant to both Cefotaxime and Ceftazidime from vegetables sold in Kefi Main Market, Nigeria

<table>
<thead>
<tr>
<th>Isolates</th>
<th>No. of resistant isolates</th>
<th>No. (%) ESBL producers</th>
<th>No. (%) ESBL genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSFE</td>
<td>8</td>
<td>8 (100.0)</td>
<td>8 (100.0)</td>
</tr>
<tr>
<td>SFE</td>
<td>12</td>
<td>12 (100.0)</td>
<td>11 (91.7)</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>20 (100.0)</td>
<td>19 (90.5)</td>
</tr>
</tbody>
</table>

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

![Figure 1](image_url): Agarose gel electrophoresis of blaSHV gene detected 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 (Ngwai et al., 2014) that have been reported to cause diarrheal diseases and haemolytic uremic syndrome most especially in children of underage.

It was observed that all the NSFE and SFE jointly resistant to both Cefotaxine 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 Cefotaxine and Ceftazidime. Although the mechanism of resistance of both Cefotaxine
and Ceftazidime by NSFE and SFE isolates were not evaluated in this study but Jacoby and Meideiros (1991) reported that resistance to Cefotaxine and Ceftazidime is due to the production of ESBL enzymes that inactivates the third generation cephalosporin mentioned above.

4. Conclusion

Sorbitol and non-sorbital 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 Cefotaxine 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.

References