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Antibiotic Suscepibility Studies on *Escherichia coli* O157:H7 Isolated from Vegetables Sold at Sokoto, North Western, 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

Aim: The study was aimed at antibiotic susceptibility studies on *Escherichia coli* O157: H7 isolated from vegetables sold at Sokoto Central Market, Northwestern, Nigeria.
Study Design: The study is a cross-sectional study involving sampling of vegetables sold at Central Market, Sokoto, Sokoto State, Nigeria.
Place and Duration of Study: The study area is Shehu Shagari Central Market Sokoto were sample are collected and analyzed at central Laboratory, Department of Medical Microbiology, Usmanu Danfodiyo University, Sokoto, Nigeria. The research was conducted over a period of six (6) months (January, 2019 – June, 2019).
Methodology: A cross-sectional study involving sampling of vegetables sold at Central Market, Sokoto, Sokoto State, Nigeria. Vegetable samples were processed and inoculated unto sorbiol MacConkey's agar (SMAC) at a pH of 7.2 for the isolation of *Escherichia coli* by streaking method. Antibiotic susceptibility test was carried out on Escherichia coli which were isolated from the samples analyzed using Kirby-Bauer disc diffusion technique. Isolates DNA was extracted using MiniPrep kit (Zymo research (ZR) California, USA) and then samples were amplified in separate reactions using the GeneAmp PCR (Polymerised chain reaction) System 9700 thermocycler for *Escherichia coli* O157: H7 specific gene detection.

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Results: The presence of *E. coli* O157:H7 in vegetable samples was 2 (1.25%) and significantly low. High sensitivity patterns were observed against ofloxacin, ciprofloxacin, ceftazidine and cefuroxine. Resistance was found to be high for amoxycillin-clavulanic acid and gentamicin.
Conclusion: Although there was significantly low prevalence of *E. coli* O157:H7 in vegetable samples, investigation of non-0157 shiga toxin producing *E. coli* (STEC) is therefore required.

Keywords: Escherichia coli 0157:H7; vegetables; Sokoto, Nigeria.

1. INTRODUCTION

Numerous outbreaks of gastroenteritis have been associated with ingestion and consumption of raw foods, foods incorporating raw ingredients or foods obtained from unsafe sources. Salmonella and *Escherichia coli* are the most widespread food-borne bacteria in almost all countries. Almost all warm-blooded and many cold-blooded animals serve as their natural hosts [1].

E. coli is a Gram negative bacillus that is often found in raw food products, including beef, raw milk, and produce. Cattle, deer and sheep may carry *E. coli* in the intestinal tracts and can thus act as vehicles in the transmission of the organism to food products consumed by humans. The Environmental protection agency (EPA) recognizes that *E. coli* is the best available indicator for fecal contamination due to its specificity to fecal material from warm-blooded animals [2].

The species *Escherichia coli*, is a coliform bacterium; which has the ability to ferment glucose and produce acid and gas during incubation at temperature of 37^0C. Pathogenic *E. coli* causes gastroenteritis, including diarrhoea and abdominal cramps, although recovery occurs for most people within a week. The effect of the toxins produced by this food pathogen can also lead to a kidney failure known as Hemolytic uremic syndrome (HUS) at a rate of 3-7% of those infected [3]. *Escherichia coli* 0157:H7 and other Shiga toxin-producing *E. coli* can cause severe illness and contamination can occur anytime from the initial harvesting of farm produce (e.g. vegetables) to processing, distribution, and to the final destination of this farm produce gives a pathway called the "farm to fork continuum" [4].

Evidence of fresh produce-associated food-borne illnesses has been growing in recent decades and the description of such outbreaks is common in many parts of the world [5], despite the prevalence of the main pathogens continuing to be low in fruit and vegetables [6]. However, outbreaks of illness associated with vegetable have been increasing in recent years, rising from 4.4% in 2013 to 7.6% in 2014 [7].

Polymerase chain reaction (PCR) has been proven to be useful in detecting pathogen in food sample rapidly and accurately [8]. It is a technique used in molecular biology to amplify a single copy or a few copies of a segment of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Multiplex PCR methods have been developed for specific detection of *Salmonella* and *E. coli*, however they are limited to qualitative determination of the organism unless they are used in conjunction with Most Probable Number (MPN) procedure for quantitative determination of this pathogens in foods [8].

In a study conducted in Lagos, Nigeria, cases of outbreak of bacterial contamination of vegetables grown in soil contaminated by *E. coli* has been reported [9]. In addition, more than 36% of the food handlers were not aware that food contaminated by germs can cause serious damage to health, 38.3% did not know that they should wash their hands after using the toilet before handling food items, while 25% did not know that theyshould wash their hands after handling money. However, there is paucity of literature on the knowledge, perception and practices related to vegetables contamination with *E. coli* in Sokoto, Sokoto State, Nigeria.

2. METHODOLOGY

2.1 Study Area

Sokoto is located in the extreme North West of Nigeria. It is located around the Sahel Savannah region with an annual temperature of 28.3^oC (82.9^oF). It is located in Northern Nigeria with the global positioning system and coordinates of Sokoto are $13^o0'$ 21.1428^0N and $5^o14'51.1872^0$E with latitude of 13.005873, and longitude of

5.247552. Sokoto has a height of 305 meters which is equivalent to 1,001ft. [10].

The study area is Shehu Shagari Central Market Sokoto, Sokoto State and the research was conducted over a period of six (6) months.

2.2 Study Samples

A total of 160 samples of raw vegetables specifically; Scent leaf (*O. gratissimum*), Cabbage (*Brassica oleracea*), Ugwu (Fluted pumpkin), Jute leaf, ewedu in Yoruba (*Corchorus olitorius*), Green leaf (*Amaranthus amaranthus*), Water leaf, crunchy spinach *(Talinum fruticosum*), Lettuce (*Lactuca sativa*) and Bitter leaf (*Vernonia amygdalina*) were analyzed.

2.3 Processing of Vegetable Materials to Obtain Microbial Sample

The vegetable samples were mashed using clean laboratory pestle and mortar with the addition of distilled water [11]. Ten (10) grams of each sample was weighed and placed in a stomacher bag and it was diluted in 90 mL of distilled water and homogenized for 2 minutes. Seven serial dilutions of the suspension were made in pre-enrichment Buffered Peptone Water (BPW) and maintained at room temperature for six (6) hours followed by taking 0.1 ml amount of 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, and 6^{th} dilution and cultured onto sorbiol MacConkey's agar (SMAC) and at a pH of 7.2 by streaking method. Cultured inoculated plates were incubated at 37 ^{o}C and 44.5 ^{o}C for heterotrophic plate and coliform count respectively for 24 hours [11]. The accepted limit of ≤ 10^5/10g was used as standard and appeared to be a realistic goal for all the food types with a high level of natural flora [12]. The various colonies observed in the plates were distinguished on the basis of their cultural characteristics [13]. All strains isolated were sub-cultured onto nutrient slant agar and stored at -60 ^{o}C (VT 307 A/S Vestfrost DK-6705 Esbjerg Ø, Denmark) for further testing [11].

2.4 Culture Characterization and Identification of Isolates using Biochemical Methods

The various colonies observed in the plates were distinguished on the basis of their cultural characteristics [12]. The isolates were further identified and characterized according to standard biochemical methods which included indole, methyl red, Voges-Proskauer, hydrogen sulphide production, citrate utilization, sugar utilization tests and microscopy of Gram stained isolates [13]. Other identification test include carbohydrate fermentation, β-galactosidase activity, gelatine hydrolysis, amino acids and enzymes activity (coagulase, Oxidase, lysine, ornithine decarboxylase, phenylalanine deamination test) [14].

2.5 Antibiotic Susceptibility Test (Kirby-Bauer Disk Diffusion Method)

Antibiotic susceptibility test was carried out on *E. coli* organisms which were isolated from the vegetable samples analyzed using Kirby-Bauer disc diffusion technique. Pure culture of the organisms were inoculated into sterile nutrient broth to a density of 9 × 10^8 cfu/ml (McFarland Standard test-tube 3) and incubated for 24 hrs at 37 ^{o}C. The suspension was used to streak for confluent growth on the surface of Muller-Hinton Agar plates with sterile swab for the disc diffusion test (Colony forming units, cfu of bacteria used and Zone sizes are measured and interpreted using Clinical and Laboratory Standards Institute (CLSI) standards [15]. Antibiotics disc used were ceftacidine (30µg/disc), cefuroxime (30µg/disc) gentamicin (10µg/disc), cefixime (15µg/disc), ofloxacin (5µg/disc), amoxycillin-clavulanic acid (30µg/disc), ciprofloxacin (5µg/disc) and erythromycin (15µg/disc) after 24 hrs incubation, the susceptibility profile pattern was obtained. Results were interpreted according to Clinical and Laboratory Standards Institute guidelines [15].

2.6 DNA Extraction Target DNA using MiniPrep kit (Zymo Research (ZR) California, USA)

Extraction and was performed in accordance with the manufacturer's instructions: One (1) µl of DNAase was added to a clean 1.5 ml tube then 200 µl of 10^4~10^8 cultured cells was applied to the tube containing proteinase K. Binding buffer 200 µl, amount was added to the sample and mix immediately by vortex mixer. The sample was completely re-suspended to achieve maximum lysis efficiency. The mixture was incubated at 60 ^{o}C for 10 min. A 100 µl amount of Isopropanol was added and mixed by pippetting. After this the preparation was briefly spin down to get the drops clinging under the lid without vortexing to obtained high DNA yield. The lysate solution few drops was carefully transferred into the upper

reservoir of the binding column tube (fit in a 2 ml tube) without wetting the rim. The tube was then closed and centrifuged at 8,000 rpm for 1 min. Each binding column tube was closed firmly to avoid aerosol formation during centrifugation. The tube was opened and the binding column tube transferred to a new 2 ml tube for filtration. A 500 µl amount of washing buffer 1 (W1) was added to it without wetting the rim, the tube closed, and centrifuged at 8,000 rpm for 1 min. The tube was then opened and the solution poured from the 2 ml tube into a disposal bottle. A 500 µl amount of Washing buffer 2 (W2) was carefully added without wetting the rim, the tube closed, and centrifuged at 8,000 rpm for 1 min. The preparation was centrifuge once more at 12,000 rpm for 1 min to completely remove ethanol, and ensured that there is no droplet clinging to the bottom of binding column tube. The binding column tube was then transferred to a new 1.5 ml tube for elution and 200 µl of elution buffer (EL, or nuclease-free water) was added onto binding column tube, and wait for at least 1 min at 15~25 ^{O}C until EL is completely absorbed into the glass fiber of binding column tube. The volume of EL added can be adjusted from 50 µl to 100 µl. And the preparation was then centrifuged at 8,000 rpm for 1 min to elute. The eluted genomic DNA is stable and stored at 4^{0}c for later analysis [16].

2.7 Amplification using GeneAmp PCR System 9700 and Detection of the Target DNA

Samples were then amplified in separate reactions using the GeneAmp PCR System 9700 thermocycler. For the reaction set-up a 4 µl amount specific UidA primers (Table 1) mixture and 3 µl of 8-methoxypsoralen (8-MOP) and 10 µl Multiplex Master Mix (contains DNA polymerase and buffer containing dNTPs) were mixed. The 17 µl reaction Mastermix was then dispensed into 0.2 ml PCR tubes and mixed with 3 µl of each sample's nucleic acid. Negative and positive controls were prepared by adding 3 µl of UidA negative and positive control respectively instead of sample's nucleic acid. Then the PCR thermocycling reactions start immediately using the following program; Pre-denaturation: 5min at 94^{o}C, Denaturation: 30sec at 94^{o}C, Annealing: 30sec at 52^{o}C, Extension: 1min at 72 ^{o}C 35 cycles. Final extension: 5min at 72^{o}C [16]. For the detection of the target DNA one percent (1%) agarose was used, solution was heated in a boiling water bath until agarose is completely dissolved. It was allowed to cool in a water bath set at 50 – 55 ^{o}C. Gel casting tray was prepared by sealing ends of gel chamber with a casting system. Appropriate number of combs was placed in gel tray. A 5 µl of ethidium bromide was added to cooled gel and it was poured into gel tray and allowed to cool for 30 minutes at room temperature. After the gel has solidified, the comb was removed, carefully not to rip the bottom of the wells. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber containing loading buffer. Of the amplified products 5 µl were transferred and allowed to separate in the 1% agarose gel electrophoresis in TAE buffer (40 mM Tris-acetate, 2 mM EDTA [pH 8.3]). After the run at 70 Volt for 2.5 hours, stained gels were examined under ultra-violet (UV) trans-illuminator. A DNA ladder of 1 Kb (Fermenters USA) was used as a molecular weight marker. The electrophoretic profiles were observed visually to determine the presence or absence of amplification band in the sample lane and on the lane for standard molecular weight. The presence of amplification band corresponding to the expected band size in relation to the result of the negative control was considered as positive [8].

2.8 Data Analysis

The data obtained were entered and analyzed using SPSS statistical package. Results were summarized and presented using frequency tables and bar charts. Data obtained were compared using Chi-square test at 95% confidence level to test the level of significance.

Table 1. Oligonucleotide primers used for PCR amplification

Target gene	Sequence (5′-3′)	Fragment size	Reference
uidA	Forward TGGTAATTACCGACGAAAACGGC primer Reverse ACGCGTGGTTACAGTCTTGCG primer	162	[23]

3. RESULTS

A total of 2 (1.25%) *E. coli* O157:H7 were isolated from 160 samples of raw vegetables specifically one each from waterleaf *(Talinum fruticosum*) and green leaf (*Amaranthus amaranthus*). Table 4, represent the result of antimicrobial susceptibility testing on two *Escherichia coli* 0157:H7isolated from foods and vegetables in Sokoto Metropolis Central Market, Sokoto State. Of total *E. coli* 0157:H7 isolates tested high sensitivity patterns were observed against ofloxacin, ciprofloxacin, ceftazidine and cefuroxine. Resistance was found to be high for amoxycillin-clavulanic acid and gentamicin. Different log concentration of HPC was recorded for the various vegetables analyzed, virtually all the vegetable items tested contain a distinctly significant higher value (normal HPC count levels in foods = ≤ 10^5).

The Agarose gel electrophoresis results of *Escherichia coli* O157:H7 detected following agarose gel electrophoresis using UidA primer band size 162bp is presented in Plate 1. Lane WCMS shows the positive amplification band of 162 bp corresponding to the expected band size for *Escherichia coli* O157:H7 species tested.

Table 2: Heterotrophic Plate Count (HPC**) and Coliform Count for Vegetable Samples**

Name of vegetable Sample	Mean HPC(CFU/ml)	Coliform count(CFU/ml	*p*-value
Cabbage	$5.0x\ 10^5$	$2.4x10^4$	0.821
Ugwu	$6.2x10^5$	$2.3x10^4$	
Jute leaf (ewedu)	$5.5x10^5$	$1.4x10^4$	
Green leaf	$6.8x10^5$	$2.9x10^4$	
Water leaf	$5.3x10^5$	$2.0x10^4$	
Lettuce	$8.2x10^5$	$3.5x10^4$	
Bitter leaf	$1.2x10^5$	$0.5x10^4$	
Scent leaf	$2.4x10^5$	$1.1x10^4$	

CFU = colony-forming units

Table 3. Distribution of *E. coli* O157:H7 from Vegetable Sample sold at Sokoto central market. (N=160)

Vegetable sample	No Examined (%)	No (%) of positive sample *E. coli* O157:H7
Water leaf	20	1(5.0)
Lettuce	20	0(0.0)
Bitter leaf	20	0(0.0)
Green leaf	20	1(5.0)
Cabbage	20	0(0.0)
Jute leaf (ewedu)	20	0(0.0)
Scent leaf	20	0(0.0)
Ugwu leaf	20	0(0.0)
Total	160	2(1.25)

Table 4 Antibiotic susceptibility pattern of *E. coli*0157:H7 Isolates from Vegetables sold in Sokoto Metropolis Central Market

Antibiotic agent	Disc content (µg)	Susceptibility n(%) n=2		
		S	I	R
Ceftazidine	(30µg)	2(100%)	0(0%)	0(0%)
Cefuroxine	(30µg)	2(100%)	0(0%)	0(0%)
Gentamicin	(10µg)	1(50%)	0(0%)	1(50%)
Cefixine	(15µg)	2(50%)	0(0%)	1(50%)
Ofloxacin	(5µg)	2(100%)	0(0%)	0(100%)
Amoxycillin-clavulanic acid	(30µg)	2(0%)	0(0%)	2(100%)
Erythromycin	(15µg)	0(0%)	0(0%)	2(100%)
Ciprofloxacin	(5µg)	2(100%)	0(0%)	0(0%)

S- Sensitive I- Intermediate R- Resistant

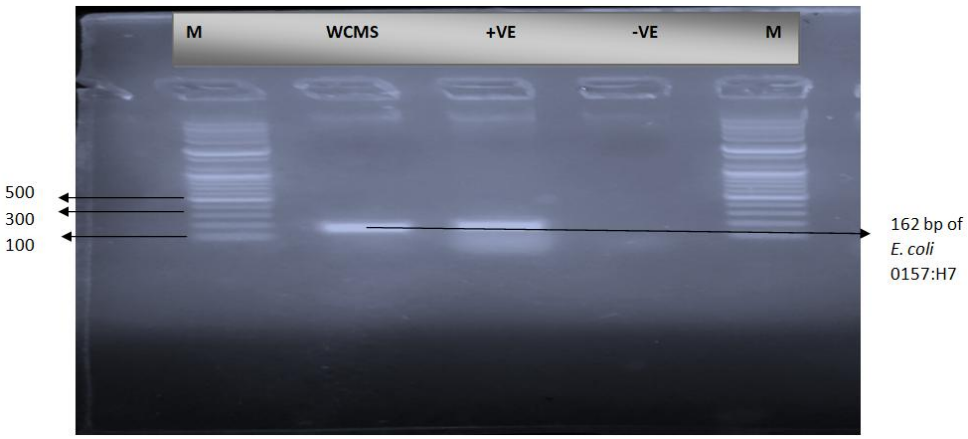

Plate 1. PCR result of *Escherichia coli*detected following agarose gel electrophoresis using Inv A primer band size 162bp

Key M= 1000bp ladder
+ve= positive InvA162bp
-ve= negative Inv1A 162bp
LCM= Lettuce sample 8 from central market

4. DISCUSSION

From this research carried out, it was observed that almost all the vegetable samples examined had bacteria count above the acceptable limit and it is microbiologically unacceptable. The high bacterial count range of 8.2 x 10^5 to 5.0 x 10^5recorded in this work for vegetable samples is dependent on the factors such as available nutrient, temperature, mechanical handling and moisture. The vegetable on display for sale are often touched by many hands of the customers and by the vendors. The customers pick and drop as many vegetables as are available to enable them make a choice. Frequent handling by unhygienic hands is a factor contributing to the high microbial load. The dusty environments of the market road coupled with water of unquestionable quality that often is used to sprinkle the vegetables to keep it fresh are contributing factors that could aid the survival and possible multiplication of microorganism on the vegetable surfaces. Many vegetables grow low to the ground where they are likely to come in contact with the soil. If the soil has been treated with improperly treated animal manure as fertilizer or irrigated with contaminated waters also attribute to the high coliform count as observed in this research for the vegetable samples. Halablab et al*.* [17] reported that coliform counts can be considered as a hygienic indicator especially for fecal contamination.

It has been recognized that vegetables can be an important vehicle for transmitting *E. coli* to humans [18] an indication that bacterial pathogens continue to be a major contributor to produce-associated food-borne diseases. In this study, 2 (1.25%) *E. coli* O157:H7 was detected from the 160 vegetable samples specifically one each from green leaf (*Amaranthus amaranthus*) and lettuce leaf (*Lactuca sativa*), the *E. coli* O157:H7 strain has been characterized by production of either one or both of the two major types of Stx proteins designated as shiga like toxin–1 (stx1toxin) and shiga like toxin–2 (stx2 toxin) and the production of the later is associated with increased risk of developing Haemolytic Uraemic Syndrome [19]. *Escherichia coli* O157:H7 strain has also been isolated from a number of environmental sources such as soil, irrigation water, manure etc [20]. The result recorded for *E.coli* O157:H7 detection from vegetable samples in this studies is slightly lower when compared with the results of Sharma and Carlson [21] who found *E. coli* O157 in 11.7% of studied vegetables and Berger et al. [5] who found prevalence of *E. coli* O157 ranged from 0% to 33% in onions and cabbage respectively but disagrees with what was recorded by Fakruddin et al. [22] who did not detect *E. coli* O157 among all isolated *E. coli* from vegetables [23].

It is worth noting that of all the vegetable samples studied, bitter leaf (*Vernonia amygdaline*) and scent leaf (*Occinum gratissmum)* did not yield any growth for the bacteria under study. This probably might be due to the phytochemical content of both leaves.

Phytochemicals (secondary plant metabolites) are naturally occurring, biologically active complex chemicals found in plants, notably in fruits and vegetables. The phytochemical screening of the leaves of bitter leaf and scent leaf has revealed the presence of alkaloids, flavonoids, steroid, tannins and carotenoid. The findings of some study have demonstrated that bitter leaf and scent leaf have an antibacterial activity against several species of bacteria and even fungi [4].

The study also showed that among the tested antibiotic, *E. coli*0157:H7 was 100% sensitive to ciprofloxacin, ofloxacin and ceftazidine, 50% sensitive to erythromycin, cefiroxine and cefixine and 100% resistance to augmentin. This shows that ciprofloxacin, ofloxacin and ceftazidine are good antibiotic therapy for the organism under study.

5. CONCLUSION

The study supported that the presence of *E. coli* O157:H7 in vegetable samples is low 2(1.25%) and investigation of non-0157 shiga toxin producing *E. coli* (STEC) is required. The appropriate measures should be taken to avoid bacterial contamination of vegetables while antibiotics susceptibility test must be sought before instituting appropriate therapy to prevent emergence of drug resistance organisms.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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