Molecular Characterization and Antibiotics Sensitivity Profiling of Enterotoxigenic Escherichia coli Strains in Samples from Okigwe, Imo State, Nigeria

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ABSTRACT

Different virulence factors produced by Enterotoxigenic Escherichia coli (ETEC) enable them to colonize and cause watery diarrhea. This study determined the prevalence and antibiogram of ETEC in cow dung, river water and human diarrheal samples collected from Okigwe zone, Imo State, Nigeria. The study was done between May and June, 2025. Results showed that out of a total of 150 samples studied, comprising of 50 cow dung samples, 50 river water samples and 50 diarrheal samples of children between 0 to 5 years, E. coli was isolated from a total of 44 samples, representing 29.33% of the total. There was E. coli in 13 cow dung samples (26.00%), 14 river water samples (28.00%) and 17 human faecal samples (34.00%) out of the total of 50 samples in each case. Results of antibiogram revealed that the highest percentage of sensitivity to drugs by the isolates from cow dung was 41.7% to Ciprofloxacin. Isolates from river water samples gave highest sensitivity of 23.1% while those from human diarrheal samples produced 26.7% highest sensitive to Ciprofloxacin. The isolates were all resistant to Cefepime, Augmentin, Ceftriaxone, Meropenem and Aztreonam. The prevalence of LT gene of ETEC was higher in cow dung samples with 50% than the ST gene with 41.7%. However, in river water and diarrheal faecal samples, the prevalence of ST gene was higher than that of LT. There was 30.8% and 30.8% prevalence of ST genes in river water samples and diarrheal faecal samples, but 7.7% and 15.4%, respectively, for LT gene. The results reported here have provided informative data regarding the prevalence of ETEC, their virulence genes and antibiotic sensitivity profiles.

Keywords: Public health, Diarrheal disease, ETEC, Molecular typing, Enterotoxins

INTRODUCTION

Escherichia coli is a member of the Enterobacteriaeceae family. It is a major contributor to human nosocomial and community-acquired infections. As a result, the organism may be isolated from a variety of specimens and is clinically significant (Nsofor and Iroegbu, 2019). Six distinct groups of pathogenic E. coli strains that possess different virulence factors that allow them to cause diarrheal disease are Enterotoxigenic E. coli (ETEC), enterohemorrhagic E. coli (EHEC), enteropathogenic E. coli (EPEC), enteroaggregative E. coli (EAEC), diffusely adherent E. coli (DAEC) and enteroinvasive E. coli (EIEC) (Amin et al., 2017). Heat-

labile and heat-stable enterotoxins can be produced by ETEC strains, which are a growing source of food-borne epidemics. They are often linked to childhood diarrhea and traveler's diarrhea (Amin et al., 2017). Enterotoxigenic Escherichia coli (ETEC) has been implicated as one of the main causes of infectious diarrhea in children. There are no approved vaccinations to prevent ETEC (Kipkirui et al., 2021). Heat-stable (ST) and/or heat-labile (LT) enterotoxins expressed by plasmids are produced by enterotoxigenic E. coli (Ifeanyichukwu et al., 2018). By increasing fluid and electrolyte secretion through downstream activation of adenylate cyclase (by LT) or guanylate cyclase (by ST) in the small intestine epithelial cells, both of these enterotoxins contribute to the disruption of intestinal fluid balance. Watery diarrhea occurs when this mechanism surpasses the bowel's absorption capacity (Kipkirui et al., 2021).

Diarrheal disease is the most prevalent health issue in impoverished nations, particularly in Africa, and the primary reason for hospitalization and occasionally even death in young children (Ugboko et al., 2020). An estimated 10.12% of child deaths in Nigeria during the first five years of life are linked to diarrheal illnesses (WHO, 2018). Diarrhea is responsible for 9% of pediatric hospital admissions and mortality in children under five (Tsehay et al., 2021). According to Nsofor and Iroegbu (2019), molecular methods have been utilized to correlate infections associated with animals with similar illnesses affecting humans and to clearly establish transferable resistant genes carried by plasmids common to both humans and animals.

Antimicrobial medications have been crucial in reducing illness and mortality from infectious diseases in both humans and animals (Daniel et al., 2012). However, the establishment and spread of drug-resistant characteristics among commensal and pathogenic bacteria have also been largely driven by the selective pressure imposed by the use of antimicrobial drugs (Aarestrup et al., 2008). It has been noted that bacterial isolates' susceptibility to antibiotics is dynamic and changes over time and in different environments. As a result, it is necessary to regularly check the antibiotic susceptibility profiles of common bacterial pathogens in various communities (Nsofor & Iroegbu, 2019).

Consequently, there are insufficient data on recent prevalence and antibiotic sensitivity profiles of ETEC in Okigwe, Imo State, comprising samples from children under the age of 5 years, environment and animals. To overcome this knowledge gap, the present study evaluated the prevalence and antibiotics sensitivity profiles of ETEC pathotype of E. coli in a variety of samples from Okigwe zone, Imo State, Nigeria.

MATERIAL AND METHODS

Study Area

The Okigwe zone of Imo State, Nigeria, was the site of this investigation. Situated in Nigeria's South-East geopolitical zone, Imo State shares borders with Anambra State to the north, Rivers State to the west and south, and Abia State to the east. Although over-farming and high population density have seriously degraded the soil, agriculture remains the main profession of the locals (Onu et al., 2008). The Okigwe zone experiences a yearly rainfall of 1,500 to 2,200 mm (60 to 80 inches) during the rainy season, which runs from April to October. Relative humidity is 75% when the average yearly temperature is higher than 20 °C (68.0 °F). Humidity levels can reach 90% during the rainy season (Njoku et al., 2017). During the dry season, which runs from late December to late February, harmattan occurs for two months. Months of January to March are the hottest months in Imo State (Okoroafor-Nwosu et al., 2017).

Samples Collection

Samples for the isolation of E. coli were collected uniformly throughout Imo State's Okigwe zone. A total of 150 samples were taken at random from the study area using sterile sample containers. These samples included 50 samples of cow dungs (C) from slaughterhouses and cow rearing facilities, human diarrheal stools from children aged 0 to 5 (H), and water samples from rivers (W). May and June of 2025 saw the collection of samples. After being labelled and placed in an ice pack, each sample was brought to the lab for prompt analysis.

Biochemical Characterization of Isolates

Eosin Methylene Blue (EMB) agar, prepared according to the manufacturer's instructions, was used to isolate E. coli. The collected samples were serially diluted under aseptic conditions prior to inoculation. After spreading 1 ml of the 10-5 dilution on the media, it was incubated for 24 h at 37 oC. To obtain pure cultures, each distinct colony was sub-cultured by streaking on new media. Morphological, biochemical, and molecular techniques were used to characterize the isolated bacteria. The method outlined by Cheesbrough (2004) and Aryal (2020) was used for Gram staining and biochemical characterization of isolates.

Antibiotic Susceptibility Test

Using the Kirby Bauer disc diffusion method, the sensitivities of the isolates were tested to the drugs: gentamicin (GEN), levofloxacin (LBC), augmentin (AUG), ceftriazone (CTR), ciprofloxacin (CIP), cefepime (CPM), meropenem (MEM) and aztreonam (AT). This was done using each cell suspension equivalent to 0.5 McFarland standards (Hudzicki, 2016).

Molecular Characterization of EPEC Isolates

Extraction of DNA

A ZR fungi/bacteria DNA mini-prep extraction kit from Inqaba South Africa was used for the extraction. First, 200 µl of isotonic buffer, pure colonies of the bacterial isolate, and 750 µl of lysis solution were introduced into the ZR Bashing Bead lysis tubes. The tubes were fastened in a bead beater with a 2 ml tube holder assembly and treated for 5 min at maximum speed. Centrifugation was performed on the ZR bashing bead lysis tubes for one minute at 10,000 rpm. After that, 400 µl of the supernatant was moved to a Zymo-Spin IV spin filter (orange top) in a collecting tube and centrifuged for one minute at 7000 rpm. The total volume in the collection tubes was then increased to 1600 µl by adding 1200 µl of fungal/bacterial DNA binding buffer to the filtrate.

Then 800 µl was then moved to a Zymo-Spin IIC column in a collection tube, centrifuged for 1 min at 10,000 rpm, and the flow through was removed from the collection tube. After being moved to the same Zymo-spin, the remaining volume was spun. Then 200 microliters of the DNA Pre-Was buffer was added to the Zymo-spin IIC in a fresh collection tube. It was spun at 10,000 rpm for 1 min. Next, 500 microliters of the fungal/bacterial DNA Wash Buffer were added, and it was centrifuged at 10,000 rpm for 1 min. To elute the DNA, the Zymo-spin IIC column was moved to a sterile 1500 µl centrifuge tube, 100 µl of DNA elution buffer was added to the column matrix, and the tube was spun for 30 seconds at 10,000 rpm. After that, the ultra-pure DNA was kept at -20 oC for further studies.

DNA quantification

Utilizing the Nanodrop 1000 spectrophotometer, the isolated genomic DNA was measured. The Nanodrop icon was double clicked to launch the equipment's software. Then 2 µl of sterile distilled water were used to initialize the apparatus, and normal saline was used to blank it. After loading 2 µl of the extracted DNA onto the lower pedestal, the upper pedestal was lowered to make contact with the extracted DNA. Clicking the "measure" button allowed the DNA concentration to be calculated.

Extended Spectrum Beta- Lactamases detection

TEM Genes Amplification

The TEMF: 5'-ATGAGTATTCAACATTTCCGTG-3' and TEMR: 5'-TTACCAATGCTTAATCAGTGAG-3' primers were used to amplify TEM genes of the isolates on an ABI 9700 Applied Biosystems thermal cycler, for 35 cycles at a final volume of 40 microliters. The PCR mixture contained 50 ng of extracted DNA as a template, primers at a concentration of 0.4M, and the X2 Dream Taq Master mix (Taq polymerase, DNTPs, and MgCl) provided by Inqaba, South Africa.

These were the PCR conditions: 95 ºC for 5 minutes for initial denaturation, 95ºC for 30 seconds for denaturation, 58 ºC for 30 seconds for annealing, 72 ºC for 30 seconds of extension for 35 cycles, and 72 ºC for 5 minutes for final extension. The product was resolved on a 1% agarose gel at 120V for 25 minutes and visualized on a UV transilluminator.

CTX-M Genes Amplification

The CTX-MF: 5'-CGCTTTGCGATGTGCAG-3' and CTX-MR: 5'-ACCGCGATATCGTTGGT-3' primers were used to amplify the CTX-M genes from the isolates on an ABI 9700 Applied Biosystems thermal cycler, for 35 cycles at a final volume of 40 µl. The PCR mixture contained 50 ng of extracted DNA as a template, primers at a concentration of 0.4M, and the X2 Dream Taq Master mix (Taq polymerase, DNTPs, and MgCl) provided by Inqaba, South Africa.

The PCR conditions were as follows: initial denaturation at 95 oC for 5 minutes; denaturation at 95 ºC for 30 sec; annealing at 52 oC for 30 seconds; extension at 72 oC for 30 seconds for 35 cycles; final extension at 72 oC for 5 minutes. The result was visualized using transilluminator after being resolved at 120V for 25 minutes on a 1% agarose gel.

SHV Genes Amplification

The SHV F: 5' CGCCTGTGTATTATCTCCCT-3' and SHV R: 5' CGAGTAGTCCACCAGATCCT-3' primers were used to amplify the SHV genes from the isolates on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 µl for 35 cycles. The PCR mixture contained 50 ng of the extracted DNA as a template, primers at a concentration of 0.4M, and the X2 Dream Taq Master mix (Taq polymerase, DNTPs, and MgCl) provided by Inqaba, South Africa.

The following were the PCR conditions: Initial denaturation at 95 oC for five minutes; denaturation at 95 oC for thirty seconds; annealing at 56 oC for forty seconds; extension at 72 oC for fifty seconds for thirty-five cycles; and final extension at 72 oC for five minutes. The result was visualized using transilluminator after being resolved at 120V for 25 minutes on a 1% agarose gel.

Identification of Isolates with Molecular Techniques

DNA Extraction Using Heating Method

Five milliliters of an overnight broth culture of the isolated bacteria in Luria Bertani (LA) media were centrifuged in 1.5 ml Eppendorf tubes at 14000 rpm for three minutes. The cells were resuspended in 500 µl of normal saline after the supernatant was disposed of. It was homogenized following vortexing to remove any remaining residue.

After centrifuging it for three minutes and discarding the supernatant once more, 300 µl of DNA elution buffer was added. After vortexing again, it was heated to 95 oC for 20 minutes on a heating block. For purification, the heated bacterial suspension was quickly cooled on ice and spun at 1400 rpm for three minutes. Then 200 µl of the DNA-containing supernatant was moved to a 1.5 ml micro centrifuge tube and kept at -20 oC for further downstream processes.

DNA Quantification

Utilizing the Nanodrop 1000 spectrophotometer, the isolated genomic DNA was measured. The Nanodrop icon was double clicked to launch the equipment's software. Then 2 µl of sterile distilled water were used to initialize the apparatus, and normal saline was used to blank it. After loading 2 µl of the extracted DNA onto the lower pedestal, the upper pedestal was lowered to make contact with the extracted DNA. Clicking the "measure" button allowed the DNA concentration to be calculated.

16S r RNA Amplification

The 27F: 51-AGAGTTTGATCMTGGCTCAG-3′ and 1492R: 51-CGGTTACCTTGTTACGACTT-3′ primers were used to amplify the 16S rRNA regions of the isolates rRNA genes using an ABI 9700 Applied Biosystem Thermal Cycler at a final volume of 40 µl for 35 cycles. The primers at a concentration of 0.4 µl, the extracted DNA as a template, and the X2 Dream Taq Master mix (Taq Polymerase, DNTPs, MgCl) provided by Inqaba, South Africa comprise the PCR mix.

The PCR conditions were as follows: initial denaturation at 95 oC for 5 minutes; denaturation at 95 oC for 30 seconds; annealing at 52 oC for 30 seconds, extension at 72 oC for 30 seconds for 35 cycles, and final extension at 72 oC for 5 minutes. A transilluminator was used to visualize the result after it was resolved on a 1% agarose gel at 130V for 25 minutes. They are compared to a 100 bp ladder marker (Amersham Pharmacia Biotech, USA) to determine the PCR product.

Sequencing

The 3510ABI sequencer from Inqaba Biotechnology in Pretoria, South Africa, was used to perform the sequencing utilizing the Big Dye Terminator kit. The 0.25 µl big dye terminator v1.1/v3.1, 2.25 µl of 5 x big dye sequencing buffer, 10 µml primer PCR primer, and 2–10 ng PCR template per 100 bp were the components used in the sequencing, which was completed at a final volume of 10 µl. These were the conditions for sequencing: 32 cycles at 96 oC for 10 seconds; 55 oC for for 5 seconds; 60 oC for 4 minutes.

RESULTS AND DISCUSSION

Morphological and Biochemical Characteristics of Isolates

Table 1 presents the morphological and biochemical characteristics of the targeted E. coli isolates obtained from cow dung, river water samples and human diarrheal samples from Okigwe zone of Imo State. Under a light microscope, they showed that the isolates were Gram negative rods. They were primarily motile, exhibiting positive responses to tests for indole, methyl red and catalase, but negative responses to tests for coagulase, oxidase, hydrogen sulfide, voges-proskaure, citrate and urease.

Their ability to produce gas was demonstrated by the yellow color of the triple sugar iron agar medium. Results showed that out of a total of 150 samples studied, which comprised of 50 cow dung samples, 50 river water samples and 50 diarrheal samples of children between 0 to 5 years, E. coli was isolated from a total of 44 samples representing 29.33% of the total.

Specifically, there was E. coli in 13 cow dung samples (26.00%), 14 river water samples (28.00%) and 17 human faecal samples (34.00%) out of the total of 50 samples in each case from Okigwe zone of Imo State. These findings are less than those of an earlier study that revealed E. coli was identified from 62% of raw milk samples overall, including 62% of dairy stores, 59% of dairy farms, and 68% of street sellers (Amin et al., 2017). The prevalence of isolated E. coli strains from O101: 51 (42.50%), O8: 18 (15.00%), O9: 10 (8.30%), O115: 6 (5.00%), and 35 (29.10%) for unknown have been reported in another investigation (Yadegari et al., 2019).

Molecular Characteristics of ETEC Isolates

Phylogenetic tree was constructed from 16S rRNA sequences of Escherichia coli obtained from animal (cow dung, C), human (H), and environmental water (W). The tree topology revealed two principal groups that's separated by a moderately supported basal node (bootstrap = 79). Within these groups, a clade with strong bootstrap support (100) that included both human (H31_Escherichia coli) and environmental (W136_Escherichia coli) isolates was observed. Likewise, a moderately supported pairing bootstrap of 65 between W40_Escherichia coli and H94_Escherichia coli was identified.

A cluster containing C61_Escherichia coli, C142_Escherichia coli, and W8_Escherichia coli had bootstrap

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values of 64 and 59. Several isolates such as H10_Escherichia coli, H123_Escherichia coli, and C144_Escherichia coli were placed on long branches suggesting greater sequence divergence.

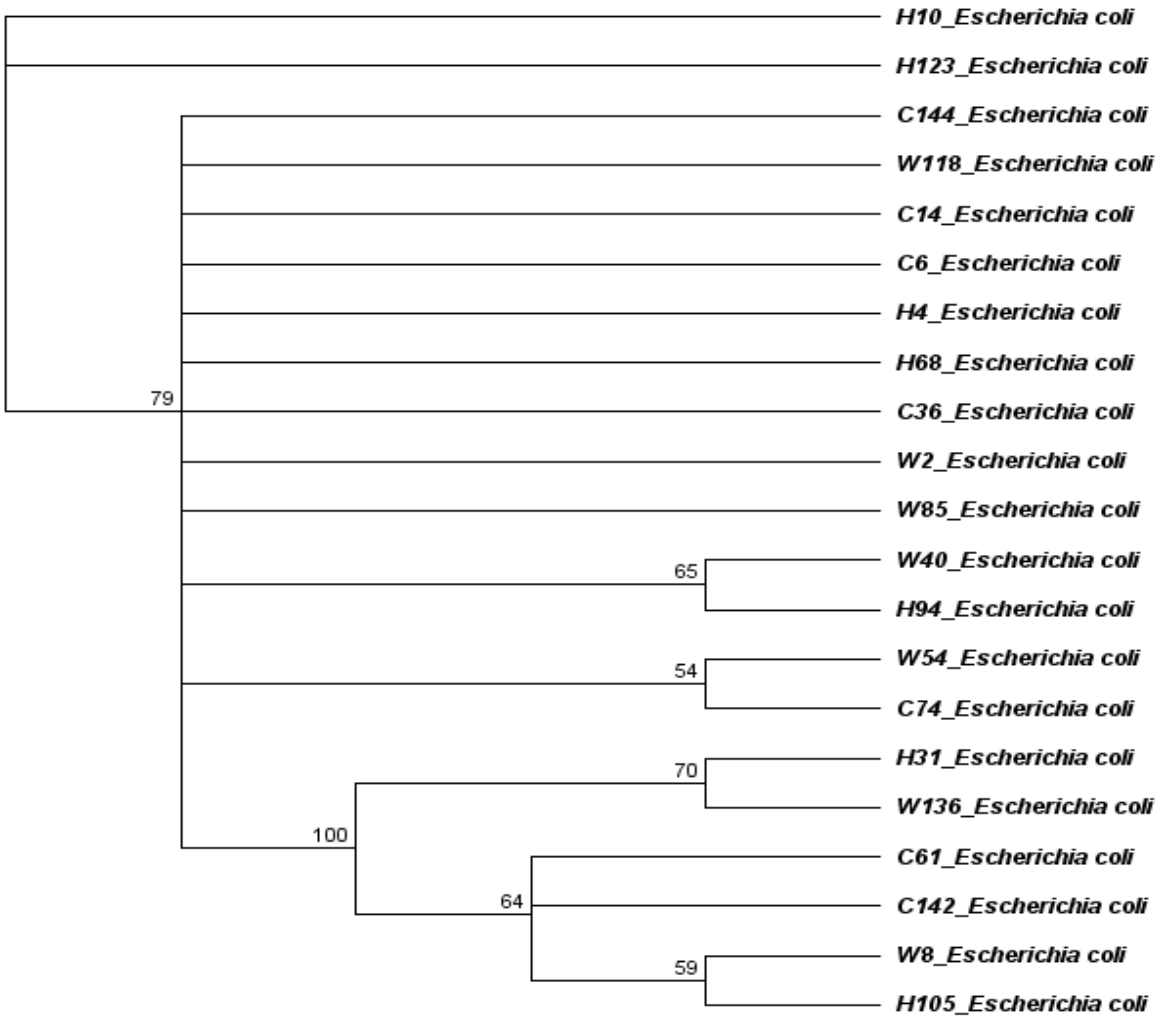


Figure 1. Phylogenetic of evolutionary relationships between ETEC isolates

Table 1. Morphological and biochemical characteristics of E. coli isolates from animal, human and environmental samples

		Biochemical Test	**Colony characteristic**
	Gram Stain	Gram-negative rod (−ve rod)	Smooth, circular, convex colonies having green metallic sheen on EMB agar
	Oxidase Test	Negative (−ve)	
	Citrate Test	Negative (−ve)	
	Indole Test	Positive (+ve)	
	H_2S Test	Negative (−ve)	
	Coagulase Test	Negative (−ve)	
	TSIA Test	Positive (+ve)	
	Urease Test	Negative (−ve)	
	Methyl Red Test	Positive (+ve)	
	Voges–Proskauer (VP) Test	Negative (−ve)	
	Motility Test	Positive (+ve)	

	Catalase Test	Positive (+ve)	
Sugar fermentation Test	**Glucose**	Positive (+ve)	
	Maltose	Negative (−ve)	
	Mannitol	Positive (+ve)	

Legends:

TSIA: Triple Sugar Iron agar medium

EMB: Eosin Methylene Blue

VP: Voges Proskauer

Antibiotics Sensitivity/Resistance Profiles of the E. coli Isolates

Figure 2 indicates the antibiotics susceptibility profiles of E. coli isolated from cow dung, river water and human diarrheal samples from Okigwe. The results indicated that each of the antibiotics used against the isolates from the locations exhibited varying zones of inhibitions. On comparison of the zones of inhibition to the guidelines of the Clinical and Laboratory Standard Institute (2017) to classify the isolates into resistant, intermediate and sensitive to each of the antibiotics, results showed that sensitivities of the isolates from each sample to the antibiotics used in this study were low. It was observed that out of 12 E. coli isolates obtained from cow dung in Okigwe zone, the highest percentage (41.7%) of the isolates that showed sensitivity was recorded with Ciprofloxacin, while they were all resistant to Augmentin, Ceftriaxone, Cefepime, and Aztreonam. In river water samples, the highest percentage (23.1%) of isolates also showed sensitivity to Ciprofloxacin, whereas isolates were resistant to Ceftriaxone, Cefepime and Aztreonam. For human diarrheal samples, the highest percentage (26.7%) of sensitive isolates was also recorded with Ciprofloxacin. As well, the isolates were all resistant to Augmentin, Ceftriaxone, Cefepime, Aztreonam and Meropenem. Statistically, at $\alpha = 0.05$ there was no significant difference in the percentages of isolates that showed sensitivity to the drugs across the samples studied. . Similarly, the antibiograms of another study of multidrug-resistant Escherichia coli isolates showed that all isolates (100%) were resistant to doxycycline (94.87%), gentamycin (79.16%), ciprofloxacin (75.48%), erythromycin (73.07%), levofloxacin (71.66%), ceftriaxone (47.36%), and tetracycline (46.66%). On the other hand, all strains of E. coli were susceptible to azithromycin (92.50%), vancomycin (92.50%), and amikacin (95%) (Haque et al., 2023).

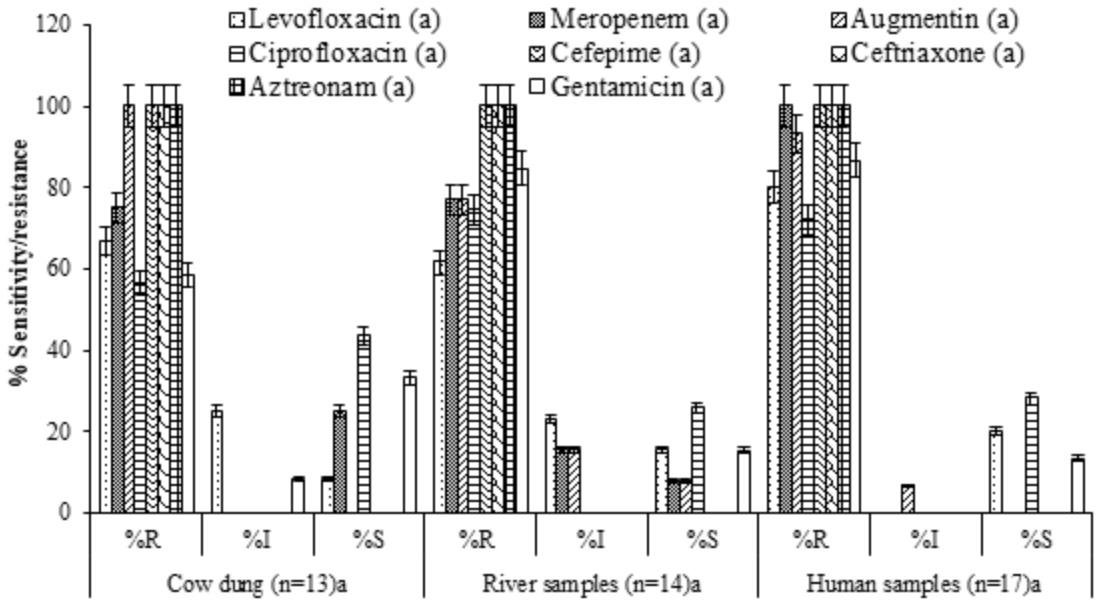


Figure 2. Antibiotics sensitivity profiles of E. coli isolates in cow dung, river water and human diarrheal

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samples from Okigwe. Legends: Parameters with different lower case letters are significantly different; R = Resistant; I = Intermediate and S = Susceptible.

Prevalence of ETEC Virulence Genes

Results obtained in this study revealed the presence of different virulence genes in the identified E. coli isolates collected from different zones of Okigwe zone, Imo State. Figure 3 is the multiplex agarose gel electrophoresis showing the LT and ST genes of ETEC in lanes 2, 3, 6 and 7. Comparing them to the reference 100 bp ladder, the sizes of LT and ST of ETEC genes were 1200 bp and 150 bp respectively. Samples in lanes 1, 4, 5 and 8 failed to produce any bands, indicating absence of the genes.

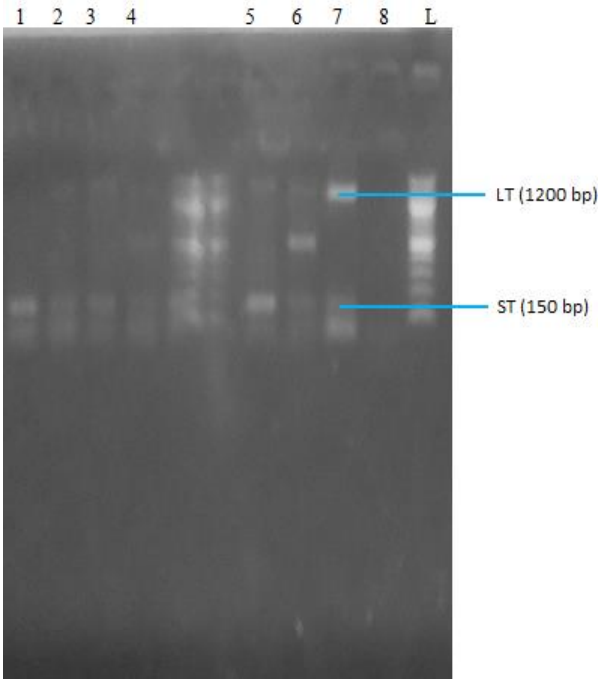


Figure 3. Enterotoxigenic strain of E. coli on multiplex agarose gel electrophoresis. Lanes 2, 3, 6 and 7 represent the ETEC strains while lane L represents the 100 bp ladder.

The prevalence of LT gene of ETEC was higher in cow dung samples with 50% than the ST gene with 41.7% (Figure 4). However, in river water and diarrheal faecal samples, the prevalence of ST gene was higher than that of LT. There was 30.8% and 30.8% prevalence of ST genes in river water samples and diarrheal faecal samples, but 7.7% and 15.4%, respectively, for LT gene. The statistical analysis of the results revealed that there is no significant difference in the prevalence of the resistance enzymes as well as the virulence genes across the three Zones.

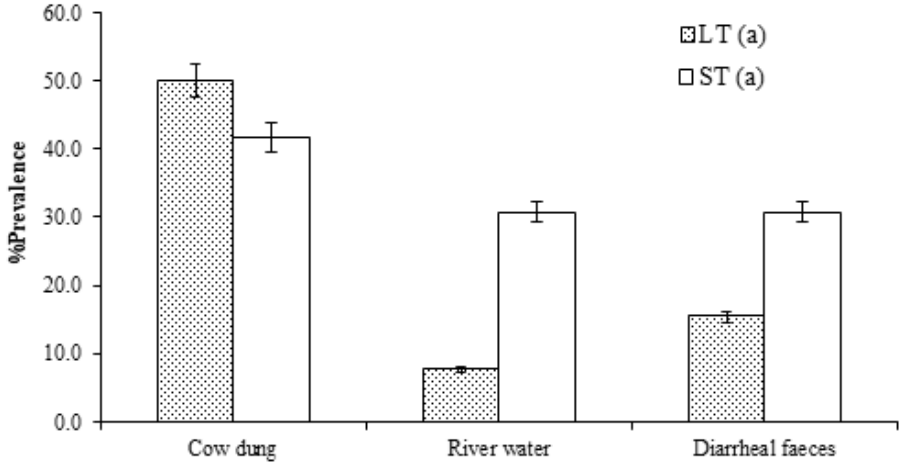


Figure 4. Virulence genes prevalence in E. coli isolates in cow dungs, river water and human diarrheal

samples from Okigwe zone. Legends: Factors with different lower case letters are significantly different.

A prior investigation revealed that 23 (10.2%) of the 225 E. coli isolates that were characterized tested positive for ETEC. The most prevalent gene found in 16 (69.6%) was the heat stable toxin (ST) gene; 5 (21.7%) of the ETEC isolates had the heat-labile toxin (LT) gene while 2 (8.7%) of the isolates had genes for both ST and LT toxins. While ETEC LT gene was equally detected in 1 (20%) of all age groups, the majority of 5 (31.25%) of ETEC with ST gene were found in children aged >36–48 months. In children aged <12–24 months and >36–48 months, ETEC containing both LT/ST genes were equally detected 1 (50%) (Kipkirui et al., 2021). In a similar vein, a different study revealed that 3.7% of all milk and cheese samples had ETEC. The incidence of ETEC in milk was 0.9% because just one isolate of E. coli from raw milk had the STh gene. According to Amin et al. (2017), ETEC accounted for 9.6% of the E. coli isolates from Kareish cheese, of which two contained the LT gene and five simply had the STh gene. This is lower than the prevalence recorded in the current study. El Nahas et al. (2015) found that Kareish cheese had 15% ETEC and milk samples had 27.27%. Additionally, a Multiplex PCR analysis of the three primary virulence genes of ETEC, F5, F41, and STa, which have respective diameters of 314 bp, 380 bp, and 190 bp, showed that only 7 (5.80%) of all patients tested positive for F5, F41, and STa. The frequency of isolates with one copy of 1800 bp was 44.16% (53/120), that of isolates with one copy of 2200 bp was 18.33% (22/120), and that of isolates with both copies was 21.66% (26/120). Moreover, 19 isolates (15.83%) did not code for a comparable region (Yadegari et al., 2019). A previous report has shown that approximately one-third of all ETEC strains isolated worldwide are ST-only, one-third are ST/LT, and one-third are LT-only (Amin et al., 2017).

CONCLUSION

The prevalence and antibiograms of ETEC in various samples from the Okigwe zone of Imo State, Nigeria, have been recorded in this study. These findings as well as the fact that they differed from some results of earlier studies conducted in other states in Nigeria, and other countries, highlight the necessity for regular monitoring and identification of ETEC toxins and their antibiotic profiling. This will improve knowledge of their prevalence and drug resistance patterns. Moreover, a thorough understanding of the nature and distribution of toxins is necessary for the development of ETEC vaccine. Thus, these findings offer valuable insights which may be used as baseline for the development of an ETEC vaccine and appropriate policy making.

Competing Interests

Authors have declared that no competing interests exist.

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