Using Molecular Techniques for the Characterization of *Escherichia coli* Isolated from Creek Road/Bonny Estuary in Rivers State, Nigeria

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Authors' contributions

This work was carried out in collaboration between both authors. Authors CPA and KCW designed the study. Author CPA performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed the literature searches. Both authors managed the analyses of the study and approved the final manuscript.

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ABSTRACT

This study characterised *Escherichia coli* isolated from Creek Road/Bonny Estuary using molecular techniques. One hundred and twenty (120) water samples were investigated for the presence of *E. coli* in Creek Road/Bonny Estuary. Most Probable Number (MPN) Technique, Eijkman test and molecular techniques were used for the isolation and detection of *E. coli*. The analysis was carried out periodically (morning and evening) and seasonally (end of rainy season (November), the start of rainy season (April) and in the middle of rainy season (July)). All *E. coli* isolates were screened for resistance genes in the pathotypes of *E. coli* (EHEC, EPEC, EAEC, ETEC and EIEC), including Sulfhydryl Variable (SHV), CTX-M, Temoniera (TEM) and MCR genes using the Polymerase chain reaction Technique. The results showed that 100% of the water samples were positive for coliforms and all the water samples harboured *E. coli*. The highest total coliforms count recorded was in the morning of April (48 MPN/100 ml). The distribution of individual genes in the *E. coli* isolates are: SHV (5.0), CTX-M (5.8), TEM (4.2), MCR (0.0%), stx1 and stx2 (4.2), esV and bfA (1.7), aaiC (4.2), elt (3.3) and invE (2.5). The CTX-M was the most found of all the resistance genes.
1. INTRODUCTION

Most *Escherichia coli* (E. coli) strains exist as part of the normal flora of endotherms, while some strains are pathogenic. The pathogenic strains are pathogenic enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), uropathogenic *E. coli* (UPEC), enteroaggregative *E. coli* and diffusely adherent *E. coli*. Aside from *E. coli*, many other microorganisms are found in the gut of warm-blooded animals as normal flora. However, *E. coli* is a major inhabitant of the large intestine [1,2]. Researchers have noted that some strains of *E. coli* are the frequent cause of life-threatening gastroenteritis (infectious diarrhoea), bloodstream infections and other common infections, such as urinary tract infections, neonatal meningitis and others [3].

According to Environmental Protection Agency (US-EPA) *Escherichia coli* is one of the indicator bacteria that if detected, may suggest the presence of faecal material in food, soil, as well as water environment [4]. Bathing in such polluted water environment could increase the chances of developing illnesses as pathogens could enter the body through different routes, such as the mouth, nose, ear or cuts on the skins. One of such illnesses is gastroenteritis. One of the main sources of *E. coli* to water bodies are building toilets on water bodies and the discharge of sewage into water environment. The use of human and animal excreta as manure could also be a source of polluting water bodies with faecal materials [5,6].

Pollution of natural water bodies with waste effluents and Heavy metals arising from various industries and oil through spills have been recorded in Nigeria. Consequently, there is the likelihood that toxicants from these sources may affect plants, enter the food chain and affect a larger human population, as well as cause mutations in the microorganisms found in the water environment. The former is because, the polluted water contents may cause changes in the microorganisms, which could lead to mutation in their genetic make-up and cumulative to antibiotic-resistant-microorganisms [7,8]. This problem is further aggravated as some residents of Rivers State (mostly in the rural areas) defecate in the water bodies and depend directly on them for drinking and other domestic purposes. Recently, the rapidly rising antimicrobial resistance rates in *E. coli* has been reported; one of such reports was on BBC News London by James Gallagher (21 December, 2015) [9]. He stated that another strain of *E. coli* that is resistant to the "last-resort" antibiotic called colistin (polymyxins), has been discovered and the gene identified to be responsible for the resistance is called Mobilized Colistin Resistance gene (MCR gene). The most recently discovered strain has been isolated from humans and food animals/poultry [9, 10].

Because of some activities, which are seen regularly such as, the building of toilets on water bodies and the discharge of untreated and improperly treated sewage into water bodies, high levels of temperature [11,12] and high concentrations of heavy metals [13] in water bodies, there is therefore need to check if there are isolates of *E. coli* from water bodies in Rivers State that have developed mutations.

2. MATERIALS AND METHODS

2.1 Study Area and Sample Collection

The present study was carried out in Rivers State, Nigeria from November, 2016 to July 2018 (end of rainy season (November), the start of rainy season (April) and the middle of rainy season (July)). A total of one hundred and twenty (120) water samples were collected from Creek Road/Bonny Estuary in sterile bottles (4 oz. (118.3 ml) capacity). After the collection, the water samples were put in a cooler filled with ice packs, transported immediately to the Department of Medical Laboratory Science Medical Microbiology Laboratory, Rivers State University, Port Harcourt, Nigeria and processed within two to three hours. The bacteriological analysis was carried out both seasonally and periodically (morning and evening).

Creek Road/Bonny Estuary is one of the main water bodies in Rivers State, Nigeria where people visit almost every day as there is a major market located very close to the water body, called Creek Road market. The choice of the water body was based on human activities, including bunkering activities in and around the...
water body, its close proximity to the market and because toilets are built on it. Creek Road/Bonny Estuary is also one of the major markets where the people of Rivers State go to purchase fishes and seafood that they consume, as it is a marine water. Most of the fishes and seafood consumed by the people of Rivers State, especially those who reside in that part of the City are from the water body. Also, some local residents depend on the water body for domestic activities (for example, washing of cloths). Also, some of the residents swim in the water body. Some of the local residents at Creek Road could be grouped as lower class.

During each season (November, April and July), the water samples were collected 10 times in a week; that is to say that each day, 2 water samples were collected in the morning and 2 in the evening, so that 4 water samples were collected in a day. Then, in 5 days, 20 water samples were collected (both in the morning and evening). In every season, 20 water samples were collected and totally (from November, 2016 to July, 2018), 120 water samples were collected.

2.2 Isolation and Identification of Escherichia coli

Escherichia coli was enumerated, isolated and identified from water samples using the standard culture technique, microscopic method, Most Probable Number (MPN) technique and the Eijkman test [14,15]. The MPN technique was analysed in a series of tubes containing a MacConkey broth culture medium. The test tubes were inoculated with test portions of samples of water. After a specified incubation time at a given temperature, each tube showing gas formation and yellow colour was regarded as positive.

The MPN of bacteria present was estimated from the number of tubes inoculated and the number of positive tubes obtained in the test, using a specially devised statistical table (MacCrady table) [16,17,18]. Moreover, for confirmation of the thermostolerant coliform bacteria (E. coli), positive tubes obtained in the test were further incubated in water bath at 45°C for 24 hours (Eijkman test). The positive tubes showed gas formation and yellow colour.

In addition, in order to further confirm, CHROMagar ECC (CHROMagar for E. coli and other coliforms) was inoculated with materials taken from the positive tubes (positive tubes incubated at 45°C). Following an appropriate incubation time, the culture plates were examined for the presence of E. coli (showed a blue colour) [19]. Finally, the pure cultures of E. coli were sent for molecular detection of the genes in the isolates of E. coli.

2.3 Molecular Detection of Genes in the E. coli Isolates

An overnight Luria Bertani (LB) broth culture of each of the E. coli isolates (5 ml) was centrifuged at 14400 revolutions per minute (rpm) for 3 minutes and the supernatant was discarded into a disinfectant jar. Then, five hundred microlitres (500 µl) of sterile normal saline was used to resuspend the cell pellet in each tube and they were heated at 95°C for 20 minutes. Then, the heated bacterial suspension was cooled on ice and spun for 3 minutes at 14000 rpm. The supernatant containing the DNA was transferred into a clean 1.5 ml microcentrifuge tube and stored at -20°C for other downstream reactions till required for Polymerase Chain Reaction (PCR). Nanodrop 1000 spectrophotometer was used to quantify the extracted genomic DNA. The primers for the study were synthesized at Inqaba Biotechnical limited, South Africa.

Sulphrydryl Variable (SHV) genes from the isolates of E. coli were amplified using the SHV F: 5' CGCCTGTGTATTATCTCCCT-3' and SHV R: 5'-CGAAGTGATCACGAGATCCT-3' primers [20] on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 30 µl for 35 cycles. The PCR mix were: the X2 Dream taq Master mix manufactured by New England BIOLABS (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 M and 50 ng of the extracted DNA as template. The following were the PCR conditions: Initial denaturation, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; annealing, 56°C for 40 seconds; extension, 72°C for 50 seconds for 35 cycles and final extension, 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 120 V for 25 minutes and visualized on a UV transilluminator for a 200 bp product size [20].

Primers, such as CTX-MF: 5'- CGCTTTGCGATGCGCAG-3' and CTX-MR: 5'- ACCGCGATACCGTGGT-3' were used to amplify CTX-M genes from the isolates [21] on an ABI 9700 Applied Biosystems thermal cycler.
The PCR conditions were as follows: Initial denaturation, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; annealing, 58°C for 30 seconds; extension, 72°C for 30 seconds for 35 cycles and final extension, 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 120 V for 25 minutes and visualized on a UV transilluminator for a 980 bp product size.

Multiplex PCRs were performed on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X5 Multiplex Master mix manufactured by New England BIOLABS (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 M and 50 ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; annealing, 52°C for 30 seconds; extension, 72°C for 30 seconds for 35 cycles and final extension, 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 120 V for 25 minutes and visualized on a UV transilluminator.

All isolates were screened for the presence of the MCR using the MCR gene. MCR-F 5'-CGGTCACTGCTTTGTTTC-3' and MCR-R 5'-CTTGGCTCGGTCTGTAGGG-3'. A 25 μl PCR reaction was carried out with the following amplification conditions 94°C 60 sec. with a final extension of 72°C for 10 min. PCR generated products were run on a 1.5% agarose gel at 120 V for 25 min. and visualised on a blue light transilluminator.

Temoniera (TEM) genes from the isolates were amplified using the TEMF: 5'-ATGAGTATTAACATTTACC-3' and TEMR: 5'-TTACCAATGCTTATACGTAG-3' primers [20] on an ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix included: the X2 Dream taq Master mix manufactured by New England BIOLABS (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.4 M and 50 ng of the extracted DNA as template. The PCR conditions were as follows: Initial denaturation, 95°C for 5 minutes; denaturation, 95°C for 30 seconds; annealing, 58°C for 30 seconds; extension, 72°C for 30 seconds for 35 cycles and final extension, 72°C for 5 minutes. The product was resolved on a 1% agarose gel at 120 V for 25 minutes and visualized on a UV transilluminator.

### 3. RESULTS

#### 3.1 Seasonal Variations of Total Coliform Count (TCC) and Total *E. coli* in November

Table 2 shows the results of the total coliform and total *E. coli* counts in the morning and evening of November. In accordance with the results, the highest coliform count was obtained on days 1 and 2 (28 MPN/100 ml). The results also showed that *E. coli* was found.

**Table 1. List of multiplex Polymerase Chain Reaction (PCR) primers [21]**

| Target genes | Primer sequence | Amplicon size (bp) |
|--------------|----------------|-------------------|
| aiC Forward  | 5'-AGAGCGGTCCACTGTCAGAGCGT-3' | 500 |
| aiC Reverse  | 5'-GGACACCTGCTTGCGTGAAAT-3' | |
| esV Forward  | 5'-TAACCGCTGCAGCATACTACCC-3' | 620 |
| esV Reverse  | 5'-TGATGCTGCGCTGCTGAG-3' | |
| bfpA Forward | 5'-TCTGCAATGGTGTGCGTTG-3' | 330 |
| bfpA Reverse | 5'-CAGTTGGCGCTCTACAGCAGGAG-3' | |
| stx1 Forward | 5'-AGCGATGCACTTAAAAA-3' | 300 |
| stx1 Reverse | 5'-GAAGAAGTCCGTGGGATTACG-3' | |
| invE Forward | 5'-GTTCAGAAATTGCCAGCAGC-5' | 600 |
| invE Reverse | 5'-GTTCAGAAATTGCCAGCAGC-5' | |
| stx2 Forward | 5'-TTAACCACACCCCCACCCGGCAG-3' | 300 |
| stx2 Reverse | 5'-GCTGGAATGCACGTCTCAGGT-3' | |
| elt Forward  | 5'-AACGGGTAACAGCTTACCAAC-3' | 400 |
| elt Reverse  | 5'-ACCTGAATATGTGGCCTACGGTCTCT-3' | |

Table 3, the highest total coliform count was obtained on day 1 (48 MPN/100ml), in the
morning of April. *Escherichia coli* was also found in the water body both in the morning and in the evening.

**Seasonal variations of Total Coliform Count (TCC) and total *E. coli* in July:** The highest TCC obtained (21 MPN/100 ml) was in the evening of day 3 in July (Table 4). *Escherichia coli* was also found both in the morning and in the evening.

**Distribution of the resistance genes detected in *E. coli* isolates:** As shown in Table 5, the gene detected most in the isolates of *E. coli* is CTX-M, followed by SHV. However, MCR gene was not detected at all, while some other resistance genes were detected in the isolates of *E. coli*.

### 4. DISCUSSION

It is estimated that every sickness in the world can be attributed to inadequate suitable water supplies and poor sanitation [22]. Industrialization, urbanization and modernization have continued to threaten natural water quality available to humans, animals and organisms. For example, the use of pesticides and fertilizers has to a great extent, made natural water highly polluted with different harmful pollutants. Moreover, the presence of coliforms and *E. coli* in water bodies has been reported by other researchers, both in Nigeria and outside [23], [24].

The present study investigated 120 water samples for the detection of *E. coli* from Creek

### Table 2. Seasonal variations of Total Coliform Count (TCC) and total *E. coli* in November

| Days | Morning | Evening |
|------|---------|---------|
| TCC MPN/100 ml | TEC MPN/100 ml | TCC MPN/100 ml | TEC MPN/100 ml |
| 1 | 28 | 7 (25.0%) | 15 | 3 (20.0%) |
| 2 | 20 | 7 (35.0%) | 28 | 7 (25.0%) |
| 3 | 11 | 3 (27.3%) | 21 | 3 (14.3%) |
| 4 | 15 | 3 (20.0%) | 20 | 3 (15.0%) |
| 5 | 21 | 4 (19.0%) | 15 | 7 (46.6%) |

*This was carried out in replicate

### Table 3. Seasonal variations of Total Coliform Count (TCC) and total *E. coli* in April

| Days | Morning | Evening |
|------|---------|---------|
| TCC MPN/100 ml | TEC MPN/100 ml | TCC MPN/100 ml | TEC MPN/100 ml |
| 1 | 48 | 7 (14.6%) | 28 | 7 (25.0%) |
| 2 | 11 | 3 (27.3%) | 11 | 3 (27.3%) |
| 3 | 21 | 3 (14.3%) | 23 | 4 (17.4%) |
| 4 | 11 | 3 (27.3%) | 11 | 3 (27.3%) |
| 5 | 15 | 7 (46.7%) | 20 | 3 (15.0%) |

*This was carried out in replicate

### Table 4. Seasonal variations of Total Coliform Count (TCC) and total *E. coli* in July

| Days | Morning | Evening |
|------|---------|---------|
| TCC MPN/100 ml | TEC MPN/100 ml | TCC MPN/100 ml | TEC MPN/100 ml |
| 1 | 15 | 3 (20.0%) | 14 | 3 (21.4%) |
| 2 | 20 | 3 (15.0%) | 11 | 7 (63.6%) |
| 3 | 7 | 4 (57.1%) | 21 | 3 (14.3%) |
| 4 | 15 | 3 (20.0%) | 15 | 7 (46.7%) |
| 5 | 11 | 3 (27.3%) | 15 | 3 (20.0%) |

*This was carried out in replicate
Table 5. Distribution of the resistance genes detected in *E. coli* isolates

| Locations          | No. of *E. coli* isolates | SHV  | CTX-M | TEM  | MCR (Colistin) | EHEC (*Stx1*; *Stx2*) | EPEC (*esV*; *bfA*) | EAEC (*aaIC*) | ETEC (*elt*) | EIEC (*invE*) |
|--------------------|---------------------------|------|-------|------|----------------|------------------------|---------------------|---------------|-------------|---------------|
| Creek Road/Bonny   | 120                       | 6 (5.0) | 7 (5.8) | 5 (4.2) | 0 (0.0 %) | 5 (4.2) | 2 (1.7) | 5 (4.2) | 4 (3.3) | 3 (2.5) |
Road/Bonny Estuary. Results obtained from the enumeration, identification and isolation of the bacteria are presented in Tables 2, 3 and 4. It was revealed that 120 out of 120 water samples harboured coliforms and E. coli (MPN/100 ml). The result confirms observations made during the sampling, where human and animal excreta were found at the banks of the water body. According to the MPN technique, the highest total coliforms count recorded was during the start of the rainy season (48 MPN/100ml), followed by the end of the rainy seasons (28 MPN/ml). The presence of coliforms, including E. coli in water bodies suggests that the water environment may have been polluted with human and animal faeces and because of waste dump that is sited very close to the water body. The assumption is because during sampling, human and animal excreta were seen at the banks of the water body. Livestock were as well observed drinking water from the water body. Also, the high number of E. coli in all likelihood reflects the indiscriminate building of toilets on the water body.

Reduced rainfall could be the major reason why coliforms and E. coli were more during the start and end of rainy than the middle of rainy season. This is because increased rainfall during the middle of rainy season might dilute the contents of the water body, which includes bacteria. Faecal pollution caused by human excreta could contain pathogens that are of human origins; such pathogens are Shigella spp., Salmonella spp., pathogenic E. coli and enteroviruses, including hepatitis A. Some researchers have noted that food animals could as well serve as a vector for important pathogens including E. coli 0157:H7 and Cryptosporidium parvum [25,24]. Further, the presence of E. coli revealed in the study is in conformity with the findings of other researchers [26,27,28]. It could be concluded that water may be an important reservoir for E. coli infection.

In this study, some resistance genes were detected (Table 5) and the most detected of all the resistance genes is the CTX-M. However, the most commonly detected genes in the pathotypes of E. coli were stx1 and stx2 (EHEC) and aaiC (EAEC). The presence of the different pathotypes of E. coli could be from individuals who are local resident of Creek Road, as they defecate in the water body. These results suggest that some of the residents of the area have E. coli infections. Some of the genes detected in isolates of E. coli in this present study have also been detected by several other researchers [23,29,27]. [30] detected the presence of different types of virulence genes of E. coli in water isolates with the use of Multiplex Polymerase Chain Reaction (mPCR) [31]. It was reported in 2017 that Shiga toxin–producing Escherichia coli O157 caused infection in children who had played in a stream flowing through a park. According to them, their results revealed that faeces from deer were most likely the source of contamination of the stream [32]. Faeces of animals and humans could harbour Shiga toxin–producing Escherichia coli O157 that is why intensive monitoring, especially of faecal pollution arising from building toilets on water bodies, the use of faeces as manure and defecating in water bodies is important in understanding the extent of this pollution problem.

Genes in Enteropathogenic E. coli (EPEC) were also detected and the pathotype is known to be the major reservoir in humans; it is known to cause watery diarrhoea mainly in children under two years old. It has been noted that EPEC persists in developing countries as a cause of diseases [33]. Some of the pathotypes of E. coli may have come from warm blooded animals as they were seen in and around the water body feeding and drinking water.

5. CONCLUSION

Coliforms and E. coli were detected in the water body and the E. coli isolates had resistance genes, which have also been detected in clinical isolates. The presence of the bacterium could be as a result of human activities in and around the water body. Siting of waste dumps, discharge of sewage and the building of toilets, and the use of humans and animals excreta as manure, may have contributed to the presence of the bacteria in the water body. Therefore, it is recommended that intensive monitoring, especially of faecal pollution arising from building toilets on Water bodies, the use of faeces as manure and the discharge of sewage be discouraged.

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COMPETING INTERESTS
Authors have declared that no competing interests exist.

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