

Original Article

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# Presence and characterisation of extended spectrum beta-lactamase producing Gram-negative bacteria obtained from water in selected storage tanks

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## ABSTRACT

**Objective:** To detect the presence of extended-spectrum beta-lactamase-producing Gram-negative bacteria in water from selected storage tanks in Ogun State, Nigeria.

**Methods:** MacConkey agar supplemented with 6 µg/mL of cefotaxime was used to isolate bacteria. Pure isolates were identified through PCR amplification. The susceptibility patterns of the isolates to ceftazidime (30 µg/mL), amoxicillin/clavulanate (30 µg/mL), tetracycline (30 µg/mL), cefotaxime (30 µg/mL), cefpodoxime (10 µg/mL), and ciprofloxacin (5 µg/mL) were evaluated using the Kirby-Bauer disc diffusion method, while the identification of beta-lactamase production was performed using the double disc synergy test.

**Results:** A total of 19 isolates belonging to four genera (*Citrobacter*, *Pseudomonas*, *Escherichia*, and *Serratia*) were obtained from the ten water samples. *Escherichia coli* and *Citrobacter* spp. had the highest presence (30.0%). The antibiotic with highest resistance was observed to be cefpodoxime (100%) followed by amoxicillin/clavulanate (89.5%) and cefotaxime (89.5%), the least was noted with tetracycline (31.6%). Additionally, 10 (52.6%) out of the 19 isolates showed beta-lactamase-producing phenotypic properties with high resistance to cefpodoxime (100%), amoxicillin/clavulanate (100%) and ceftazidime (90.0%). Additionally, this study showed the link between bacterial strains identified by 16S rRNA and related human diseases, highlighting the importance of genetic identification in managing infections.

**Conclusions:** This study highlights the prevalence of beta-lactam-resistant bacteria, emphasizing the urgent need for proactive measures to address this issue.

**KEYWORDS:** Antibiotic resistance; Gram-negative bacteria; Extended-spectrum beta-lactamase; Water

## 1. Introduction

As the saying goes, "Water is life". This phrase underscores the essential role of water in various facets of human activity, including agriculture, pharmaceuticals, food production, and industry[1–4]. Although water is as valuable a resource to humans as it is, the availability of potable water remains a major concern globally. Nigeria, for example, having an estimate of over 190 million people is considered to be the largest populous country in Africa but it is categorized among the most vulnerable states in the world in terms of

**Significance**

Access to potable water remains a critical issue in rural areas where citizens often rely on wells and boreholes as their primary sources of water and store it in tanks. These storage tanks harbor pathogenic microorganisms that possess mechanisms to inactivate common antibiotics. This study identifies the presence of beta-lactamase-producing Gram-negative bacteria, highlighting the prevalence of these resistant strains and underscoring the urgent need for interventions to protect water quality and prevent the spread of antibiotic resistance.

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water, sanitation and health (WASH)[5,6]. Water is one of the pressing issues whose severity varies from region to region within the country[7].

The government of Nigeria is responsible for providing safe water supply and sanitation systems. Unfortunately, as a result of governance constraints, individuals are left to provide their source of water themselves; people with the means construct boreholes, while those facing financial constraints opt for well, contributing to Nigeria's limited advancement in providing widespread access to drinking water. Although urban population has been steadily increasing, rural areas still face challenges, as only a few households have access to water distribution systems via piped lines or groundwater wells[8,9].

Isara community in Ogun State of Nigeria is riddled with wells and boreholes indiscriminately dug and connected to storage tanks which are used to close the gap of unavailability of water supply to self and surrounding houses. Although these storage tanks have alleviated water shortages in the community, studies have revealed that they face numerous challenges, raising concerns about the quality of the water stored within them[10,11]. Studies have also shown that water tanks used for storage harbour various microorganisms, which are opportunistic pathogens that results in diseases[12–15]. This is mainly because of poor storage conditions including the types of containers used for storage and poor maintenance culture which results in formation of biofilm that contributes to increased presence of microorganisms in stored water[16–18].

Waterborne diseases are a major group of diseases that is responsible for several health challenges in several regions in the world and reports revealed that it resulted in about 829 000 deaths per year and a study by WHO in 2023 estimated that 1.4 million deaths could be prevented each year through access to clean water[19, 20]. Various diseases which include typhoid fever, diarrhea, bacillary dysentery, urinary tract infections, cholera, and others use water as a mode of transmission. One of the major bacterial families of concern is the Enterobacteriaceae and Pseudomonadaceae which are Gram-negative bacteria, tagged opportunistic pathogens. These bacteria have been commonly isolated from water and have the ability to tolerate the action of antibiotics, a mechanism disseminated among various bacteria[21–23]. One of the widely disseminated and important mechanisms hindering the effects of antibiotics is the production of a subset of the enzyme beta-lactamase which is the extended spectrum beta-lactamase (ESBL). This enzyme breaks the ring of beta-lactam antibiotics (such as cephalosporins, carbapenems and monobactams), rendering them inactive to their target bacteria hence leading to resistance[24]. The class of antibiotics known as beta-lactams make up about 50%-70% of the type of antibiotics used in various countries in the world including Nigeria[25–27]. The possibility of bacteria which has the ability to resist antibiotics being present in stored water is a significant threat. Hence, this study aims at isolating and characterizing bacteria which are resistant to a selected panel of antibiotics and checking for the production of beta-lactamase.

## 2. Materials and methods

### *2.1. Study area and sample collection*

Isara is a community located in the Remo North Local Government Area of Ogun State, Nigeria, with a latitude of 6° 58' 27" N, a longitude of 3° 40' 58" E of the Greenwich Meridian and an altitude of 106.28 M. It shares a boundary with Ode-Remo and Ipara which are both located in Remo-North Local government. Samples of water were aseptically obtained from ten randomly chosen water storage tanks using the random cluster technique. The water samples were aseptically collected into sterile sample bottles. The water was used to rinse the bottles twice consecutively, before the bottles were filled and capped carefully[28]. Samples were labelled appropriately and transported to the laboratory for further analysis.

### *2.2. Laboratory analysis*

#### *2.2.1. Isolation of bacteria*

Microbiological analyses of the obtained water samples were done with the pour plate method; 1 mL aliquot of the water samples were transferred aseptically into Petri dishes containing MacConkey Agar incorporated with 6 μg/mL of cefotaxime and gently swirled. The petri plates were left to sit at room temperature, inverted and incubated for 24 h-48 h at 37℃[29]. After the period of incubation, presumptive Gram-negative bacteria colonies were selected. All distinct colonies were subcultured by streaking on freshly prepared MacConkey Agar to obtain pure cultures.

#### *2.2.2 Kirby– Bauer disc diffusion test*

The bacterial isolates were subjected to a panel of antibiotics to determine their susceptibility pattern. The disc diffusion method developed by Kirby- Bauer on Muller-Hilton was employed. Briefly, an inoculating loop was used to inoculate into 5 mL normal saline from 18-24 h old cultures of each bacterium using aseptic

procedure. The bacterial suspension's turbidity was matched with McFarland standard (0.5). Using a swab stick(sterile), the surface of the Muller-Hilton agar plate was seeded with the standardized inoculum. Six antibiotic discs were used and these include ceftazidime (30 μg/mL), amoxicillin/clavulanate (30 μg/mL), tetracycline (30 μg/mL), cefotaxime (30 μg/mL), cefpodoxime (10 μg/mL), and ciprofloxacin (5 μg/mL). Forceps were aseptically used to place antibiotic discs on agar plates that have been inoculated with bacteria and were incubated at 37 ℃ for 18 h-24 h. The inhibition zone was measured using standard recommendations[30].

### 2.2.3. Double disc synergy test for ESBL production

Disc diffusion method on Muller-Hilton was employed as previously described but here four antibiotic discs were used which include amoxicillin/clavulanate (30 μg/mL), cefotaxime (30 μg/mL), cefpodoxime (10 μg/mL) and ceftazidime (30 μg/mL). The selected antibiotic discs were positioned 20 mm apart on agar plates which have been inoculated in a cross-like shape, and incubated for 24 h at 37 ℃. Phenotypic ESBL production was considered positive if the zone of inhibition expanded towards the amoxicillin clavulanate antibiotic disc[30,31].

## 2.3. Characterization of isolates

### 2.3.1. Extraction of DNA

DNA were extracted from the bacterial isolates using the DNA Preparation Kit-column-based JENA Bioscience according to the instruction manual. Bacteria broth culture (500 μL aliquot) were centrifuged at 10 000 rpm for 1 min. The supernatant was removed and 300 μL of Re-suspension Buffer and 2 μL of lysozyme solution was added. The tube was homogenized and incubated at 37 ℃ for 1 h. Thereafter, the tube was centrifuged and re-suspended cells were obtained and 300 μL of lysis buffer was added along with 2 RNase A and 8 μL proteinase K solution, this was incubated for 10 min at 60 ℃. The mixture after incubation was allowed to cool for 5 min using ice; to the mixture, a 300 binding buffer was added and mixed by a vortex mixer. The vortexed mixture was cooled again on ice and centrifuged with a duration of 5 min at 10 000 rpm. The DNA was then recovered by the transfer of the supernatant from the centrifuged vortexed mixture into a spin column which was centrifuged at 10 000 rpm for 1 min. Washing buffer was applied to recovered DNA and eluted into clean Eppendorf tubes with 50 elution buffer[32].

### 2.3.2 Polymerase chain reaction (PCR)

The PCR master mix consists of 12.5 μL of PCR master mix, 1 μL each of the forward and reverse primers (27F: 5' AGA GTT TGA TCM TGG CTC AG 3' forward and 1492R: 5' TAC GGY TAC CTT GTT ACG ACT T 3' reverse), 1 μL of DNA template, and 9.5 μL of PCR-grade water, resulting in a total volume of 25 μL. The reaction was denatured at 94 ℃ for 3 min and then at 94 ℃ for an additional 45 s. The primers were allowed to anneal to the target sequence for 60 s at 55 ℃, followed by an extension step at 72 ℃ for 10 min. Amplification of the PCR was performed using an Applied Biosystems 2720 Thermocycler[33].

### 2.3.3. Gel electrophoresis and sequencing

The 2% agarose gel consisting of the dye ethidium bromide was used to visualize PCR products using a blue led transilluminator and the size of the amplicons were measured using a DNAmarker (200 bp) and this was done alongside the visualization of the PCR products. The Sanger sequencing method was used for sequencing the PCR products using AB13730XL sequencer and done by lnqaba Biotec, Pretoria-South Africa[34]. The DNA sequences obtained for each bacterial isolate in fasta format was subjected to Basic Local Alignment Search Tool (BLAST) using the National Center for Biotechnology Information BLAST tool.

## 3. Results

Nineteen Gram-negative bacteria able to resist 6 μg/mL of cefotaxime were obtained from the ten sampled storage tanks. Table 1 shows the 19 isolates obtained from water samples and the distribution of resistance patterns among them. The bacterial isolates exhibited high resistance to amoxicillin/clavulanate and ceftazidime but lowest resistance was seen in tetracycline. Out of the 19 isolates, 10 were positive for phenotypic production of ESBL. Table 2 depicts the distribution pattern of resistance to the selected panel of antibiotics by the ESBL-producers. The ESBL-positive isolates showed notable resistance to ciprofloxacin (90.0%), amoxicillin/clavulanate (100%) and cefpodoxime (100%). The PCR products which depict the presence of target DNA fragments, confirming the genetic characteristic of the isolates at approximately 1 500 bp were visualized (Figure 1). The sequence results of the PCR product on the basis of the BLAST public database of homologous genes on the National Center for Biotechnology Information (NCBI) server showed that the 10 ESBL-producing isolates belonged to 4 genera (Figure 2). The isolates included *Citrobacter*, *Pseudomonas*, *Escherichia* and *Serratia*; and the gene sequences obtained showed

an average of 95% identity match to the sequence in GenBank Database. Figure 2 also shows the phylogenetic tree which highlights the evolutionary relationship and the relative distances between the genera obtained. Table 3 highlights the correlation between bacterial strains identified through 16S rRNA homology and associated human diseases, emphasizing the role of genetic identification in understanding and managing infections.

**Table 1.** Antibiotics resistance profile of bacterial isolates.

| Isolate codes | AMC | CPD | CTX | CAZ | CIP | TE |
|---|---|---|---|---|---|---|
| B1 | R | R | R | R | R | R |
| C2 | R | R | R | R | R | S |
| A1ii | R | R | R | R | R | R |
| D1ii | R | R | R | S | R | S |
| A1i | R | R | R | R | R | R |
| A | R | R | R | R | S | S |
| E | R | R | R | R | R | S |
| C1 | S | R | R | R | R | S |
| B2ii | R | R | R | R | S | S |
| F | S | R | R | R | R | S |
| E1i | R | R | R | R | R | R |
| E1ii | R | R | S | S | R | S |
| B2i | R | R | R | R | R | R |
| E2ii | R | R | R | R | S | S |
| B | R | R | R | R | R | S |
| C | R | R | S | R | R | S |
| D | R | R | R | S | R | S |
| A2i | R | R | R | R | R | S |
| D1 | R | R | R | R | R | R |

Note: B1: *Serratia marcescens*; C2: *Citrobacter farmeri*; A1ii: *Escherichia coli*; D1ii: *Citrobacter farmeri*; A1i: *Pseudomonas aeruginosa*; A: *Citrobacter farmeri*; E: *Escherichia coli*; C1: *Escherichia coli*; B2ii: *Escherichia coli*; F: *Escherichia coli*; E1i: *Escherichia coli*; E1ii: *Citrobacter farmeri*; B2i: *Serratia marcescen*; E2ii: *Pseudomonas aeruginosa*; B: *Citrobacter farmeri*; C: *Serratia marcescens*; D: *Pseudomonas aeruginosa*; A2i: *Pseudomonas aeruginosa*; D1: *Citrobacter farmeri*; R: Resistant; S: Sensitive; CPD: Cefpodoxime; CTX: Cefotaxime; AMC: Amoxicillin clavulanate; CIP: Ciprofloxacin; CAZ: Ceftazidime; TE: Tetracycline.

**Table 2.** Antibacterial susceptibility test of ESBL-producing bacterial isolates.

| Isolate codes | AMC | CPD | CTX | CAZ | CIP | TE |
|---|---|---|---|---|---|---|
| C | R | R | R | R | R | R |
| E | R | R | R | R | R | R |
| A1ii | R | R | R | R | R | R |
| B | R | R | R | R | S | S |
| A1i | R | R | S | R | R | S |
| D1 | R | R | R | R | R | R |
| A | R | R | R | R | R | S |
| B1 | R | R | S | R | R | S |
| D | R | R | R | S | R | S |
| E1i | R | R | R | R | R | R |

Note: C: *Serratia marcescens*; E: *Escherichia coli*; A1ii: *Escherichia coli*; B: *Citrobacter farmeri*; A1i: *Pseudomonas aeruginosa*; D1: *Citrobacter farmeri*; A: *Citrobacter farmeri*; B1: *Serratia marcescens*; D: *Pseudomonas aeruginosa*;E1i: *Escherichia coli*; R: Resistant; S: Sensitive; CPD: Cefpodoxime; CTX: Cefotaxime; AMC: Amoxicillin clavulanate; CAZ: Ceftazidime; TE: Tetracycline; CIP: Ciprofloxacin.

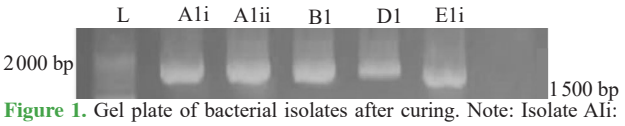



**Figure 1.** Gel plate of bacterial isolates after curing. Note: Isolate AIi: *Pseudomonas aeruginosa*; Isolate AIii: *Escherichia coli*; Isolate BI: *Serratia marcescens*; Isolate DI: *Citrobacter farmeri*; Isolate EIi: *Escherichia coli*.

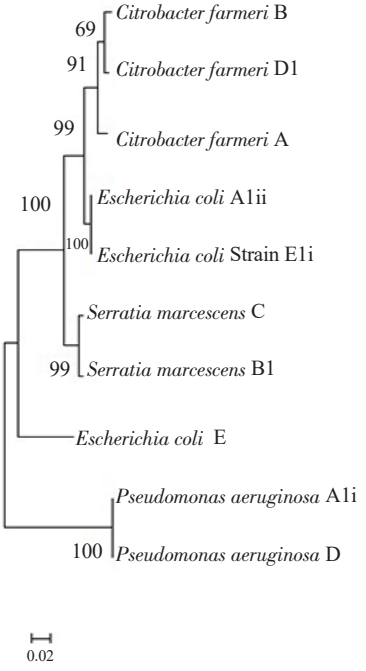



**Figure 2.** Phylogenetic tree of obtained bacteria isolates based on 16sRNA sequences constructed using Mega software.

## 4. Discussion

In Isara-Remo community, the major storage option for water is plastic water tanks. This study was founded on the premise that poor storage practices and inadequate maintenance can compromise water quality, making it a significant source of Gram-negative bacteria that produce ESBL and contribute to disease exacerbation. In the present investigation, a total of nineteen cefpodoxime resistant isolates were obtained and this is similar with the research work by Adeyemi *et al.*[35] who reported 13 isolates from borehole water sources in Oyo State. The variation in the number of isolates obtained could be due to factors specific to the sample sites analyzed.

The bacterial isolates obtained in this study belong to four different genera which include *Citrobacter*, *Pseudomonas*, *Escherichia* and *Serratia*. This is similar to the reports of previous works[35–37] as they also obtained these different genera. However, the most predominant genera detected were *Citrobacter*, *Escherichia* and

**Table 3.** GenBank accession number of bacterial strains isolated and their human- associated diseases

| Isolate codes | 16S rRNA strain homology | GenBank accession No. | Human- associated diseases |
|---|---|---|---|
| B1 | *Serratia marcescens* | KX527573.1 | Urinary tract infections, wound, infections |
| A1ii | *Escherichia coli* | MH071520.1 | Urinary tract infections, gastroenteritis |
| A1i | *Pseudomonas aeruginosa* | FJ972534.1 | Respiratory infections, wound infections, gastrointestinal infections |
| B | *Citrobacter farmeri* | AB504755.1 | Gastrointestinal infections, blood stream infections |
| E | *Escherichia coli* | MH197074.1 | Urinary tract infections, gastroenteritis |
| D1 | *Citrobacter farmeri* | EU030438.1 | Gastrointestinal infections, blood stream infections |
| A | *Citrobacter farmeri* | LK021069.1 | Gastrointestinal infections, blood stream infections |
| C | *Serratia marcescens* | KF528829.1 | Urinary tract infections, wound, infections |
| D | *Pseudomonas aeruginosa* | FJ972533.1 | Respiratory infections, wound infections, gastrointestinal infections |
| E1i | *Escherichia coli* | MH071520.1 | Urinary tract infections, gastroenteritis |

*Pseudomonas*. The presence of these genera could be an indication of feacal contamination and the potential of water to cause several gastrointestinal diseases commonly called gastroenteritis[38–40]. *Citrobacter* sp. and *Escherichia coli* were the most frequently occurring organisms across all sampled tanks. This observation corroborates with Daoud *et al*.[41] who reported a high occurrence of *Citrobacter* sp. (83.0%) in tank water samples in Westbank Palestine. *Citrobacter* species is responsible for infections like septicemia, surgical site infections and lower respiratory tract infections. The presence of such bacteria may result in diseases and poor health for the people consuming it. *Serratia*, despite being the least organism identified in this water that has various domestic uses, still, is a cause for alarm because it is known as human opportunistic pathogen. These bacteria isolates are associated with infections targeted at gastrointestinal tract such as dysentery, diarrhoea[42,43]. They are also associated with infections of the lungs and the urinary tract, being prevalent in individuals whose immune system has being compromised[44]. The phylogenetic tree shows a close proximity between *Citrobacter*, *Escherichia* and *Serratia* with *Pseudomonas* being more distantly related. Although the relative distances are quite distinct, they are interconnected and responsible for the ease of the dissemination of antibiotic resistance through horizontal gene transfer. This makes the identified genera in this study cause diseases that are still a challenge for several African countries and has been further complicated with the discovery of the ability of these genera to resist antibiotics which are the first line of treatment of the diseases that these pathogens cause.

Most of the 19 isolates obtained showed high resistance to cefpodoxime(100%), amoxicillin/clavulanate (89.5%) and cefotaxime (89.5%), which is consistent with the results of Babalola *et al.* and Adelowo *et al.*[37,45] who obtained isolates which were resistant to amoxicillin/clavulanate (92.0%) and ceftazidime (100%), respectively. However, our finding is in contrary with the work of Banjo *et al.*[46] who obtained isolates that exhibited high level of resistance to ceftazidime (93.8%) but had lower resistance to amoxicillin/clavulanate (68.8%), and cefotaxime (75.0%), though varied by sample source. The bacterial isolates obtained in this study were relatively tolerant to ciprofloxacin (84.2%) and poorly resistant to tetracycline (31.6%) and this does not agree with the works of Babalola *et al.*[37] whose isolates obtained from water distribution tanks showed lower resistance to ciprofloxacin (46.0%). The moderately high resistance observed in the findings of this study could infer that there is an accelerated rate of misuse and overuse of antibiotics in the study area and the bacteria strains have developed multi-drug resistance due to environmental pressure.

Furthermore, this study revealed that some of the bacterial isolates produced ESBL. The production of ESBL was observed in 10 (52.6%) of the isolates and this was higher than the report of similar study (17.0%) by Mahmud *et al.*[47] from household water samples in Bangladesh. This could imply that the distribution of organisms that produce ESBL varies from one nation to another. It could also be due to an increase in population which results in a simultaneous increase in anthropogenic activities that produce wastes that are sources of these ESBL-producing organisms which are released into surface waters and well waters through percolation and run-off. The most prevalent ESBL- producing isolates identified were *Escherichia coli* and *Citrobacter* spp. This is in contrast to the reports of Adesoji *et al.* and Falodun *et al.* who observed a high occurrence of *Klebsiella* species in selected water sources in Southwestern Nigeria[48,49]. *Escherichia coli* and *Citrobacter* sp. occurring the most among the ESBL- producing organisms obtained is of great public health concern because of the varying infections they can cause which include community-acquired and nosocomial infections.

Bacterial isolates that produce the ESBL enzyme are capable of rendering beta-lactam antibiotics inactive leaving little to no treatment options left after infection[50]. The antibacterial

susceptibility tests revealed high resistance or tolerance of the ESBL-producing isolates to cefpodoxime, amoxicillin/clavulanate and low resistance to tetracycline (50.0%) which was used as an alternative treatment in this study. This corroborates the study of Zhang *et al.*[51] whose isolates exhibited high resistance to beta-lactam antibiotics but does not corrobarate with that of Adelowo *et al.*[45] whose isolates obtained from river sources showed higher resistance to tetracycline (88.9%). Tetracycline are inexpensive antibiotics which are used in treatment of various animal and human infections and also as growth promoters for animals as they are incorporated in their feed[52,53]. Therefore, it is not far-fetched that this antibiotic becomes ineffective along the run, making tetracycline-resistant pathogens more prevalent.

## 5. Conclusions

The isolates identified in the sampled drinking water tanks are of great public health threat. These isolates are not only multiple antibiotic resistant but also ESBL-producers. This demonstrates the significant function played by the environment in the spread and distribution of multidrug-resistant bacteria to various water source including drinking and domestic purpose. This study highlights the need for the appropriate agencies, especially in our country, Nigeria, to act now and quickly in the struggle against bacterial resistance to antibiotics in order to curtail the future dangers of antibiotic resistance, which is becoming an increasingly critical issue. For an effective approach to this issue, all areas of concern: Animal, human, and environmental health under the One Health concept must be actively engaged.

## Conflict of interest statement

The authors claim there is no conflict of interest.

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This study receives no extramural funding.

## Data availability statement

The data supporting the findings of this study are available from the corresponding author upon request.

## Authors' contributions

Olajide OE and Nwadike BI conceptualized the study, designed the experimental frame work; Oluwole AP and Omotosho O performed the laboratory experiments and contributed to the data collection. Nwadike BI and Oladide EO were involved in data analysis, preliminary draft and critical review of the manuscript. All authors read, revised and approved the final manuscript.

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