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High prevalence of *Salmonella* Typhi from well water harboring CTX-M and *tet*A resistance gene

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Abstract

The presence of antibiotic-resistance genes in well water is a threat to community inhabitants. Thus, our study tends to identify the prevalence of *Salmonella* Typhi from well water harboring CTX-M and *tet*A resistance genes. A total of ten well water samples were collected from different wells at Onueke metropolis and cultured for the presence of *Salmonella* Typhi. Antimicrobial resistance studies of *Salmonella* Typhi were determined using the Kirby–Bauer disk diffusion method. PCR was employed to screen the distribution of the resistance genes (*bla*$_{CTX-M}$ and *tet*A) among the recovered *Salmonella* serovar Typhi. A total of 112 isolates of *Salmonella* Typhi were isolated from different well water comprising WW1 (n=6), WW2 (n=10), WW3 (n=15), WW4 (n=7), WW5 (n=21), WW6 (n=5), WW7 (14), WW8 (n=11), WW9 (n=15), and WW10 (n=8). *Salmonella* Typhi demonstrated high resistance to tetracycline 100 %, Aztreonam 100%, cephalosporin 79.5-100 %, amoxicillin-clavulanic acid 82.1 %, but were susceptible to imipenem 100%, Azithromycin 89.3 %, ciprofloxacin 91.1 % and gentamicin 84.8 %. PCR amplification of the resistance gene in *Salmonella* Typhi revealed the overall occurrence of *bla*$_{CTX-M}$ and *tet*A gene at 69.7% and 71.4 % respectively. Our study reports the presence of *bla*$_{CTX-M}$ and *tet*A genotype in *Salmonella* Typhi from well water and, may become responsible for intestinal infectious diseases in humans that are often considered as healthy carriers. There is a high tendency to transfer antimicrobial-resistant *Salmonella* strains to humans through the water source posing a public health threat that can result in higher morbidity and mortality, as well as increased cost of treatment. Improvement of water quality is of primary concern, well water depth inspection, and more stringent chlorine disinfection need to be taken into consideration to prevent resistant bacteria, and waste-water effluent should be properly treated before discharge to avoid percolation of ARG into groundwater. Also, there should be awareness/ management strategies for the spread of antibiotic-resistant bacteria in water

Keywords: *Salmonella* Typhi; *bla*$_{CTX-M}$; *tet*A; Well water

1. Introduction

Salmonella enterica serovar Typhi (*S*. Typhi), is a rod-shape Gram-negative bacteria responsible for typhoid fever (a serious intestinal tract and bloodstream infection) [1, 2]. In developing countries, *S*. Typhi has been a significant cause of morbidity and mortality [3, 4]. According to a Global Burden of Disease Study, an estimated 9.25 million cases of

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typhoid fever resulted in 110,000 deaths in 2019, of which the majority of the cases were in South Asia and sub-Saharan Africa. The incidence of typhoid fever is more common in low-and middle-income countries in South Asia and Africa than in developed countries [2, 3, 5].

S. Typhi infects humans through ingestion of food or water contaminated with the organism's excrement. Affected persons usually develop symptoms after an asymptomatic interval of 7 to 14 days following ingestion of *S*. Typhi contaminated food or water. After the initial asymptomatic phase, patients may develop an influenza-like illness accompanied by fever, nausea, vomiting, and diarrhea [1, 6]. The endemic nature of *S*. Typhi is mainly present in areas with inadequate portable water sources, poor hygiene, or low socioeconomic status. In most areas in Ebonyi state, *S*. Typhi is easily contacted through contaminated drinking water sources such as well water commonly found in most rural communities [7]. The frequent incidence of typhoid fever among the people of Onueke communities has been attributed to a lack of good potable water sources. The majority of the dwellers resort to well water as the source of water supply for many homes in this area.

Numerous cases of *S*. Typhi infection are often treated with antibiotics, which reduces the morbidity and mortality of many infectious diseases [8] and has been utilized as an essential arsenal to fight typhoid fever.

However, the widespread and indiscriminate use of antibiotics in human medicine has resulted in the rapid evolution and spread of antimicrobial resistance (AMR) in *S*. Typhi strains [4, 9]. Several studies have previously documented the AMR patterns of *Salmonella* serovars to several antibiotic classes, particularly sulfonamides, aminoglycosides, tetracyclines, and penicillins [4, 10]. The AMR phenotype are facilitated by various antibiotic-resistance-related genes found in *S*. Typhi including *tet*A (conferring resistance to tetracycline) [11], $bla_{\text{CTX-M}}$ (resistant to beta-lactam antibiotic) [2].

In most cases, easy access to certain antimicrobial agents prompts victims to resort to self-administration of antibiotics, such as tetracycline. This action has resulted in the irrational use of tetracycline in most Nigerian communities seen today. Many studies have documented the link between irresponsible antibiotic usage and antibiotic resistance, drawing the attention of relevant authorities worldwide [12]. Additionally, resistance to tetracycline can occur by several mechanisms: antibiotic efflux or modification, protection of the binding site, or modification of 16S rRNA at the tetracycline-binding site [13]. To date, several *tet* genes involved in tetracycline resistance in *Salmonella* spp. have been identified. The most common *tet* genes are classified as A, B, C, D, and G. Many of these genes can be localized within mobile portions of the *Salmonella* genome, such as transposons or plasmids, and this allows them to be easily disseminated in the environment and transferred to other bacteria [12, 14].

As tetracycline resistance persist, alternative antimicrobial treatments, including third-generation cephalosporin (ceftriaxone) or azithromycin, are increasingly used as first-line therapies [15]. Recently, resistance to these newer drugs has been reported in *Salmonellae* implicated in cases of enteric fever [4, 16, 17, 18]. While these cases have largely been sporadic, a large-scale extensively drug-resistant (XDR) *S*. Typhi outbreak began in Pakistan in 2016, harboring resistance to third-generation cephalosporins [19]. Resistance to ceftriaxone or other extended-spectrum ß-lactams is usually due to the production of extended-spectrum-ß-lactamases (ESBLs) of which $bla_{\text{CTX-M}}$ type ESBLs are one of the determinants for cephalosporin resistance in *Salmonella* [2, 5]. Many $bla_{\text{CTX-M}}$ variants are described in the literature, with $bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-14}}$, and $bla_{\text{CTX-M-15}}$ being the most commonly reported [20, 21, 22]. The $bla_{\text{CTX-M}}$ type ESBLs are usually encoded by transmissible plasmids [23, 24]. As a result, routine surveillance of resistance determinants is essential to understand when and where populations may be affected.

Hence, this study was performed to evaluate the prevalence of CTX-M ESBL and tetracycline (*tet*A) resistance gene in *S*. Typhi isolated from the well water found in human inhabitants.

2. Materials and Methods

2.1. Study Area

The well water samples were collected from Onueke. Onueke is located at latitude 6.1323° N, and longitude 8.0220° E and is the ancestral headquarters of the Ezza, Ezzas are the most populous clans in the Ebonyi State of Nigeria [7]. Ezza people live in virtually all three senatorial zones of Ebonyi State and beyond but are concentrated in the Ezza North, Ezza South, Onicha, Ishielu, and Ohaukwu Local Government Areas. The Ezza people in Ohaukwu dominate in Effium community inhabiting the minority communities of Inikiri Umuezeoka, Umuezeokaoha, and Kpakpaji. The majority of the people are farmers, the Ezaas produce yams, cassava, rice, cocoyams, and many other crops in abundance.

2.2. Sample collection and Analysis

A total of ten water samples of 500ml volume were collected from different wells at Onueke metropolis. The samples were vortexed, and 0.5ml of each well water samples were spread plate on the solidified CHROMagar ™ *Salmonella* Typhi (Difco, USA) plate. The plates were incubated at 37^{o}C for 18-24 h. After overnight incubation, the appearance of mauve colonies infers *Salmonella* Typhi. The isolated bacteria were further characterized using the VITEX 2 Automated system [2, 4].

2.3. Antibiotics Susceptibility Studies of *Salmonella* Typhi

This was conducted using antibiotic disks of Azithromycin (10µg), aztreonam (10µg), imipenem (10µg), amoxicillin-clavulanic acid (20/10µg), cefepime (30µg), ceftriaxone (30µg), ceftazidime (30µg), ciprofloxacin, (5µg), gentamicin (10µg) and tetracycline (10µg) [Oxoid, UK] on MH agar plate swabbed with bacterial suspension (0.5 McFarland turbidity standards) [7]. Susceptibility plates were incubated at 37^{o}C for 24h; and isolates were inferred as susceptible or resistant using the antibiotic breakpoints of CLSI [25, 26].

2.4. PCR Screening of resistance genes in *Salmonella* serovars Typhi

PCR was used to screen the distribution of the resistance genes (*bla*$_{CTX-M}$, *tet*A) among the recovered *Salmonella* serovars. The *g*DNA of the tested *Salmonella* serovars was extracted using a genomic DNA extraction Kit (Invitrogen, Carlsbad, USA). Moreover, positive (positive strains obtained from the A.H.R.I, Egypt) and negative controls (reactions with DNA-free reactions); were used. The used primers (Thermo Fisher Scientific, Karlsruhe, Germany) and PCR protocols were clarified in Table 1. The amplified PCR products were screened by the agar gel electrophoresis (1.5% agarose stained with 10 mg/ml ethidium bromide). Afterward, the gel was photographed.

Table 1 PCR primers used resistance gene in this study

Target Gene	Primers sequences	Product size (bp)	PCR thermal profile (35 cycles)			References
			Denaturation	Annealing	Extension	
*tet*A	F:GGT TCA CTC GAA CGA CGT CA	576	94 °C, 30 s	55 °C, 40 s	72 °C, 45 s	[27]
	R:CTG TCC GAC AAG TTG CAT GA					
bla$_{CTX-M}$	R:CTG TCC GAC AAG TTG CAT GA	593	94 °C, 30 s	54 °C, 40 s	72 °C, 45 s	[28]
	R:TGG GTR AAR TAR GTS ACC AGA AYC AGC GG					

3. Result

A total of 112 isolate of *Salmonella* Typhi were isolated from different well water from Onueke. The proportion of the bacteria comprising of WW1 (n=6), WW2 (n=10), WW3 (n=15), WW4 (n=7), WW5 (n=21), WW6 (n=5), WW7 (14), WW8 (n=11), WW9 (n=15), WW10 (n=8).

Azithromycin [Susceptible 100(89.3 %) and Resistance 12(10.7 %)], imipenem [Susceptible 112(100 %) and Resistance 0(0.0 %)] Aztreonam [Susceptible 0(0.0 %) and Resistance 112(100 %)], amoxicillin-clavulanic acid [Susceptible 20(17.9%) and Resistance 92 (82.1 %)], cefepime [Susceptible 4 (3.6 %) and Resistance 108(96.4 %)], ceftriaxone [Susceptible 23(20.5%) and Resistance 89(79.5 %)], ceftazidime [Susceptible 38(33.9 %) and Resistance 74(63.4 %)], tetracycline [Susceptible 0(0.0 %) and Resistance 112(100 %)], cefotaxime 100%, ciprofloxacin [Susceptible 102 (91.1 %) and Resistance 10(8.9 %)], gentamicin [Susceptible 95(84.8 %) and Resistance 17(15.2 %)] as shown in Figure 1.

PCR amplification of resistance gene of *Salmonella* Typhi were as follows; WW1 [CTX-M n=6/6 (100 %)], *tet*A n=6/6 (100 %)], WW2 [CTX-M n=5/10 (50 %)], *tet*A n=10/10 (100 %)],WW3 (n=15), [CTX-M n=15/15 (100 %)], *tet*A n=7/15 (46.7 %)], WW4 (n=7), [CTX-M n=3/7 (42.8 %)], *tet*A n=7/7 (100 %)], WW5 (n=21), [CTX-M n=15/21 (71.4 %)], *tet*A n=10/21 (47.6 %)], WW6 (n=5) [CTX-M n=1/5 (20 %)], *tet*A n=4/5 (80 %)], WW7 (n=14), [CTX-M n=14/14 (100 %)], *tet*A n=9/14 (64.3 %)], WW8 (n=11), [CTX-M n=11/11 (100 %)], *tet*A n=11/11 (100 %)], WW9 (n=15), [CTX-M n=5/15 (33.3 %)], *tet*A n=9/15 (60 %)], WW10 (n=8), [CTX-M n=3/8 (37.5 %)], *tet*A n=7/8 (87.5 %)] as shown in Figure 2.

The overall occurrence of CTX-M and *tet*A was 78 (69.7%) and 80 (71.4 %) respectively Figure 3

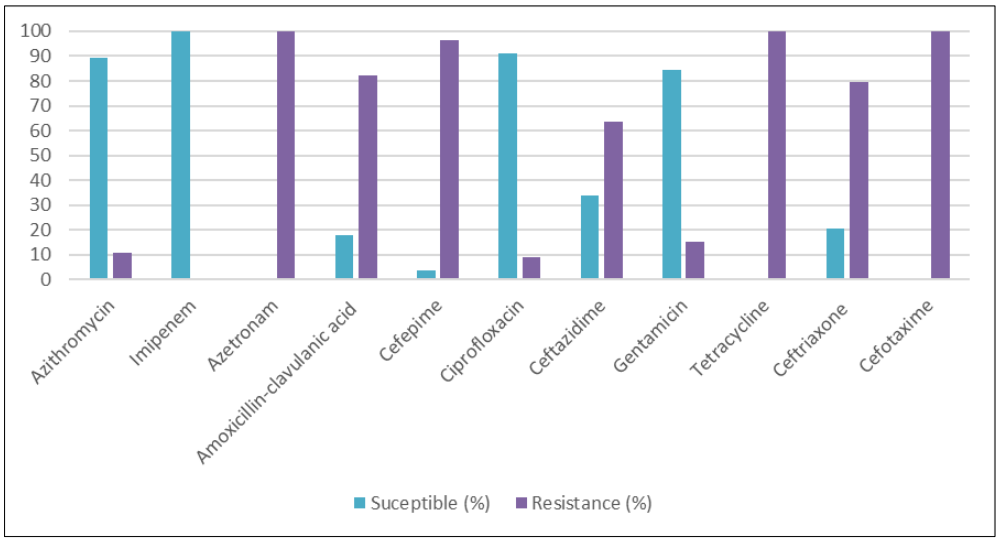

Figure 1 Susceptibility and resistance of *Salmonella* Typhi

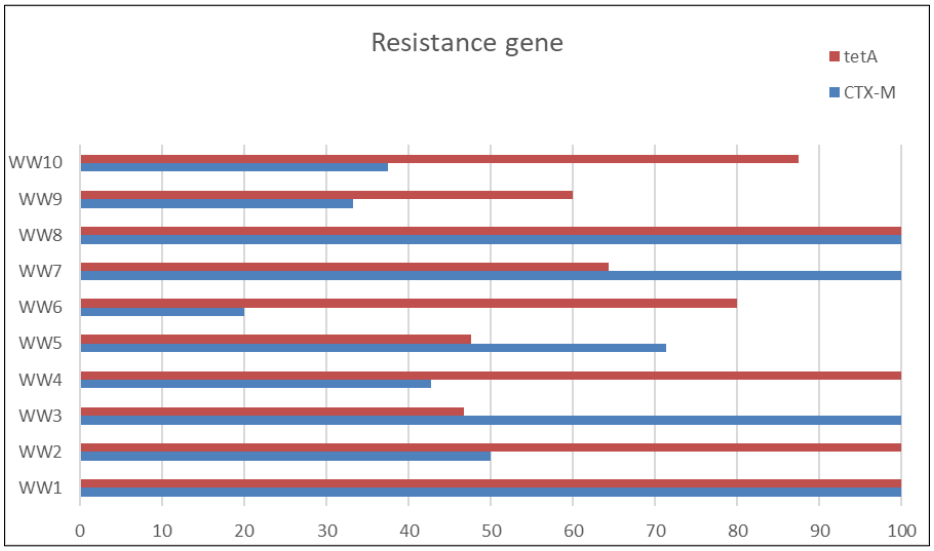

Figure 2 Distribution of *Salmonella Typhi* from well water harboring CTX-M and *tet*A resistance gene

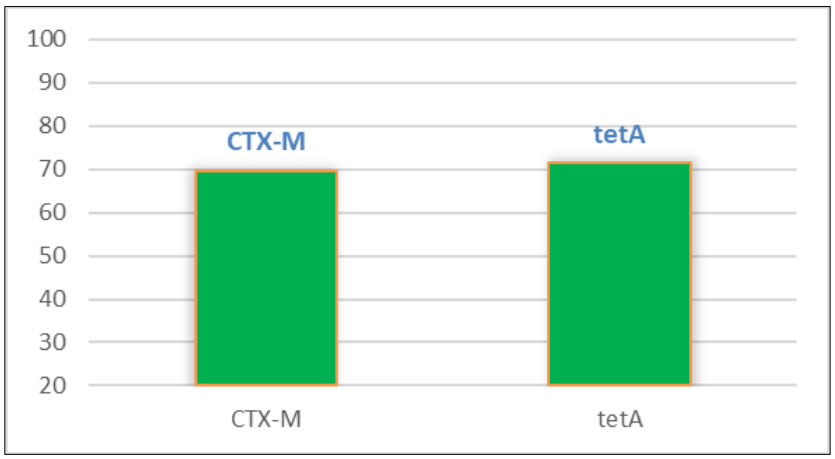

Figure 3 Overall Occurrence of Resistance Genes

4. Discussion

The CHROMagar culture identified 112 *Salmonella* Typhi isolates from the well water. The significantly high prevalence of *Salmonella* Typhi in the examined sample poses a concern to the area's inhabitants. However, well water is believed to be naturally filtered by soil and to have acceptable microbiological quality (i.e., less microorganisms). However, it may be undermined or compromised by inferior construction or insufficient depth of the well and may be contaminated by nearby latrines, septic tanks leaching fields, wastewater discharge into the land, and rainfalls [29, 30, 31]. *Salmonella* contamination of groundwater is mainly a concern of developing countries, especially in rural areas, due to poor hygienic conditions, deficiently structured water supply systems, and inadequate disinfection treatment [7, 32], but it occasionally occurs in developed countries [33, 34]. *Salmonella enterica* serotype Typhi has been implicated in numerous cases of Typhoid fever. Typhoid fever is a systemic infection caused by *Salmonella enterica* serotype Typhi is a highly adapted human-specific pathogen that possesses a remarkable mechanism for persistence in the host [35]. *S*. Typhi continues to be a major cause of typhoid fever in Nigeria, where antimicrobial therapy remains the cornerstone of treatment and management of the disease burden. However, populations of *S.* Typhi rapidly develop resistance whenever a new antimicrobial is introduced for the treatment of typhoid fever

Most of our studied *S. Typhi* strains were resistant to cephalosporin notably 2nd, 3rd and 4th generation. Several studies have revealed similar patterns of *S.* Typhi resistance [4, 7, 36, 37, 38, 39]. The expansion of plasmid-mediated ampC or ESBL genes has been reported to be associated with an increase in ceftriaxone resistance in *Salmonella* species [2, 36, 39]. Third-generation cephalosporin-resistant *S.* Typhi have been previously isolated in India and associated with the presence of the ESBL gene *bla*SHV^{-12} [37].

Cephalosporins are essential drugs for the treatment of major bacterial infections in humans [40, 41, 42]. In recent years, there has been an increase in reports of cephalosporin-resistant *Salmonella* in humans and food-producing animals worldwide [41, 43]. The common mechanism of resistance to cephalosporins is via the production of extended-spectrum β-lactamase (ESBL) and plasmid-mediated AmpC β-lactamase (pAmpC) [28, 36], which can hydrolyze extended-spectrum cephalosporins. ESBL- and pAmpC-producing *Salmonella* present a serious threat to global public health because it leads to treatment failure in humans [37, 39, 41, 44]. Nevertheless, all isolates in our study were extremely resistant to cephalosporin and it could not be an option to treat typhoid fever in Onueke metropolis. Due to the wide use of third-generation cephalosporins, especially ceftriaxone, and cefotaxime may be associated with high mobilization of the CTX-M encoding genes and can express resistance to a diversity of beta-lactams including ampicillin, and a combination of amoxicillin and amoxicillin/clavulanic acid.

The isolate was extremely resistant to tetracycline and its resistance to tetracycline has been widely reported [11]. In a study carried out by Adzitey *et al*. [45] in Ghana, 86% of *Salmonella* species were tetracycline resistant while two studies in Nigeria reported 33% and 67% respectively [46, 47].

Tetracycline is one of the most regularly utilized antibiotics in human and veterinary healthcare settings in Nigeria [48]. This widespread use aided the emergence of tetracycline resistance in a diverse group of bacteria, limiting the clinical value of these drugs. Thus, our phenotypic tetracycline-resistant *S*. Typhi is not surprising, given that drivers of antimicrobial resistance e.g., misuse and abuse of antibiotics are common in societies where agents of propagation and spread of antimicrobial-resistant organisms exist, such as a lack of clean water, sanitation, and inadequate infection prevention and control measures.

This study found significant resistance among *Salmonella* to β-lactam antibiotics and tetracycline, which requires immediate attention. Differences in resistance percentages between nations may be attributed to a variety of factors, including the source of environmental samples and the persistent nature of the resistant determinant, both of which are known to be related to frequent antibiotic usage.

Antimicrobial susceptibility tests show that imipenem, gentamicin, and ciprofloxacin are effective against the isolate. Among the drugs, all *S*. Typhi isolates were completely sensitive to imipenem. This is consistent with an earlier study in which imipenem, gentamicin, and ciprofloxacin were the most effective drugs against *S*. Typhi. [7, 49]. As a result, we recommend that the use of imipenem, gentamicin, and ciprofloxacin to treat typhoid fever be limited to multi-drug resistant strain and complicated cases and that antibiotic susceptibility tests be performed to ensure that these drugs remain effective for a longer period.

Our study reports an overall proportion of *tet*A gene 71.4 %. The phenotypic tetracycline resistance shows that *Salmonella* species resistance to tetracycline is frequently observed in studied samples, and this resistance is attributed mostly to the presence of *tet* genes in these bacteria. The *tet*A gene has been found as the most prevalent genetic

component in tetracycline-resistant *Escherichia coli* and *Salmonella* species [50] and is typically found in mobile genetic components such as integrons, transposons, and plasmids, where the gene can easily be transported to various bacteria. [51]. Similar to our study, earlier reports detected *tet*A in drinking water distribution sources [52]. Zhang *et al*. [53] reported that among 105 tetracycline-resistant *Salmonella*, the *tet*A gene was most frequently detected (80.9%). Zhang *et al* [54] reported that *tet*A genes are widely detected in fecal coliforms from rivers and animal sources.

The most common tetracycline resistance mechanism is antibiotic efflux pumps, in which *tet* genes encode the membrane-associated efflux proteins, which exchange a proton for a tetracycline-cation complex against a concentration gradient, exporting the drug to outside bacterial cells. These genes are generally associated with plasmids, transposons, or both and are often conjugative [11, 52]. However, this study did not assess the conjugative ability of plasmids through conjugation experiments. The ability of the *tet*A gene to spread freely in farm animals compared with other *tet* genes has been widely demonstrated; Deekshit *et al*. [55] found that the *tet*A gene in strains of *Salmonella* species isolated from seafood in India was located on a plasmid and this gene was identical to *tetA* detected in other bacterial species including *Escherichia coli* and *Vibrio cholerae*. According to Vital *et al*. [56], large conjugative resistance plasmids have been detected in *Salmonella* food isolates from several countries. In drinking water sources, Conjugation experiments showed that five of 17 *tet*A positive bacteria successfully transferred the gene to the recipient host using the conditions employed in this study (three *Alcaligenes* and two *E. coli* [52]. Conjugative plasmids can transfer several resistance genes between different bacterial species, and the presence of multiple antibiotic resistance genes facilitates their host survival despite intense antibiotic selection [58].

In addition to their high pathogenic potential, bacteria of the *Salmonella* genus are of particular interest for their contribution to the spread of antibiotic resistance, as they can accumulate other ARGs like EBSL genes. In our study, we recorded a 69.7% CTX-M ESBL genotype in *Salmonella* Typhi. However, since the early 2000s, CTX-M enzymes have emerged as the most commonly encountered ESBLs [2, 59]. CTX-M genes are classified as class A ESBLs and encompass multiple variants that currently dominate ESBL prevalence in clinical and community settings [2, 59]. CTX-M possesses the ability to hydrolyze expanded-spectrum cephalosporins, such as cefotaxime, ceftriaxone, ceftazidime, and cefepime, as well as monobactams like aztreonam [23, 59] as noted evidence in our antimicrobial susceptibility testing. CTX-M genes are worldwide in distribution among enterobacteria in aquatic environments [28]; Muringani *et al*. [60], found that from 65 water samples, 18 were positive for CTX-M. Adesoji and Ogunjobi. [57] reported CTX-M among enterobacteria except *Salmonella* from Drinking Water Distribution Channels in Southwestern Nigeria.

Altayb *et al.* [61] detected a high prevalence of blaCTX-M-1 group in *E. coli* isolated from drinking water (40%). Also, an earlier study that isolated *E. coli* from water environments in northern Thailand, stated that the most common extended-spectrum β-lactamase-encoding gene was $bla_{\text{CTX-M}}$ group 1 (75%) followed by $bla_{\text{CTX-M}}$ group 9 (13.2%) [62]. Furthermore, this finding is more than a previous report in northern Tanzania; there it was found that the CTX-M gene was present in 17.7% of *E. coli* isolated from drinking water [63]. Furthermore, in China, a study conducted by Gao *et al*. [64] reported 14.8% of ESBL-producing *E. coli* from downstream water. In South Africa, Muringani *et al*. [60] found that 28% (18/65) of water samples were positive for CTX-M. The high prevalence of $bla_{\text{CTX-M}}$ carrier *Salmonella* Typhi in Onueke well water could be one of the main reasons for the dissemination of CTX-M-positive clinical isolates of Enterobacteria in Onueke and require surveillance.

To the best of our knowledge, this is the first report of the presence of CTX-M and *tet*A genotype in well water in Southeastern, Nigeria. The presence of ESBL and tetracycline resistance genes is concerning, as *Salmonella* can survive for months in drinking water and humans, becoming responsible for intestinal infectious illnesses in humans who are frequently regarded as healthy carriers. There is a considerable risk of antibiotic-resistant *Salmonella* strains being transferred to humans via water sources, providing a public health risk that can result in increased morbidity and mortality, as well as increased treatment costs. It may also lead to cross-resistance to other antibiotics with similar mechanisms of action. For these reasons, relevant healthcare authorities must investigate feasible methods of reducing such tendencies to prevent the situation from deteriorating further.

Our limitation was that we did not have a control population to evaluate other variables of interest to determine other potential domestic sources of bacterial and resistance gene transmission into the well water. Additionally, the limited class of antimicrobial agents and the limited number of resistant genes screened in this study were considered limitations

5. Conclusion

The results of this investigation have led to several recommendations. Immediate action is required to reduce the burden of antibiotic resistance in the environment, such as the prudent use of antibiotics in human and veterinary

treatment, as well as agriculture. Improving water quality is of main concern, such as implementing more stringent chlorine disinfection guidelines to prevent resistant bacteria from entering the aquatic environment. Following the emergence of antibiotic-resistant bacteria in well water, governments should devise management techniques to prevent such incidents.

Additional research into antimicrobial treatment use and management procedures in clinical and food animals could help us better understand which factors contribute most to the establishment, persistence, and transmission of resistance genes like *tet*A and bla_{CTX-M}. New attempts to sequence the entire genome of all *Salmonella* isolates at public health laboratories across the country will help assess whether plasmid-mediated *tet*A and bla_{CTX-M} have spread to additional *Salmonella* serotypes.

Enhanced surveillance and more studies on humans and food animals may aid in identifying the sources of illness and implementing prevention and control strategies. Meanwhile, community dwellers and healthcare professionals should be aware of the dangers and consequences of infection with this strain, particularly the possibility of antibiotic treatment failure.

Compliance with ethical standards

Disclosure of conflict of interest

No Conflict of Interest to be disclosed.

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