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ARTICLE



Prevalence of multidrug-resistant bacteria in groundwater supplies in Osogbo, Southwest Nigeria

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ABSTRACT

The occurrence of multidrug-resistant (MDR) bacteria in groundwater samples from Osogbo, southwest Nigeria was investigated in this study. Bacteria isolated from the water samples were tested against antibiotics by disc diffusion method. Out of the 113 gram-negative bacteria isolated, *E. coli* (28.32%) was the most predominant and the least was *Serratia* spp. (2.65%). All the isolates were 100% resistant to cefuroxime and <20% susceptible to ceftazidime and cefixime. More than 99% of the isolates were MDR and 33 (29.20%) were resistant to at least five classes of antibiotics. High prevalence of MDR bacteria in the groundwater indicates that the water sources are reservoirs of antibiotic resistance.



KEYWORDS

Antibiotics; groundwater; gram-negative; bacteria; Osogbo; Nigeria

Introduction

Safe water is a basic human need. Although the Millennium Development Goals include provision of safe drinking water and adequate sanitation, many countries lack these. About 2 billion people worldwide depend on water contaminated with faeces. Most of these people live in the developing countries and 2.4 billion do not have access to sanitation [1]. Ground and surface waters are the main sources of water supplies in the developing countries. Disease-causing microorganisms could contaminate these water sources via runoffs, infiltration from septic tanks, wastewater, and human and animal wastes [2,3]. Water-borne diseases resulting from consumption of microbiologically contaminated water are widespread in many developing countries where there is inadequate or no waste disposal. Common disease causing bacteria that have been implicated in well and surface waters are the Gram-negatives, which include members of the *Enterobacteriaceae* family and *Pseudomonas* spp. Some of these bacterial species are intrinsically resistant to antibiotics. Others acquire resistance by horizontal gene transfer [4,5].

Increased occurrences of antibiotic-resistant bacteria (ARB) have been attributed to the consumption of water contaminated with ARB [6]. This follows from the uncontrolled use of antibiotics by human beings and in animal breeding [7,8], coupled with the fact that antibiotics are not fully metabolised in the body but are excreted in the faeces

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[9]. This has led to the acquisition of antimicrobial resistance in aquatic organisms. ARB can transfer resistant genes between different species, thus increasing the incidence of multidrug-resistant (MDR) strains in the environment [10,11]. Several studies have confirmed the presence of ARB and antibiotic resistant genes in drinking waters [12–16]. Pathogenic bacteria ingested via contaminated drinking water can colonise the gastrointestinal tracts, where they could cause infection and transfer resistance to normal intestinal flora.

Lack of piped water supply in Nigeria has resulted in dependence on shallow ground-water and surface waters for drinking [17,18]. There have been repeated outbreaks of gastrointestinal diseases in some regions of Nigeria and this can be associated with poor sanitation and consumption of faecal-contaminated food and water [19,20]. Assessing the microbial quality of drinking water in major towns and cities in Nigeria will provide decision-makers with relevant information. This study aimed to determine the microbial quality and prevalence of multidrug resistant gram-negative bacteria in drinking water samples in a city in southwest Nigeria.

Materials and methods

Study area and sample collection

Osogbo is the state capital of Osun State and one of the major cities in southwest Nigeria with an estimated population of 749,750 by 2022 and an annual growth rate of 3% [21]. It is located between Latitude 7°42'20'' and 7°49'20'' and Longitude 4°30'20'' and 4°38'20''E at an altitude of about 320 m above sea level and a geographical area of 2875 km² [21]. The city experiences both wet and dry seasons with the wet season occurring between March and October and the dry season extending from November to February. The mean day and night temperatures vary between 22 and 25°C and 28 and 31°C, respectively. The mean annual precipitation varies between 1750 and 1900 mm. The relative humidity is about 75% during the rainy season and 30% during the dry season. The main source of drinking water in Osogbo is private hand-dug wells that are often shallow and indiscriminately sited wherever land is available without regard to adjacent sewage channels and not being treated before consumption.

Water samples were collected from 38 private hand-dug wells within Osogbo metropolis, southwest Nigeria, between September 2019 and October 2020. Only wells used mainly for drinking and other domestic purposes were considered in this study and this was based on availability (Figure 1). One hundred millilitres of groundwater samples were collected into sterile screw-capped bottles, labelled accordingly and taken to the laboratory immediately and analysed within 2 h of collection.

Isolation of gram-negative bacteria from water samples

Gram-negative bacteria were isolated from the water samples by enrichment technique and by plating on Petri dishes containing nutrient agar, eosin methylene blue (EMB) agar, salmonella-shigella agar, cetrimide agar and MacConkey agar (Oxoid, UK). The plates were incubated at 37 ± 2°C for 24 h and observed for discrete colonies. Based on

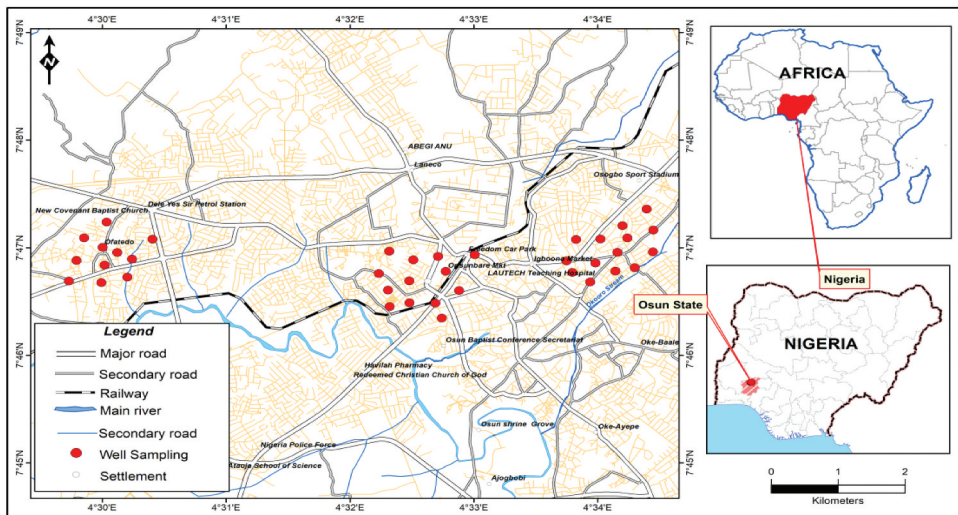


Figure 1. Groundwater sampling points within Osogbo, southwest Nigeria.

distinct morphology, selected bacteria colonies from the agar plates were streaked on nutrient agar, purified by repeated streaking and kept on nutrient agar slants at 4°C. The isolates were identified by standard biochemical tests [22].

Antibiotic susceptibility of the bacteria isolates

Disc diffusion method as described by Bauer et al. [23] was used for antibiotic susceptibility of the bacteria isolated from the water samples. Pure culture of the isolate was introduced into sterile peptone water and incubated overnight at 37°C. The overnight broth was spread on Muller Hinton agar (MHA) plates, using sterile swab stick and antibiotic discs were placed on the agar plates with the aid of sterile forceps and incubated at 37°C for 18–24 h. Five classes of commonly used antibiotics were used for this study, which includes cephalosporins – ceftazidime (30 µg), cefuroxime (30 µg), and cefixime (5 µg); penicillins – augmentin (30 µg); aminoglycoside – gentamicin (10 µg); fluoroquinolones – ciprofloxacin (5 µg), ofloxacin (5 µg); and nitrofurantoin – nitrofurantoin (300 µg). The procedure was carried out in duplicates. The diameters of zones of inhibition around the antibiotic discs were measured to the nearest millilitre as described by Clinical and Laboratory Standard Institute (CLSI) guidelines [24].

Multiple antibiotics resistance index (MARI)

Multiple antibiotics resistance index (MARI) is useful in determining health risk associated with antibiotics in a study area. The MARI of the isolates against the antibiotics was calculated as described by Krumpelman [25] using the formula a/b , where a represents the number of antibiotics the isolate was resistant to and b is the number of antibiotics evaluated. MARI values of ≥ 0.2 imply a high health risk in the environment where the antibiotics are often used.

Screening for extended-spectrum beta-lactamase (ESBL) producing gram-negative bacteria

Isolates which showed a zone of inhibition diameter of less than 21 mm for ceftazidime and less than 19 mm for cefixime were chosen for the ESBL screening. The test was carried out by modifying the method described by Kaur *et al.* [26]. The modified double-disc synergy test (MDDST) which includes the use of augmentin (amoxicillin/clavulanate – 20/10 µg), third-generation cephalosporins – 3GC (ceftazidime and cefixime) and fourth-generation cephalosporins – 4GC (cefepime) was used for this study. The MDDST was chosen because it is more sensitive than the ordinary double-disc synergy test (DDST) which requires the use of 3GC alone. Overnight, broth culture of the bacterial isolates was spread on MHA plate. The augmentin disc was placed at the centre of the plate. The 3GC and 4GC discs were placed 15 mm centre to centre of the augmentin disc. An extension in the zone of inhibition towards the augmentin disc was considered positive for ESBL production.

Statistical analysis

Descriptive statistics were achieved using Excel 2010 and results were presented as mean and percentages. Data were analysed using the Statistical Package for Social Sciences (SPSS) software programme for Windows (Version 21.0). Pearson's chi-square test with $p < 0.001$ was used to evaluate the level of association between the antibiotic resistant phenotype from the drinking water sources.

Results

A total of 113 gram-negative bacteria were isolated from the groundwater samples. The most frequent bacteria species were *Escherichia coli* (28.32%), followed by *Klebsiella* spp. (23.01%). Others were *Pseudomonas aeruginosa* (18.58%), *Proteus* spp. (7.96%), *Citrobacter* sp. (7.08%) *Salmonella* spp. (5.31%), *Shigella flexneri* (3.54%), *Enterobacter* spp. (3.54%), and *Serratia* spp. (2.65%) (Figure 2).

The bacterial isolates were classified according to the guidelines of the Clinical and Laboratory Standards Institute (2009). Table 1 shows the results. Out of the 113 gram-negative bacteria tested, 86 (76.11%) were susceptible to ofloxacin, 65 (57.52%) were susceptible to ciprofloxacin, but none was sensitive to cefuroxime and only one (0.88%) was sensitive to ceftazidime. Forty-three (38.05%) of the isolates displayed intermediate resistance to gentamicin. High complete resistance was shown against augmentin (92.04%), cefuroxime (90.27%), cefixime (89.38%), and ceftazidime (86.73%). Only three isolates (2.65%) showed complete resistance to ofloxacin.

Table 2 shows the distributions of antibiotic resistance among the gram-negative bacteria isolated from the groundwater samples (intermediate resistance was classified to be resistance to the antibiotics). All the isolates were 100% resistant to ceftazidime and cefuroxime, except the *Enterobacter* spp. showing 75% resistance to ceftazidime. The isolates showed less than 20% susceptibility to cefixime antibiotic. *Citrobacter freundii* and *Shigella flexneri* obtained from the water samples were 25% resistant to gentamicin. All other isolates exhibited $\geq 50\%$ resistance.

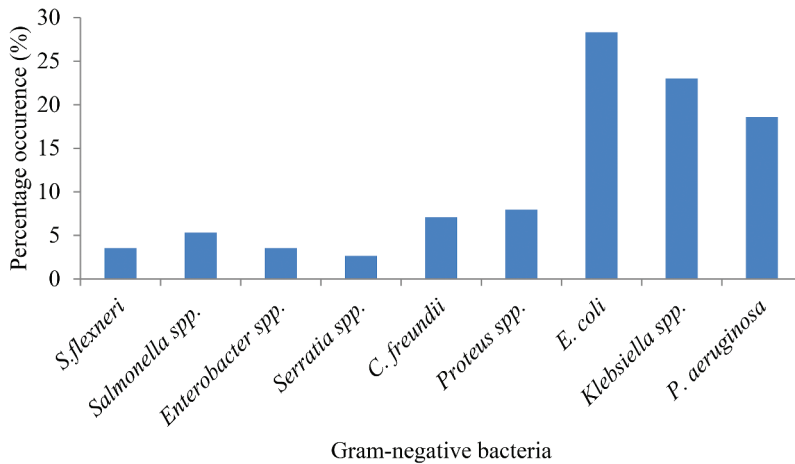


Figure 2. Percentage occurrence of gram-negative bacteria isolated from groundwater samples.

Table 1. Susceptibility profile of gram-negative bacteria isolated from groundwater samples.

Antibiotics	Antibiotics susceptibility pattern (N = 113)		
	Sensitive n (%)	Intermediate n (%)	Resistant n (%)
Nitrofurantoin (300 µg)	28 (24.78)	7 (6.19)	78 (69.03)
Ceftazidime (30 µg)	1 (0.88)	14 (12.39)	98 (86.73)
Cefixime (300 µg)	4 (3.54)	8 (7.08)	101 (89.38)
Augmentin (30 µg)	2 (1.77)	7 (6.19)	104 (92.04)
Gentamicin (10 µg)	51 (45.13)	43 (38.05)	19 (16.81)
Cefuroxime (5 µg)	0 (0)	11 (9.73)	102 (90.27)
Ofloxacin (5 µg)	86 (76.11)	24 (21.23)	3 (2.65)
Ciprofloxacin (5 µg)	65 (57.52)	32 (28.32)	16 (14.16)

N Overall number of tested isolates, n Number of isolates.

All the isolates also showed more than 95% resistance to augmentin. Ofloxacin seems to be the least resisted drug among the bacteria isolates and ciprofloxacin was very effective against many of the *E. coli* isolated from the water sample. The order of resistance to antibiotics among the isolates as shown in Table 2 were cefuroxime (100%) > augmentin (99.12%) > ceftazidime (97.22%) > cefixime (94.98%) > nitrofurantoin (72.07%) > gentamicin (60.44%) > ciprofloxacin (58.82%) > ofloxacin (34.18%). The result showed that all the bacterial isolates have high MARI values greater than 0.2 with the highest values indicated in *Salmonella* spp. and *Pseudomonas aeruginosa* (Table 2).

Table 3 shows that the most prevalent multidrug-resistant pattern of the gram-negative bacteria isolated from the groundwater samples was GEN/CRX/NIT/CAZ/CXM/AUG (18.58%) followed by CRX/NIT/CAZ/CXM/AUG (13.27%) and GEN/CRX/NIT/CAZ/CXM/CRP/AUG (13.27%). All the isolates displayed resistance to two or more antibiotics, of which 29.20% were resistant to the five classes of antibiotics tested, 36.28% were resistant to four classes of antibiotics, 34.51% to three classes and 8.85% to two classes of antibiotics. Four isolates, comprising *Proteus* sp., *Serratia* sp., *Salmonella* sp., and *Citrobacter freundii*

Table 2. Distribution of antibiotic resistance among gram-negative bacteria isolated from water samples.

Isolates (N)	Distribution of antibiotics resistance among Gram-negative isolates [N (%)]										MARI
	Nitrofurantoin	Ceftazidime	Cefixime	Augmentin	Gentamicin	Cefuroxime	Ofloxacin	Ciprofloxacin			
<i>Shigella flexneri</i> (N = 4)	1 (25)	4 (100)	4 (100)	4 (100)	1 (25)	4 (100)	1 (25)	3 (75)			0.5-0.9
<i>Salmonella</i> spp. (N = 6)	3 (40)	6 (100)	5 (80)	6 (100)	4 (60)	6 (100)	4 (60)	5 (80)			0.7-1.0
<i>Enterobacter</i> spp. (N = 4)	4 (100)	3 (75)	4(100)	4 (100)	4 (100)	4 (100)	0 (0)	4 (100)			0.7-0.9
<i>Serratia</i> spp. (N = 3)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	3 (100)	1 (33.33)			0.7-0.9
<i>Citrobacter</i> sp. (N = 8)	2 (25)	8 (100)	7 (87.5)	8 (100)	2 (25)	8 (100)	2 (25)	5 (62.50)			0.5-0.9
<i>Proteus</i> spp. (N = 9)	9 (100)	9 (100)	9 (100)	9 (100)	7 (77.77)	9 (100)	2 (23.57)	8 (88.88)			0.8-0.9
<i>Escherichia coli</i> (N = 32)	31 (96.88)	32 (100)	31 (96.88)	31 (96.88)	16 (50)	32 (100)	5 (15.63)	6 (18.75)			0.3-0.9
<i>Klebsiella</i> spp. (N = 26)	21 (80.77)	26 (100)	26 (100)	26 (100)	14 (53.85)	26 (100)	9 (34.62)	11 (42.31)			0.5-0.9
<i>Pseudomonas aeruginosa</i> (N = 21)	17 (80.95)	21 (100)	19 (90.48)	20 (95.24)	11 (52.38)	21 (100)	5 (23.81)	6 (28.57)			0.5-1.0
Percentage mean resistance	10.11 (72.07)	12.44 (97.22)	12 (94.98)	12.33 (99.12)	6.89 (60.44)	12.56 (100)	3.44 (34.18)	5.44 (58.82)			

N Number of isolates; MARI – Multiple antibiotics resistance index *intermediate resistance was classified to be resistance to the antibiotics.

Table 3. Phenotypic multidrug resistant pattern of gram-negative bacteria isolated from groundwater samples.

Antibiotype	Isolates					No of organisms	No of antibiotic class	Resistant profile
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>Klebsiella</i> spp.	<i>Proteus</i> spp.	Others*			
1					1	1	2	CRX/CAZ/AUG
2	1					1	3	GEN/CRX/CAZ/AUG
3	3	3	1		2	9	2	CRX/CAZ/CXM/AUG
4		1	2		4	7	3	CRX/CAZ/CXM/CRP/AUG
5	1				2	3	4	CRX/OFL/CAZ/CXM/AUG
6	1					1	3	OFL/CAZ/CXM/CRP/AUG
7	8	5	1		1	15	3	CRX/NIT/CAZ/CXM/AUG
8		1				1	3	CRX/OFL/NIT/CAZ/CPR
9			1			1	4	CRX/OFL/AUG/NIT/CAZ/CXM
10	1					1	3	GEN/CRX/NIT/CAZ/CXM
11	1		1			2	3	GEN/CRX/CAZ/CXM/AUG
12					1	1	4	GEN/CRX/NIT/CXM/CRP/AUG
13	1		2	1	1	5	4	CRX/NIT/CAZ/CXM/CRP/AUG
14					1	1	4	GEN/CRX/CAZ/CXM/CRP/AUG
15	9	3	8	1		21	4	GEN/CRX/NIT/CAZ/CXM/AUG
16		1			1	2	5	GEN/CRX/NIT/CAZ/CRP/AUG
17			1			1	3	CRX/OFL/CAZ/CXM/CRP/AUG
18	2	3	2	5	3	15	5	GEN/CRX/NIT/CAZ/CXM/CRP/AUG
19					2	2	4	GEN/CRX/OFL/CAZ/CXM/CRP/AUG
20	2	4	3		3	12	5	GEN/CRX/OFL/NIT/CAZ/CXM/AUG
21	2		4	1		7	4	CRX/OFL/NIT/CAZ/CXM/CRP/AUG
22				1	3	4	5	GEN/CRX/OFL/NIT/CAZ/CXM/CRP/AUG
Total	32	21	26	9	25	113		

Ceftazidime – CAZ (30 µg), cefuroxime – CRX (30 µg), gentamicin – GEN (10 µg), ciprofloxacin – CRP (5 µg), ofloxacin – OFL (5 µg), nitrofurantoin – NIT (300 µg), cefixime – CXM (5 µg), and augmentin – AUG (30 µg). Other*- *Shigella flexneri*, *Salmonella* sp., *Enterobacter* sp., *Serratia* sp. *Citrobacter* sp.

exhibited resistance to all the five classes of tested antibiotics. More than 99% of the isolates were resistant to at least two cephalosporin antibiotics and 98.23% were resistant to penicillin. Bacterial isolate resistance to three or more classes of antibiotics means ‘multi-drug resistance’. The result showed that all the isolates were multidrug resistant strains, except a strain of *Citrobacter freundii*, which was resistant to only two classes of antibiotics.

Modified double-disc synergy test indicated production of extended-spectrum beta-lactamase in 18 (15.93%) isolates (Figure 3), which include the genera; *Shigella flexneri*, *Citrobacter freundii*, *Enterobacter* sp., *Klebsiella* spp., *P. aeruginosa*, *Proteus* sp., and *Escherichia coli*.

The correlation matrix of association between antibiotic resistance phenotypes of gram-negative bacteria isolated from the groundwater samples showed that there was a strong relationship between resistance of the isolates to gentamicin and nitrofurantoin and between penicillin and cefixime at $p < 0.001$.

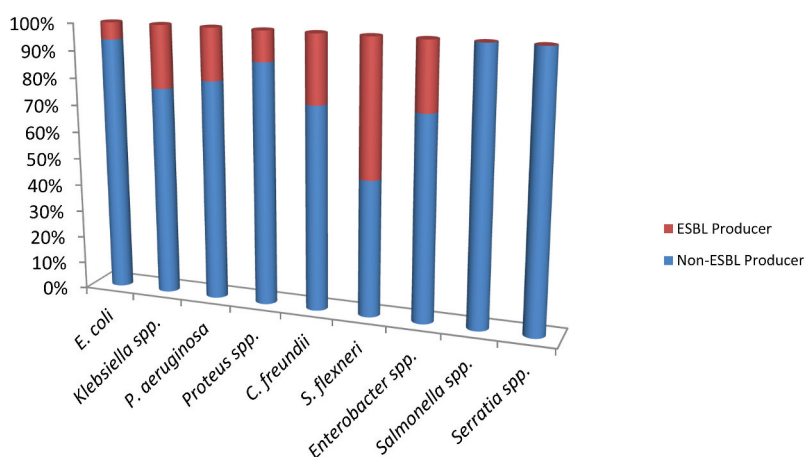


Figure 3. Extended-spectrum beta-lactamase producing gram-negative bacteria isolated from groundwater samples.

Discussion

The lack of safe water and sanitation are major contributory factors to increased risks of waterborne diseases. It is therefore necessary to carry out routine checks on the microbial quality of drinking water sources in any community, so as to ascertain the safety of the water consumed by the inhabitants, in order to prevent the spread of waterborne diseases. This study aimed at investigating the prevalence of multidrug resistant and extended-spectrum beta-lactamase producing gram-negative bacteria in underground waters used mainly for drinking purposes in Osogbo, southwest Nigeria.

A total of 113 gram-negative bacteria were isolated and identified from the private wells. *Escherichia coli* was found to be the most frequent bacterium in the groundwater samples and this is similar to the report of many researchers, who identified bacteria from surface and ground waters [27,28]. The presence of *E. coli* in drinking water is associated with faecal pollution and indicates that there could be pathogenic microorganisms in the water samples [29]. Yelle *et al.* [30] found *Escherichia coli*, *Klebsiella spp.*, *Enterobacter spp.*, *Salmonella spp.* and *Serratia spp.* in drinking water in South Mumbai, similar to the findings of this study. These organisms have been implicated in gastrointestinal diseases, such as diarrhoea and typhoid fever, a serious threat in developing countries. In some developed countries, the genera *Klebsiella*, *Citrobacter*, *Escherichia*, *Enterobacter*, *Salmonella*, *Shigella* and *Pseudomonas* are also implicated in drinking water [31–33]. In many communities within the study area, septic tanks are located near the residential buildings and these sewage systems contain bacteria that may seep through the groundwater channels, contaminating the water source. This is of great concern because the faecal materials may carry pathogenic microorganisms [34,35]. Many of the communities where the water samples were collected usually consume the water without any form of treatment and without concern for possible seepage from the underground septic tanks. Microbial contamination of water poses serious public health challenges when bacteria found in drinking water harbour antibiotic-resistant genes and could colonise the intestinal tracts and transfer resistance to the normal intestinal flora [31]. Monitoring

the presence of ARB in drinking water sources is essential, as is also disseminating information on the presence of ARB in the environment, avoidance of the use of un-prescribed drugs and the need to abide by laws governing the construction of wells. There have been several reports on the presence of ARB in drinking water sources [13,15,28].

The present study showed that all the gram-negative bacteria isolated from the drinking wells were resistant to two or more antibiotics and about 94–100% resistance to beta-lactams were also observed. Presumably, increased resistance to β -lactam antibiotics can simply be attributed to its widespread use by human beings and in animal breeding [11]. This study showed that $\geq 50\%$ of the isolates were resistant to gentamicin which seemingly used to be very effective against bacteria from environmental samples [27]. An earlier study by Odumosu and Akintimehin [12] reported that bacteria isolated from some wells in Ogun State, southwest Nigeria were 75–85.70% susceptible to fluoroquinolones, which is in disagreement with the findings of this study. Although fluoroquinolones are often the last line of therapy, they are gradually becoming ineffective. There is clear evidence from this study that the drinking water sources are reservoirs of ARB. High MARI reported in this study is in agreement with other studies carried out in Nigeria [36–38]. Onuoha [37] also reported high MARI value (0.75) in *Salmonella* spp. isolated from surface water in eastern Nigeria which is similar to this study. High MARI values reported in this study can be attributed to the over-use and abuse of antibiotics.

The most prevalent phenotypic multi-drug resistant pattern of the gram-negative bacteria isolated from water samples was GEN/CRX/NIT/CAZ/CXM/AUG followed by CRX/NIT/CAZ/CXM/AUG and GEN/CRX/NIT/CAZ/CXM/CRP/AUG. This indicates that gentamicin, cefuroxime, nitrofurantoin, ceftazidime, cefixime and augmentin are becoming useless. This suggests a need for more effective drugs, but antibiotic resistance is now a worldwide problem. Titilawo *et al.* [39] reported high prevalence of MDR *E. coli* in some rivers within Osun State. Similarly, Nzima *et al.* [40] reported 100% resistance to penicillin in *E. coli* isolated from surface water in South Africa, which are in agreement with the findings of this study. More than 99% of the bacteria isolated in this study were MDR which is higher than previous studies in Nigeria reporting 75% and 77% MDR strains in drinking water in southwest Nigeria [12,41]. Likewise, Nzima *et al.* [40] and Lenart-Boron [8] reported 100% MDR bacteria in environmental samples from South Africa and Poland, respectively.

Extended spectrum beta-lactamase producing bacteria are becoming a major global threat; because infections are now difficult to treat. Resistance to third-generation beta-lactam antibiotics usually indicates the expression of ESBL genes [29,40]. Although drinking water has been described as one of the reservoirs of ESBL-producing bacteria, there is a dearth of information on their occurrence in drinking water in Nigeria. Eighteen bacteria isolates which include members of the genera *Shigella flexneri*, *Citrobacter freundii*, *Enterobacter* spp., *Klebsiella* spp., *P. aeruginosa*, *Proteus* spp., and *Escherichia coli* were found positive for ESBL production in this study. This showed that 15.93% of the bacteria isolates were ESBL producers which is more than 5.3% and 7.14% as reported by De Boeck *et al.* [42] and Odumosu and Akintimehin [12], respectively but similar to more recent studies by Falodun *et al.* [28], who reported 17.9% ESBL producing bacteria from surface water in Ibadan, southwest Nigeria. The presence of ESBL implies that drinking water could be a source of

community-acquired infections with ESBL-producing bacteria [39]. The strong correlation observed among the bacteria resistance to different antibiotics could be owing to the fact that many antibiotic genes are found on the same mobile genetic element [43,44].

Conclusion

The high incidence of antibiotic-resistant bacteria observed in drinking water sources in many communities within Osogbo indicated that the water sources are polluted and unsafe for human consumption and this could contribute to the burden of infectious diseases. The overall result of this study showed that 99.12% of the isolates were multi-drug resistant strains. The presence of extended-spectrum beta-lactamase producing bacteria in the drinking water samples could be attributed to the abundance of beta-lactam antibiotics in the environment as a result of over-use. There is a need for government at all three levels in Nigeria to take responsibility for solving these problems, by improving drinking water quality and sanitation in the study area. The use of unprescribed drugs should also be discouraged.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Authors' contribution

Olukemi Aromolaran conceptualised, designed the study, supervised the work, analysed the data and drafted the manuscript. Ayansina ADV made critical revisions and improved the manuscript. Aromolaran O, Adegbam AA, Oshanisi PO, and Oladoyin DM carried out the research work in the laboratory. All authors approved the final manuscript.

Data availability

Data associated with this work are available upon request.

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