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RESEARCH ARTICLE

Extended Spectrum Beta-Lactamase (ESBL)-producing bacteria isolated from hospital wastewaters, rivers and aquaculture sources in Nigeria

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Abstract Untreated wastewater is a risk factor for the spread of antibiotic resistance in the environment. However, little is known about the contribution of untreated wastewater to the burden of antibiotic resistance in the Nigerian environment. In this study, a total of 143 ceftazidime-/cefpodoxime-resistant bacteria isolated from untreated wastewater and untreated wastewater-contaminated surface and groundwater in Nigeria were screened for extended-spectrum β-lactamase (ESBL) genes, integrons and integron gene cassettes by PCR. The genetic environment of $bla_{\text{CTX-M-15}}$ was mapped by PCR and potentially conjugative plasmids were detected among the isolates by degenerate primer MOB typing (DPMT). ESBL production was confirmed in 114 (79.7%) isolates and ESBL genes (bla_{SHV}, $bla_{\text{CTX-M-15}}$ and bla_{TEM}) were detected in 85 (74.6%) ESBL-producing isolates. $bla_{\text{CTX-M-15}}$ was associated with IS*Ecp1* and with *orf477* in 12 isolates and with IS*Ecp1*, IS*26* and *orf477* in six others. To the best of our knowledge, this is the first report of $bla_{\text{CTX-M-15}}$ in hand-dug wells and borehole serving as sources of drinking water and a first report of the genetic environment of $bla_{\text{CTX-M-15}}$ in environmental bacteria from Nigeria. The results of this study confirm untreated wastewater as an important medium for the spread of ESBL-producing bacteria within the Nigerian environment. Hence, the widespread practice of discharging untreated wastewater into the aquatic ecosystem in Nigeria is a serious risk to public health.

Keywords Antibiotic resistance · β-Lactamases · bla_{SHV} · bla_{TEM} · $bla_{\text{CTX-M-15}}$ · Conjugative relaxase · Untreated wastewater

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Introduction

The widespread occurrence of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in the environment is currently a major global public health issue. Previously, the spread of resistance has been associated with selection pressure derived from the clinical use of antibiotics, but recent studies have shown that natural environmental reservoirs contribute significantly to the global proliferation of antibiotic resistance (Canton 2009; Finley et al. 2013). In particular, municipal wastewater treatment systems which concentrate wastewaters containing residual antibiotics, ARB and ARGs of different origins are hotspots of antibiotic resistance and a direct source for the dissemination of ARB and ARGs into the environment (Berendonk et al. 2015; Di Cesare et al. 2016). Hence, several recent studies have investigated the important role played by wastewater and wastewater treatment systems in environmental contamination with ARB and ARGs (LaPara et al. 2011; Munir et al. 2011; Czekalski et al. 2014; Du et al. 2014; Rodriguez-Mozaz et al. 2015). Most of these studies are however carried out in countries with well-established wastewater treatment systems. Studies investigating environmental contamination with ARB and ARGs from wastewater and/or wastewater treatment systems in developing countries including Nigeria are still not very common.

In many developing countries like Nigeria, wastewaters from hospitals and industrial, domestic and agricultural sources are discharged into open sewers, rivers and lakes without prior treatment. This practice will likely create high selection-pressure zones within recipient aquatic ecosystem where residual antibiotics, ARB and ARGs in the untreated wastewater and the resident microflora of the receiving ecosystem are continually mixed. Such ecosystems are likely to act as hotspots for the emergence, proliferation and eventual dissemination of ARB and ARGs into the human population through the water-human route. There is thus an acute need for studies evaluating the contribution of untreated wastewater to environmental contamination with ARB and ARGs in developing countries. Such studies are particularly important because in the absence of wastewater treatment systems as it is presently the case in many developing countries, untreated wastewater remains an important risk factor in environmental contamination with ARB and ARGs.

Presently, one of the mechanisms of resistance reducing the effect of modern antibiotics is the extended-spectrum β-lactamases (ESBLs). ESBL genes are often carried on mobile genetic elements and control the production of enzymes that inactivate cephalosporins via hydrolysis of the β-lactam ring. Beta-lactams are a class of antibiotics accounting for up to 50–70% of the total antibiotic use in several countries of the world (Korzeniewska and Harnisz 2013) including Nigeria (Ogbolu et al. 2013). Worldwide, resistance to β-lactams has been reported in bacterial strains isolated from human clinical sources, animals, food products and the environment (Lupo et al. 2012).

However, very little information exists on the occurrence and spread of ESBLs in the Nigerian aquatic ecosystems. More importantly, few studies, if any, have investigated the role of untreated wastewater as a source of contamination of the Nigerian aquatic ecosystem with ESBL genes. Available studies on ESBLs in Nigeria focused on clinical bacteria isolates (Soge et al. 2006; Ogbolu et al. 2011, 2013). This study therefore investigated the occurrence of ESBL genes in gram-negative bacteria isolated from untreated wastewater and untreated wastewater-impacted aquatic ecosystems in Nigeria. Our primary aim is to assess the possible contribution of untreated wastewater to the contamination of Nigeria's aquatic ecosystem with clinically relevant ARB and ARGs such as ESBLs.

Materials and methods

Bacterial isolates

A total of 143 bacteria with resistance to ceftazidime (6 μg/mL) or cefpodoxime (6 μg/mL) were included in this study. The bacterial isolates were collected between 2009 and 2013 from six states located in three geo-political regions of Nigeria. The bacteria were collected within the framework of a larger project examining the role of untreated wastewater commonly released into the Nigerian environment from several point and non-point sources as a potential medium for the spread of ARB and ARGs into the Nigerian environment. The project focused on wastewater from three different sources considered of interest to Nigeria, namely (a) hospital sources, (b) domestic sources and (c) aquaculture.

Samples from hospital sources consisted of untreated wastewater and groundwater (from hand-dug wells and boreholes) within the vicinity of the wastewater discharge points of seven hospitals located in Ogun State ($n = 5$), southwestern Nigeria, and Benue State ($n = 2$), North Central Nigeria. The Ogun State samples were made up of untreated wastewater and groundwater collected from boreholes and wells between September 2009 and August 2011 while untreated wastewater samples were collected from the two hospitals in Benue State in March and April 2013. Aquaculture samples consisting of effluents from three fish farms located in Ibadan (IBD 1 and IBD 2; with seven and two ponds, respectively) and Ilesha (ILE1 with six ponds) in the southwest of Nigeria were collected in the second and fourth weeks of March 2013. Up till the time of sample collection, oxytetracycline and neocloxin (a mixture of oxytetracycline, chloramphenicol and neomycin) were commonly used in IBD 1 and IBD 2 ponds to treat fish infections while no previous history of antibiotic use was known at ILE 1. Partially treated wastewater from domestic sources was collected from the wastewater oxidation pond at the University of Ibadan, Oyo State, Nigeria. Additionally, water from three polluted rivers receiving untreated wastewater from domestic and industrial sources in the south of Nigeria was also collected. The three rivers were as follows: the Zik River flowing through the Campus of the University of Ibadan in Oyo State, southwest of Nigeria, which receives untreated wastewater from the university's hostel facilities, and the Ikpoba River and the River Cross located in Edo and Cross River States in the south-south region of Nigeria which receives untreated wastewater from domestic and industrial sources. Samples were collected monthly from the Zik River between August 2012 and February 2013 and from the wastewater oxidation pond in March and April 2013. Samples from Ikpoba and Cross Rivers were collected during an environmental impact assessment trip in December 2012.

Bacteria isolation, antimicrobial susceptibility testing and screening for ESBL production

Bacteria were isolated on Muller Hinton agar (MHA) or Eosin Methylene Blue Agar (EMB) amended with ceftazidime (6 μg/mL) or cefpodoxime (6 μg/mL). Samples were serially diluted in sterile normal saline and aliquots (200 μl) of diluted samples plated on ceftazidime- or cefpodoxime-supplemented

EMB and MHA. Non-duplicate colonies of each morphological types observed on the plates after overnight incubation at 35 °C were picked and streaked on fresh plates to obtain pure cultures which were stored frozen in glycerol broth (15%) pending transfer to the Institute of Hydrobiology, Technical University of Dresden, Dresden, Germany, for genotyping. Isolates selected on MHA plates were subjected to gram staining and 3% KOH test (Buck 1982) to confirm them as gram negatives before inclusion in the study. Where two isolates from the same sample selected from EMB and MHA plates showed exactly the same pattern of resistance and identity, only one was included in the study. Bacterial isolates were tested for susceptibility to four antibiotics (Oxoid™): sulphamethoxazole/trimethoprim (SXT, 25 μg), tetracycline (TET, 30 μg), ciprofloxacin (CIP, 5 μg) and ceftazidime (CAZ, 30 μg) by the agar disc diffusion method. Zones of growth inhibition around each disc were measured and interpreted as described by the Clinical and Laboratory Standards Institute (CLSI 2011). Isolates confirmed as showing resistance or intermediate resistance to ceftazidime were selected for double disc synergy test (DDST) (CLSI 2011) to confirm ESBL production.

Bacteria identification

Suspected *Escherichia coli* (colonies showing greenish metallic sheen on EMB agar) were identified by streaking on chromogenic selective Brilliance™ *E. coli*/coliform agar while all other isolates, including isolates showing ambiguous colony appearance on Brilliance *E. coli*/coliform agar, were identified by PCR amplification and sequencing (GATC Biotech, Köln, Germany) of the 16S rDNA (Lane 1991). Sequences obtained were blasted against reference sequences in the GenBank (http://blast.ncbi.nlm.nih.gov/blast.cgi) to identify the bacteria strains. Genomic DNA for 16S rDNA amplification was extracted with the microwave method (Orsini and Romano-Spica 2001).

PCR detection of ESBL genes, integrons and conjugative relaxases

Presumptive ESBL-producing bacteria were screened by standard PCR for the presence of bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, ampC gene bla_{FOX} and integrons classes 1, 2 and 3 as described (Pérez-Pérez and Hanson 2002; Mulvey et al. 2003; Machado et al. 2005; Calbo et al. 2011). Gene cassettes were amplified with primers targeting the variable regions of class 1 integrons (Machado et al. 2005). The genetic contexts of detected $bla_{CTX\text{-}M}$ were mapped as described by Dihanji et al. (2011). Plasmids of ESBL-producing isolates were extracted with a Nucleospin plasmid purification kit (Qiagen) according to the manufacturer's instructions. Degenerate primers targeting ten relaxase MOB families MOB F11, F12, P11, P12, P13, P14, P3, P4, H121, and Q11 were used to amplify conjugative relaxase genes as described by Alvarado et al. (2012) using the extracted plasmid DNA as templates. The selected primer pairs targeted relaxases of the plasmid incompatibility groups IncN, IncW, IncP9, IncF complex, Inc9, IncP1 complex, Incl1 complex, IncK, IncB/O, IncL/M, IncQ1, IncQ2, IncP6, IncX, IncA/C and IncU (Alvarado et al. 2012). Representatives of correct size amplicons of all detected genes were sequenced to confirm their identity.

Results

Identification of ESBL-producing bacteria and their antibiotic susceptibility

A total of one hundred and forty-three (143) gram-negative bacteria with reduced susceptibility to ceftazidime or cefpodoxime were isolated from the different samples analysed in this study. DDST screening revealed the production of ESBLs in 114 isolates (79.7%) where 3.5% ($n = 4$) originated from the oxidation pond, 7.9% ($n = 9$) from rivers, 40.4% ($n = 46$) from hospitals and 48.2% ($n = 55$) from fish farm effluents.

ESBL-producing isolates of hospital/river/domestic wastewater origin were identified as mostly members of the family *Enterobacteriaceae*, while non-lactose fermenting gram-negative bacteria predominated in the fish farm effluents (Table 1). In the wastewater from the hospitals, the majority of isolates were identified as *E. coli* and *Proteus mirabilis*, the water samples from the river/domestic wastewater oxidation pond instead displayed a higher bacterial diversity but *E. coli* was unrepresented in these samples. In the fish farm effluents, *Stenotrophomonas maltophilia* dominated among the ESBL-producing bacteria.

All the 114 isolates showed resistance to CAZ while 75, 90, 93.5 and 94.5% of isolates from oxidation pond, rivers, hospital and fish farms were resistant to TET. From the same samples, 75, 66.7, 89 and 52.7% showed resistance to CIP, and 75, 66.7, 93.5 and 11% are resistant to SXT (Fig. 1). Most of the isolates showed resistance to more than one of the tested antibiotics with 95.5, 92.3 and 72.7% of isolates from hospital, river/domestic wastewaters and fish farms displaying a multi-resistance phenotype (Table 1). Interestingly, isolates of *Stenotrophomonas* from the fish farms showed low levels of resistance to sulphamethoxazole/trimethoprim (SXT) (Fig. 1). This is in contrast to other reports, describing the recent global emergence of resistance to sulphamethoxazole/trimethoprim in species of *Stenotrophomonas* (Toleman et al. 2007; Huang et al. 2014).

Table 1 Phenotypic pattern of resistance and occurrence of ß-lactamases among gram-negative ESBL-producing bacteria isolates

Isolate	Sampling site	Sampling date	Phenotypic pattern of resistance	Resistance genes	Integron/gene cassettes
Rivers					
Klebsiella pneumoniae subsp. *pneumoniae* OZ63	Zik River, Ibadan	02/2013	CAZ, TET	bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}15}$	NID
Citrobacter freundii OZ9	"	10/2012	CAZ, CIP, SXT, TET	bla_{TEM}, bla_{SHV}	[a]*Intl1*
Escherichia fergusonii OZ24	"	08/2012	CAZ, CIP, SXT, TET	$bla_{CTX\text{-}M\text{-}15}$	*Intl1*/*dfr*A17|*aad*A5
Stenotrophomonas maltophilia IK37	Ikpoba River	12/2012	CAZ, CIP, TET	bla_{SHV}	NID
K. varicola IK16	"	12/2012	CAZ, CIP, SXT, TET	bla_{SHV}	NID
Enterobacter asburiae IK42	"	12/2012	CAZ, CIP, SXT, TET	bla_{SHV}	NID
Providencia rettgeri CR230	Cross River	12/2012	CAZ, CIP	ND	*Intl1*/*dfr*A32|*ere*A|*aad*A2
P. rettgeri CR4	"	12/2012	CAZ, SXT, TET	bla_{SHV}	*Intl1*/*dfr*A32|*ere*A|*aad*A2
Wastewater oxidation pond					
K. pneumoniae subsp. *rhinoscleromatis* OZ49	Wastewater flow channel	04/2013	CAZ	bla_{SHV}	*Intl1*/*dfr*A16|*aad*A2
Acinetobacter baumannii OZ43	Wastewater oxidation pond	04/2013	CAZ, CIP, SXT, TET	ND	NID
Aeromonas punctata OZ53	"	05/2013	CAZ, CIP, SXT, TET	ND	[a]*Intl1*
S. maltophilia OZ85	"	04/2013	CAZ, CIP, SXT, TET	bla_{SHV}	NID
Hospital sources					
Proteus mirabilis MK220	Wastewater, Benue State	03/2013	CAZ, CIP, SXT, TET	bla_{TEM}, bla_{SHV}	*Intl1*/*dfr*A17|*aad*A5
P. mirabilis MK221	"	03/2013	CAZ, CIP, SXT	bla_{TEM}, bla_{SHV}	*Intl1*/*dfr*A7
P. mirabilis MK222	"	03/2013	CAZ, TET	bla_{TEM}, bla_{SHV}	*Intl1*/*dfr*A7
P. mirabilis MK223	"	03/2013	CAZ, SXT, TET	bla_{TEM}, bla_{SHV}	*Intl1*/*dfr*A17|*aad*A5
P. mirabilis MK224	"	03/2013	CAZ, CIP, TET	bla_{TEM}, bla_{SHV}	*Intl1*/*dfr*A7
P. mirabilis MK226	"	04/2013	CAZ, CIP, SXT	bla_{TEM}	*Intl1*/*dfr*A17|*aad*A5, *Intl2*[b]
P. mirabilis MK227	"	04/2013	CAZ, SXT	bla_{TEM}	[a]*Intl1*, *Intl2*[b]
Shigella flexneri MK228	"	04/2013	CAZ, CIP, TET	$bla_{CTX\text{-}M\text{-}15}$	*Intl1*/*dfr*A7
E. coli EOd1	Wastewater, Ijebu-Ode, Ogun State	09/2009	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EOd5	"	03/2010	CAZ, CIP, SXT, TET	bla_{TEM}	NID
E. coli EOd8	"	03/2010	CAZ, CIP, SXT, TET	bla_{TEM}, $bla_{CTX\text{-}M\text{-}15}$	[a]*Intl1*
E. coli EOd9	"	03/2010	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EOd11	"	07/2010	CAZ, CIP, SXT, TET	bla_{TEM}, $bla_{CTX\text{-}M\text{-}15}$	[a]*Intl1*
E. coli EOd12	"	09/2010	CAZ, CIP, SXT, TET	bla_{TEM}, $bla_{CTX\text{-}M\text{-}15}$	[a]*Intl1*
E. coli EOd14	"	09/2010	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EOd17	"	02/2011	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EOd19	"	02/2011	CAZ, CIP, SXT, TET	$bla_{CTX\text{-}M\text{-}15}$	*Intl1*/*dfrA17*|*aadA5*
E. coli EOd20	"	03/2011	CAZ, CIP, SXT, TET	$bla_{CTX\text{-}M\text{-}15}$	*Intl1*/*dfrA17*|*aadA5*
E. coli EOd21	"	07/2011	CAZ, CIP, SXT, TET	$bla_{CTX\text{-}M\text{-}15}$	*Intl1*/*dfrA17*|*aadA5*
E. coli EId3	Wastewater, Ijebu-Igbo, Ogun State	10/2009	CAZ, CIP, SXT, TET	bla_{TEM}, $bla_{CTX\text{-}M\text{-}15}$	*Intl1*/*aadA1*
E. coli EId9	"	07/2010	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EId10	"	07/2011	CAZ, CIP, SXT, TET	bla_{TEM}, $bla_{CTX\text{-}M\text{-}15}$	[a]*Intl1*
R. ornithinolytica EId13	"	07/2011	CAZ, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EIw2	Well water, Ijebu-Igbo, Ogun State	09/2010	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
Ent. amnigenus EIw4	,,	09/2010	CAZ, CIP, SXT, TET	bla_{TEM}	*Intl1*/*aad*A1
E. coli EIw10	"	08/2011	CAZ, SXT, TET		[a]*Intl1*

Table 1 (continued)

Isolate	Sampling site	Sampling date	Phenotypic pattern of resistance	Resistance genes	Integron/gene cassettes
				bla_{TEM}, $bla_{CTX-M-15}$	
E. coli EAd2	Wastewater, Ago-Iwoye, Ogun State	09/2009	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EAd9	"	03/2010	CAZ, CIP, SXT, TET	$bla_{CTX-M-15}$	*Intl1*/*dfr*A17|*aad*A5
E. coli EAd10	"	07/2010	CAZ, CIP, SXT, TET	$bla_{CTX-M-15}$	*Intl1*/*dfr*A17|*aad*A5
E. coli EAd14	"	02/2011	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EAd15	"	07/2011	CAZ, CIP, SXT, TET	bla_{TEM}, bla_{SHV}, $bla_{CTX-M-15}$	*Intl1*/*aad*A1
E. coli EAd16	"	07/2011	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. hermannii EAd19	"	07/2011	CAZ, CIP, SXT, TET	bla_{TEM}, bla_{SHV}, $bla_{CTX-M-15}$	*Intl1*/*aad*A1
S. sonnei EAw8	Well water, Ago-Iwoye, Ogun State	07/2011	CAZ, CIP, SXT, TET	$bla_{CTX-M-15}$	*Intl1*/*dfr*A17|*aad*A5
E. coli EWd1	Wastewater, Ibiade, Ogun State	01/2010	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EWd5	"	04/2010	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EWd9	"	07/2010	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EWd13	"	12/2010	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EWb2	Borehole water, Ibiade, Ogun State	08/2010	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*
E. coli EWb3	"	09/2010	CAZ, CIP, SXT, TET	bla_{SHV}, $bla_{CTX-M-15}$	*Intl1*/*dfr*A17|*aad*A5
Fish farms					
S. maltophilia IBD1–58	Pond 1	12/03/2013	CAZ, TET	ND	[a]*Intl1*
S. maltophilia IBD1–235	"	"	CAZ, CIP	bla_{SHV}	NID
S. maltophilia IBD1–75	Pond 2	"	CAZ, CIP, TET	bla_{TEM}	NID
S. maltophilia IBD1–168	"	"	CAZ, TET	bla_{SHV}	NID
S. maltophilia IBD1–55	Pond 3	"	CAZ, CIP, TET	ND	[a]*Intl1*
Chryseobacterium jejuense IBD1–47	Pond 4	"	CAZ, TET	bla_{TEM}	NID
S. maltophilia IBD1–54	"	"	CAZ, TET	ND	[a]*Intl1*
Pseudomonas hibiscicola IBD1–85	Pond 5	"	CAZ, TET	ND	[a]*Intl1*
S. maltophilia IBD1–120	"	"	CAZ, CIP, TET	bla_{SHV}	NID
S. maltophilia IBD1–125	"	"	CAZ, TET	bla_{SHV}	NID
S. maltophilia IBD1–108	Pond 6	"	CAZ, CIP, TET	bla_{SHV}	NID
S. maltophilia IBD1–141	"	"	CAZ, CIP, SXT, TET	bla_{SHV}	NID
S. maltophilia IBD1–84	Pond 7	"	CAZ, CIP, TET	bla_{SHV}	NID
S. maltophilia IBD1–127	Stream	"	CAZ, TET	bla_{SHV}	NID
Stenotrophomonas spp. IBD1–239	"	"	CAZ, TET	bla_{SHV}	[a]*Intl1*
S. maltophilia IBD2–98	Pond A	"	CAZ, TET	bla_{SHV}	NID
Stenotrophomonas spp. IBD2–234	"	"	CAZ, TET	bla_{SHV}	NID
Stenotrophomonas spp. IBD2–236	"	"	CAZ, CIP, TET	bla_{SHV}	NID
S. maltophilia ILE1–70	Stream	"	CAZ, TET	ND	[a]*Intl1*
S. maltophilia IBD1–104	Pond 1	26/03/2013	CAZ, CIP, TET	bla_{SHV}	NID
S. maltophilia IBD1–139	"	"	CAZ, TET	bla_{SHV}	NID
S. maltophilia IBD1–138	Pond 2	"	CAZ, CIP, TET	bla_{SHV}	NID
S. maltophilia IBD1–198	"	"	CAZ, TET	bla_{SHV}	NID
S. maltophilia IBD1–113	Pond 3	"	CAZ, CIP, SXT, TET	bla_{SHV}	NID
S. maltophilia IBD1–193	"	"	CAZ, CIP, TET	bla_{SHV}	NID
S. maltophilia IBD1–196	"	"	CAZ	bla_{SHV}	NID
S. maltophilia IBD1–74	Pond 4	"	CAZ, CIP, SXT, TET	bla_{TEM}	[a]*Intl1*

Table 1 (continued)

Isolate	Sampling site	Sampling date	Phenotypic pattern of resistance	Resistance genes	Integron/gene cassettes
S. maltophilia IBD1–90	"	"	CAZ, CIP, TET	bla_{SHV}	NID
S. maltophilia IBD1–142	Pond 5	"	CAZ, TET	bla_{SHV}	NID
S. dysenteriae IBD1–175	"	"	CAZ, TET	bla_{TEM}	*Intl1*/*dfr*A7
S. maltophilia IBD1–137	"	"	CAZ, CIP, TET	bla_{SHV}	NID
S. maltophilia IBD1–169	"	"	CAZ, CIP, TET	bla_{TEM}	NID
S. maltophilia IBD1–218	"	"	CAZ, CIP, SXT, TET	bla_{SHV}	NID
S. maltophilia IBD1–151	Pond 6	"	CAZ, CIP, SXT, TET	bla_{SHV}	NID
S. maltophilia IBD1–185	"	"	CAZ, CIP, TET	bla_{SHV}	NID
S. maltophilia IBD1–200	"	"	CAZ, TET	bla_{SHV}	NID
S. maltophilia IBD1–132	Pond 7	"	CAZ, CIP, TET	bla_{SHV}	NID
S. maltophilia IBD1–204	Stream	"	CAZ,	bla_{SHV}	NID
S. maltophilia IBD2–99	Pond A	"	CAZ, TET	bla_{SHV}	NID
S. maltophilia IBD2–186	"	"	CAZ, SXT, TET	bla_{SHV}	NID
S. maltophilia ILE1–164	Pond F	"	CAZ, CIP, TET	bla_{SHV}	NID
S. maltophilia ILE1–79	Stream	"	CAZ, TET	ND	NID

ND no ESBL gene detected, *NID* no integron detected, *CAZ* ceftazidime, *CIP* ciprofloxacin, *SXT* sulphamethoxazole/trimethoprim, *TET* tetracycline, [a] *Intl1* class 1 integrons without gene cassettes, *Intl2*[b] class 2 integrons were not searched for gene cassettes, *IBD1* fish farm 1, Ibadan, *IBD2* fish farm 2, Ibadan, *ILE1* fish farm at Ilesha

Characterisation of ESBL determinants and their relationship to aquatic habitats

Eighty-five of 114 (74.6%) isolates with reduced susceptibility to ceftazidime and positive in DDST carried at least one of the ESBL genes bla_{SHV}, bla_{TEM}, bla_{CTX-M} included in this study. None of the isolates carried a bla_{FOX} gene (Table 1). *bla* genes were detected in 40 of 46 (86.9%) isolates from hospital wastewater, 36 of 55 (65.5%) isolates from fish farm ponds and 9 of 13 (69.2%) river/domestic wastewater isolates. Overall, bla_{SHV} was detected in 47 (55.3%) isolates, bla_{TEM} in 39 (45.9%) and bla_{CTX-M} in 18 (21.2%). The majority of the bla_{CTX-M}-positive isolates were detected in the wastewater from hospitals, specifically in *Shigella flexnerii* from the north central region hospitals and in *E. coli*, *E. hermannii* and *Shigella sonnei* from the hospitals of the southwest region (Table 1).

None of the isolates from fish farms carried bla_{CTX-M} but the gene was present in two isolates (*Escherichia fergusonii* and *Klebsiella pneumoniae* subsp. *pneumoniae*) originating

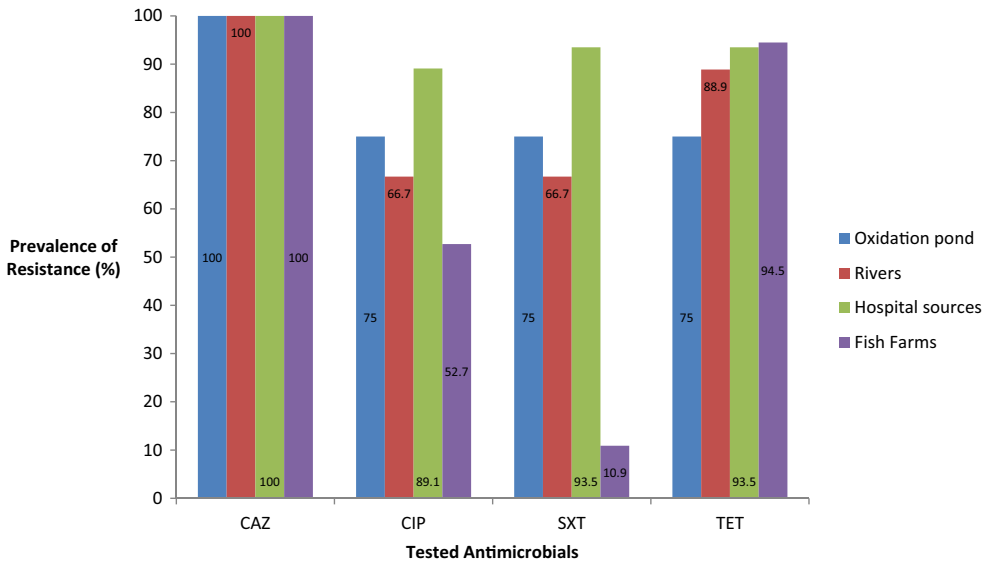

Fig. 1 Phenotypic pattern of antibiotic resistance among ESBL-positive isolates from the different sampling environments. CAZ ceftazidime, CIP ciprofloxacin, SXT sulphamethoxazole/trimethoprim, TET tetracycline

from the Zik River. Interestingly, two ESBL-producing bacteria carrying bla_{CTX-M} (*Shigella sonnei* and the *E. coli*) were also isolated from borehole and hand-dug wells near the hospital wastewater collection points in the southwest region, highlighting the potential public health risk associated with the use of these waters for domestic purposes. bla_{SHV} occurred most frequently among isolates from rivers/domestic wastewaters (61.5%) and fish farm ponds (73.8%) but less frequently in the wastewater from hospitals (20%). Sequencing of representative amplicons confirmed the identity of the detected ß-lactam genes. All bla_{CTX-M} and the selected bla_{TEM} amplicons sequenced shared sequence identities (99–100%) with $bla_{CTX-M-15}$ (accession no: KJ451410.1) and bla_{TEM-1b} (accession no: KF 976462.2) respectively in the GenBank. In contrast, sequencing of seven representative bla_{SHV} amplicons showed they shared 99–100% sequence identities with bla_{SHV-1}, bla_{SHV-11}, bla_{SHV-12} and $bla_{SHV-121}$ (GenBank accession no. KC699840.1, GU083599.1, DQ219473.1, KF585135.1, KJ083256.1, NG_050005.1).

Occurrence of multiple ß-lactam determinants in ESBL-producing bacteria

The occurrence of multiple ß-lactam resistance genes was detected in 18.8% of the ESBL-positive isolates (Table 1). bla_{TEM} and bla_{SHV} co-occurred in six isolates from hospital wastewater (*Proteus mirabilis* $n = 5$) and *Citrobacter freundii* isolated from the Zik River. Co-occurrence of bla_{TEM} and $bla_{CTX-M-15}$ was mainly found in hospital wastewater (*E. coli* $n = 5$) and water from neighbouring hand-dug wells (*E. coli* $n = 1$) in the southwest region of Nigeria. $bla_{CTX-M-15}$ and bla_{SHV} occurred together in one *E. coli* isolated from borehole water also in the southwest of Nigeria. Co-occurrence of the three *bla* genes was found in three isolates from hospital wastewater (*E. coli* and *E. hermannii*) and river water (*K. pneumoniae* subsp. *pneumoniae*) from the southwest of Nigeria. No isolate from fish farms carried multiple ß-lactam resistance determinants (Table 1).

Genetic environment of $bla_{CTX-M-15}$

The $bla_{CTX-M-15}$ was flanked upstream and downstream by IS*Ecp1* and *orf*477 in 12 isolates similar to the international $bla_{CTX-M-15}$ genetic environment commonly reported for clinical bacterial strains. In the remaining six isolates, the $bla_{CTX-M-15}$ was flanked upstream by a combination of IS*Ecp1* and IS*26* and downstream by *orf477*. The six isolates where $bla_{CTX-M-15}$ is associated with IS*Ecp1* and IS*26* are from wastewater (*E. coli*: $n = 4$), well water (*E. coli*: $n = 1$) and borehole water (*S. sonnei*: $n = 1$) from Ogun State, southwest Nigeria (Table 2). IS*Ecp1* has been previously reported upstream of $bla_{CTX-M-15}$ in bacteria from clinical (Soge et al. 2006; Aibinu et al. 2012; Iroha et al. 2012; Inwezerua et al. 2014; Fortini et al. 2015 and Raji et al. 2015) and food animal sources (Ojo et al. 2016) in Nigeria. However, to the best of our knowledge, this is the first time the genetic environment of $bla_{CTX-M-15}$ is being reported in bacteria from environmental sources in Nigeria.

PCR-mapping of class 1 integron

Overall, integrons were detected in 54 (63.5%) of the isolates. *Int1* was the most prevalent class in hospital and domestic/river water and was associated with the presence of at least one ESBL gene in 46 isolates (Table 1). Integrons were rarely detected in fish farm isolates, being found in 8 of 55 isolates including 5 isolates where none of the tested *bla* genes was detected. *Int2* was detected only in two isolates from hospital wastewater which were also positive for *Int1*. *Int3* was not detected in any isolate in this study. Different sized amplicons were obtained with primers targeting the variable region of class 1 integrons in 23 (42.6%) of class 1 integron-positive isolates, indicating that the remaining isolates are either carrying empty integrons without gene cassettes or are harbouring *Tn*5090-like class 1 integron lacking the 3′conserved end. The amplicons were randomly assigned to four classes (classes 1–4) based on size of the products and the products sequenced (GATC Biotech, Konstanz, Germany).

Group 1 gene cassettes (ca. 1.5 kb) occurred in 12 isolates with 11 (3 *P. mirabilis* from Makurdi, 6 *E. coli* and 1 *S. sonnei* from Ogun state, and 1 *E. fergusonni* from the Zik River) sharing 99% sequence identity with *dfrA*17 and *aadA*5 on class 1 integron of reference strains in the GenBank (accession no: KX573886.1 and JQ823009.1). The last member of this group found in *K. pneumoniae* subsp. *rhinoscleromatis* OZ49 isolated from oxidation pond in Ibadan shared 99% sequence identity with *drfA*16 and *aadA*2 present on class 1 integron In36 of *E. coli* plasmid pHSH1 (AY259085.1). All group 2 gene cassettes (ca. 850 bp) shared 99% sequence identity with *dfrA*7 on class 1 integron of *E. coli* G8 (KR952342.1) and *Salmonella enterica* subsp. enterica serovar *Enteritidis* D7795 (LN879484.1). Sequences of group 3 cassettes (> 850 bp) obtained from four isolates shared 98–99% identity with *aadA*1 on class I integrons of *S. enterica* subsp. enterica serovar Typhimurium (LN794248.1) and *E. coli* EC4951 (JN814922.1). Only two isolates, *P. rettgeri* CR4 and CR230, from Cross River carried group 4 (> 1.5 kb) cassette which shared 99% sequence identity with class 1 integron of *Aeromonas hydrophila* M-X4A containing *drfA*32, *ereA* and *aadA*2 (KJ543558.1) (Table 1).

Conjugative relaxase typing

Plasmid bands were detected in 56 (63.5%) of the ESBL-producing bacteria, more frequently in hospitals and rivers/domestic wastewaters than in fish farm isolates. Twenty-

Table 2 Genotypic characterisation of $bla_{CTX\text{-}M\text{-}15}$ genetic environments

Isolate	Sampling site	$bla_{CTX\text{-}M\text{-}15}$ genetic environments
Klebsiella pneumoniae subsp.		
Pneumoniae OZ63	Zik River, Ibadan	IS*Ecp1*|*orf477*
Escherichia fergusonii OZ24	"	IS*Ecp1*|*orf477*
Shigella flexneri MK228	Wastewater, Benue State	IS*Ecp1*|*orf477*
E. coli EOd8	Wastewater, Ijebu-Ode, Ogun State	IS*Ecp1*|*orf477*
E. coli EOd11	"	IS*Ecp1*|*orf477*
E. coli EOd12	"	IS*Ecp1*|*orf477*
E. coli EOd19	"	IS*Ecp1*|*IS*26|*orf477*
E. coli EOd20	"	IS*Ecp1*|*IS*26|*orf477*
E. coli EOd21	"	IS*Ecp1*|orf477
E. coli EId3	Wastewater, Ijebu-Igbo, Ogun State	IS*Ecp1*|orf477
E. coli EId10	"	IS*Ecp1*|*orf477*
E. coli EIw10	Well water, Ijebu-Igbo, Ogun State	IS*Ecp1*|*orf477*
E. coli EAd9	Wastewater, Ago-Iwoye, Ogun State	IS*Ecp1*|*IS*26|*orf477*
E. coli EAd10	"	IS*Ecp1*|*IS*26|*orf477*
E. coli EAd15	"	IS*Ecp1*|*orf477*
E. hermannii EAd19	"	IS*Ecp1*|*orf477*
S. sonnei EAw8	Well water, Ago-Iwoye, Ogun State	IS*Ecp1*|*IS*26|*orf477*
E. coli EWb3	Borehole water, Ibiade, Ogun State	IS*Ecp1*|*IS*26|*orf477*

three (23) of the detected plasmids could not be typed with degenerate primer MOB typing (DPMT) described by Alvarado et al. (2012) while PCR using extracted plasmids DNA as template yielded amplicons for 33 isolates. Relaxases of the MOB F12 family were most prevalent and were found in 81.8% of the 33 typable plasmids, while MOB F11 and P11 were found in 24.2 and 21.2% of the isolates respectively (Table 3). All isolates of *Stenotrophomonas* positive for relaxase genes were from a single fish farm (IBD1) (Table 3). Multiple relaxases were detected in 7 (20.0%) of the isolates, all of which showed multiple plasmid detection bands in gel electrophoresis. Representative amplicons of the DPMT sequenced shared 100% identity with conjugative transfer relaxases TraI (Accession no KF732966.1).

Discussion

Direct discharge of untreated wastewater from domestic, industrial, hospital and agricultural sources into surface waters is a common practice in Nigeria. However, to the best of our knowledge, few studies have evaluated the risk of environmental contamination with ESBL-producing bacteria resulting from such untreated wastewater discharge in Nigeria. In this study, we demonstrated that discharge of untreated wastewater from hospitals and domestic and aquaculture sources is a risk factor for the release of ESBL-producing bacteria into the Nigerian aquatic ecosystem. Only recently, Obasi et al. (2017) reported the detection of ESBL-producing *K. pneumoniae* in wastewater collected from six pharmaceutical manufacturing industries in southwestern Nigeria. The authors however did not investigate surrounding aquatic ecosystems of the wastewater collection points and only detected ESBL genes in species of *Klebsiella*. Interestingly, even though sample types are different, we also detected the ESBL genes $bla_{CTX\text{-}M\text{-}15}$ (detected in *K. pneumoniae* subsp. *pneumoniae* OZ63; *E. coli* EOd8, EOd11, EOd12, EOd19, EOd20, EOd21, EId3, EId10, EIw10,EAd9, EAd10, EWb3; *E. fergusonii* OZ24; *Ent. hermannii* EAd19; *S. flexneri* MK228 and *S. sonnei* EAw8) and $bla_{SHV\text{-}12}$ (detected in *E. coli* EAd15), and the broad spectrum beta-lactamases $bla_{SHV\text{-}1}$ (*Ent. asburiae* IK42, *P. mirabilis* MK221, *S. maltophilia* OZ85), $bla_{SHV\text{-}11}$ (*K. pneumoniae* subsp. *rhinoscleromatis* OZ49, *S. maltophilia* IBD1–108) and $bla_{TEM\text{-}1}$ among our isolates similar to the results of Obasi et al. (2017). A previous study has also detected $bla_{SHV\text{-}12}$ in an *Enterobaacter aerogenes* isolated from the blood of a 2-year-old patient admitted to a tertiary hospital in southwestern Nigeria (Kasap et al. 2010), the same as the region of the present study and the study of Obasi et al. (2017). However, in contrast to a single species (*K. pneumoniae*) reported by Obasi et al. (2017), we detected the aforementioned genes in 18 different species of bacteria isolated from untreated wastewater and contaminated surface and groundwater in the proximity of our wastewater collection points indicating possible cross-contamination of the water sources. It has previously been hypothesised that human infections and faecal carriage of ESBL-producing *Enterobacteriaceae* are a major source of

Table 3 Conjugative relaxases detected in this study

Relaxase	Isolates[a]	Isolate source
MOB F11	*K. varicola* IK16	Ikpoba River
	S. maltophilia IBD1–168[a]	Pond 2 farm 1, Ibadan
	S. maltophilia IBD1–75	Pond 2 farm 1, Ibadan
	E. coli EOd17[a]	Wastewater, Ijebu-Ode, Ogun State
	E. coli EIw2[a]	Well water, Ijebu-Igbo, Ogun State
	E. coli EAd16[a]	Wastewater, Ago-Iwoye, Ogun State
	E. hermannii EAd19	Wastewater, Ago-Iwoye, Ogun State
	E. coli EWb2[a]	Borehole water, Ibiade, Ogun State
MOBF12	*P. rettgeri* CR230	Cross River
	R. ornithinolytica EId13[a]	Wastewater, Ijebu-Igbo, Ogun State
	Ent. amnigenus EIw4	Well water, Ijebu-Igbo, Ogun State
	S. sonnei EAw8	Well water, Ago-Iwoye, Ogun State
	S. maltophilia IBD1–169[a]	Pond 5 IBD1, Ibadan
	E. coli EOd1	Wastewater, Ijebu-Ode, Ogun State
	E. coli EOd5	Wastewater, Ijebu-Ode, Ogun State
	E. coli EOd8	Wastewater, Ijebu-Ode, Ogun State
	E. coli EOd9	Wastewater, Ijebu-Ode, Ogun State
	E. coli EOd11	Wastewater, Ijebu-Ode, Ogun State
	E. coli EOd12	Wastewater, Ijebu-Ode, Ogun State
	E. coli EOd14	Wastewater, Ijebu-Ode, Ogun State
	E. coli EOd17[a]	Wastewater, Ijebu-Ode, Ogun State
	E. coli EOd19	Wastewater, Ijebu-Ode, Ogun State
	E. coli EOd20	Wastewater, Ijebu-Ode, Ogun State
	E. coli EOd21	Wastewater, Ijebu-Ode, Ogun State
	E. coli EId3	Wastewater, Ijebu-Igbo, Ogun State
	E. coli EIw10	Well water, Ijebu-Igbo, Ogun State
	E. coli EAd2	Wastewater, Ago-Iwoye, Ogun State
	E. coli EAd10	Wastewater, Ago-Iwoye, Ogun State
	E. coli EAd15	Wastewater, Ago-Iwoye, Ogun State
	E. coli EAd16[a]	Wastewater, Ago-Iwoye, Ogun State
	E. coli EWd1	Wastewater, Ibiade, Ogun State
	E. coli EWd5	Wastewater, Ibiade, Ogun State
	E. coli EWd9	Wastewater, Ibiade, Ogun State
	E. coli EWd13	Wastewater, Ibiade, Ogun State
	E. coli EWb2[a]	Borehole water, Ibiade, Ogun State
MOBP11	*S. maltophilia* IBD1–168[a]	Pond 2 IBD1, Ibadan
	S. maltophilia IBD1–169[a]	Pond 5 IBD1, Ibadan
	S. dysenteriae IBD1–175	Pond 5 IBD1, Ibadan
	R. ornithinolytica EId13[a]	Wastewater, Ijebu-Igbo, Ogun State
	E. coli EOd17[a]	Wastewater, Ijebu-Ode, Ogun State
	E. coli EAd16[a]	Wastewater, Ago-Iwoye, Ogun State
	E. coli EIw2[a]	Well water, Ijebu-Igbo, Ogun State

[a] Isolates with multiple relaxases

ESBL-producing gram-negative bacteria in wastewaters (Dolejska et al. 2011).

This is the first report of $bla_{\text{CTX-M-15}}$ genes in hospital wastewaters, rivers, wells and boreholes used for domestic purposes in Nigeria with some bacteria isolates of public health importance such as *E. coli*, *C. freundii* and *K. pneumoniae* subsp. *pneumoniae* carrying additional *bla*-resistance genes. The presence of $bla_{\text{CTX-M-15}}$ in bacteria from these sources is worrisome since this gene is the most common ESBL in ESBL-producing bacteria causing human

infections. Moreover, the genetic environment of the detected $bla_{CTX\text{-}M\text{-}15}$ is similar to that commonly found in clinical isolates. The isolation of these bacteria from the untreated wastewater-contaminated aquatic environment is an indication of the important role played by untreated wastewater in the spread of ESBL-producing bacteria into the Nigerian environment. Previous studies have ascribed a role for anthropogenic pollution (Tacão et al. 2012) and especially wastewater effluent (Amos et al. 2014) in the dissemination of $bla_{CTX\text{-}M\text{-}15}$ in the aquatic environment. Additionally, the screening of wastewater from different sources showed that different beta-lactamase types dominated the different samples. Bacteria isolated from hospital wastewater and environments polluted with hospital wastewater displayed mostly $bla_{CTX\text{-}M\text{-}15}$ and bla_{TEM} genes while isolates from rivers and fish farms were mostly carrying bla_{SHV} genes independent of the geographical location.

More importantly, all bla_{SHV} carriers from the fish farms and one bla_{SHV} carrier each from rivers and wastewater oxidation pond were identified as members of the genus *Stenotrophomonas.* This gene, which originated in and is mostly found among *Enterobacteriaceae* causing clinical infections, is now being reported in both *Enterobacteriaceae* and non-*Enterobacteriaceae* from different epidemiological settings covering human, animals, food and the environment (Pouget et al. 2013; Maravic et al. 2015; Rocha-Gracia et al. 2015; Zurfluh et al. 2013, 2015; Alcala et al. 2016), thus suggesting a changing epidemiology of this gene. The unusual widespread detection of bla_{SHV}-producing *Stenotrophomonas* in this study is similar to the detection of bla_{SHV} in *S. maltophilia* from a recreational lake in Serbia (Novovic et al. 2015). However, while plasmids were detected in 71.4% bla_{SHV}-producing Enterobacteria isolates ($n = 14$) in this study consistent with the association of the gene with plasmids in *Enterobacteriaceae*, plasmids were detected in only 18% of bla_{SHV}-producing *Stenotrophomonas* isolates ($n = 33$), suggesting that most of the allelic variants detected among the bla_{SHV}-producing *Stenotrophomonas* may be chromosome associated. Remarkably, the detection and occurrence of bla_{SHV} in *Stenotrophomonas* in the present study coincided with the first identification of bla_{SHV} gene in *S. maltophilia* in Nigerian clinical settings (Ogbolu et al. 2013) raising concerns about the role of this bacterium as an opportunistic pathogen (Brooke 2012) and a reservoir of novel antibiotic resistance genes (Tada et al. 2014; Maravic et al. 2014). The presence of multi-resistant *S. maltophilia* in the fish farms is also worrisome because of the likelihood of its introduction into the food chain and subsequently humans (Ozaktas et al. 2012; Abgottspon et al. 2014).

To our knowledge, this study was the first attempt to use DPMT to investigate plasmid diversity in a polluted aquatic ecosystem. However, conjugative relaxases were detected mostly in isolated members of the *Enterobacteriaceae* family. Design of specific primers for relaxase characterisation among environmental microbial community is therefore needed before adopting this method in environmental and public health surveillance (Alvarado et al. 2012).

In conclusion, we demonstrated the important role of untreated wastewater as a source of environmental contamination with ESBL-producing bacteria in Nigeria. The widespread occurrence of ESBL producers across multiple bacterial hosts in Nigerian waters though worrisome was however not unexpected, but thus far had not been well documented.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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