

## Phylogenetic characterization, antimicrobial susceptibilities, and mechanisms of resistance in bacteria isolates from a poultry waste-polluted river, southwestern Nigeria

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Received: 05.11.2010

**Abstract:** Bacteria resistance to antibiotics has become a worldwide problem impacted partly by antibiotics use in livestock production. The present study investigated the pattern and mechanisms of resistance in 21 bacteria species isolated from a river used as a waste-dump by a poultry farm in Ogbomoso, southwestern Nigeria. Bacteria were isolated on nutrient agar supplemented with 50 µg/mL of tetracycline, their identities were determined by PCR amplification, and sequencing of their 16S rRNA genes and their susceptibility to 6 antimicrobials determined by broth dilution. The molecular basis of resistance among the isolates was also investigated by the PCR amplification of class 1 and 2 integrons, *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetL*, *tetW*, *tetM*, *tet37*, *bla<sub>TEM</sub>*, *aadA1*, *strA*, *strB*, *sul2*, *sul3* and a 550bp gene fragment encoding a multidrug resistance efflux transporter of the resistance nodulation division (RND) family. All the isolates were resistant to the tested antimicrobials with MIC ranging from <16 µg/mL to 512 µg/mL. class 2 integrons *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetL*, *tetW*, *tetM*, *tet37*, *aadA1*, and *sul3* could not be detected in any of the isolates. However, 2 isolates were positive for class 1 integrons, 1 each for *bla<sub>TEM</sub>* and *tetA*, 3 for *sul2*, and 2 for *strA* and *strB*. Seven of the isolates were positive for the RND efflux pump. Results of the present study showed that polluted aquatic ecosystems may serve as a reservoir of transferable antibiotic resistance.

**Key words:** 16S rRNA, resistance genes, RND transporter, integrons

### Introduction

The development of resistance to antimicrobial drugs has become a serious problem worldwide threatening man's ability to treat infections in animals and humans. This is partly as a result of antimicrobials usage in livestock production (1,2). Pharmaceutical compounds including antibiotics, hormones, and steroids are widely used to prevent and/or treat diseases and to promote animal growth in livestock production facilities (3,4). In Nigeria, indiscriminate

use of antibiotics in animal husbandry is widespread, particularly as feed additives (5,6). A significant amount of these antibiotics (which is up to 75%) is excreted as active metabolites. Thus, animal waste presents a major potential source of antibiotic input to the environment (3). A growing concern about the presence of antibiotics in the environment is that they may contribute to the emergence of resistant pathogenic bacteria strains (7). This is because exposure to antimicrobials may result in a selective

advantage and, consequently, increased abundance of resistant bacteria in animals, their excreta, and surroundings (8,9).

Livestock, such as swine, poultry, and beef and dairy cattle, are major sources of fecal pollution that can introduce human pathogens, as well as chemical pollutants, into surface and ground waters. Fecal contamination of water occurs when manure is directly deposited in streams, is transported via land runoff, and/or migrates into ground water (10). Hence, unless best management practices are applied, the disposal of animal wastes on land is a potential non-point source of water degradation (11). Little is known about the effects of antibiotics in animal waste on resistance levels of environmental bacteria (12). Moreover, the fate of antibiotic resistant bacteria, in particular their resistance genes and mobile genetic elements introduced via manure into soil and aquatic ecosystems, is not well studied (13,12).

A large variety of genes conferring resistance to antimicrobials have been reported among bacteria from animal waste impacted ecosystems, but there are a lack of reliable data on the occurrence and distribution of antimicrobial resistance in the Nigerian environment. More importantly, few studies have investigated the molecular basis of antimicrobial resistance among resistant environmental bacterial isolates from Nigeria as a step in surveillance of resistance in the Nigerian environment. The primary purpose of the present study was to investigate the diversity of, and antibiotic resistance among, bacteria isolates from a poultry waste-polluted river in southwestern Nigeria by the PCR amplification and sequencing of their 16S rRNA and selected resistance genes.

## Materials and methods

### Sampling site

Antibiotic resistant bacteria were isolated from water samples collected from a river flowing through the waste dump of a poultry farm in Ogbomoso, a semi-urban agricultural town of southwestern Nigeria. The organisms were identified by PCR amplification and sequencing of their 16S rRNA and their susceptibility to selected antibiotics determined by the broth dilution method of the European Committee for

Antimicrobial Susceptibility testing (14). Details of isolation, 16S rRNA amplification and sequencing using the primers 27F (5'-agagtttgatcctggctca-3') and 1541R (5'-aaggagtgatccagccgca-3') (AUGCT Biotechnology Synthesis Laboratory, Beijing, PRC), and antimicrobial susceptibility testing are as reported previously (15).

### Plasmid isolation

Plasmids DNA was isolated from all the organisms by culturing them overnight in LB broth. Cells were pelleted by centrifugation and plasmid DNA extracted by the rapid method described by Kado and Liu (16). Plasmids were detected by agarose gel (0.8% w/v) electrophoresis.

### PCR amplification of resistance genes

Ten organisms representing all the gram-negative species among the isolates from the sampled river were selected for the PCR amplification of class 1 and 2 integrons, *bla*-<sub>TEM</sub>, *strA*, *strB*, *sul2*, *sul3*, *tetA*, *tetB*, *tetC*, *tetD*, *tetE*, and *tetG* with primers and conditions described before (17). The organisms are *Brevundimonas diminuta* XW2a and XW11a, *Alcaligenes faecalis* XW13, *Stenotrophomonas maltophilia* XW61, *Roultella ornithinolytica* XW721, *Citrobacter freundii* XW722, *Ochrobactrum intermedium* XW92, *R. planticola* XW10, *Enterobacter hormachei* XW122, and *Providencia rettgeri* XW16. To identify the gene cassettes encoded on class 1 integrons, the variable region was amplified with primers pairs Att-1-F/3'CS-B and Sul-1B/qacEΔ1-F (18). Primers A24f2 and A577r2 (19) (AUGCT Biotechnology Synthesis Laboratory, Beijing, PRC) were also used to amplify a fragment of Hydrophobe/Amphiphile Efflux 1 (HAE1) family multidrug resistance transporter of the RND superfamily from the genomic DNA extracted from the test organisms. The HAE1 family includes all known drug and solvent resistance RND transporters (19). The PCR products were purified from agarose gels using the TIANgel Midi Purification Kit (TIANgen Biotech, Beijing, PRC) and sequenced (Sinogenomax, Beijing, PRC).

### Sequence and phylogenetic analysis

Deduced amino acid sequences of amplified HAE1 transporters were compared with amino acid sequences of 22 other HAE1 family proteins

downloaded from GenBank. The protein sequences and their accession numbers are as follows: HAE1 family proteins of *Delftia acidovorans* SPH – 1 (ZP\_01582207), *Acidovorax* sp. JS42 (YP\_987054), *Comamonas testosteroni* KF – 1 (ZP\_01521360), *Acidovorax avenae* subsp. *citrulli* AACOO – 1 (YP\_970466), *Pseudomonas syringae* pv. *syringae* B728a (YP\_235939), *Caulobacter* sp. K31 (ZP\_01420882), *Burkholderia phytofirmans* PsJN (ZP\_01511220), *Burkholderia xenovorans* LB400 (YP\_560277), *Yersinia pseudotuberculosis* IP 31758 (YP\_001402539), *Psychrobacter* sp. PRWF – 1 (YP\_001279788), *St. maltophilia* R551 – 3 (ZP\_01645609), *Psychrobacter cryohalolentis* K5 (YP\_581019), *E. coli* B (ZP\_01699829), *Enterobacter* sp. 638 (YP\_001178413), *Serratia proteamaculans* 568 (YP\_001477358), AcrB/AcrD/AcrF family protein, *Caulobacter crescentus* CB15 (NP\_419624), EefB, *Enterobacter aerogenes* (CAD48862), EefB *Klebsiella pneumoniae* (ABV24995), RND protein, *St. maltophilia* (CAG34257), MdtF, Rpos – dependent *E. coli* APEC01 (YP\_859120), YhiV, *E. coli* UTI89 (YP\_543006), and SdeY of *Serratia marcescens* (BAC77251).

The sequences were aligned by multiple sequence alignment technique using CLUSTAL W (20) and a phylogenetic tree constructed by the neighbor joining method (21) using MEGA version 3.1 (22). AdeC, like the outer membrane protein of *Acinetobacter* sp ADP1 (YP\_047482), was used as outgroup.

## Results and discussion

### Characterization of isolates

A total of 21 organisms were isolated from the water sample collected from the study site. Analysis of the 16s rRNA gene fragments amplified from the genomic DNA extracted from the 21 bacterial isolates by PCR resulted in characteristic bands (1500 bp) in 0.8% agarose gels. BLAST results showed that the isolated organisms can be classified into 5 distinct phylogenetic groups with high sequence identities ( $\geq 98\%$ ) with type strains (Table 1). The 4 gram-positive isolates belong to 2 classes: the class *Bacillus* (n = 4) and the class *Actinobacteria* (n = 3), while the gram-negative isolates belong to 3 distinct classes of the phylum *Proteobacteria*: the  $\alpha$ -proteobacteria (n = 3), the  $\beta$ -proteobacteria (n = 4), and the  $\gamma$ -proteobacteria (n = 6).

Table 1. Phylogenetic identities of the resistant bacteria isolated from the study site.

Bacterial division	Best phylogenetic match (Accession Number)	% Identity isolates	Number of
Firmicutes	<i>B. thuringiensis</i> Strain Al Hakam (CP000485.1)	99%	2
	<i>B. cereus</i> Strain CCM2010 (DQ207729.2)	100%	2
	<i>Lysinibacillus fusiformis</i> Strain SW-B9 (AY907676.1)	99%	1
$\alpha$ - Proteobacteria	<i>Brevundimonas diminuta</i> (X87274.1/BD16S1635)	99%/100%	2
	<i>Ochrobactrum intermedium</i> Strain RMA 16449 (AM409326.1)	100%	
$\beta$ - Proteobacteria	<i>Alcaligenes</i> sp. Strain IS – 92 (AY346141.1)	99%	4
$\gamma$ - Proteobacteria	<i>Stenotrophomonas maltophilia</i> Strain LMG958-T (X95923.1)	99%	1
	<i>Citrobacter freundii</i> strain SSCT56 (AB210978.1)	99%	1
	<i>Enterobacter</i> sp. Strain B901-2 (AB210978.1)	99%	1
	<i>Klebsiella</i> sp. (AY363386.2)	99%	1
	<i>Raoultella planticola</i>	99%	1
	Strain ATCC33558T (AF129444.1)		
Actinobacteria	<i>Providencia</i> sp. Strain OP1 (AM040495.1)	99%	1
	<i>Cellulosimicrobium cellulans</i> Strain AS4.1333 (AY665978.1)	99%	2
	(AY114178.1)	99%	1

# Accession numbers in parentheses

Susceptibility to antimicrobial agents

There is a high level of resistance to the tested antimicrobial agents among the isolated organisms (Table 2), which generally followed the pattern of drug usage on the farm where the present study was carried out. The least resistance was observed to ciprofloxacin, which was not mentioned among the antibiotics used on the farm since it started operation with MIC ranging from <16 to 128 µg/mL. The highest level of resistance was observed to tetracycline, streptomycin, and erythromycin with MICs ranging between 128 and 512 µg/mL. These 3 antibiotics have been in constant use on the farm since its inception. However, even though trimethoprim has never been used on the farm, there is a high level resistance to this drug among bacteria isolated from the farm.

Plasmid profile

Of the organisms, 42.9% carried various sizes of plasmids; however, only one isolate, *Enterobacter* sp. XW122, carried more than one plasmid in its genome (Figure 1).

Detection and analysis of resistance genes

Analysis of the PCR products of the target genes in 0.8% agarose gel and sequencing showed that class 2 integrons, *sul3*, *aadA1*, *tetB*, *tetC*, *tetD*, *tetE* and *tetG* could not be detected in any of the tested organisms. However, 2 organisms, *Alc. faecalis* XW13 and *Cit. freundii* XW722, were positive for the presence of class 1 integrons (Table 2). Two distinct bands were detected in the variable region of the amplified class 1 integron from the 2 organisms. The 1.5 kb amplicon from XW722 has 98% sequence identity with *aadA2* region of *Cit. freundii* strain CIT1 class 1 integron and insertion sequence ISCR1 with accession number FJ646630. The 1 kb amplicon from XW13 showed 99% sequence identity with *aadA2* gene of *E. coli* class 1 integron with accession number FJ855127. The representative amplicon of the product of *sul1B/qacEΔ1-F* from the 2 isolates showed 99% sequence identity with the *sul1* region of *Pseudomonas aeruginosa* strain PA-SL2 transposon Tn1721 with accession number GQ396666. *bla*<sub>-TEM</sub>, the only β-lactamase encoding gene tested in the present

Table 2. Pattern of resistance and resistance genes profile of the tested isolates.

Organisms	MIC (µg mL <sup>-1</sup> )						Resistance genes
	Cip	Tet	Ery	Tri	Str	Car	
<i>B. thuringiensis</i> XW1	32	<128	>512	256	>512	>256	ND
<i>Brev. diminuta</i> XW2a	16	<256	>512	256	>512	>128	<i>HAE1</i>
<i>B. cereus</i> XW2b	<32	128	>512	<256	>512	>256	ND
<i>C. cellulans</i> XW31	>64	32	128	256	>512	64	ND
<i>Alc. faecalis</i> XW5	32	<256	128	256	>512	256	ND
<i>St. maltophilia</i> XW61	<32	<256	>512	>256	>512	512	<i>HAE1</i>
<i>R. ornithinolytica</i> XW721	128	256	>512	256	>512	>256	<i>HAE1</i>
<i>C. freundii</i> XW722	128	<256	>512	<256	>512	64	<i>Class1 integron</i> , * <i>sul1</i> , <i>sul2</i> , * <i>aadA2</i> , <i>HAE1</i>
<i>O. intermedium</i> XW92	16	<256	>512	256	>512	>256	No genes detected
<i>R. planticola</i> XW10	<32	256	>64	256	>512	256	No genes detected
<i>Brev. diminuta</i> XW11a	<64	256	<512	256	>512	256	<i>sul2</i> , <i>strA</i> , <i>strB</i>
<i>Ent. hormachei</i> XW122	<32	<256	>512	256	>512	>256	<i>bla</i> <sub>-TEM</sub> , <i>sul2</i> , <i>strB</i> , <i>HAE1</i>
<i>Alc. faecalis</i> XW13	32	<256	128	256	>512	256	<i>Class1 integron</i> , * <i>sul1</i> , * <i>aadA2</i> , <i>tetA</i> , <i>HAE1</i>
<i>P. rettgeri</i> XW16	<32	<512	>512	256	>512	>256	<i>HAE1</i>
<i>L. fusiformis</i> XW18	16	>64	128	512	>512	64	ND

Cip: Ciprofloxacin, Tet: Tetracycline, Ery: Erythromycin, Str: Streptomycin, Tri: Trimethoprim, Car: Carbenicillin, ND Not determined, \*detected as part of class 1 integron.



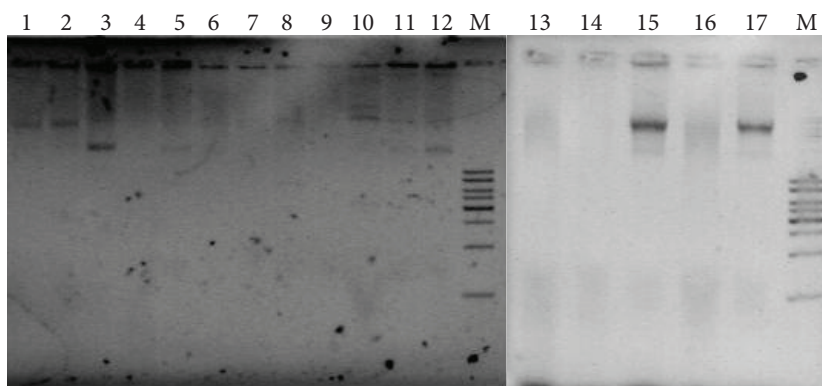


Figure 1. Plasmid profiles of the organisms in 0.8% agarose gels; Lanes 1 *B. thuringiensis* XW1, 2 *B. cereus* XW2b, 3 *Alcaligenes* sp. XW5, 5 *R. ornithinolytica* sp. XW721, 8 *Ochrobactrum intermedium* XW92, 10 *Enterobacter* sp. XW122, 12 *Alcaligenes* sp. XW13, 15 *Providencia* sp. XW16, and 17 *Lysinibacillus fusiformis* XW18 M 1 kb DNA marker.

study, was detected in only one isolate, *E. hormachei* XW122. The product showed 99% sequence identity with *E. cloacae* plasmid pWB280  $\beta$ -lactamase TEM1 gene with accession number GQ336884. Similarly, the only tetracycline resistance gene detected in this study, *tetA*, was detected in *Alc. faecalis* XW13. The product showed 100% identity with the *tetA* region of *Acinetobacter baumannii* Tn1721-like transposon transposase (tnPA) gene with accession number AY196695.

The sulphonamide resistance gene *sul2* was detected in 3 organisms: *B. diminuta* XW11a, *E. hormachei* XW122, and *Cit. freundii* XW722. The sequenced representative amplicon from *B. diminuta* XW11 showed 99% identity with *Riemerella anatipestifer* dihydropteroate synthase protein *sul2* gene with accession number FJ711653. Similarly, the streptomycin phosphotransferase genes *strA* and *strB* were detected in 2 organisms: *E. hormachei* XW122 and *B. diminuta* XW11a. Of these 2 organisms, *B. diminuta* XW11a carried both *strA* and *strB*. The sequenced representative amplicons showed 100% and 98% sequence identity respectively with the *strA* portion of *Alcaligenes faecalis* transposon Tn 5393d with accession number AJ627643 and *strB* portion of *Salmonella enterica* subsp. *Enterica* serovar Typhimurium plasmid pSRC15 with accession number GQ379901.

Resistance in 6 organisms appeared to be conferred by genetic determinants other than those tested in the present study. No resistance gene was detected in *S. maltophilia* XW61, *O. intermedium* XW92, *B. diminuta* XW2a, *R. ornithinolytica* XW721, *R. planticola* XW10, or *P. retgerri* XW16. Thus, all the isolates were tested for the presence of multidrug resistance efflux pumps of the RND family by PCR.

DNA fragments with the expected sizes (500bp) were successfully amplified from 7 of the test organisms, viz. *B. diminuta* XW2a, *St. maltophilia* XW61, *R. ornithinolytica* XW721, *Cit. freundii* XW722, *E. hormachei* XW122, *Alc. faecalis* XW13, and *P. retgerri* XW16. To confirm if the amplified fragments actually contained HAE1 gene fragments, each purified amplicon was sequenced. BLAST analysis of the obtained sequences showed that most of the amplified fragments were affiliated with HAE1 family transporters with sequence identities of 74% to 86% with HAE1 sequences stored in GenBank (Table 3). Phylogenetic and evolutionary analysis of deduced protein sequences of the HAE1 genes showed that they formed 4 distinct clusters (Figure 2). The deduced protein sequence of the HAE1 fragment obtained from *B. diminuta* XW2a formed a separate cluster with the YhiV of *E. coli* UTI 89 (YP\_543006) and MdtF, Rpos-dependent of *E. coli* APEC01 (YP\_859120), while the sequence from *E.*

Table 3. HAE1 sequences obtained from the organisms.

Organisms	Highest Match in the Database #	% Identity
<i>B. diminuta</i> XW 2a	Putative RpoS – Dependent multidrug transporter <i>MdtF</i> of <i>E. coli</i> APEC01 (CP000468.1)	79
<i>St. maltophilia</i> XW 61	Conserved Hypothetical Protein of <i>X. campestris</i> pv. <i>campestris</i> strain ATCC33913 (AE012303.1)	74
<i>R. ornithinolytica</i> sp. XW 721	<i>E. aerogenes</i> tripartite efflux pump operon <i>eefABC</i> (AJ508047)	84
<i>Cit. freundii</i> XW 722	Putative RpoS – Dependent multidrug transporter <i>MdtF</i> of <i>E. coli</i> APEC01 (CP000468.1)	79
<i>Enterobacter</i> sp. XW 122	HAE1 Transporter of <i>Delftia acidovorans</i> SPH - 1 (CP000884.1)	86
<i>Alcaligenes</i> sp. XW 13	HAE1 Transporter of <i>Delftia acidovorans</i> SPH - 1 (CP000884.1)	86
<i>Providencia</i> sp. XW 16	HAE1 Transporter of <i>Delftia acidovorans</i> SPH - 1 (CP000884.1)	86

# Accession numbers in parentheses

*hormachei* XW122, *Alc. faecalis* XW13, and *P. retgerri* XW16 formed a separate cluster. The sequences from *R. ornithinolytica* XW721 and *Cit. freundii* XW722 formed a cluster with the EefB of *K. pneumoniae* (ABV24995) and *E. aerogenes* (CAD48862) and HAE1 proteins of *St. maltophilia* R551 – 3 (ZP\_01645609) and *Yersinia pseudotuberculosis* IP31758 (YP\_001402539). The fourth cluster was formed by the sequence from *St. maltophilia* XW61.

Fecal contaminations of natural water bodies have emerged as a major challenge in the developing world (23). Fecal wastes from intensive animal production facilities often contain resistant bacteria strains carrying a variety of resistance genes (8,9). Antibiotic resistance in bacteria associated with food and water has been a global concern as the presence of resistance genes together with the acquisition of virulence genes can lead to clonal expansion and spread of resistant pathogenic bacteria strains (23). Hence, study of resistance in pathogenic and commensal bacteria associated with food animals and their environment is considered very important as this can provide valuable insights into the panoptic dynamics of antimicrobial resistance (24). However, addition of tetracycline to the isolation medium used in the present study may be responsible for the limited number of isolates as the antimicrobial agent might have been selected against tetracycline susceptible species that are resistant to other antimicrobials.

Among the isolates in the present study there is a generally high level of resistance to the 6 antimicrobials tested. The pattern of resistance

correlates with patterns of drug use in the farm of study. The organisms are highly resistant to antimicrobial drugs used in large volumes in the farm of study and are least susceptible to ciprofloxacin, which at present with other fluoroquinolones, are not commonly used in animal husbandry in Nigeria. However, while trimethoprim is also not widely used, sulphonamides are used widely in poultry farms in Nigeria; we, therefore, suspected that resistance to trimethoprim might have co-evolved with resistance to sulphonamides since they both act on the same pathway of foliate synthesis.

Nearly half of the present isolates were found to carry plasmids of various sizes, while 2 of the isolates were also positive for the presence of class 1 integrons. The role of plasmids and integrons in the dissemination of antibiotic resistance genes among bacterial isolates of food animal production farms has been well documented (25,26).

The widespread occurrence of RND transporters among the gram-negative isolates of the present study suggests that energy-dependent efflux systems may play an important role in the observed drug resistance phenotypes of the organisms. Four organisms among the 6 organisms not positive for any of the resistance genes tested in the present study (*St. maltophilia* XW61, *B. diminuta* XW2a, *R. ornithinolytica* XW721 and *Providencia* sp. XW16) are positive for the HAE1 gene. Multidrug resistance efflux systems have been implicated in the acquired and intrinsic multidrug resistance of several bacteria of environmental and public health importance and significantly contribute

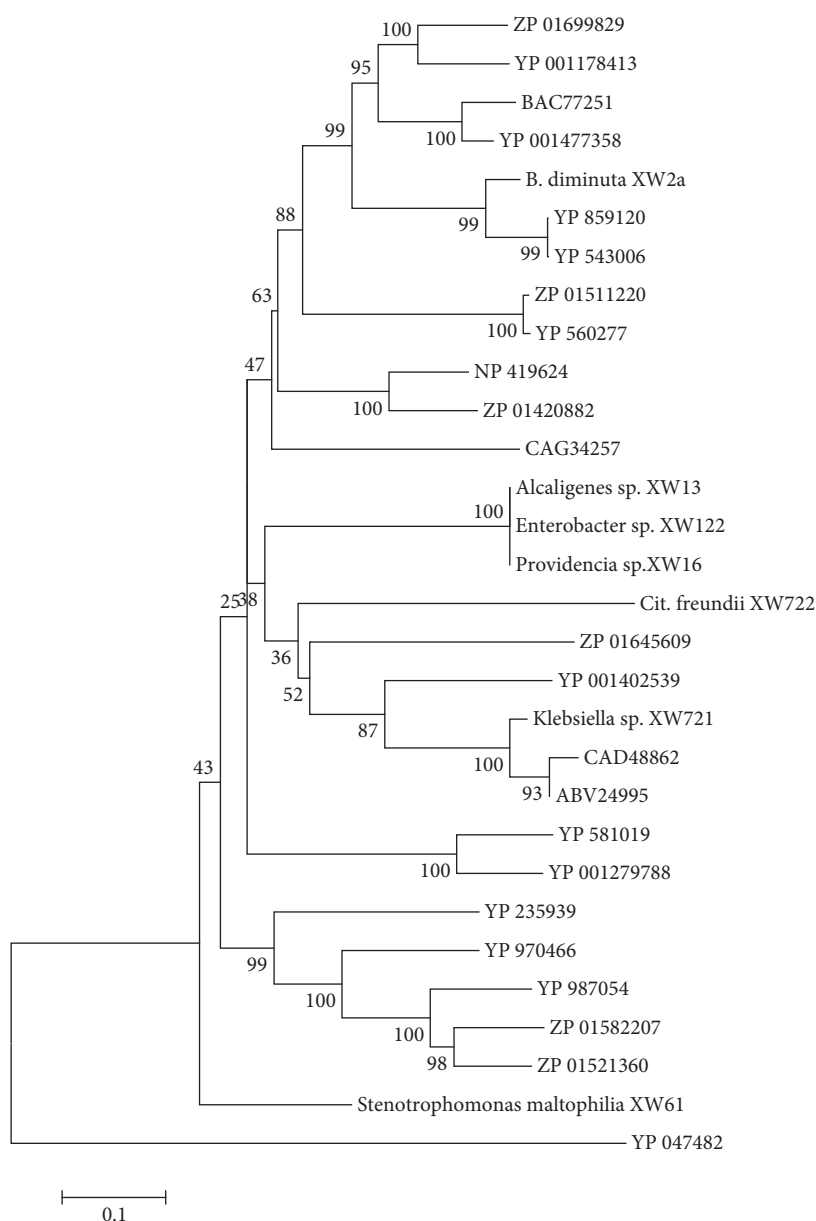


Figure 2. Phylogenetic relationships of the deduced amino acid sequences of the amplified HAE1 transporters. Nucleotide positions at which any of the sequences had a gap or an ambiguous base were not included in the phylogenetic calculations. Bootstrap values (per 100 trials) are shown at the branch nodes. AdeC-like outer membrane protein of *Acinetobacter* sp. ADP1 (YP\_047482) was used as outgroup.

to the emergence and spread of multidrug resistance pathogens (27-29).

However, active efflux may be acting in conjunction with other resistance mechanisms to mediate the high level resistance observed in the

present study since active efflux systems confer low to moderate level resistance to antimicrobial agents (30). Alternatively, the concomitant expression of several efflux pumps in the organisms may lead to apparently high level resistance phenotypes (30,31).

## Conclusion

The high level of antimicrobial drug resistance observed among isolates in the present study suggests that agricultural waste polluted aquatic ecosystem can serve as an environment for the development and dissemination of clinically relevant antimicrobial drug resistance among bacterial species. The presence of plasmids and integrons may also increase the horizontal transfer of resistance genes among the bacterial species. In addition, the presence of HAE1 gene fragments in the organisms suggests that RND transporters may likely play important roles in antibiotic resistance among the organisms. However, since the complete RND efflux pump systems encoded in the genome of the organisms are not fully characterized, their contributions to the observed resistance are currently unclear. It is important to note that although the results of the study provided an insight into the nature of the resistant bacteria community of the polluted river and the occurrence of RND transporters among the gram-negative isolates, a wider study may be required to make a more generalized conclusion on the nature of the resistant bacteria community associated with agricultural waste-polluted aquatic ecosystems in Nigeria and the diversity of resistance determinants encoded in their genome.

## GenBank submission

The 16s rRNA sequences and the sequences of the HAE1 transporter genes described in this study has been submitted to GenBank under accession numbers EU545396 – EU545408 and EU515242 – EU515247.

## Acknowledgements

The authors are grateful to the Academy of Sciences for the Developing World (TWAS) and the Chinese Academy of Sciences (CAS) for a 1-year fellowship to the first author to carry out part of this work at the Environmental Microbiology and Biotechnology Research Center, Chinese Academy of Sciences, Beijing, People's Republic of China.

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