Asian Journal of Biotechnology and Genetic Engineering

1(1): 1-10, 2018; Article no.AJBGE.38009

Metagenomic Assessment of Antibiotics Resistance Genes from Four Ecosystems in the Niger Delta Area of Nigeria

U. O. Edet[1,2*], S. P. Antai[2], A. A. Brooks[2] and A. D. Asitok[2]

[1]Department of Microbiology, Faculty of Natural and Applied Sciences, Obong University, Obong Ntak, Etim Ekpo LGA, Akwa Ibom State, Nigeria.
[2]Department of Microbiology, Faculty of Biological Sciences, University of Calabar, Calabar, Cross River State, Nigeria.

Authors' contributions

This work was carried out in collaboration between all the authors. All the authors were involved in the design of the study. Author EUO performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. All the authors managed the analyses of the study. Author EUO managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/AJBGE/2018/38009
Editor(s):
(1) Tsygankova Victoria Anatolyivna, Professor, Department for Chemistry of Bioactive Nitrogen-Containing Heterocyclic Compounds, Institute of Bioorganic Chemistry and Petrochemistry of National Academy of Sciences of Ukraine, Ukraine.
Reviewers:
(1) Bamidele Tajudeen, Nigerian Institute of Medical Research, Nigeria.
(2) Abhishek Kumar, Nitte University, India.
(3) Gokben Ozbey, Firat University, Vocational School of Health Services, Turkey.
Complete Peer review History: http://prh.sdiarticle3.com/review-history/22170

Original Research Article

Received 6th November 2017
Accepted 24th November 2017
Published 6th December 2017

ABSTRACT

Antibiotics resistance genes (ARGs) in environmental samples have been implicated in the clinical spread of resistance to antibiotics. This study was therefore aimed at the metagenomic assessment of ARGs from various environmental samples. Benthic, epipellic, estuary and soil samples were collected and analyzed for physicochemical parameters using standard techniques and ARGs via metagenomics. Metagenomic DNA was extracted from the various samples and sequenced on Miseq Illumina platform. Following next generation sequencing, gene calling was performed on the assembled sequence reads using FragGeneScan to predict open reading frames (ORFs), which were functionally annotated to various taxonomic groups using the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Ghost KOALA databases. Results of physicochemical analysis showed

**Corresponding author: E-mail: uwemedet27@gmail.com;*

anthropogenic influence in the various environments. Furthermore, a total of 12 types of ARGs were identified, that belongs to the RND and ABC superfamilies of ARGs. These were dominated by five classes of bacteria which were *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria*, and the *Firmicutes (Clostridia)*. Our findings suggest that ARGs diversity differs with samples; with soil being the most abundant and only sample with the multidrug resistant efflux pumps. Further studies aimed at building a repository of ARGs genes in various environments should be carried out.

Keywords: Antibiotics resistance genes; environment; efflux pumps; ABC transporters; metagenomics.

1. INTRODUCTION

The discovery of antibiotics at the onset of the twentieth century was a turning point in human history [1]. Since then, they have played important roles in health and agriculture [2]. Sadly, the gains of this novel discovery are threatened by widespread antibiotics resistance [3-6]. Resistance to antibiotics has been described as an evolutionary adaptation by microbes and can easily be spread by plasmids across species in the same genus [3,7-8]. Microorganisms utilize a number of resistance mechanisms singly or in combination such as hydrolysis, efflux pumps, alteration of targets, nucleotidylation and reprogramming peptidoglycan biosynthesis to mention just a few [9]. The widespread and indiscriminate use of antibiotics by humans in health care and agriculture [10-11] and their arbitrary release into various environments have given antibiotics resistance genes (ADRs) a global public health dimension [2,12-13].

Bacteria are always involved in a constant and dynamic transfer of ARGs in the environment and this has the potential to spread to pathogens and commensal of humans or animals with eventual amplification and spread of these genes [1]. Sediments, waste water, surface water, hospital effluent and ground water are reservoirs of resistance genes such as ampC, mecA, vanA, and gentamicin resistant genes [11]. Studies have shown a significantly positive correlation between the copy numbers of ARGs and total concentration of antibiotics in environments exposed to a high level of antibiotics [14-16]. Furthermore, there is evidence on the environmental origin of some clinically relevant resistance genes [17].

Efflux pumps and their proteins are present in both antibiotics susceptible and resistant bacteria [18]. Based on a number of criteria, bacterial efflux pumps are placed into five families. These families include the resistance-nodulation-division (RND) family, the ATP (adenosine triphosphate)-binding cassette (ABC) superfamily, the major facilitator superfamily (MFS), the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family [13,18-20]. The RND family is common to the Gram negative bacteria while the other families are well distributed amongst the Gram positive and negative bacteria [7,13,19,21-22].

A number of methodologies exists that can be used to study microbial resistance and their genes and these can be divided into cultural and molecular methods. They include isolation and culture [10], polymerase chain reaction (PCR), quantitative PCR, DNA microarray [2][5], and metagenomics [12]. In Nigeria, a number of studies exist that are based on cultural and molecular methods that have used culturable microorganisms to describe resistance and ARGs [8,10,23-24]. The main challenge with these methodologies is that the results are based on the culturable minority and it does not account for the unculturable majorities in these environments. In Nigeria, there is a dearth of information on the metagenomic assessment of antibiotics resistance genes (ADRs) on various environments. Thus, this study was therefore aimed at assessment of ADRs in various ecosystems in the Niger Delta Region of Nigeria using metagenomics.

2. MATERIALS AND METHODS

2.1 Sampling Site Description

The samples used in this study were collected from an oil producing community (Eastern Obolo Local Government Area of Akwa Ibom State) located in the Niger Delta Region of Nigeria. The coordinates of the study site were latitudes 4° 32′ 0″ N & longitude 7°42′ 0″ E. The study location has a total land mass of 117,008 km^2 with an

estimated shoreline of about 184 km. The major occupation of the indigenous population is majorly subsistent farming and fishing. Sampling was carried out in March 2017 which is the start of raining season in the Nigeria.

2.2 Collection and Pre-processing of Samples

Estuary water, soil, benthic and epipellic sediments samples were all collected from the study site from five different locations. Estuary water samples were collected using a sterile water sample bottles. The water samples were then pooled together to obtain a composite water sample. Soil and epipellic samples were collected using a sterile 22cm long hand held Dutch auger at each collection point. The soil samples were collected from a depth of 10-15cm after getting rid of all surface debris. The soil samples were then made into a composite sample after sieving it through a 2 mm mesh sieve. The sieved composite soil sample was then stored in a sterile plastic bag. The same procedures were also repeated for the epipellic sediment sample. For collection of the benthic sediment, a Shepek (Wiidco) mud grab was used. From a depth of 5-10 m below sea level and from the various locations, the benthic sediment samples were collected by lowering the mud grab to collect the benthic sediments and then placed in plastic bags. The samples were then sieved and also made into a composite sample. All samples were collected at low tide, stored and transported at -4°C to the laboratory for further analysis. These were done as previously described [25-27].

2.3 Physicochemical Analysis

The samples were analyzed for physicochemical parameters such as pH, temperature, electrical conductivity, ammonia, phosphorus, sulphide, N-nitrate and N-nitrite. These were done as previously described [28-29].

2.4 Metagenomic DNA Extraction, PCR Amplification and Library Construction

Following sampling, genomic DNAs were extracted from all the four composite samples. These were done using ZYMO soil DNA extraction Kit (Model D 6001, Zymo Research, USA). The extractions were done using 0.25 g soil and sediments samples and 1 ml of the water sample. Extraction of genomic DNAs were done by strictly following the manufacturer's manual instructions. Following successful DNA extraction from the samples, the genomic DNA extract was subjected to gel electrophoresis to check for purity. Labelled Eppendorf tubes with sample codes and primer name on the top and sides were used for amplification. The amplification were done using the universal primer pair 341 Forward (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG-3′) and 785 Reverse (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3′) together with Illumina specific adapter added to the 5′ end of the primer [26]. Polymerase chain reactions were carried out using 25 µl reaction volume. The reaction volume was made up of 2.5 µl genomic DNA, 5 µl of each primer, and 12.5 µl of 2× KAPA Ready Mix. Ac control, PCR reaction mix holding 2.5 µl distilled water was used as control. The PCR was then carried out using a 96 well thermal cycler (2E^{TM} UK) with cycling conditions set as follows: initial denaturation at 95°C for 3 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min before cooling to 4°C. Construction of libraries, purification and validation, and other preprocessing steps were carried out as described previously [26]. Each of the samples gave approximately 0.4 kb (37ng) of genomic DNA on amplification.

2.5 Next Generation DNA Sequencing and Analysis

Sequencing was performed on the Illumina MiSeq platform, using MiSeq Reagent kit v3 (600 cycles). Overall bioinformatic analysis was done using NCBI-BLAST-2.2.24 and CLC bio Genomics workbench v7.5.1. The pair-end of the four metagenomes was assembled individually using PEAR (Paired-end read Merger) v.0.9.6.0 (Zhang et al. 2013). The assembled sequence reads were processed as previously described using RDP pipeline [30-32].

2.6 Antibiotics Resistance Gene and Data Analysis

KEGG and GhostKOALA [33] were employed for functional analyses of the metagenomes. Gene calling was performed on the assembled sequenced reads using FragGeneScan [34] to predict open reading frames (ORFs), which were

functionally annotated and assigned to the KEGG and GhostKOALA. In GhostKOALA, each query gene is assigned a taxonomic category according to the best-hit gene in the Cd-hit cluster supplemented version of the non-redundant pangenome dataset [35].

3. RESULTS

The results of the study are presented in Tables 1 and 2, and Figs. 1 to 5. Fig. 1 shows the frequency of annotation of the ARGs to various bacterial classes. Table 1 shows the physicochemical analysis results across the various ecosystems. Physicochemical analysis results indicate anthropogenic interferences in the environments sampled. Table 2 shows the KEGG annotation and the bacteria classes. The ARGs assigned to beta lactamase were the oligopeptide transport system substrate-binding protein (oppA, mppA and oppD) and was elaborated by the firmicutes (clostridia) in both soil and epipellic sediment samples and alphaproteobacteria in estuary water sample, respectively. Others include the beta-lactam resistance (AmpC system), aminoglycoside resistance, virginiamycin B lyase, and class A pen P as shown in Table 2. The various efflux pump systems (RND and ABC promoters families) were elaborated only by the class betaproteobacteria.

Top 100 classes from all the metagenome were selected and merging based on frequency of occurrence. The results indicate that soil, epipellic sediment, benthic sediment and estuary water metagenome gave a total of 19, 10, 15 and 18 classes, respectively. Soil metagenome was the most diverse followed closely by estuary water. Both sediment metagenomes were in between with the epipellic sediment having the least.

Table 1. Physicochemical analysis of the various samples

Parameters	Soil	Epipellic	Benthic	Estuary water
pH	5.60±0.07	7.30±0.14	6.80±1.14	7.06±0.01
Temperature (^{o}C)	28.70±0.14	27.00±0.71	27.70±0.14	29.5±0.14
Electrical conductivity (μs/cm)	11.80±0.14	51.40±0.14	100.40±0.14	38.30±0.14
N Ammonia (mg/L)	0.08±0.01	0.11±0.01	0.50±0.40	0.24±0.01
Phosphorus (mg/L)	28.50±0.14	20.40±0.14	25.60±0.14	ND
N-nitrite (mg/L)	41.20±0.14	0.05±0.01	0.05±0.01	2.60±0.14
N-nitrate (mg/L)	0.040±0.01	102.00±1.41	54.00±1.41	0.03±0.01
Sulphide (mg/L)	6.00±1.41	201.00±1.41	118.00±1.41	BDL

ND = Not determined; BDL = Below detection level

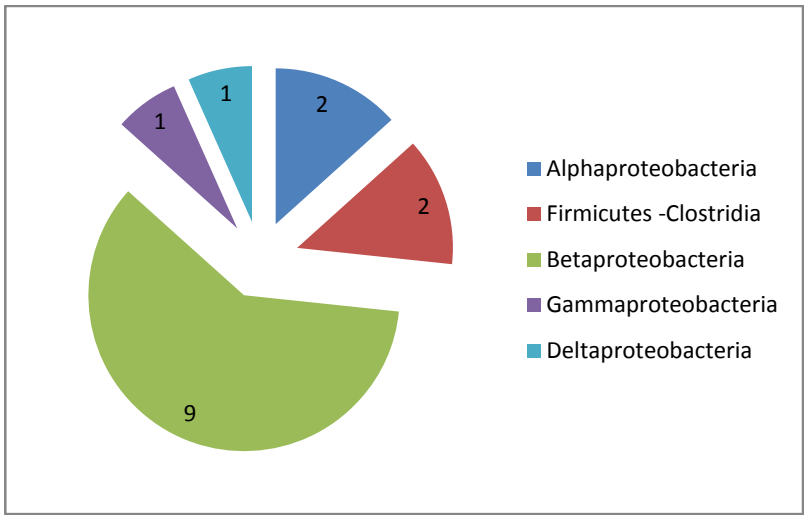

Fig. 1. Frequency of annotation of the ARGs to bacteria classes

4. DISCUSSION

The benthic metagenome top 100 classes had a unique class woesearchaeota, an archae while every other class across all the metagenomes were all of bacteria domain. The top five frequent classes were alphaproteobacteria, actinobacteria, acidobacteria, thermomicrobia and deltaproteobacteria for soil. In the epipellic sediment, they were flavobacteriia, percubacteria, zetaproteobacteria, gammaproteobacteria and cytophagia. For benthic sediment, it was gammaproteobacteria, alphaproteobacteria, deltaproteobacteria, planctomycetia and actinobacteria. Estuary metagenome revealed gammaproteobacteria, alphaproteobacteria, actinobacteria, planctomycetia and deltaproteobacteria. The characterized ARGs classes were all amongst the top 100 classes in all the metagenomes.

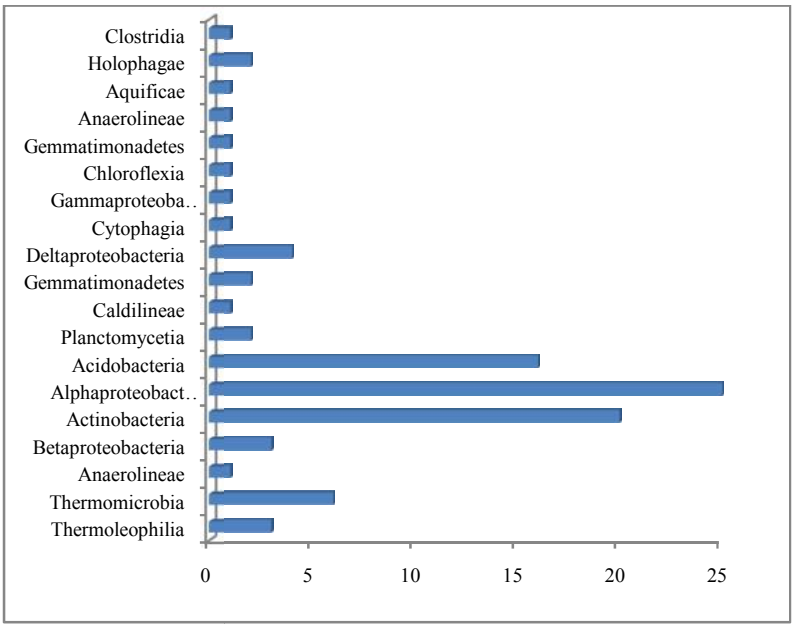

Fig. 2. Top 100 classes from soil metagenome

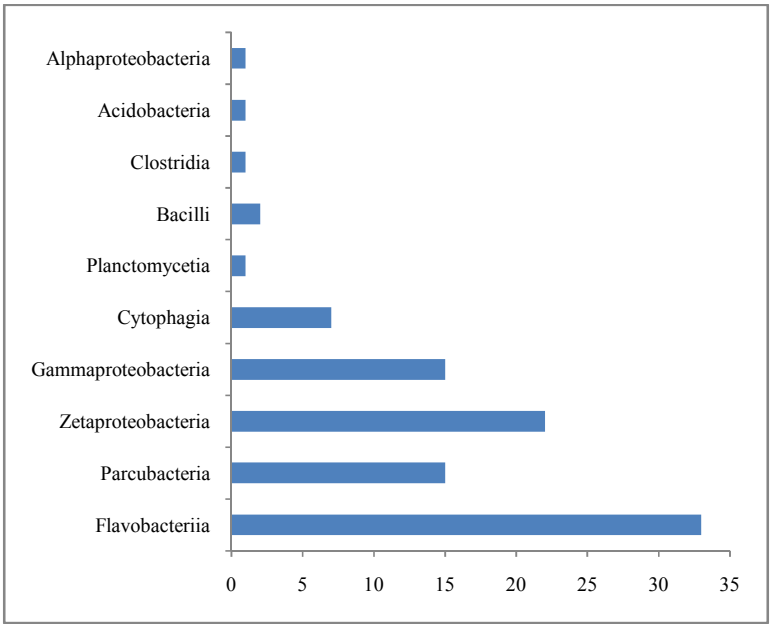

Fig. 3. Top 100 classes from epipellic metagenome

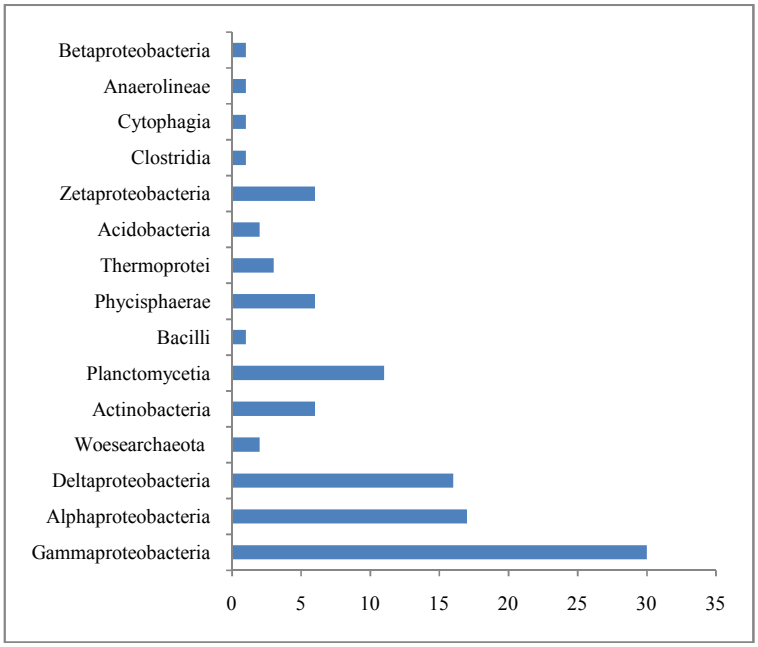

Fig. 4. Top 100 classes from benthic metagenome

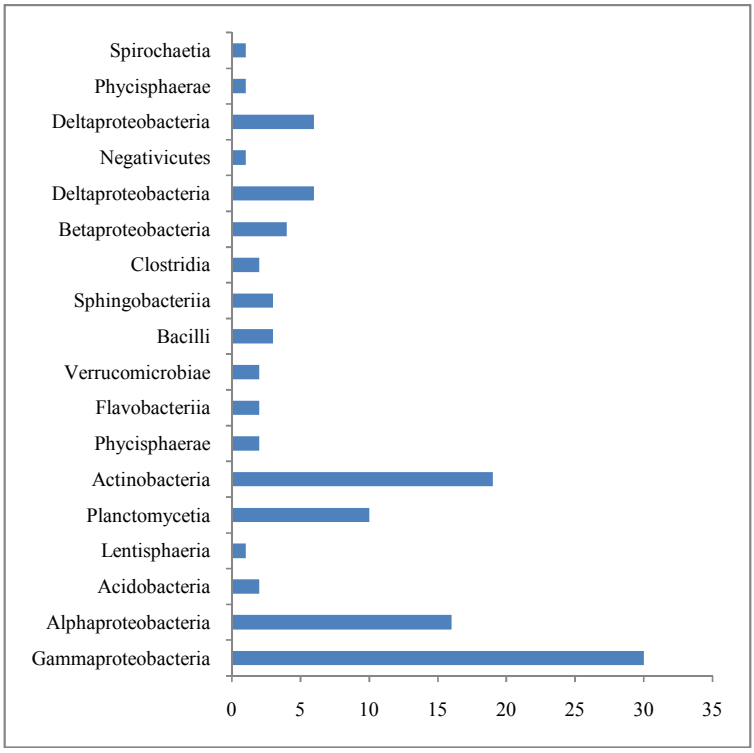

Fig. 5. Top 100 classes from estuary metagenome

The results of the physicochemical analysis show anthropogenic interference with the sampled environments. Xiao et al. [12] showed that pH was the most strongly correlated physicochemistry parameter with ARGs in addition to high abundance of ARGS in paddy

soils from south of China. Soil sample elaborated the highest number of ARGs in this study. This could be due to the fact soil is known to hold the highest amount of prokaryotic diversity. Furthermore, the nitrate and phosphate levels indicate anthropogenic inference with these environments most notably from farming and crude oil exploration. This is further supported by an earlier study that showed varying levels of hydrocarbon contaminants [25]. A recent study that examined ARGs from various environmental samples (n = 369) showed that that water soil and sediment generally have low relative abundance and few varieties of known ARGs[36] and explains the low abundance and diversity of ARGs in non-soil samples in this study.

The efflux pump families recovered from our metagenome were evenly distributed amongst two families mainly: RND and ABC superfamilies. The adenosine triphosphate-binding cassette (ABC) superfamily pumps were oppA, mppA, Ame ABC and Amp C transport systems. While the resistance-nodulation-division (RND) family efflux pumps were mtr C, cme A, Acr A, mex A, Ade I, sme DEF and TolC efflux pumps. These two families are associated with the extrusion of numerous drugs in both Gram positive and negative organisms [19]. The ARGs were distributed across five classes namely alphaproteobacteria, betaproteobacteria, gammaproteobacteria, deltaproteobacteria and the firmicutes (clostridia).

Table 2. KEGG annotation of ARGs and proteins

Category/subclass	Bacteria groups	Antibiotics resistance genes and proteins
Soil metagenome		
Beta-Lactam resistance	Firmicutes – Clostridia	Oligopeptide transport system substrate-binding protein (oppA, mppA)
	Betaproteobacteria	Beta-lactamase class A (penP)
	Betaproteobacteria	acrA, mexA, adeI, smeD, mtrC, cmeA; membrane fusion protein, multidrug efflux system
Cationic antimicrobial peptide (CAMP) resistance	Betaproteobacteria	Multidrug efflux system (acrA, mexA, adeI, smeD, mtrC, cmeA)
Antimicrobial resistance genes	Betaproteobacteria	Beta-lactamase class A (penP)
	Betaproteobacteria	Multidrug efflux system (acrA, mexA, adeI, smeD, mtrC, cmeA)
Drug efflux transporter/pump	Betaproteobacteria	Multidrug resistance efflux pump (AcrAD-TolC (acrA, mexA, adeI, smeD, mtrC, cmeA;
	Betaproteobacteria	Multidrug resistance, efflux pump (AcrAB-TolC/SmeDEF (acrA, mexA, adeI, smeD, mtrC, cmeA)
	Betaproteobacteria	Multidrug resistance efflux pump (AmeABC, acrA, mexA, adeI, smeD, mtrC, cmeA)
Drug resistance	Betaproteobacteria	Beta-lactam resistance, AmpC system (penP; beta-lactamase class A)
	Alphaproteobacteria	Aminoglycoside resistance, protease FtsH
Epipellic sediment metagenome		
Beta-Lactam resistance	Firmicutes - Clostridia	Oligopeptide transport system substrate-binding protein (oppA, mppA).
Antimicrobial resistance genes	Gammaproteobacteria	Virginiamycin B lyase (vgb)
Benthic sediment metagenome		
Vancomycin resistance	Deltaproteobacteria	Alanine racemase (alr)
Estuary water		
Beta-Lactam resistance	Alphaproteobacteria	Oligopeptide transport system (oppD)

Efflux pumps have poly substrate specificity and can expel a broad range of antibiotics. Also worrisome is the fact that they can drive the acquisition of other resistance mechanisms via accumulation of mutation and lowering antibiotics concentration inside cells. Their expression is tightly regulated and is always in response to various environmental and physiological signals [13]. In addition to resistance, they have a role in the colonization and the persistence of bacteria in hosts [18], cell to cell communication (MexAB-OprM) and component of membrane stress response (MexCD-OprJ) amongst others [37]

The mex A efflux pump is elaborated by *Pseudomonas aeruginosa*, while the Acr A is elaborated by the *Esherichia coli* and Salmonella *typhimurium.* Others such as cmeA, mtrC, and sme DEF are common amongst the *Campylobacter jejuni, Neisseria gonorrhoeae* and *Stenotrophomonas maltophilia.* For the Cme ABC pumps, the inducible signal is bile salt and paraquat while it is triclosan (a biocide) for the Sme DEF pumps. The mexA pumps are induced by superoxide stress. Furthermore, most of these pumps excrete the quinolones. In an earlier study, it was shown that overexpressed *mexA* or *ampC* and reduced *oprD* were associated with b-lactam resistance [38].

The AcrAB-TolC and Acr AD- Tol D systems which belongs to the RND family which were also recovered in our metagenomes remains the most characterized efflux pump in *E. coli.* The pump is over expressed in clinical isolates and has been implicated in the export of several drugs and compounds including chloramphenicol, tetracycline, fluoroquinolones, fusidic acid, ethidium bromide, rifampicin and bile salts [19,39- 41].

The AmpC is linked to beta lactamase resistance which recent evidence suggests that it is inducible by major molecular mechanisms (theAmpG–AmpR–AmpC pathway and BlrAB-like two-component regulatory system) in Gram-negative bacteria [42].

Aminoglycoside resistance linked to protease FtsH expression was also detected in our metagenome. Hinz et al. [43] showed that proteolysis particularly that controlled by the membrane protease FtsH, is a major driver of resistance of aminoglycosides. Furthermore, they proposed that that the network of proteases provides strong defense from aminoglycosides and other substances via the removal of membrane-disruptive mistranslation products.

5. CONCLUSION

Across the Niger Delta Region and to the best of our knowledge, this is the first documented investigation of different antibiotics resistance genes in soil, estuary water, benthic and epipellic sediments using metagenomic on Illumina platform. Based on our findings, soil sample was the richest in ADRs compared to other environments. Furthermore, two out of the five efflux pumps systems were observed in our study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Aminov RI. A brief history of the antibiotic era: lessons learned and challenges for the future. Frontiers in Microbiology. 2010;1:1-7.
2. Ghosh S, LaPara TM. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J. 2007;1:191–203.
3. Ventola CL. The antibiotics resistance crisis part 1. Pharm and Ther. 2005;40: 277-283.
4. Procopio REDL, Silva IRDS, Martins MK, Azevedo J LD, Araujo MD. Antibiotics produced by Streptomyces. The Brazilian Journal of Infectious Disease. 2012; 16:466-471.
5. D'Costa VM, McGrann KM, Hughes DW, Wright GD. Sampling the antibiotic resistome. Science. 2006;311:374–377.
6. Centers for Disease Control and Prevention, Office of Infectious Disease. Antibiotic resistance threats in the United States; 2013.
Available:http://www.cdc.gov/drugresistance/threat-report-2013
(Assessed 2017 28 August)
7. Poole K. Efflux pumps as antimicrobial resistance mechanisms. Ann. Med. 2007;39:162–176.
8. Mbim EN, Mboto CI, Edet UO. Plasmid profile analysis and curing of multidrug resistant bacteria isolated from two

hospital environments in Calabar metropolis. Nigeria. Asian Journal of Medicine and Health. 2016;1:1-11.
9. Davies J, Davies D. Origins and evolution of antibiotic resistance. Microbiology and Molecular Biology Reviews. 2010;7:417–433.
10. Adelowo OO, Fagade OE, Agersø Y. Antibiotic resistance and resistance genes in *Escherichia coli* from poultry farms, southwest Nigeria. Journal of Infection in Developing Countries. 2014;8:1103-1112.
11. Kummerer K. Resistance in the environment. Journal of Antimicrobial Chemotherapy. 2004;54:311–320.
12. Xiao KQ, Li B, Ma L, Bao P, Zhou X, Zhnag T, Zhu YG. Metagenomic profiles of antibiotic resistance genes in paddy soils from South China. FEMS Microbiology Ecology. 2016;92:1-6.
13. Sun J, Deng Z, Yan A. Bacterial multidrug efflux pumps: Mechanisms, physiology and pharmacological exploitations. Biochemical and Biophysical Research Communications. 2014;45:254–267.
14. Wu N, Qiao M, Zhang B et al. Abundance and diversity of tetracycline resistance genes in soils adjacent to representative swine feedlots in China. Environ Sci Technol. 2010; 44:6933–9.
15. Gao P, Munir M, Xagoraraki I. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. Sci Total Environ. 2012;421:173–83.
16. Ji X, Shen Q, Liu F, et al. Antibiotic resistance gene abundances associated with antibiotics and heavy metals in animal manures and agricultural soils adjacent to feedlots in Shanghai; China. J Hazard Mater. 2012;235–6:178–85.
17. Wright GD. Antibiotic resistance in the environment: A link to the clinic? Curr. Opin. Microbiol. 2010;13:589–594.
18. Piddock IJV. Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria. Clinical Microbiology Reviews. 2006; 19:382–402.
19. Soto SM. Role of efflux pumps in the antibiotic resistance of bacteria embedded in a biofilm. Virulence. 2014;4:223–229.
20. Kumar SK, Mukherjee MM, Varela M. Modulation of bacterial multidrug resistance efflux pumps of the major facilitator superfamily. International Journal of Bacteriology. 2013;1-15.
21. Putman M, van Veen HW, Konings WN. Molecular properties of bacterial multidrug transporters. Microbiol. Mol. Biol. Rev. 2000;64:672–693.
22. Handzlik J, Matys A, Kiec´-Kononowicz K. Recent advances in multi-drug resistance (MDR) efflux pump inhibitors of Gram-positive bacteria *S. aureus*, Antibiotics. 2013;2:28–45.
23. Anugasi FB, Multidrug resistance profiles of clinical and environmental isolates of *Pseudomonas aeruginosa* and *Escherichia coli.* An MSc thesis submitted to University of Nuskka; 2015.
24. Ayandiran TA, Ayandele AA, Dahunsi SO, Ajala OO. Microbial assessment and prevalence of antibiotic resistance in polluted Oluwa River, Nigeria. Egyptian Journal of Aquatic Research. 2014; 40:291–299.
25. Udotong IR, Eduok SI, Essien JP, Ita BN. Density of hydrocarbonoclastic bacteria and polycyclic aromatic hydrocarbon accumulation in Iko River Mangrove Ecosystem, Nigeria. World Academy of Science, Engineering and Technology. 2008;44:8830-836.
26. Salam LB, Obayori SO, Nwaokorie FO, Suleiman A, Mustapha R. Metagenomic insights into effects of spent engine oil perturbation on the microbial community composition and function in a tropical agricultural soil. Environmental Science Pollution Resource; 2017.
DOI: 10.1007/s11356-017-8364-3
27. Udotong IR, Uko MP, Udotong JIR. Microbial diversity of a remote aviation fuel contaminated sediment of a lentic ecosystem in Ibeno, Nigeria. Journal of Environmental and Analytical Toxicology. 2015;5(320):1-7.
28. Day PR. Particle fractionation and particle-size analysis. In: Black CA et al., editors. Methods of soil analysis, part 1. Madison (Wisconsin, USA): American Society of Agronomy Inc. 1965;545-567.
29. Odu CT, Babalola O, Udo EJ, Ogunkunle AO, Bakere TA, Adeoye GO. Laboratory manual for agronomic studies in soil, plan and microorganisms. University of Ibadan, Ibadan press Ltd; 1986.
30. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM. Ribosomal database project: Data and tools for high

throughput rRNA analysis. Nucleic Acids Research. 2014; 42:D633–D642.

31. Wang Q, Garrity GM, Tiedje JM, Cole JR. Nai¨ve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy. Applied and Environmental Microbiology. 2007; 73:5261–5267.
32. Zhang J, Kobert K, Flouri T, Stamatakis A. PEAR: A fast and accurate Illumina Paired-End reAd merger. Bioinformatics. 2013; 30:614–620.
33. Tatusov RL, Galperin MY. Natale DA, Koonin EV. The COG database: A tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Research. 2000; 28:33-36.
34. Rho M, Tang H, Ye Y. FragGeneScan: predicting genes in short and error-prone reads. Nucleic Acids Research. 2010; 38:1-12.
35. Kanehisa M, Sato Y. Morishima K. BlastKOALA and GhostKOALA: KEGG tools for functional characterization of genome and metagenome sequences. Journal of Molecular Biology. 2016;726-731.
36. Pal C, Bengtsson-Palme J, Kristiansson E, Larsson DGJ. The structure and diversity of human, animal and environmental resistomes. Microbiome. 2016;4:1-15.
37. Poole K. Bacterial multidrug efflux pumps serve other functions. Microbes. 2008;3: 179-185.
38. Tomas M, Doumith M, Warner, M, Turton JF, Beceiro A, Bou G, Livermore DM, Woodford N. Efflux Pumps, OprD Porin, AmpC _-Lactamase, and Multiresistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Antimicrobial Agents and Chemotherapy. 2010;2219–2224.
39. Webber MA, Piddock LJ. Absence of mutations in mar-RAB or soxRS in acrB-overexpressing fluoroquinolone resistant clinical and veterinary isolates of Escherichia coli. Antimicrob Agents Chemother. 2001;45;1550-1552.
40. Fernandes P, Ferreira BS, Cabral JM. Solvent tolerance in bacteria: Role of efflux pumps and cross resistance with antibiotics. Int J Antimicrob Agents. 2003; 22:211-6.
41. Anderson JL, He GH, Kakarla P, Ranjana KC, Kumar S, Lakra WS, Mukherjee M M, Ranaweera I, Shrestha U, Tran T, Varela MF. Multidrug efflux pumps from Enterobacteriaceae, *Vibrio cholerae* and *Staphylococcus aureus* bacterial food pathogens. Int. J. Environ. Res. Public Health. 2015;12:1487-1547.
42. Zeng X, Lin J. Beta-lactamase induction and cell wall metabolism in gram-negative bacteria. Frontiers in Microbiology. 2013; 4:1-9.
43. Hinz A, Lee S, Jacoby K, Manoil C. Membrane proteases and aminoglycoside antibiotic resistance. Journal of Bacteriology. 2011;193: 4790–4797.

Peer-review history:
The peer review history for this paper can be accessed here:
http://prh.sdiarticle3.com/review-history/22170