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Heavy Metal Tolerance profile among Bacterial species Isolated from Hydrocarbon polluted sites and their mobile genetic elements

Antai Sylvester Peter[1], Agbor Reagan Bessong[2*], Iwatt Godwin Dickson[1], Ubi Stanley Emmanuel[3]

[1]Department of Microbiology, University of Calabar, Calabar, Cross River State, Nigeria
[2]Department of Genetics and Biotechnology, University of Calabar, Calabar, Cross River State, Nigeria
[3]Department of Civil Engineering, Cross River University of Technology, Calabar, Cross River State, Nigeria

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ABSTRACT

This present study evaluated the plasmid incidence in bacteria and their genetic elements in heavy metals tolerant-antibiotics resistant microbes isolated from petroleum hydrocarbon polluted sites. The plasmid isolation was carried out using the fermentas Genejet plasmid miniprep kit (Thermofisher Scientific Inc, USA). Screening for class 1, 2, and 3 integrons, incompatibility group P testing, plasmid replicon typing, plasmid restriction analysis, and other analysis was performed using standard laboratory procedures. Plasmid incidences were higher among multiple heavy metal-tolerant bacterial species from hydrocarbon-polluted sites than those from the pristine site. Further, Class 1 integron incidence was significantly higher among the integrons in heavy metal tolerant bacterial isolates isolated from the polluted ecosystems than those from pristine ecosystems. Plasmid replicon type of bacteria with multiple heavy metal tolerance and antibiotics resistance indexes revealed that IncN plasmid replicon type carrying class 1 integron. This encodes resistance to sulphamethazole/trimethoprim, ampicillin, and tolerance to Cd, Ni, and Cu in *Klebsiella pneumoniae* isolate from petroleum-polluted soil. This is the first report of IncN plasmid in environmental bacteria in Nigeria, particularly from petroleum polluted environment. The conjugation experiment confirmed the possible transferability of antibiotic resistance determinants among isolates in polluted ecosystems. From the results of this study, it can be concluded that petroleum hydrocarbon pollution vis-a-vis heavy metal selective pressure with the abundance of mobile genetic elements amongst isolates from polluted ecosystems could contribute to the dispersing of antibiotic resistance genes, thus posing a serious public health concern.

* Corresponding author
E-mail: agborreagan@yahoo.com (Agbor Reagan Bessong)

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1 Introduction

Over the years, petroleum hydrocarbons have improved several nations' socioeconomic standards. But the mismanagement of this essential commodity due to pipeline vandalization, accidental discharge, and refining processes has resulted in severe environmental threats. Many polluted soil and aquatic ecosystems have been abandoned because of ineffective reclamation methods (Ekpo et al. 2012). However, various reports have shown that excess heavy metals, especially in soil environments, reduce microorganisms' growth rate and replication. Heavy metals are found naturally in the ecosystem, but their abundance and prevalence are associated with various anthropogenic activities such as industries (Petrochemical, Mining, and gas flaring) and Agricultural sectors (Fertilizer plan, Fermentation industries, milling companies). The bioavailability of heavy metals at low concentrations may not cause a serious environmental threat, but higher accumulation in aquatic and soil ecosystems may cause a significant challenge to public health (Azarbad et al. 2015; Berg et al. 2005). They have some heavy metals that might act as essential micronutrients and are involved in cell metabolism, but bioaccumulation of these heavy metals in high proportion, such as Zn and Pb uptakes, could be toxic and cause danger to its accumulator. The biodiversity of microbial communities could be altered when the excess accumulation of heavy metals occurs in the terrestrial and aquatic ecosystems (Epelde et al. 2015).

Plasmids are autonomous self-replicating extra-chromosomal DNA elements (Chen et al. 2015), which are not beneficial for bacterial growth and proliferation but play essential roles in various other metabolic activities like drug and heavy metal resistance. Plasmids are highly mobile and spread widely between the same or different bacteria genera and even eukaryotes (Hu et al. 2017). The family *Enterobacteriaceae* is one of the typical bacterial family that has been used in the transfer of plasmids from one specie to another (Knapp et al. 2017), between enteric and other gram-negative bacteria (Chen et al. 2015) and between or within non-enteric gram-negative organisms (Sawut et al. 2018). However, plasmids are smaller but have a vast carrying capacity of not only transferring and replicating genes but also coding for antibiotic resistance, metabolic enzymes, and bacteriocin production (Gati et al. 2016). Making a copy of plasmid in a cell is determined by replication of origin, also known as the replicon. The inability of a single cell to maintain different plasmids with the same replication mechanism has given rise to incompatibility (Inc) grouping of plasmids. Incompatibility classifies plasmids by their ability to coexist stably with other plasmids in the same bacterial strain. The number of incompatibility plasmid groups increases from the 26 known Inc groups occurring among the *Enterobacteriaceae* (Frost et al. 2005). This study aimed to evaluate plasmid incidence in bacterial and its genetic elements in heavy metals tolerant-antibiotics resistant microbes from petroleum hydrocarbon polluted sites.

2 Materials and Methods

2.1 Heavy metal resistance screening in bacteria isolates

As described by Lee et al. (2009), the agar dilution method for bacterial isolation was adopted with slight modifications. A loopful of 12-16 hr bacteria culture in TSB was streaked on Mueller Hinton agar (Hardy, Diagnostic, USA) supplemented with heavy metal salts to achieve 100µg/ml each of cadmium, Chromium, cobalt, nickel, vanadium, and 600 µg/ml each of lead and copper. The rest procedure is as described by Lee et al. (2009)

2.2 Detection of Bacterial plasmid

Plasmid DNA was obtained using the Fermentas Genejet plasmid miniprep kit (Thermo-Fisher Scientific Inc, USA). New bacterial colonies were inoculated into sterile 10 ml Lauria Bertani medium (Merck, Germany) in a 50 ml capacity tube and incubated for 12-16 hours at 37°C at a shaker with 200 rpm rotations. It was followed by harvesting the bacterial cells by centrifugation at 8000 rpm using microcentrifuge model 5415 R (Eppendorf, Germany). Pelleted bacterial cells were resuspended and subjected to SDS/alkaline lysis. Two hundred and fifty microlitres (250µl) of resuspension solution with RNase A was added to the pelleted cells and vortex by using a Labinco Model L46 vortex mixer (Labinco BV, Netherlands). 250µl of lysis solution was mixed thoroughly by inverting the tube 4-6 times until the solution became viscous and slightly transparent. Vortexing can be avoided in other not to shear chromosomal DNA. The lysis process did not exceed five minutes to avoid supercoiled plasmid DNA denaturing. It was followed by adding a neutralization solution (350µl) and inverting the tube 4-6 times. The tube was centrifuged for 5 minutes at 13000 rpm to remove pellet cell debris and chromosomal DNA. After this, pipetting was done to extract the supernatant to the Gene-Jet spin column, the transferred supernatant was centrifuged for one minute, and the flow-through was discarded. Further, five hundred microliters (500µl) of the wash solution was added to the Gene-Jet spin column, centrifuged for 30-60 seconds, and the flow-through was discarded. Again the washing step was performed twice. After the last washing, the Gene-Jet column was centrifuged for 60 seconds to remove the residual wash solution. Following centrifugation, the Gene-Jet spin column was transferred into a fresh 1.5ml microcentrifuge tube, and 50µl of the prewarmed (at 70°C) elution buffer was added to the center of the Gene-Jet column membrane to elude the plasmid DNA. The column was incubated at room temperature for two minutes and centrifuged for two minutes. The isolated plasmid DNA was stored at -20°C. Using the standard method, plasmid DNA was detected using 1% agarose gel in lx TAE buffer.

2.3 Screening for class 1, 2, and 3 integrons

Moura et al. (2007) developed PCR to screen bacterial isolates with genes *int* 11, *int* 12, and *int* 13. One microliter (1µl) of bacterial genomic DNA was used as a template for PCR while 4µl of boiled cells of positive control strains, i.e., *Salmonella enterica serovar typhimurium* (*int*11), *Escherichia coli* (*int*11) and *Klebsiella pneumoniae* (*int*13) were used as templates. These were added to the reaction mixture containing 14.25µl of sterile milli-Q water, 3µl of 25mM MgCb (Fermentas, USA), 2.5µl of Taq buffer with $(NH_4)_2S0_4$ (Fermentas, USA), 1.25µl of DMSO (Eurobio, France), 1µl of dNTPs (BIORON, Germany), 0.75µl each of primer pairs and 0.5p.l of U Taq Polymerase (Fermentas, USA) to achieve a total volume of 25µl. Amplification was performed in a Thermal Cycler (BIORAD, USA) with the following PCR program 94°C for 9 min, 30 cycles of 94°C for 30 sec, 55°C or 50°C for 30 sec (as appropriate), and 72°C for 45 sec, with a final extension at 72°C for 10 min. PCR reaction products were analyzed on 1.5% agarose gel in lx TAE buffer, run at 80V for 60min. Amplicons were visualized after staining in ethidium bromide using a molecular imager.

2.4 Detection of Class 1 integron variable region

Class 1 integron variable region was also detected by PCR amplification (Levesques et al. 2005) using primer pairs targeting class 1 integron variable region (5'-CS: GGC ATC CAA GCA GCA AG and 3'-CS: AAG CAG ACT TGA CCT GA). The PCR mixture contained 10µl of Extensor long PCR master mix (ABgene-Thermo Scientific, UK), 1µl each of primer pairs, 7µl of milli-Q water, and lµl DNA. The amplification protocol used was denaturation at 94°C for 5min followed by 30 cycles of 94°C for 30 sec; 58.5°C for 30 sec and 68°C for 3 min with a final extension at 68°C for 10 min (Table 1). Reaction products were analyzed as described above.

2.5 Testing of incompatibility group P (IncP-1)

All positive plasmid DNAs were screened for broad host range (3HR), plasmid IncP-1 subgroups a, p, s, *y*, and 5 by PCR technique. Three primer pairs developed by Bahl et al. (2009) were used to amplify the 281 bp homologous fragments of the *trfA* gene from plasmids belonging to the different IncP-1 subgroups. The primer pairs and positive controls are presented in Table 2. Each PCR mixture contained 12.55µl of sterile milli-Q water, *2.5*µl of Buffer (Promega, USA), 2.5µl of $MgCl_2$ (Promega, USA), 2.5µl of 200µM dNTPs (BIORON, Germany); 1.25µl of each primer, *1.25*µl of DMSO (Eurobio, France), 0.2µl of GoTaq Flexi DNA Polymerase-5µl (Promega, USA) and 1µl of DNA template. The total volume of each PCR mixture was 25 µl. The PCR program was initially denatured at 98°C for 30 sec. followed by 35 cycles of 98°C for 20 sec., 67°C for 20 sec, and 72°C for 30 sec. with a final extension at 72°C for 5min. Reaction products were analyzed in 1.5% Agarose Gel in lx TAE buffer, run at 80V for 70min, and viewed using a molecular imager after staining in ethidium bromide.

2.6 Plasmid replicon typing

Three panels multiplex PCR was carried out to detect 18 plasmid replicons, as Johnson et al. (2007) described, with some required modifications in the PCR. The PCR mixture contained the primer pairs for each plasmid replicon (Table 2). The PCR condition used in this study is as follows: 5 minutes at 94°C; 30 cycles of 30 sec at 94°C, 30 sec at 60°C, and 90 sec at 72°C and a final extension of 5 mins at 72°C. Amplicons were visualized on 1.5% Agarose Gel in l X TAE buffer alongside a 1-kb DNA ladder.

Table 1 Oligonucleotides used for integrase gene detection

Primer pair	Target	Sequence (5' -3')	Annealing Temp. (°C)	Amplicon size (bp)
int1F int11R	*intI1*	CCT CCC GCA CGA TGA TC CCT CCC GCA CGA TGA TC	55	280
intI2F intI2R	*intI 2*	TTA TTG CTG GGA TTA GGC ACG GCT ACC CTC TGT TATC	50	233
intBF intI3R	*intI 3*	AGT GGG TGG CGA ATG AGT G TGT TCT TGT ATC CGC AGG TG	50	600

Source: Liu et al. (2018)

Table 2 Primer pairs for IncP-1 subgroups

Subgroup	Name	Forward primer (5'-3')	Reverse primer (5'-3')
α, β, ε	TrfA	TTCACSTTCTACGAGMTKTGCCAGGAC	GWCAGC1TGCGGTACTTCTCCCA
γ	trfA-y	TTCACTTTTTACGAGCTTTGCAGCGAC	GTCAGCTCGCGGTACTTCTCCCA
&	trfA5-&	TTCACGTTCTACGAGCTTTGCACAGAC	G AC AGCTCGCGGT ACTTTTCCC A

PCR was selected as per Bahl et al. (2009).

2.7 Conjugation experiment

The filter paper mating assay described by Moura et al. (2007) was adopted for the conjugation experiment. Four class 1 integron-positive bacterial isolates (Gl, G2 containing InC-N plasmid and ITS5, 'TS1 containing InC-P plasmid) were included as donors, while *Escherichia coli* CV601 resistant to kanamycin and rifampicin but sensitive to ampicillin was used as a recipient cell. Donor strains were resistant to ampicillin and trimethoprim/sulphamethazole. Donors and recipient strains were grown separately in Luaria Bertani broth Merck, Germany) and incubated with agitation (200rpm) at 37°C for 24 hours. The optical density of cells was adjusted at 600nm to 1 OD, equivalent to 8 x 10 cells/ml. The concentration of cells needed for mating was determined. Donor and recipient strains were mixed in 0.9% NaCl solution and filtered through 0.45 µm pore size nitrocellulose filters. Filters were placed on TSA plates and incubated at 37°C for 24 hours. Vortexing in 10 mL of 0.9% NaCl was carried out to wash off cells on the filter. Serial dilutions were prepared, and aliquots of 100µL were spread-plated on TSA plates supplemented with rifampicin (100 mg/L), kanamycin (30 mg/L), and ampicillin (50 mg/L). Aliquots of 5µl were also inoculated into 1ml of TSB containing rifampicin (100 mg/L), kanamycin (30 mg/L), and a disc of trimethoprim/sulphamethazole(25mg). Donor and recipient were also inoculated on the selective plates and broths for mutant detection. Assays were carried out in duplicate. Transconjugants were confirmed by repetitive extragenic palindromic sequence polymerase chain reaction (Rep-PCR), InC-N and P plasmid typing, and class 1 integron detection.

2.8 Plasmid restriction analysis

Plasmid DNA from donor cells (G1 and G2) and transconjugants were extracted using the Gene-jet plasmid miniprep kit. Plasmid DNA was double-digested using two 6-cutter enzymes: *Pst*1(CTGCA↓G) and *Bst* 17701 (GTA↓TAC), according to manufacturer's instructions (Fermentas, Lithuania). The restriction profile was analyzed in 0.8% Agarose Gel and electrophoresis was run at 40V for 3hr. The gel was stained in ethidium bromide and viewed using a molecular imager.

2.9 Rep-PCR protocol

For this, the following primers: Rep 2I-(NCG ICT TAT CIG GCC TAC) and Rep 2I-(III ICG ICG ICA TCI GGC) were used (Versalovic et al. 1991). Cell suspension of each donor, recipient, and supposed transconjugants was prepared in 100µl of sterile distilled water without boiling and used as a DNA template. The PCR mixture was made up of 11.15µl of sterile MilliQ-water, 3µl of MgCl (Promega, USA), 5µl of NH_4^+ buffer (Promega, USA), 1.5 µ1 of dNTPs (Bioron, Germany), 1.25 µl (of DMSO(Eurobio, France), 1 µl each of primer pair, 0.1µl of GoTaq Flexi DNA Polymerase (Promega, USA) and 1 µl of DNA template.

2.10 16S rDNA PCR

The protocol of Manni et al. (2008) was adopted using universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') (Lane 1991) and 1492R (5'GGTTACCTTGTTACGACTT-3'). PCR mixture consists of 14.25µl of sterile MilliQ-water, 2.5µl of lx buffer (Fermentas, USA), 3µl of MgCb fermentas, USA), 1.5µl of dNTPs (Bioron, Germany), 0.75µl each of primers, 15µl of DMSO (Eurobio, France), 0.5µl of Taq DNA polymerase (Fermentas, USA) and 0.5µl of DNA template. The PCR condition was initially denatured at 94°C for 3 mins and followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 mins at 72°C. The final extension was at 72°C for 10 mins. PCR products were run on 1% Agarose Gel in lx 7AE buffer at 80V for 80 min. Amplicons were visualized after staining in ethidium bromide. Products with an expected amplicon size of approximately 1400bp were purified and sent for sequencing.

2.11 PCR product purification

The PCR product was purified using JETQUICK spin column techniques (Genomed, USA) as per Manufacturer's protocol with slight conditional modification. Four hundred microlitres (400µl) of solution HI (binding solution containing guanidine hydrochloride and isopropanol) was added to 20µl of PCR product and mixed thoroughly. A JETQUICK spin column was placed into a 2ml Eppendorf tube, loaded with the mixture, and centrifuged at 13,000xg for 1 minute. After centrifugation, the flow through was discarded. The spin column was inserted into an empty receiver tube, and 500µl of reconstituted solution H_2 (ethanol, NaCl, EDTA, and Tris-HCl) was added. The column was centrifuged at 13,000xg for 1 minute. The flow-through was discarded, and the spin column was placed back in the same receiver tube and centrifuge at maximum speed for 1 minute. DNA was eluted from the spin column with 20µl of sterile dH_20 pre-warmed to 65^0C and centrifuged at 13,000xg for 2 minutes. Eluted DNA was stored in the freezer at -20°C and further used for sequencing.

2.12 DNA sequencing

All purified PCR products were sent for commercial sequencing. Sequences were compared to the NCBI nucleotide sequence database using The Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST).

2.13 Statistical analysis

Data collected were subjected to Analytical software (SPSS version 16, Quick Calcs online GraphPad, and Microsoft Excel). The chi-square test was used to compare the differences in the number of heavy metal-tolerant bacterial isolates from pristine and hydrocarbon-polluted ecosystems. The rate of occurrence of

plasmids and class 1 integron in bacterial isolates from both ecosystems were compared using Fisher exact test. Correlation analysis was performed to establish a relationship between heavy metal tolerant profiles of bacterial isolates, the incidence of plasmids, and class 1 integron. All statistical testing was performed at a 95% confidence level.

3 Results

3.1 Heavy metal tolerance testing

A total of 345 bacterial isolates were isolated and screened for their ability to tolerate l00µg/ml of Ni, Cr, Cd, Co, V, and 600µg/ml of Pb and Cu, respectively. The result of the study are presented in Table 3; among the 152 isolates from pristine ecosystems, the percentage tolerance to the different heavy metals was 69.7% for Cu; 66.4% for Pb; 41.4% for Cd; 36.2% for Co; 34.9% for Cr; 6.6% for Ni and 5.9% for V. Further, among the 193 isolates from polluted ecosystems (Itu in Akwa Ibom, Odukpani and Calabar in Cross River), the percentage tolerance to all heavy metals tested was above 60% and were as follows: 90.4% for Cu; 89.5% for Pb; 82.4% for Cr; 81.4% for Co; 77.2% for Cd; 74.6% for Ni and 63.4% for V. Comparatively, tolerance to heavy metals among bacterial isolates from polluted ecosystems was significantly greater than ($p<0.01$) those from pristine ecosystems (Table 3).

3.2 Plasmid and integron detection in selected isolates

Table 4 presents the rate of plasmids and Class 1 integrons occurrence among the selected bacteria isolates. However, of the 37 selected heavy metal-resistant isolates from polluted ecosystems, plasmids were detected in 19 (51.4%), while class 1 integrons were detected in 32 (86.5%). As for isolates from pristine ecosystems, plasmids were detected in 12(30.8%) out of 39, while class 1 integrons were detected in 18(42.6%). About 10.3% of isolates from pristine ecosystems harbored plasmids and class 1 integrons whereas 43.2% from polluted ecosystems harbored plasmids and class 1 integrons. The percentage occurrence of plasmids positive-class 1 integrons negative was 20.5% and 43.2% for isolates from pristine and polluted ecosystems, respectively, while plasmid negative-class 1 integrons positive occurrence was 35.9%, and 43.2% for isolates from pristine and polluted ecosystems respectively. The overall pattern/level of plasmids and class 1 integron occurrence was greater amongst isolates from polluted than from pristine ecosystems. The difference in plasmid occurrence among isolates from pristine and polluted ecosystems was not statistically significant. The difference in the number of isolates that harbored class 1 integrons with those that harbored both plasmids and class 1 integrons was statistically significant *($p=0.0003$* and *$p=0$*.0002-Fisher exact test). In addition, plasmid positive-class 1 integrons negative isolates were significantly higher among isolates from polluted ecosystems than those from

Table 3 Heavy metal tolerance among bacteria isolates from pristine and polluted ecosystems
(Number and Percentage of tolerant bacterial isolates)

	Pristine (N=152)	Polluted (N=193)	p-value (X^2)
Pb(600ug/ml)	101(66.4)	173(89.5)	<0.0001
Ni(100ug/ml)	10(6.6)	144(74.6)	<0.0001
Cr(100ug/ml)	53(34.9)	159(82.4)	<0.0001
Cd(100ug/ml)	63(41.4)	149(77.2)	<0.0001
Co(100ug/ml)	55(36.2)	157(81.4)	<0.0001
Cu(600ug/ml)	106(69.7)	175(90.4)	<0.0001
V(100ug/ml)	9(5.9)	122(63.4)	<0.0001

Table 4 Rate of occurrence of plasmids and class 1 integron in bacterial isolates from pristine and polluted ecosystems
(Number and Percentage of occurrence)

Types	Pristine (N=39)	Polluted (N=37)	p-value (Fisher exact test)
Plasmid positive	12(30.8)	19(51.4)	0.1018
Intl 1 Positive	18(46.2)	32(86.5)	0.0003*
Plasmids positive-Intl 1 Positive	4(10.3)	16(43.2)	0.0002*
Plasmids positive- Intl 1 negative	8(20.5)	16(43.2)	0.0481*
Plasmids negative- Intl 1 Positive	14(35.9)	16(43.2)	0.6395

pristine ecosystems. Class 2 or 3 integrons were detected among the selected isolates.

Figure 1 presents the correlation between the incidence of plasmids and heavy metal tolerance. A positive correlation (r=0.786) was observed between the number of bacteria isolates from the polluted ecosystem that harbored plasmids and their ability to tolerate multiple heavy metals, but this correlation was not significant (*P>0.05*). Bacteria isolates from pristine ecosystems that harbored plasmids were also found to tolerate multiple heavy metals, but this correlation was poorly significant (r=-0.414, *P>0.05).* A significant positive correlation (P<0.05, r=0.926) between the number of isolates from the polluted ecosystem that harbored class 1 integrons and their ability to tolerate multiple heavy metals was found (Figure 2). Conversely, a negative non-significant correlation (r=-0.184, *P>0.05*) was the case for class 1 integrons negative isolates from pristine ecosystems and their multiple heavy metal tolerability (Figure 2).

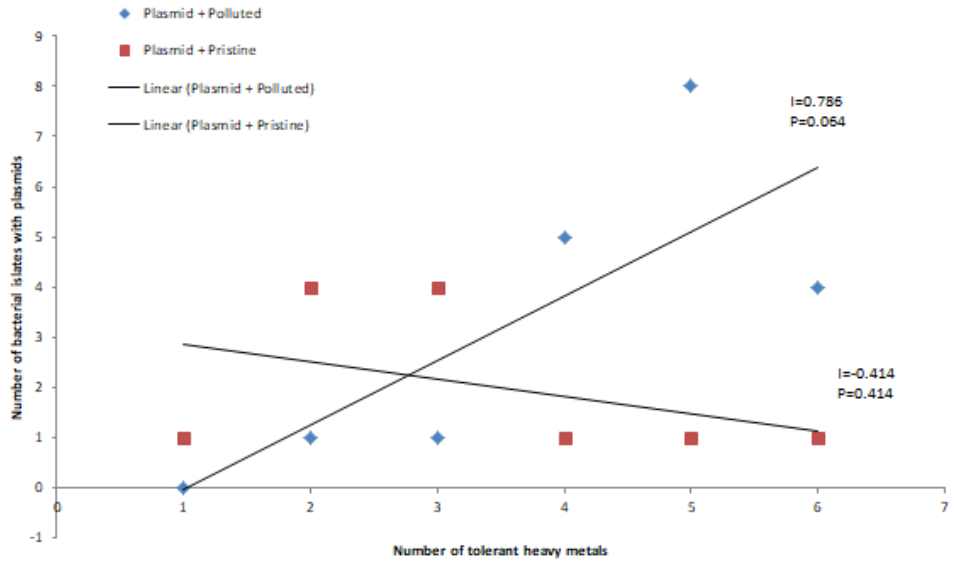

Figure 1 Correlation between heavy metal tolerant profiles with plasmids incidence among bacterial isolates isolated from pristine and polluted ecosystems

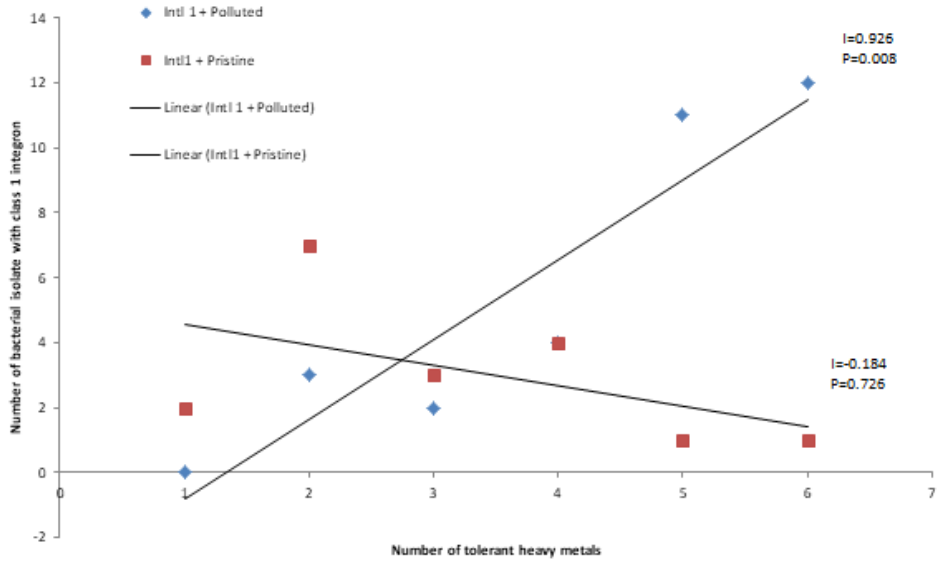

Figure 2 Correlation between heavy metal tolerant profile with the occurrence of class 1 integrons among bacterial isolates from pristine and polluted ecosystems

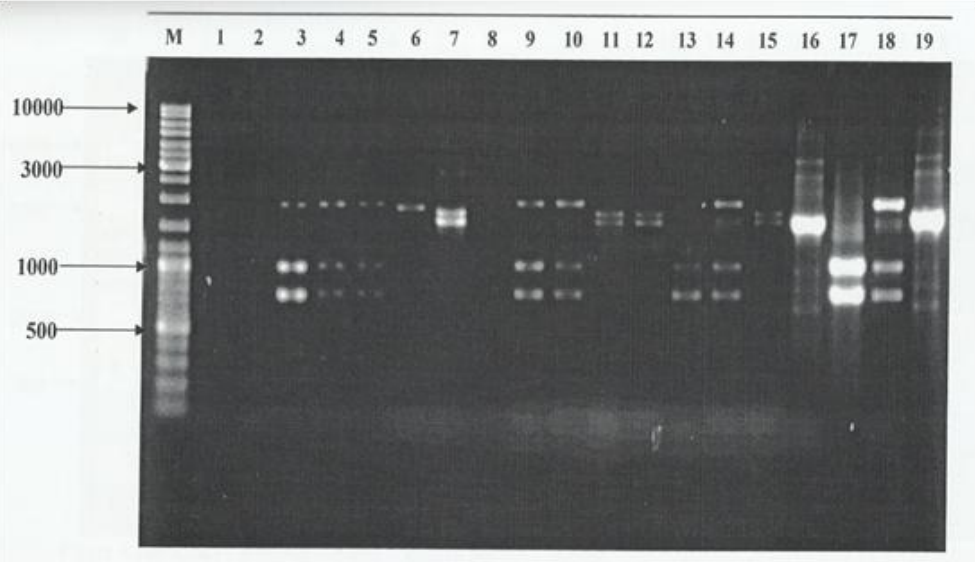

Figure 3 Class 1 integron variable region of bacterial isolates from the pristine ecosystem (M= Molecular maker (bp), 1= *Serratia sp* 4R, 2= *Pseudomonas* sp NSW6, 3= *Serratia* sp CRWI, 4= *Serratia* sp CRSI, 5= *Serratia sp* CRSI4, 6=*Pseudomonas* sp EEW8, 7=*Klebsiell*a sp CRSA13, 8= *Pseudomonas* OKW4, 9 = *Klebsiella sp* OKW2, 10=*Serratia* sp NSS11, 11=*Enterobacter* sp OKW1, 12=*Klebsiella* sp NSW5, 13=*Yersinia* sp NSS1, 14= *Bacillus* sp NSS14, 15= *Enterobacter* spp CRS9, 16= *Enterobacter* sp ITS2, 17= *Aeromonas* sp ITS1, 18 = *Enterobacter* sp ITS5)

Table 5 Distribution of class 1 integron variable region among bacterial isolate from prinstine and polluted ecosystems (Number and Percentage of occurrence)

Variable size (kbp)	Pristine n=18	Polluted n=32	Total (n=50)
6.0	-	1(3.1%)	1(12%)
4.0	-	9(28%)	9(18%)
2.0	7(39%)	7(22%)	14(28%)
1.5	5(28%)	12(38%)	17(34%)
1.2	-	7(22%)	7(14%)
1.0	9(50%)	6(19%)	15(30%)
0.7	9(50%)	3(9.4%)	12(24%)

3.3 Class 1 integron variable region detection

Class 1 integron variable region of bacterial isolates from pristine and polluted ecosystems are presented in Figure 3. Variable sizes between 0.7 and 2.0kb were detected among isolates from pristine ecosystems, while 0.7 to 6.0kb sizes were detected among isolates from petroleum hydrocarbon-polluted ecosystems. The 0.7 and l.0kb regions were highly prevalent among isolates from pristine ecosystems, followed by the 2.0kb region. Among the isolates from polluted ecosystems, the 1.5kb region was the most prevalent and was detected in 12 (38%) out of 32 isolates. Other variable regions detected were 4.0kb (28%), 2.0kb (22%), 1.2kb (22%), 1.0kb (19%), 0.7kb (9.4%), and 6.0kb (3.1%). The 1.5kb region was the most prevalent among isolates from both ecosystems (Table 5).

3.4 Plasmid analysis and typing

Plasmid profiles of some bacterial isolates from pristine and petroleum hydrocarbon-polluted ecosystems are presented in Figure 4. Each plasmid-containing isolate from both pristine and polluted ecosystems harbored at least one plasmid with a size ranging from approximately less than 2.1 kbp to 55 kbp. Most of these plasmids were of low molecular weights, with a few isolates (ITS5, ITS 1, Gl, G2, and G13) harboring high molecular weight plasmids. Plasmid positives isolates which were gram negatives and belonged to the *Enterobactericeae* group examined for the presence of plasmids of IncP-1 subgroups *trfA-α, β,ε, trfy, trfβ,* and 18 plasmid replicon types. The expected amplicon size, specific for IncP-1 subgroups, was not detected. However, plasmid replicon typing PCR revealed the presence of IncN plasmid replicon in

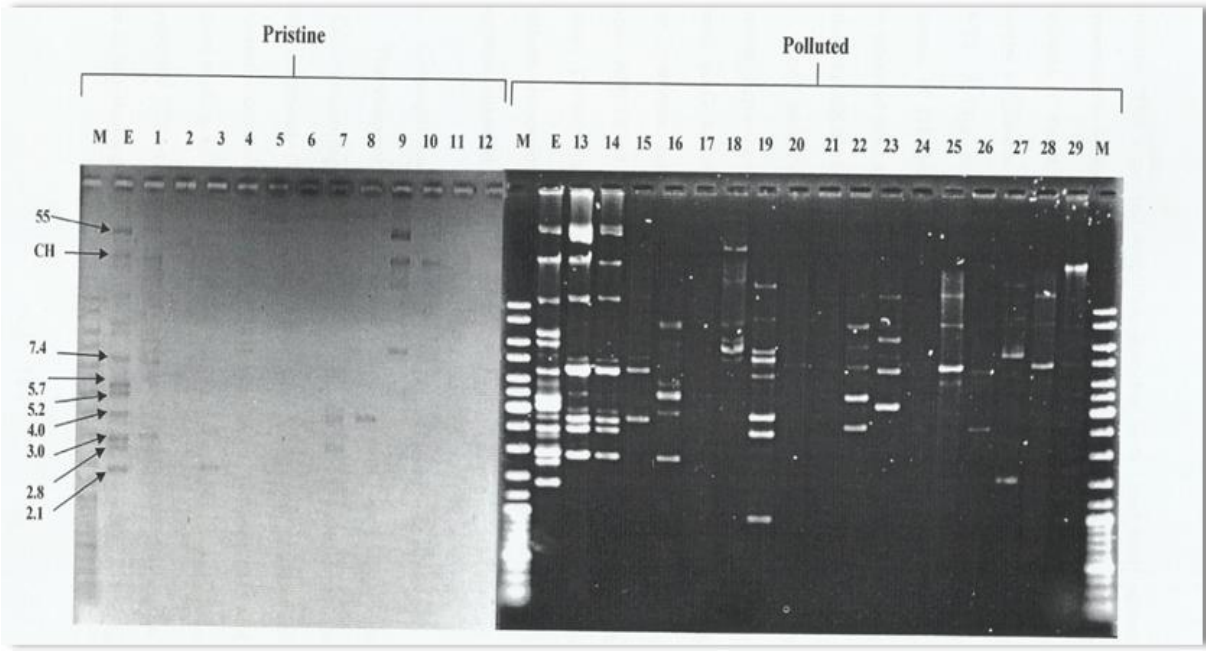

Figure 4 Plasmid profile of some bacteria isolates from pristine and petroleum hydrocarbon polluted ecosystems (M=100bp DNA ladder, E= *E.coli* CV517, 1=ITSS, 2=CRS12, 3= CRS3, 4=CRS8, 5=CRW1, 6=CRW2, 7=EEWI, 8=ITS3, 9=ITSI, 10=GENOMIC DNA, 12=NSS2, 13=GI, 14=G2, 15=G6, 16=F3, 17=E14, 18=G13, 19=G5, 20=HO6W5, 21= HO6W6, 22=G7, 23=E4, 24=F12, 25=FIS5, 26=A10, 28=F11, 29= genomic DNA)

isolates Gl and G2 from petroleum hydrocarbon polluted soil and IncP plasmid replicon in isolates ITS5 and ITS1 from pristine sediments. The expected amplicon size for IncN and IncP plasmid replicons was approximately 559bp and 534bp, respectively. These isolates' IncN and IncP plasmid amplicon sequences (Gl, G2, ITS5, and ITS 1) were analyzed by performing Clustalw alignment and comparing the aligned sequence to the NCBI database using BLAST. A high degree of similarity (100%) was observed between the IncN plasmid sequence of Gl (pCHNGl) and G2 (pCHNG2) and that of completely sequenced IncN plasmid-pNL 194 of *Klebsiella pneumoniae* strain NL194 (GenBank accession number: GU585907.1). IncP plasmid sequence of isolates-ITSl and ITS5 showed 99% similarity to complete sequences of *Salmonella enterica subsp. enteric serovar* Dublin strain 853 plasmid pSD_88 (GenBank accession number: JF267652.1), *Salmonella enterica subsp. enterica serovar Typhimurium* plasmid pYTl DNA (GenBank accession number AB576781.1) uncultured bacterium plasmids PSP21 (GenBank accession number: CP002153.1), PB11 (GenBank accession number: CP002152.1), PB5 (GenBank accession number: CP002151.1) and partial sequence of *Pseudomonas aeruginosa* plasmid R1033 (GenBank accession number: HM804085.1).

3.5 Conjugal transfer of antibiotic resistance and class 1 integron

The fingerprints of suspected transconjugants are presented in Figure 5; Gl and G2 transconjugant fingerprints (Lane 4 and 5) were similar to that of *E. coli* CV601 recipient (Lane 2), indicating that there was a transfer of ampicillin and sulphamethoxazole/trimethoprim resistance from Isolates Gl and G2 to *E. coli* recipient leading to its survival in the presence of selective antibiotics. The fingerprint of suspected ITS 1-transconjugant (Lane 3) differed from that of the *E. coli* recipient (Lane 2) but showed semblance to isolate ITS1 (Lane 2) fingerprint, indicating no conjugal transfer of ampicillin and trimethoprim/sulphamethazole resistance between ITS1 and *E. coli* recipient. Conjugal transfer of class 1 integron from isolates G1 and G2 to *E. coli* recipient was also observed. The expected amplicon size of approximately 280bp was detected in the transconjugants but not in the *E coli* recipient. This signifies the conjugal transfer of class 1 integron, which could be plasmid-borne. As analyzed, the transconjugants' class 1 integron variable region showed the presence of a 1.5 kbp region. It shows the presence of transferred high molecular weight plasmid of approximately 55kbp in G1 and G2-transconjugants (Lane 3 and 5) as compared to plasmid profiles of donors (Gl= Lane 2 and G2= Lane 4) with seven plasmids a piece. The transconjugants' plasmids were further screened to detect the plasmid of the incompatibility group N (IncN). The expected amplicon size of 559bp was detected in transconjugants indicating that the IncN plasmids of donor strains (G1 and G2) were transferred via conjugation. Restriction analysis of the transconjugants plasmids showed identical restriction profiles.

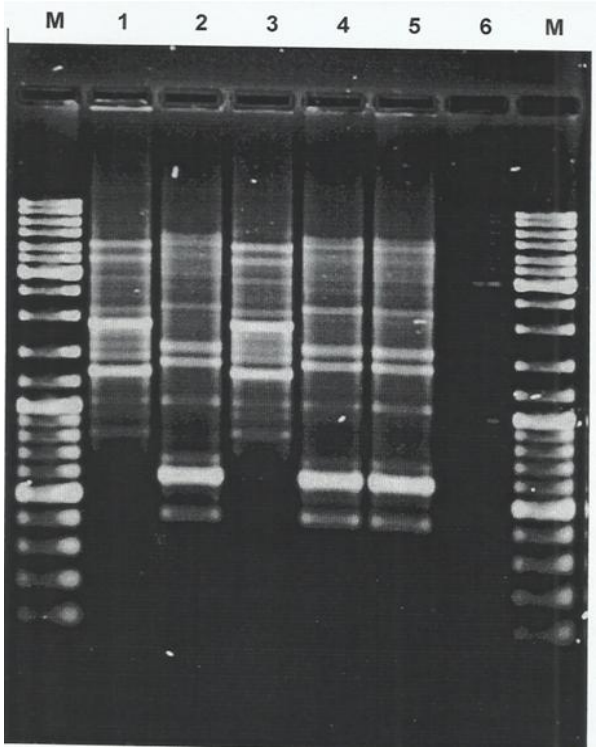

Figure 5 Rep-PCR fingerprint showing conjugal transfer of antibiotics resistance (M=DNA ladder, 1=ITSI, 2=*E.coli* recipient, 3=suspected ITSI= transconjugant, 4= G1- transconjugant, 5=G2- transconjugant, 6=negative control).

4 Discussion

Plasmid incidence in the multiple heavy metal tolerant bacterial isolates obtained from petroleum hydrocarbon polluted ecosystems was higher than their counterpart from the pristine (without pollution) ecosystem, but this difference was not statistically significant. A highly positive but non-significant correlation was observed between the incidence of plasmid and heavy metal tolerance among the bacterial isolates isolated from polluted ecosystems. This insignificant increase and correlation could be due to a mixed population of diverse bacteria genera being studied. Plasmid incidence and the deposal of environmental pollutants have been evaluated and correlated by several authors at various experimental sites. Ndeddy Aka and Babalola (2017) found no significant difference in bacterial plasmid incidence between polluted and unpolluted sites but reported an increase in the frequency of catabolic plasmids in *Pseudomonas*-like isolates in polluted marine and freshwater ecosystems than in unpolluted ecosystems. Similar observations were reported by Epelda et al. (2015) that *Vibrio* spp. from oil-polluted water had a higher incidence of plasmid-bearing strains than isolates from unpolluted water. A similar difference between bacteria isolated from toxic waste-contaminated water and bacteria isolated from either uncontaminated or domestic sewage-affected waters was reported by Tan et al. (2018). The increased incidence of plasmid among the selected bacteria from polluted ecosystems obtained in this study is not significant but suggests plasmid-mediated adaptation in the polluted ecosystems.

On the contrary, Class 1 integron incidence was significantly higher in heavy metal tolerant bacterial isolates from polluted ecosystems than those from the pristine ecosystem. This incidence correlated with the ability of these isolates to tolerate multiple heavy metals. Interestingly, bacterial isolates that harbored both plasmids and class 1 integron were significantly higher in polluted than in pristine samples, suggesting the possibility of the class 1 integron being carried on the plasmid. The diversity of integrons and integron-transferred genes in heavy-metal-contaminated mine tailings has been studied by Nemergut et al. (2004). In their study, they sequenced a gene that codes for a step in a pathway for nitro-aromatic catabolism, a group of compounds associated with mining activity. This implies that integrons may serve a significant purpose during gene transfer in response to selective environmental pressures other than the availability of antibiotics. Significantly high incidence of class 1 integron and its correlation with multiple heavy metal tolerance in bacteria from polluted ecosystems suggest a possible role of gene transfer in response to petroleum hydrocarbon pollution with concomitant heavy metal contamination. The abundance of class 1 integrons and plasmids in this study, confirms the influence of industrial pollution on the mobile genetic elements.

The low incidence of plasmids and integrons, as seen amongst bacteria from the pristine ecosystem, could not be associated with these isolates' heavy metal and multidrug drug resistance profiles. This suggests that other resistance determinants, such as the efflux pump, could have been responsible for the resistance recorded. Bratu et al. (2008) and Nemec et al. (2007) reported the role of efflux pumps in conferring multidrug resistance to gram-negative bacteria. Other works also confirm that efflux pumps confer resistance against many antibacterial agents, including beta-lactams, aminoglycosides, tetracyclines, trimethoprim, fluoroquinolones, and chloramphenicol (Su et al. 2005). Amplified class 1 integron variable region showed great diversity between isolates from pristine and petroleum hydrocarbon-polluted ecosystems. Variable region size diversity for isolates from pristine ecosystems was 0.7kbp to 2.0kbp, while for isolates from polluted ecosystems, the size varied between 0.7kbp and 6.0kbp. This suggests differences and diversity in types of heavy metal tolerance and antibiotic resistance coding gene cassettes in the studied isolates. Li et al. (2017) proposed that class 1 Integrons comprise conserved and stable variable regions, with resistance genes transferred more often as part of the entire integron structure than as individual cassettes. The high prevalence of large class 1 variable region sizes among isolates from petroleum hydrocarbon polluted ecosystems suggests the presence of complex gene cassettes possibly attributable to the complex nature of pollutants in the ecosystems.

Plasmid analysis showed the presence of multiple plasmids in both isolates from pristine and petroleum-polluted ecosystems, but the prevalence was higher among bacterial isolates from petroleum hydrocarbon-polluted ecosystems. Multiplasmid bacterial strains were first described for clinical isolates (Chen et al. 2018). Subsequently, various environmental strains with a high incidence of different plasmids were isolated (Gati et al. 2016). A significant proportion of plasmids ranges from different sizes, copy numbers, and genetic equipment in a bacterium might increase its fitness (Kado, 1998) by accepting adaption to special ecological zones such as petroleum hydrocarbon pollution with concomitant-heavy metal contamination. It is well known that clinical isolates of *E. Coli* usually possess multiple plasmids with different sizes due to exposure to various antibiotics in the treatment process (Jan et al., 2009). Chen et al. (2015) correlate the presence of multiple plasmids in a bacterial strain to the prolonged use of antibiotics. In this study, multiple plasmids in bacterial isolates from polluted ecosystems could be attributed to the long-term exposure of these bacterial isolates to petroleum hydrocarbon and heavy metal pollutants in the environment. Plasmid replicons typing PCR revealed the presence of IncN plasmid replicons in isolates G1 and G2 from petroleum hydrocarbon-polluted soil sample and IncP plasmid replicons in isolates ITS1 and ITS5 from pristine sediment sample. Analysis of the IncN plasmid sequence of G1 (pCHNGl) and G2 (pCHNG2) showed maximum similarity between them and also showed similarities with the IncN plasmid- pNL194 of *Klebsiella pneumonia* strain NL194 (GenBank accession number: GU585907.1). Incompatibility group N plasmid (IncN) is one of the most frequently encountered resistance plasmid types in *Enterobacteriaceae* of human and animal origin (Carattoli 2009). IncN plasmids have been associated with genes conferring resistance to a variety of antibiotics among pathogenic *Klebsiella pneumoniae*, *K. oxytoca*, and *E. coli* strains worldwide (Novais et al. 2007; Shen et al. 2008; Carattoli 2009; Diestra et al. 2009; Gootz et al. 2009; Bortolaia et al. 2010; Cullik et al. 2010; Poirel et al. 2011). This study is the first report on the presence of IncN plasmid in bacterial isolates in Nigeria, particularly from petroleum hydrocarbon polluted ecosystem. The 16S rRNA sequence analysis of isolates G1 and G2 identified preliminary as *Klebsiella* spp using phenotypic and biochemical characteristics showed 99% similarity to the complete sequence of *K. pneumoniae* strain DSM 30104 (GenBank accession number. NR_036794.1). This also further confirms the prevalence of IncN plasmid type in *Enterobacteriaceae.* Though isolates G1 and G2 were multiplasmidic (with seven plasmids a piece), we observed that G1 and G2 transconjugants showed the presence of high molecular weight plasmid of approximately 55kbp which encoded resistance to ampicillin and trimethoprim/sulphamethazole and tolerance to heavy metals (Cd, Ni, and Cu). Analysis of the G1 and G2 transconjugants for IncN plasmid and class 1 integron showed their presence and further confirmed earlier suggestion of plasmid-borne class 1 integron among isolates from petroleum hydrocarbon polluted ecosystems.

Conclusion

Results of the study can be concluded that petroleum hydrocarbon pollution vis-a-vis heavy metals have selective pressure with the abundance of mobile genetic elements amongst isolates from polluted ecosystems could contribute to the dissemination of antibiotic resistance genes, thus posing a severe public health concern.

Declaration

The article's preprint is available on Research square with DOI: https://doi.org/10.21203/rs.3.rs-1771685/v1. The authors declare that this is their original article, and they have taken consent from the Research square team to publish this article in the journal entitled "Journal of Experimental Biology and Agricultural Sciences eissn 2320-8694". If any conflict of interest or plagiarism arises by anybody, journal management can retract this article.

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Ethical Approval

No ethical approval was obtained for the research.

Consent to Participate

This research was a Ph.D. dissertation in which the supervision was carried out among the supervisors (Prof. S.P Antai and Dr. G.D Iwatt).

Authors Contributions

Prof. S.P Antai and Dr. G.D Iwatt designed the Topic and Methodology, while Dr. Agbor R.B, and Dr. Ubi S. E. did the lab work and wrote the manuscript.

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Competing Interests

No competing interests.

Reference

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