JJBS

Volume 11, Number 4,September 2018
ISSN 1995-6673
Pages 361 - 368

Jordan Journal of Biological Sciences

The Correlation between Plasmid and Metal and Multi-Drug Resistance in Bacteria from Streams Impacted by Husbandry and Wastewaters in Akure, Nigeria

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Received November 30, 2017; Revised January 10, 2018; Accepted January 29, 2018

Abstract

Freshwaters are continuously subjected to natural and anthropogenic influences including heavy metals and antibiotic wastes that do not only pose a high level of multigenetic adaptations in microorganisms, but also forecast life-threatening consequences such as chronic public health risks and ineffective disease managements in human and veterinary medicines. The current study investigated bacterial isolates from surface waters and profiled them for metal, antibiotic resistance and plasmids. Antibiotics' susceptibility test was carried out using the disk diffusion technique and the metal resistance test by incorporating different concentrations of Zn, Fe, Cd and Ni solutions into Mueller-Hinton agar plates. The plasmid extraction and electrophoresis of the DNA were carried out on 0.8 % agarose gel in a 0.5X concentration of Tris-Borate-EDTA (TBE) buffer. To demonstrate the role of plasmid in resistance, the isolates were subjected to plasmid curing using sodium dodecyl sulfate (SDS). The identities of the isolates revealed by the 16S rRNA sequencing analysis are: *Proteus mirabilis*, *Alcaligenes faecalis*, *Alcaligenes faecalis faecalis*, *Bacillus cereus*, *Stenotrophomonas acidaminiphila*, *Proteus penneri*, *Lysinibacillus macrolides* and *Serratia* sp. Results revealed that all the isolates were multidrug-resistant to the different antibiotics tested, and that 62.5 % of the isolates were found to harbor plasmids. Multi-resistance to the antibiotics was conservatively plasmid-borne or chromosomally-mediated. Multi-resistance to the antibiotics was conservatively plasmid-borne or chromosomally-mediated. Multiple broad spectrum antibiotic resistance indices of the isolates ranged from 0.357 to 0.786. Plasmid-mediated heavy metal resistance was recorded in *A. faecalis faecalis, P. mirabilis and B. cereus* to varying metals concentration, while heavy-metal-multi-resistance was chromosomally-mediated in *P. penneri, S. acidaminiphila* and *A. faecalis faecalis* to all the metals. The study forewarns against the health risks of contracting surface water bacteria especially by the users of the water resources and through food chains.

Keywords: Surface water, Bacteria, Multi-drug resistance, Metal resistance, Plasmid.

1. Introduction

The inaccessibility to wholesome sources of water supply has necessitated the use of freshwaters in most regions of the world especially developing countries. In the recent past, the expanding human population, industrialization, intensive agricultural practices and discharges of massive amounts of wastewater into the rivers and streams have resulted in the deterioration of water quality (Nurcihan and Basaran, 2009).

The impact of these anthropogenic activities has been so extensive that water bodies have lost their self-purification capacity to a large extent (Sood *et. al*., 2008).

According to Shoeb (2006), the main sources of heavy-metal pollution in aquatic bodies are usually linked with areas with congested industries and high automobile use. Heavy metals as natural components of the earth's crust are increasingly found in microbial habitats due to several natural and anthropogenic processes. However, microbes have developed mechanisms to tolerate the presence of heavy metals either by efflux, complexation or reduction of metal ions, or through using them as terminal electron acceptors in anaerobic respiration (Mgbemena *et al.*, 2012).

Human and animal wastes serve as major sources of entry of pathogens into surface waters. Entry of pathogens into rivers can occur through rainwater surface run-offs, storm sewer spillages or overflow, discharge of untreated or partially-treated effluents from wastewater treatment plants (Petersen *et al.*, 2005; Donovan *et al.*, 2008). Nigoyi, (2005) stated that the impact of water pollution on human health depends mainly on the water uses, as well as the concentration of pathogens in the water.

Due to the overuse of antibiotics to treat microbial infections, microorganisms are becoming highly resistant to the available antibiotics. The uncontrolled or inappropriate use of antibiotic drugs in both industrialized and developing countries have contributed to the rapid development of resistance in pathogenic microbes.

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Plasmids are extra chromosomal DNA molecules. They are circular DNA molecules that replicate independently of the bacterial chromosome (Wang *et al*., 2011; Adeyemo *et al*., 2015).

The plasmids that carry genes conferring on the host cell resistance to antibiotics are called as 'R factors.' R factors are encountered in certain strains of almost all pathogenic bacteria. These R factors can be transferred to other bacteria in the environment and these extrachromosomal DNA sequences are responsible for the emergence of multiple drug resistant (MDR) strains (Barrow *et al.,* 1986).

Studies have shown the growing concerns regarding water borne diseases as a result of human interaction with surface water. In the report of Bolaji *et al.,* (2011), the greatest threat to the use of antibiotics is the emergence and spread of resistance in pathogenic bacteria that consequently cannot be treated by previously successful regimens. Available therapeutic options for antibiotic resistant organisms are severely limited, as these organisms frequently display a multidrug resistant (MDR) phenotype (Okonko *et al.,* 2009). It is thought that a correlation exists between metal tolerance and antibiotic resistance in bacteria as Mgbemena *et al.,* (2012) and Amalesh *et al.,* (2012) have previously isolated and investigated multidrug resistance and metal resistance in bacteria from municipal waste.

The issue of the emergence of the resistance phenomenon in bacteria justifies research on the multidrug and metal resistance of bacteria to serve as a pathway to the development of new possible anti-infective agents or resistance modifiers, and also give a lead on the ability of some bacteria that have potentials to act as a bioremediating agent in metal- polluted environments.

2. Materials and Methods

2.1. Surface Water Bacterial Isolates

Eight isolates previously isolated from surface waters in an earlier investigation were selected for this study among stocked cultures. The selected surface waters (FUTA and Onyearugbulem streams) have been highly impacted with husbandry and abattoir wastewater effluents due to location of major city abattoirs 10m away from the streams. The isolates have shown varying degrees of resistance to conventional antibiotics (data not shown).

2.2. Resuscitation and Presumptive Identification of Surface Water Isolates

The bacterial isolates were sub-cultured onto a freshly prepared nutrient agar plates, and were incubated at 37^{o}C for twenty-four hours. Pure culture of the isolates was further confirmed before preservation on agar slants at 4^{o}C for further analysis (Cheesebrough, 2006).

2.3. Molecular Characterization of the Isolates

A PCR-based characterization of the isolates was carried out. Genomic DNA of the isolates was extracted and purified according to Gurakan *et al.,* (2008). DNA concentration was then estimated spectrophotometrically by comparing band intensities with the ladder of known concentration on agarose gel. Also, the 16S rRNA gene of the isolates was amplified as described by Sambrook *et al.,* (2001) using the primer pair 27F-5′-AGAGT TTGATCCTGGCTCAG-3′, and 1492R 5′GGTTACC TTGTTACGACTT-3′.

2.4. Sequencing and Analysis of 16SrRNA Gene Report

The 16SrRNA gene amplified products were purified with Exo sap, and were then sent for DNA Sanger sequencing. The sequence data were analysed using the ABI Sequencing Analysis software (version 5.2), and were subjected to the basic local alignment search tool on the NCBI Genbank website (www.ncbi.nlm.nih.gov). Nucleotide sequences generated from each amplified 16S rRNA gene were submitted to the Genbank Nucleotide Sequence Database.

2.5. Plasmid Extraction

Plasmid extraction was carried out using the method described by Ojo and Oso (2009). Pure isolates were inoculated on tars broth and were incubated. The grown cells were harvested and suspended in 200 µL of solution A [100 mm glucose-50 mm tris hydrochloride (pH8)-10mm EDTA containing 10mg tanolysin and were incubated for thirty minutes at 37ºC in a dodecyl sulphate; 0.2 µL NaOH was added, and the samples were mixed by inverting the tubes. Exactly 300µL of a 30 % potassium acetate solution (pH 4.8) was added, and the samples were mixed by vortex. The supernatant was removed, extracted once with a phenol – chloroform mixture (1:1), and precipitated with an equal volume of isopropanol. The plasmid DNA was then dissolved in 100 µL of tris-EDTA buffer. Electrophoresis of the DNA was carried out on a 0.8 % agarose gel in a 0.5×concentration of trisborate-EDTA buffer. After boiling, the solution was allowed to cool; 10 µL of ethidium bromide was added to the cooled agarose solution. This was poured into a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 30 minutes, and the comb was carefully removed. Exactly 20 µL of the plasmid DNA samples were then loaded into the wells after mixing with 2 µL of bromophenol blue. The gels were thereafter electrophoresed in a horizontal tank at a constant voltage of 60V for about 90 minutes. After electrophoresis, plasmid DNA bands were viewed by the fluorescence of bound ethidium bromide under a short wave ultraviolet light transilluminator, and the photographs were taken using a digital camera.

2.6. Plasmid Curing

The plasmids were cured by treatment with sodium dodecyl sulphate (SDS) according to the method of Al Sa'ady *et al.,* (2014). The nutrient broth was prepared and supplemented with 3.0 % concentration of SDS. An overnight culture of the bacteria cultures was added into the curing agent supplemented broth. The samples were then incubated at 37 °C for 24 hours under constant agitation. The isolates were subcultured onto Mueller Hinton agar, and the plasmid extraction was repeated on the microorganisms to verify if the plasmids were successfully cured.

2.7. Metal and Multidrug Profiling

2.7.1. Determination of Antibiotics Resistance

The disk diffusion technique was employed for antibiogram testing (Willey *et al.* 2008). Standardized

inoculum size (0.5 McFarlands) corresponding to 1.5 x 10^8 CFU/mL of both wild and the plasmid-cured isolates was swabbed onto Mueller Hinton agar (Biolab) plates to give rise to uniform bacterial lawn. Afterward, antibiotic discs were positioned on the plates, and were incubated at 37^{o}C for 24 hours. Thereafter, the discs were observed for zones of inhibition, and the diameter of the zones was recorded (Cheesebrough, 2006). Antibiotics panels used were primarily broad spectrum antibiotics (BSAs) namely, ofloxacin (10 µg), streptomycin (30 µg), cotrimoxazole (30 µg), chloramphenicol (30 µg), sparfloxacin (10 µg), ciprofloxacin (10 µg), amoxicillin (30 µg), amoxicillin/clavulanate potassium (30 µg), gentamicin (10 µg), erythromycin (15 µg), pefloxacin (30 µg), ampiclox (30 µg), cefuroxime(25 µg), and ceftriaxone (25 µg). The susceptibility profiles of the isolates were defined according to the CLSI standards (CLSI, 2017). Intermediate-resistance was regarded as resistance. An isolate was designated as multidrug resistant (MDR) if found resistant to ≥3 structurally different antibiotic classes (Oteo, 2002).

2.7.2. Multiple Broad Spectrum Antibiotics Resistance Indexing of Surface Water Isolates

Multiple broad spectrum antibiotic resistance index (MBSARI) was estimated as the ratio of the number of BSAs to which a surface water isolate was resistant to the sum of all BSAs panel to which the isolate was subjected.

2.7.3. Determination of Metal Resistance

The isolates (wild and cured) were subjected to different metal concentrations (0.001 to 1M) according to De Magalhaes *et al.* (2008) and Mohania *et al.* (2008). Briefly, appropriate dilutions (0.001M, 0.01M, 0.1M and 1M) of iron, zinc, cadmium and nickel were incorporated into Mueller-Hinton agar plates. Then, the isolates were inoculated and incubated in the plates at 37^{o}C for 48 hours. Finally, the test isolates were observed for metal susceptibility.

2.8. Data Analysis

The data obtained were subjected to analysis of variance (ANOVA), and treatment means were separated using Duncan's New Multiple Range Test at 95 % confidence level with the aid of SPSS (version 23).

3. Results

The correlations between plasmids and multidrug and metal resistance in surface water isolates were investigated. The outcome of 16S rRNA sequencing analysis revealed the identities of the isolates as *Proteus mirabilis*, *Alcaligenes. faecalis*, *Alcaligenes faecalis faecalis*, *Bacillus cereus*, *Stenotrophomonas acidaminiphila*, *Proteus penneri*, and *Lysinibacillus macrolide* sunder the accession numbers KY345400 to KY345404from the sites. Figure 1 represents the plasmid profile obtained after gel electrophoresis. *P. mirabilis* has a plasmid of 2kb pair, *A. faecalis* has a plasmid of 9kb pair, *Serratia* sp. has 2kb pair, *L. macroides* has a plasmid of 9kb, *B. cereus* has two plasmids of 4kb and 6kb pair. *S. acidaminiphila*, *A. faecalis faecalis* and *P. prennei* showed no band after electrophoresis indicating the absence of plasmid.

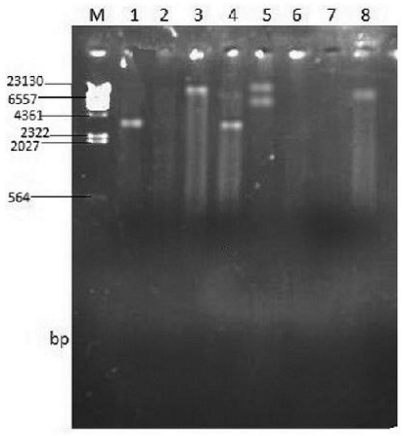

Figure 1. Gel plate showing the Plasmid profile of bacterial isolates before curing (1 – 8 = *P. mirabilis, S. acidaminiphila, A. faecalis, Serratia sp., B. cereus, P. penneri, A. faecalis faecalis* and *L. macroide*)

Figure 2 represents the plasmid analysis after the curing procedure. It was observed that there was no visible band showing that the bacteria have been effectively cured.

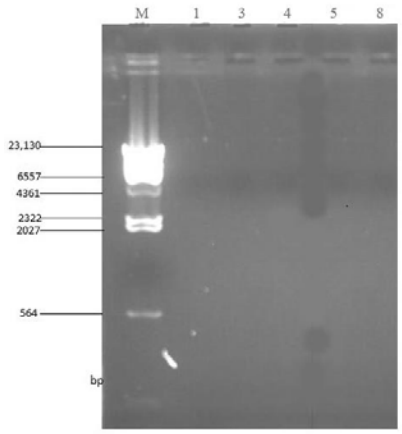

Figure 2. Gel plate showing the Plasmid profile of bacterial isolates after curing (1 – 8 = *P. mirabilis, S. acidaminiphila, A. faecalis, Serratia spp., B. cereus, P. penneri, A. faecalis faecalis* and *L. macroide*)

The antibiogram patterns of the wild and subsequent plasmid-cured isolates are presented in Tables 1 and 2. It showed that some of the microorganisms were multidrug resistant as they exhibited varying degrees of resistance to the antibiotics as follows:- *A. faecalis* (50 %), *P. mirabilis* (57.1 %), *Proteus penneri* (42.85 %), *B. cereus* (28.57 %), *A. faecalis faecalis* (42.86 %), *Serratia* sp. (64.3 %) and *L. macroides* (64.3 %); to the fourteen antibiotics tested and after the curing, *P. mirabilis* lost all its resistance, *A. faecalis* resistance to the antibiotics was reduced to 14.3 %, *P. penneri* retained its resistance, *B. cereus* resistance to the antibiotics was reduced to 21.4%, *A. faecalis faecalis* retained its resistance, *Serratia* sp. resistance to the antibiotics was reduced to 35.7 % and *L. macriodes* resistance to the antibiotics was reduced to 35.7%. The results of the metal resistance test shown in Tables 4 and 5 indicate that there was a decline in the growth of the organisms as concentrations of the metals increased in contrast to the situation in the control i.e., 0.0M of the metals, whereas there was a profuse/heavy growth by all

the organisms except for *Proteus mirabilis* that did not grow at 0.0M cadmium. At 0.001M to 0.1M of nickel concentration, there was growth of all the microorganisms. There was heavy growth of all the microorganisms at a concentration of 0.001 to 0.01M of zinc; however, *B. cereus* still exhibited heavy growth at a 0.1M concentration of zinc. All organisms resisted iron to a concentration of 0.01M at varying degree as expressed by the magnitude of their growth. For the cadmium metal, the organisms showed resistance at a minimal concentration of 0M. After the curing, *A. faecalis* and *P. mirabilis* lost all ability to resist metals; *Bacillus cereus* also retained its ability to resist the metals it previously resisted except cadmium at the same concentration before the curing.

The freshwater isolates foreshadowed a grave and higher risk in terms of multiple broad spectrum antibiotic resistance indices. The MBSARI (%) varied with the species from *B. cereus* (35.7), *A. faecalis* (42.9), *P. mirabilis* (50.0), *P. penneri* (57.1), *S. acidiminiphila* (57.1), *A. faecalis faecalis* (71.4), *L. macroides* (71.4) to *Serratia* sp (78.6) before the curing as shown in Table 3.

Table 1. Antibiotics sensitivity of bacterial isolates before the curing

Antibiotics	*P. mirabilis*	*B. cereus*	*P. penneri*	*Alcaligenes faecalis*	*S. acidiminiphila*	*Alcaligenes faecalis faecalis*	*Serratia* sp.	*L. macroides*
Perfloxacin	23±1.5	23±1.5	0±0.0	21±0.5	23±0.5	18±1.0	0±0.0	0±1.0
Erythromycin	0±0.00	18±1.5	0±0.5	13±1.0	22±2.0	10±1.5	0±1.0	22±1.5
Cotrimoxazole	0±0.00	20±2.0	0±1.5	0±5.0	0±0.0	0±0.0	0±4.0	0±1.0
Streptomycin.	0±0.0	22±0.5	0±1.0	13±4.5	0±1.5	0±0.0	0±1.5	0±0.0
Ciproflaxin	25±2.0	23±0.0	16±3.0	20±2.0	20±2.0	24±2.0	19±0.0	0±0.0
Ceftriaxone	18±2.5	19±1.0	12±4.0	15±2.5	20±0.0	23±0.0	0±0.0	22±2.0
Amoxicillin	21±1.5	22±0.0	19±1.5	16±3.0	0±0.0	0±0.0	14±1.5	0±0.0
Cefuroxime	0±0.5	0±0.0	11±1.5	0±0.0	0±1.0	12±0.0	0±1.0	18±1.5
Ampliclox	0±0.0	0±0.0	0±0.0	0±0.0	0±1.5	8±2.5	0±0.0	24±2.5
Gentamycin	0±0.0	0±0.0	10±1.5	0±1.5	0±2.5	0±0.0	0±0.0	0±1.0
Ofloxacin	18±0.5	18±0.5	0±1.5	0±0.0	18±1.5	16±0.0	15±1.5	0±1.5
Sparfloxacin	16±1.5	20±1.5	20±2.0	11±0.0	20±2.0	21±1.5	12±0.0	0±0.0
Amoxicillin/clavu-lanate potassium	0±0.0	0±0.5	23±0.0	0±1.5	0±1.5	0±0.0	0±0.0	0±0.0
Chloramphenicol	18±0.0	0±1.0	22±0.5	0±2.0	14±2.0	0±0.0	0±1.5	8±0.5

Table 2. Antibiotics sensitivity of the five bacterial isolates with plasmids after the curing

Antibiotics	*P. mirabilis*	*B. cereus*	*Alcaligene faecalis*	*Serratia* sp.	*L. macroides*
Perfloxacin	23±0.0	23±1.0	21±1.0	21±1.0	18±0.0
Erythromycin	15±4.0	18±0.0	14±1.0	16±0.5	20±1.0
Cotrimoxazole	20±1.5	23±0.5	17±0.0	0±1.5	0±0.0
Streptomycin	20±3.0	23±1.0	20±0.5	0±0.5	12±0.5
Ciproflaxin	25±1.0	23±0.0	20±0.0	21±0.0	21±1.0
Ceftriaxone	20±1.5	19±0.5	20±1.5	18±3.5	22±1.0
Amoxicillin	21±1.0	22±1.5	16±1.0	18±1.0	0±0.5
Cefuroxime	16±3.5	0±0.0	18±0.5	16±1.0	18±0.5
Ampliclox	21±0.0	0±0.0	0±2.0	0±0.0	24±0.5
Gentamycin	20±1.5	0±0.0	0±0.0	0±0.5	0±0.0
Ofloxacin	18±1.0	18±0.5	9±0.5	15±2.0	0±0.0
Sparfloxacin	16±2.0	20±0.0	11±0.0	12±0.0	17±1.0
Amoxicillin/ Clavulanate potassium	15±0.0	0±0.5	16±1.5	18±0.0	0±0.0
Chloramphenicol	15±1.0	18±1.0	18±0.0	0±0.0	12±0.5

Table 3. Multiple broad spectrum antibiotic resistance index (MBSARI) of surface water bacteria

Bacteria	MBSARI	MBSARI (%)
P. mirabilis	0.500	50.0
B. cereus	0.357	35.7
P. penneri	0.571	57.1
A. faecalis	0.429	42.9
S. acidiminiphila	0.571	57.1
A. faecalisfaecalis	0.714	71.4
L. macroides	0.714	71.4
Serratia sp.	0.786	78.6

Table 4. Resistance of bacterial isolates to heavy metals

Heavy	Bacterial isolates	Heavy metal concentration			
		0.001M	0.01M	0.1M	1M
Nickel	*P. mirabilis*	+	--	--	--
	A.faecalis faecalis	++	++	--	--
	A. faecalis	+++	+	--	--
	S. acidaminiphila	++	++	--	--
	P. penneri	++	+	--	--
	B. cereus	+++	++	--	--
	L.macroides	+++	++	--	--
	Serratiasp	+++	++	--	--
Cadmium	*P. mirabilis*	+	--	--	--
	A. faecalis faecalis	++	++	--	--
	A. faecalis	+	+	--	--
	S. acidaminiphila	+	+	--	--
	P. penneri	++	+	--	--
	B. cereus	+	+	--	--
	L.macroides	++	+	--	--
	Serratiasp	++	+	--	--
Zinc	*P. mirabilis*	+++	+++	--	--
	A. faecalis faecalis	+++	+++	--	--
	A. faecalis	+++	+++	--	--
	S. acidaminiphila	+++	+++	--	--
	P. penneri	+++	+++	--	--
	B. cereus	+++	+++	++	--
	L.macroides	+++	+++	--	--
	Serratiasp	+++	+++	--	--
Iron	*P. mirabilis*	+++	+++	--	--
	A. faecalis faecalis	+++	++	--	--
	A. faecalis	+++	++	--	--
	S. acidaminiphila	++	+	--	--
	P. penneri	+++	+++	--	--
	B. cereus	+++	+++	--	--
	L.macroides	+++	+++	--	--
	*Serratia*sp	+++	+++	--	--

Keys +++ = Heavy growth, ++ = Moderate growth, + = Scanty growth, -- = No growth

Table 5. Resistance of bacterial isolates to heavy metals.

Heavy	Bacterial isolates	Heavy metal concentration			
		0.001M	0.01M	0.1M	1M
Nickel	*P. mirabilis*	--	--	--	--
	A. faecalis	--	--	--	--
	B. cereus	++	+	--	--
	L.macroides	--	--	--	--
	*Serratia*sp	++	+	--	--
Cadmium	*P. mirabilis*	--	--	--	--
	A. faecalis	+			
	B. cereus	--	--	--	--
	L. macrolides	--	--	--	--
	Serratiasp	--	--	--	--
Zinc	*P. mirabilis*	++	+	--	--
	A. faecalis	--	--	--	--
	B. cereus	--	--	--	--
	L. macrolides	+	+	--	--
	*Serratia*sp				
Iron	*P. mirabilis*	--	--	--	--
	A. faecalis	++	++	--	--
	B. cereus	+	+	--	--
	L.macroides	+	+	--	--
	*Serratia*sp	++	++	--	--

Keys +++ = Heavy growth, ++ = Moderate growth, + = Scanty growth, -- = No growth

4. Discussion

This study identified bacterial isolates obtained from Onyearubulem and FUTA streams in Akure. The correlation between multidrug and metal resistance were determined and further profiled for plasmids and plasmid curing was carried out. The isolates obtained and identified by the 16S rRNA sequencing included *Proteus mirabilis, Alcaligenes faecalis, A. faecalis* sub *faecalis, Bacillus cereus, Stenotrophomonas acidaminiphila, Proteus penneri* and *Lysinibacillus macroides*. Pindi *et al*., (2013) reported the isolation of these bacteria from drinking water samples as potential pathogenic microorganisms. The isolation of *Alcaligenes faecalis faecalis, Proteus penneri, Proteus mirabilis* is in agreement with the investigation of Scot *et al.* (2003) and Nigoyi *et al.* (2005) who reported faecal contaminants as the major sources of microbial entry into surface waters. Okonko *et al.* (2008) also reported that the presence of coliforms in the water samples generally suggests that a certain portion of the water may have been contaminated by faeces of either human or animal origin. The presence of *B. cereus, L. macroides* and *S. acidaminiphila* also supports the fact that non-point source of water pollution such as run-offs from the surrounding environment is a cause of pollution of the surface water (Petersen *et al.*, 2005, Donovan *et al.*, 2008). These microorganisms are known to be majorly associated with the environment close to the surface water; the proximity of the surface water and the environment allows for the run-off of water which allows for the transfer of microorganisms from the environment to the water;

Kampfer *et al.* (2013) had previously isolated members of *Lysinibacillus* from surface water.

The isolates showed varying degrees of antimicrobial resistance to the twelve conventional antibiotics tested. *A. faecalis faecalis* showed 42.85 % resistance, *S. acidaminiphilia* showed 50 % resistance, *Proteus mirabilis* showed 57.1 % resistance, *Proteus penneri* showed 50 % resistance, *B. cereus* showed 35.7 %, *A. faecalis* showed 50 % resistance, *Lysinibacillus macrolides* showed 64.3 % resistance, and *Serratia* sp. showed 64.3 % resistance to the antibiotics tested.

During this study, it was observed that a higher level of multidrug resistance was exhibited by the Gram negative bacteria than their Gram positive counterpart; this is in consonance with Ronald *et al.*, (2002) who found out that a high level of antibiotic resistance was seen in all studied Gram negative bacteria isolated from the rivers of United States.

The resistance level of microorganisms to heavy metals was measured by turbidity in the growth media. Badar *et al*. (2000) stated that the microbial load decreases with an increase in the concentration of heavy metals indicating the toxic effect of the heavy metals on the growth of microorganisms. On all the heavy metals (Cd, Ni, Zn and Fe) tested there was no observable growth of microorganisms at the highest concentration (1.0M). This supports the theory stated by Konopka *et al.* (1999) that a lethal toxic effect is usually observed when bacteria are exposed to a high concentration of metal ions as their resistance mechanisms do not offer protection at extremely high levels of free metal ions. The resistance pattern indicated that among the four experimented heavy metals, the bacteria isolates showed highest resistance to zinc and iron metals; *B. cereus* was resistant to 0.1M zinc metal, whereas isolates had the highest level of susceptibility on cadmium metal, as the lowest growth was observed at 0.01M concentration. This may be because Zn and iron offered low toxicity to the isolates while Ni and Cd expressed more toxicity. However, according to

Malik and Jaiswal, (2000), there are no current acceptable concentrations of metal ions which can be used to distinguish metal resistant and metal sensitive bacteria.

The plasmid profile of the bacterial isolates revealed the presence of plasmids in all the isolates except *S. acidaminiphila, A. faecalis faecalis* and *P. prennei.* This finding supports Ronald *et al.* (2002) who reported that more than 40 % of multiple antibiotic-resistant bacteria have at least one plasmid.

P. penneri showed no band after electrophoresis, which shows that the resistance displayed by the organism to antibiotics and heavy metals was chromosomal mediated. This is in agreement with a previous finding by Kishore, (2012) that *P. penneri* strains are multiple-drug resistant based on their ability to chromosomally induce the production of β-lactamase Hug A.

The absence of plasmids showed that the resistance exhibited by *S. acidaminiphila* is chromosomally induced. This is in agreement with previous studies (Ryan *et al*., 2009 and Shivani *et al*., 2016) which indicated that *S. acidaminiphila* have great genetic versatility and adaptation, and this enhances their ability to survive in varying conditions such as surface water polluted with industrial wastewater.

The resistance of *P. mirabilis* to gentamycin, chloramphenicol, and streptomycin has been reported by Feglo *et al*. (2010). Similar results were also reported by Newman et *al*., (2006) and Bashawan and Shafey, (2013).

After the curing, the isolates showed different susceptibility patterns to the antibiotics it resisted before the curing whereas some maintained their resistance.

Proteus mirabilis lost all its resistances to the antibiotics it previously resisted, it also lost all its resistance to cadmium, nickel and iron, but still showed scanty growth on plates containing zinc. This indicates that the gene responsible for its resistance to the antibiotics and metal are plasmid borne. It has been reported by several studies (Bashawan and Shafey, 2013, Yao and Moellering, 1999) which are in consonance with this study that *Proteus mirabilis* has intrinsic resistance to nitrofurantoin and tetracycline, but is generally susceptible to the ampicillin, amoxicillin, gentamicin and ciprofloxacin. However, resistances to these aforementioned antibiotics occur when these agents have been misused. The loss of multidrug resistance ability of *P. mirabilis* after the curing has also been reported by several investigators. Dharmadhikari and Peshwe (2009), Adeniyi *et al*., (2006), Stankowska *et al*., (2008) and Yah *et al*., (2007) all reported *Proteus mirabilis* carrying plasmids and inferred that the plasmids were able to move genetic antibiotic resistant materials among various bacterial strains, and contribute to the overall pathogenic potential of the bacteria, and when the plasmids were cured, the plasmids were lost as well as the resistances to β-lactam antibiotics as ampicillin, amoxicillin, and gentamycin. *A. faecalis* lost its resistance to cotrimoxazole, cefuroxime, ofloxacin and chloramphenicol, and also lost all its ability to resist all metals it previously resisted. It, however, maintained its resistance to ampliclox and gentamycin. This result indicates that the gene responsible for its resistance to cotrimoxazole, cefuroxime, ofloxacin and chloramphenicol and also zinc, nickel, iron and cadmium metal is plasmid-mediated; however, resistance to ampliclox and gentamycin are chromosomally-mediated.

After the plasmid curing, *B. cereus* showed no considerable decrease in antibiotics' resistance, however, it became susceptible to previously resisted chloramphenicol. A considerable change in metal resistance, namely zinc and cadmium was observed. The significant loss of *Bacillus cereus* tolerance to the metals indicates that its ability to resist metals was plasmid-mediated. This is in agreement with the findings of Amalesh *et al.*, (2012). The investigation of Amalesh *et al.*, (2012) had earlier reported the presence of plasmids in *B. cereus* isolated from industrial waste water, in their report, the presence of plasmids in the bacteria mostly enhanced their metal resistance, while the antibiotics resistance was relatively low.

After the curing *L. macriodes* was susceptible to streptomycin, ciproflaxin, sparfloxacin, perfloxacin and chloramphenicol, but it retained its resistance to cotrimoxazole, amoxicillin, gentamycin, ofloxacin and amoxicillin/ clavulanate potassium. It also lost its resistance to the cadmium and nickel metals. This result indicates that the resistance to cotrimoxazole, amoxicillin, gentamycin, ofloxacin and amoxicillin/clavulanate potassium exhibited by *L. macriodes* was chromosomally-

mediated, whereas its resistance to streptomycin, ciproflaxin, sparfloxacin, perfloxacin and chloramphenicol together with the resistance to metals were mediated by resistant genes borne on the plasmid.

Serratia sp. lost its resistance to perfloxacin, erythromycin, ceftriaxone and cefuroxime, but retained resistance to cotrimoxazole, streptomycin, ampliclox, chloramphenicol and gentamycin. It also lost the ability to resist cadmium; however it retained its resistance to the zinc, nickel and iron metals. This result indicates that the genes responsible for resistances to cotrimoxazole, streptomycin, ampliclox, chloramphenicol and gentamycin together with that responsible for resistance to nickel, iron and zinc metal are chromosomally- based.

The resistances to metal and antibiotics and subsequent loss of resistance after the curing procedure exhibited by these organisms confirm the correlation between metal ions and antibiotics' resistance. This has also been reported by several other researchers on bacterial species from different sources (Rajbanshi, 2008, Mgbemena, 2012). Many have speculated and even shown this to be as a result of the likelihood that resistance genes to both antibiotics and heavy metals could be closely located on the same plasmid in the bacteria, and are thus more likely to be transferred together in the environment (Nies, 1999). Tsai, (2006) reported that many antibiotic resistant genes are located on mobile genetic elements (e.g., plasmids, transposons and integrons), some of which are easily exchanged among phylogenetically distant bacteria. Many of these mobile genetic elements encode resistance to multiple antibiotics, heavy metals and other compounds. This phenomenon is further aided by the process of conjugation where a bacterium in the population serves as donor, and transfers the gene to the other recipients in the population.

5. Conclusion

Microbes have adapted to tolerate the presence of metals and actions of certain antimicrobials. As a result of this, they have become insensitive to the action of antimicrobials and the presence of metals through various mechanisms. Their potential to resist metals not only ends by being able to absorb and use them for growth, but also extends to their untapped potentials to clean up metal-contaminated sites. Other implications of this resistance are not beneficial as the increase in antimicrobials portends future health risks and challenges. Hence, there is a need to manipulate them to either harness or destroy their resistance potentials.

Acknowledgements

We are grateful to the Department of Microbiology, Federal University of Technology, Akure, Nigeria for the facilities used for this study. We are also grateful to FOWN Biotechnology Limited, Lagos, Nigeria for the molecular studies performed in their laboratory.

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