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Antibiotic Susceptibility Patterns And Resistant Gene Determinant Of Bacterial Isolates From Drinking Water Systems Of Some Higher Institutions In Edo State, Nigeria

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Abstract: *This study was aimed at assessing the antibiotic susceptibility profiles and determination of the gene encoding resistance to bacterial isolates, recovered from drinking water distribution systems. Five (5) institutions were used for this study selected from the three senatorial districts, (Edo South (ES1 and ES2), Edo Central (EC3 and EC4) and Edo North (EN5). Forty five water biofilm water samples were obtained from the designated sampling points on three occasions, within the period of February, 2017 and April, 2017. The antibiotic susceptibility tests of bacterial isolates were determined by the disc diffusion methods. Multidrug resistant isolates were screened for the presence of resistant genes in chromosomes and plasmids by using the polymerase chain reaction (PCR). The PCR products or amplicons were verified by agarose gel electrophoresis and viewed under gel documentation system with UV-transilluminator. The phenotypically identified bacterial isolates were Citrobacter, Klebsiella, Bacillus, Pseudomonas, Proteus and Providencia. There was high resistance to antibiotics particularly, cefixime (CEF) 95.2 % and nitrofurantoin (NIT) 85.7 %. The ofloxacin (OFL) and cefuroxime (CEF) recorded the least percentage resistance (4.8 % and 9.5 %). The resistant TEM-gene was present in the chromosomal extracts of Pseudomonas hibiscicola strain R-F06-24 and Bacillus cereus strain R- G07-19 and in the plasmid extract of Proteus penneri strain R-G07-19. As a result of global challenge due to multi-drug resistant bacteria, the relevant authority has an ardent interest in the proliferation and spreading of genes conferring resistance to antibiotics. In this regard, molecular approach at substantiating for the presence of genetic elements liable for the dissemination of bacterial resistance to antimicrobial agents is necessary.*

Key words: *biofilm, bacterial isolates, antibiotic susceptibility test, polymerase chain reaction and resistant gene.*

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I. Introduction

Biofilm is the attachment of microorganisms on surface and formation of aggregates in a self mediated polymeric matrix commonly referred to as extracellular polymeric substance (EPS) composed of polysaccharides, proteins, glycolipids, nucleic acids, organic and inorganic compounds. This resultant structure is characterized by establishment of phenotypic variants or persister cells, slow antimicrobial penetration, emergence of genetically encoded resistance to biocides and antibiotics and lateral or horizontal gene transfer (WHO, 2004; Farcas, 2012).

Bacterial biofilms on surfaces costs nations huge amount of money annually in replacement of damaged equipment, product contamination, energy losses and health care facilities. Bacteria striving in biofilms are more resistant to antimicrobial substances and are shielded from the host immune responses leading to chronic infections that are recalcitrant to eliminate (Geldreich, 1999).

The frequent methods of eliminating bacteria, such as antibiotics and disinfection are often not effective with biofilm bacteria. The high concentrations of antimicrobials needed to reduce systems of biofilm bacterial are environmentally not feasible. This s is not permitted by environmental laws and medically unacceptable, as what would take to kill the biofilm bacteria would also harm the patient (WHO, 2004).

Consequently, new strategies based on a broader knowledge of how bacterial resistance and gene dissemination can be avoided come into focus. Gene cassettes conferring resistance to antimicrobials (atb), quaternary ammonium compounds (qac) and integron-integrase (intli) genes characteristics for class-1 TEM, class 1 integron were recently obtained from environmental samples and in water treatment plant (Gillings et al., 2008).

II. Materials and Methods

Samples Collections

The biofilm water samples were collected from the reservoir stations (storage tanks), distribution pipelines and collection points (taps) from the corresponding location sites by scrapping the surfaces or walls in contact with water with sterile steel blades.

III. Microbiological Analyses

Isolation and Enumeration of bacteria

Isolation of bacteria from biofilm water samples was performed by standard methods of pour plating using nutrient agar and MacConkey agar (Barrow and Feltham, 2003). The plates were incubated at 28 ± 2 ^{o}C for 24 hr and distinct bacterial colonies in the nutrient agar and MacConkey agar plates were used to respectively deduce the heterotrophic bacteria counts (HBC) and total coliform counts (TCC).

Identification and characterization of bacteria

Six bacterial colonies were picked based on their different colonial morphologies and each of them was phenotypically characterized with prescribed standard methods (Barrow and Feltham, 2003; APHA, 2005).

Antibiotic susceptibility test

The following antibiotic discs (Mast diagnosis, UK) were used at the final concentrations that are indicated: augmentin (AUG)-30 µg, cefixime (CEF)-30 µg, cepfuroxime (CFX)- 30 µg, ceptazidime (CEP) -30µg, ciprofloxacin (CIP)-5µg, gentamycin (GEN)-10µg, nitrofurantoin- 30 µg and ofloxacin (OFL)- 5 µg.

A colony picked from each sample was transferred into 3 ml of sterile distilled water to prepare a bacterial suspension. One milliliter (1.0 mL) from each suspension was spread plated on to Muller-Hinton agar plates. Multiple antibiotic discs were applied to the plates using sterile needles, incubated at 28 ± 2 ^{o}C for 24 hr. The diameter (mm) of the zones of inhibition was measured and multiple antibiotic resistance (MAR) patterns were generated for isolates that showed resistance to four or more antibiotics (Liarrull et al., 2009).

Resistant gene determination

The determination of resistant gene present in pure culture of the bacterial isolates from the biofilm water samples with multidrug resistance were further screened by the polymerase chain reaction (PCR), amplification and electrophoretic processes (Stokes and Gillings, 2011).

The following outlines the key steps in the methods taken.

DNA extraction

The chromosomal DNA was extracted using Zymo Pure Miniprep Kit (Zymo Research Centre, Johannesburg, South Africa) and Chen and Ghatak, (2013). The DNA extract was purified by adding 5.0 µL nuclease-free water and incubated for 30 min. The extracted DNA products were eluted in 25 µL DNA elution buffer and stored at -20 ^{o}C as DNA template, ready for use in PCR process.

Plasmid Extraction

This was done according to manufacturer's instructions (Zymo Pure Miniprep Kit, South Africa).

Amplification of 16S rRNA gene

The 16S rRNA gene from the chromosomal DNA was PCR amplified using universal primer sets (27F (5 AGA GTT TGA TCC TGG CTC AG-3) and 1492R 5 TAC GGT CTA CTT GTT ACG TA-3). The PCR master mix contained the following components of up to 25 µL: One taq master mix, 12.5 µL; Forward and Reverse primers, 1.25 µL; Nuclease free water, 5.0 µL and DNA template, 5.0 µL. The process was performed in Gene PCR Thermo Cycler with the recommended guidelines: Initial denaturation at 94 ^{o}C for 30 min; denaturation at 94 ^{o}C for 1 min; annealing at 50 ^{o}C for 1 min, extension at 72 ^{o}C for 1 min; final extension at 72 ^{o}C for 7 min and hold at 4 ^{o}C .

Electrophoresis

The PCR products or amplicons were verified by agarose gel (1.0 % wt/v) electrophoresis. Ten microlitre (10 µL) of the ready to use DNA Ladder (molecular marker) and the PCR products, stained with Ethidium bromide dye were loaded in the wells of solidified gel immersed in Tris Borate EDTA (TBE- buffer) in gel electrophoresis chamber processed at 90 Volt for 1 hr, viewed under gel documentation system with UV-transilluminator (Chen and Ghatak, 2013).

IV. Results

The bacterial isolates phenotypically identified were Citrobacter, Klebsiella, Bacillus, Pseudomonas, Proteus and Providencia species.

Table1. Phenotypic characterization of isolates obtained from biofilm water samples

Morphological Examination			Biochemical Examination							
Colonial Characteristics on nutrient agar (NA)	Colonial Characteristics on MacConkey agar (MA)	Gram Staining	Catalase	Oxidase	Indole	Methyl Red	Citrate	Voges proskauer	Lactose	Isolates
Large gray colony with serrated margin	Colourless colony with serrated margin	negative rods	+	-	+	+	+	+	-	*Providencia* sp.
Opaque colony with serrated margin	Pinkish colony with serrated margin	negative rods	+	-	-	+	+	-	+	*Citrobacter* sp.
Dry colony with serrated margin	Pinkish colony with serrated margin	positive rods	+	+	-	-	+	+	+	*Bacillus* sp.
Greenish pigmented colony with an entire margin	Colourless colony with an entire margin	negative rods	+	+	-	-	+	-	-	*Pseudomonas* sp.
Mucoid swarming colony with an entire margin	Colourless colony with an entire margin	negative rods.	+	-	-	+	+	-	-	*Proteus* sp
Mucoid colony with an entire margin	Pinkish colony with an entire margin	negative rods	+	-	-	-	-	+	+	*Klebsiella* sp.
Mucoid colony with entire margin	Colourless colony with entire margin	positive rods	+	+	-	-	+	+	+	*Bacillus* sp.
Mucoid swarming colony with an entire margin	Colourless colony with an entire margin	negative rods	+	-	-	+	+	-	-	*Proteus* sp.

The results of the antibiotic susceptibility tests revealed that 18 bacterial isolates were resistant to nitrofurantoin, while 20 isolates showed resistance to cefixine. Two (02) bacterial isolates were recorded to indicate resistance to ofloxacin and 03 showed resistance to cefuroxime.

Table 2: Antibiotic susceptibility profile of bacterial isolates

S/N	NIT	AUG	OFL	CEF	GEN	CEP	CIP	CFR	ISOLATES
1	R	R	S	R	R	R	R	S	*Pseudomonas* sp.
	S	R	S	R	S	S	R	R	*Bacillus* sp.
2	R	S	S	R	S	R	S	S	*Klebsiella* sp.
3	R	R	S	R	S	R	S	S	*Citrobacter* sp.
4	R	R	S	R	S	R	S	S	*Proteus* sp.
5	R	R	S	R	S	R	S	S	*Citrobacter* sp.
	R	S	S	R	S	R	R	R	*Pseudomonas* sp.
6	R	S	S	R	S	R	S	S	*Providencia* sp.
7	R	R	S	R	S	R	S	S	*Citrobacter* sp.
	R	R	R	R	S	R	R	S	*Bacillus* sp.
8	R	R	S	R	S	R	S	S	*Pseudomonas* sp.
9	R	R	S	R	R	R	R	S	*Proteus* sp.
	R	R	S	R	S	R	R	S	*Klebsiella* sp.
10	R	S	S	R	R	R	S	S	*Bacillus* sp.
	R	R	S	R	R	S	R	S	*Proteus* sp.
11	R	S	S	R	S	S	S	S	*Klebsiella* sp.
12	R	R	S	R	S	R	R	S	*Bacillus*.sp
13	R	R	S	R	S	R	S	S	*Bacillus* sp.
14	S	R	S	R	S	R	R	R	*Pseudomonas* sp.

Key: Nitrofurantoin (NIT) 30mg, Augmentin (AUG) 30mg, Ofloxacin (OFL) 5 mg, (Cefixine (CEF) 5mg, Gentamycin (GEN)10mg, Ceptazidine (CEP) 30mg, (Ciprofloxacin (CIP) 5mg and Cefuroxime (CFX) 30mg .NCCL baseline (mm): R = resistance (1 – 12), I = intermediate resistance (13 – 17) and S = susceptibility (≥ 18).

The results of the antibiotic resistance percentage of bacterial isolates revealed that the isolates investigated recorded 85.7% resistance level to nitrofurantoin (NIT) and 95.2 % resistance to cefixine. There was 4.80 % and 14.3 % resistance levels to ofloxacin and cefuroxime respectively.

Table 3: Antibiotics resistance percentage of bacterial isolates

Antibiotics	Resistance (R) %	Susceptibility (S) %
NIT	18 (85.7)	03 (14.3)
AUG	15 (71.4)	4 (19.0)
OFL	01 (4.8)	20 (95.2)
CEF	20 (95.2)	01 (4.80)
GEN	03 (14.3)	16 (71.4)
CEP	17 (81.0)	01 (4.80)
CIP	09 (42.9)	8 (38.0)
CFX	03 (14.3)	18 (85.7)

Key:
Nitrofurantoin (NIT) 30mg, Augmentin (AUG) 30mg, Ofloxacin (OFL), (Cefixine (CEF) 5mg, Gentamycin (GEN)
10mg, Ceptazidine (CEP) 30mg, (Ciprofloxacin (CIP) 5mg and Cefuroxime (CFX) 30mg.

The results of the antibiotic resistance pattern showed NIT – AUG – CEF – CEP, the most prevalent with distribution (percentage) of 08 (38.1%). The MAR index ranged from 0.4 to 0.6 greater than standard value (0.2).

Table 4.12 Antibiotic resistance pattern / multiple antibiotic resistant (MAR) Index of isolates

S/N	Isolates	Multiple antibiotic resistance (MAR)	MAR. Index
1	Pseudomonas sp.	NIT - AUG – CEF - GEN - CEP -NIT-CEF-CIP-CFR	0.6
	Pseudomonas sp.		0.5
2	Pseudomonas sp.	NIT - CEF - CEP	0.4
3	Pseudomonas sp.	NIT - AUG – CEF - CEP	0.5
4	Pseudomonas sp.	NIT - AUG – CEF - CEP	0.5
5	Bacillus sp.	NIT - AUG – CEF - CEP	0.5
	Bacillus sp.	NIT - CEF - CEP - CIP - CFR	0.6
6	Bacillus sp	NIT – OFR- CEF - CEP	0.5
7	Bacillus sp	NIT – AUG- CEF - CEP	0.5
	Bacillus sp.	NIT- AUG- OFL - CEF - CEP	0.6
8	Citrobacter sp.	NIT - AUG – CEF - CEP	0.5
9	Citrobacter sp.	NIT - AUG - CEF - GEN - CIP	0.6
	Citrobacter sp.	NIT - AUG – CEF - CEP	0.5
10	Klebsiella sp.	NIT - AUG – CEF - CEP	0.5
	Klebsiella sp.	NIT - AUG – CEF – CIP	0.5
11	Klebsiella sp.	NIT – CEF-CEP	0.4
12	Klebsiella sp.	NIT –AUG- CEF - CEP – CIP	0.6
13	Proteus sp.	NIT - AUG – CEF - CEP	0.5
14	Proteus sp.	AUG - CEF - CEP –CIP	0.5
	Proteus sp.	AUG – CEF-CEP	0.4
15	Providencia sp.	NIT - AUG – CEP - CIP	0.5

The polymerase chain reactions for bacterial isolates analyzed with1.0 % agarose gel electrophoresis showed that tubes 1 and 4 were positive for class1- TEM resistant gene with bands at 867bp indicating that the TEM- gene was present in the chromosomal extract of Pseudomonas hibiscicola strain R-HO6-24 and Bacillus cereus strain R-G07-19 (Plate 1).

Tem Resistant Gene

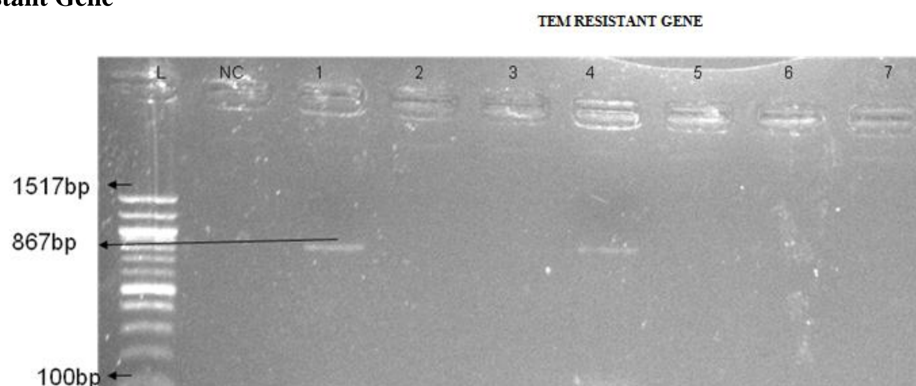

Plate1: Polymerase chain reaction results for bacterial isolates analyzed with 1.0% agarose gel electrophoresis stained with ethidium bromide. L is 100bp-1517bp DNA ladder (molecular marker).
Numbers 1 and 4 (representing samples 15 and 13) are positive for TEM resistant gene with bands at 867bp, while samples 2, 3, 5, 6 and 7 are\ negative for TEM resistant gene. NC is a no DNA template control.
Key:
1= Pseudomonas hibiscicola strain 907-R-F06-24
2= Klebsiella sp.
3= Bacillus cereus strain 907-R-G07-19
4= Bacillus cereus strain 907- R-GO7-19
5= Bacillus sp. strain 907-R-H07-22
6= Pseudomonas aeruginosa strain 907-R-A08-02
7= Proteus penneri strain 907-R-B08-05FFL8

The plasmid profiling of multiple drugs resistant bacteria isolates analyzed with 0.8 % agarose gel electrophoresis revealed that all the isolates (tubes 1 to 7) were negative for plasmid resistant gene (plate 2). The result of bacterial isolate of tube 9 was positive for plasmid genes with band at 48.5kb, revealing the presence of resistant genes in plasmid of Proteus penneri strain R-GO7-19
(Plate3).

Plasmid

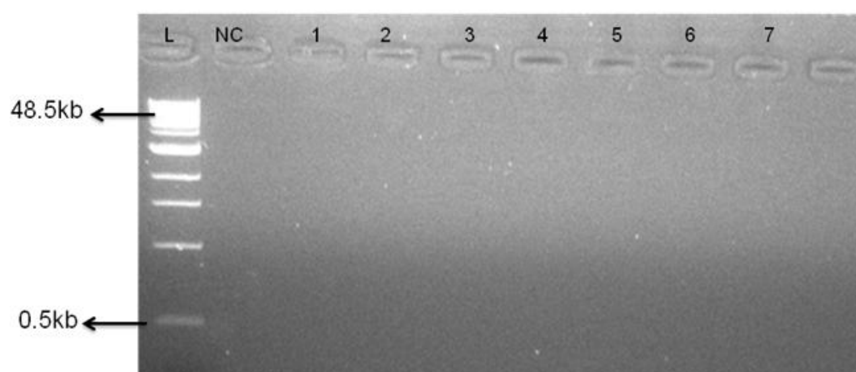

Plate 2: Plasmid profiling of multiple drug resistant bacteria isolates analyzed with 0.8% agarose gel electrophoresis stained with ethidium bromide. L is 0.5kb-48.5kb DNA ladder (molecular marker). Isolates 1, 2, 3, 4, 5, 6 and 7 are negative for plasmid genes. NC is a no plasmid DNA control.
Key:
1= Pseudomonas hibiscicola strain 907-R-F06-24
2= Klebsiella sp.
3= Bacillus cereus strain 907-R-G07-19
4= Bacillus cereus strain 907-R-GO7-19
5= Bacillus sp. strain 907-R-H07-22
6= Pseudomonas aeruginosa907R-A08-02
7= Proteus penneri strain 907-R-B08-05

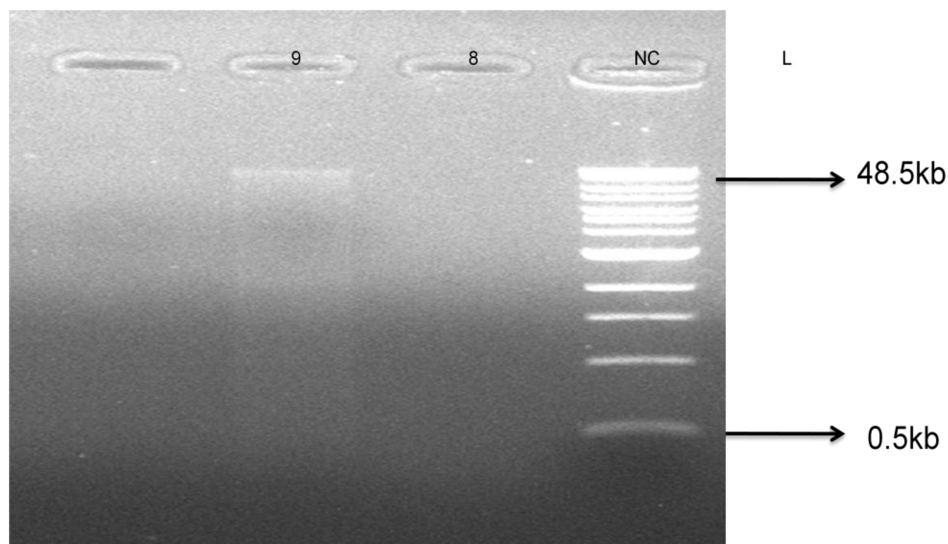

Plate3: Plasmid profiling of multiple drug resistant bacteria isolates analyzed with 0.8% agarose gel electrophoresis stained with ethidium bromide. L is 0.5kb-48.5kb DNA ladder (molecular marker). Isolate 9 is positive for plasmid genes with band at 48.5kb while isolate 8 is negative for plasmid genes. NC is a no plasmid DNA control.

Key:
8= Pseudomonas aeruginosa strain 907-R-F07-16
9= Proteus sp. strain 907-R-G07-19

V. Discussion

The bacterial isolates phenotypically identified were Citrobacter, Klebsiella, Bacillus, Pseudomonas, Proteus and Providencia. The investigated biofilms proved to be extremely active bacteria consortia with high concentrations of cultivable bacteria: heterotrophs and opportunistic bacteria. The Pseudomonas species are opportunistic pathogens that caused nosocomial infections in susceptible patients and very difficult to eradicate because of their high intrinsic resistance to a variety of antibiotics, including β - lactams, aminoglycosides and fluroquinolones. The Bacillus has been implicated as causative agent of endocarditis, neurological and human gastrointestinal infections and particularly recalcitrant to antibiotic (Liarrull et al., 2009).

In the assessment of the antimicrobial susceptibility level, using 8 antibiotics of clinical importance, all bacterial isolates were resistant to nitrofurantoin, augmentin, cefixime, gentamycin, cepfuroxime and ciproflaxacin. The lowest levels of resistance were observed in ofloxacin (4.80 %) and cefuroxime (14.3%) (Table2). From this result, it is evident that, these drugs were the most effective, because a large proportion (inhibition zone) of isolates were killed or cleared to both, indicating that these organisms would not be able to cause diseases in humans.

Subsequently, the highest levels of resistant were observed in cefixine (95.2 %) and nitrofurantoin (85.7 %) (Table3). This however, indicated that these drugs were not potent enough to destroy or kill the pathogens and therefore made the organisms to survive and proliferate in the distribution system and capable of causing diseases and becoming threats to public health. In the determination of the most prevalent antibiotic susceptibility patterns, five different multiple antibiotic resistance (MAR) patterns were observed and the most common was NIT-AUG-CEF-CEP. The (MAR) indices were generally greater than 0.2. This indicated that these drugs have been abused or misused according to public health standards (WHO, 2004). The biofilm grown organisms may serve as a reservoir for antibiotic-resistant organisms and therefore may have the potential to cause infection. This outcome is a cause for concern particularly for infants, the elderly and immune-compromised individuals in University communities (APHA, 2005; Stokes and Gillings, 2011).

The identities of organisms carrying the class 1- TEM-gene that conferred resistance to antibiotics were confirmed by the polymerase chain reaction (PCR). The TEM-genes are βeta-lactamase enzymes, produced by bacteria that provide multi-resistance to antibiotics by breaking the antibiotic four-ring carbon structure, known as βeta Lactam. The TEM-gene is an example of a new type of β- lactamase, called extended spectrum beta-lactamase (ESBL). They are frequently plasmid mediated and plasmids responsible for ESBL production commonly carry genes encoding resistance to other drugs (Farcas, 2012).

The application of the PCR technique to target TEM-genes is an excellent molecular chronometer for screening potentially resistant environmental samples. The desired gene fragments were successfully amplified,

which indicated the presence of the resistance gene in bacterial isolates, identified as Pseudomonas hibiscicola and Bacillus cereus strains respectively (Plate1) when analyzed with 1.0 % agarose gel electrophoresis.

Subsequently, Plates 2 and 3 showed the plasmid profile of multiple drug resistant bacterial isolates and the results revealed that only isolate 9 (Proteus penneri strain) was positive for plasmid genes with bands at 48.5 kb, while the other isolates (1 – 8) were negative for plasmid genes. However, NC was a no plasmid DNA control.

These findings revealed that, the TEM-resistant gene is both chromosomal and plasmid mediated and additional efforts to characterize the inactivation rate of bacterial by different antimicrobial strategies are needed and will be useful in improving our understanding of antibiotic resistant bacteria.

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