Detection of Heavy Metal-Resistance Gene in Bacteria Isolated from Clinical and Environmental Sources Using Polymerase Chain Reaction (PCR)

Danny Sendolo[1], Glory I. Baysah[1]*, and Cajethan Onyebuchi Ezeamagu[2]

1. Adventist University of West Africa, Liberia
2. Babcock University, Nigeria

Abstract

Background: Global proliferation of the pharmaceutical industry in response to devastating health challenges has led to several ecological and environmental problems. Heavy metal contamination is one of the most dangerous factors that affect ecosystems and human health. This study aimed to detect the presence of heavy-metal resistance genes in clinical and environmental sources using polymerase chain reaction (PCR).

Methods: This study employed an experimental design. The samples included 40 clinical isolates (blood, urine, throat, wound, and sputum) and 40 environmental isolates (pharmaceutical effluents).The isolates were collected and subcultured in nutrient broth for analysis.

Results: The result shows that heavy metal-resistant genes were not detected in the clinical isolates whereas 65% and 60% of the environmental isolates harbored silver (Ag) and arsenic (As) respectively. However, the clinical isolates in this study showed higher rates of resistance than the environmental isolates did. This may be due to the frequent use of broad-spectrum antibiotics during clinical sessions, leading to the development of antibiotic-resistant strains.

Conclusion: Heavy metal-resistant genes are present in bacteria in the environment and may cause severe health issues (drug resistance) when these genes are transmitted to pathogenic organisms that affect humans and animals. The association between bacterial drug resistance and heavy metal acquisition may greatly influence treatment outcomes in the event of infection. Therefore, control measures should be implemented to reduce the dissemination of these metals in the environment.

Keywords: Antibiotics, heavy metal-resistant genes, pharmaceutical effluent, bacteria

Introduction

In recent decades, uncontrolled urbanization due to sewage and industrial wastewater treatment has become a serious source of pollution. Water pollution is a major problem in both developing and industrialized countries. Wastewater treatment is the process of removing contaminants from sewage. This includes the removal of physical, chemical, and biological contaminants and aims to create waste streams and sludge that can be disposed of or returned to the environment. This material is often unintentionally contaminated with toxic organic and inorganic compounds. However, other contaminants such as heavy metals are difficult to remove from the environment (Ren et al., 2009). Heavy metals have densities greater than 4–5 g/cm3 (Nies, 1999). There are approx-

*Corresponding author. Email: baysahg@auwa.edu.lr

imately 30 heavy metals and metalloid groups, including zinc (Zn), mercury (Hg), lead (Pb), copper (Cu), silver (Ag), arsenic (As), and chromium (Cr). Metals are found in almost all types of soil, sediment, and water (Zhao et al., 2012; Tang et al., 2014; Gati et al., 2016).

Heavy metals are micronutrients essential for various cellular functions (Seiler & Berendonk, 2012) but can become toxic above a certain level (Zhao et al.., 2012). Heavy metals can enter the body through breathing in polluted air, eating contaminated food (rain, air, and soil), or drinking contaminated water, which can cause illness. The toxic effects of metals include deflocculation (Kobayashi, 2005), decreased mud permeability (Ipeaiyeda, 2016), and poor drainage quality.

Multidrug resistance (MDR) is an organism's ability to resist more drugs in two different antimicrobial classes. Increased bacterial resistance among common pathogens is emerging as a serious public health concern (Fish & Oblinger, 2006). The most current medical significance today is the far-reaching spectrum beta-lactamase (ESBL), produced mainly by the gram-negative bacteria Enterobacteriaceae, found in both communities and hospitals. This is often associated with clinical treatment failure (Pitout et al., 2005). Human activities such as the chemical use of fertilizers and mining can release large amounts of heavy metals into the biosphere (Adewole &Uchegbu, 2010). A decline in metal biocides has been suggested as a possible solution for antibiotic resistance (Lemire et al., 2013). Bacteria resistant to heavy metals are economically valuable for bioremediation and biomining (Nies, 1999). On the one hand, however, they pose a serious threat to human health. This study was conducted to detect heavy metal resistance genes in bacteria from clinical and environmental sources using PCR.

Materials and Methods

An experimental design is used in this study. The sample collection included 80 bacterial isolates that were previously isolated, identified, and stored. Thus, 40 clinical isolates (blood, urine, throat, wound, and sputum isolates) and 40 environmental isolates (pharmaceutical effluents) were collected and subcultured in nutrient broth for analysis. This study was conducted at the Babcock University Microbiology Laboratory, Nigeria.

Antibiotic Sensitivity Testing

Antibiotic testing was performed using the Agar Diffusion method and was interpreted according to the Clinical Laboratory Standards Institute (CLSI, 2017). The antibiotic discs (Abtek Biologicals Limited Gram-negative discs) used in this study contained ceftazidime (30µg). Cefuroxime (30µg); gentamicin (10µg); ofloxacin (5µg); amoxicillin/clavulanic acid (30ug); nitrofurantoin (30µg); cefixime (5µg); and ciprofloxacin (5µg); Briefly, test tubes were inoculated with a single colony of a pure isolate 1 ml of nutrient

broth and incubated overnight at 37°C. The culture broth was standardized to match the 0.5 McFarland standard. A sterile swab stick was soaked in the standardized suspension and spread on the surface of a prepared Muller-Hilton agar plate antibiotic disc. They were then placed on the agar surface and maintained at an edge-to-edge distance of 30 mm. The Plates were then incubated at 37°C for 24 hours. The apparent zone of inhibition was measured in milliliters using a meter rule adjacent to the diameter.

DNA Extraction

Bacterial DNA was extracted using a Quick-DNA™ Miniprep Plus Kit (Zymo Research, Biolab, USA) according to the manufacturer's protocol. Agarose electrophoresis was used to confirm DNA quality before the polymerase chain reaction.

Detection of Heavy Metal-Resistant Genes

The presence of heavy metal resistance genes in the isolates was evaluated using PCR. Briefly, the PCR mixture (25µL) containing 12.5µL solution of the master mix (New England Biolabs), 9.5µl H20, 0.5µL of each liquid primer and 2.0µL of DNA template. The extracted DNA was amplified using the G-STORG PCR system (Gene Technology Co., Ltd.), Program setup: initial denaturation at 940C for 3 minutes, denature at 940C for 30 seconds, annealing at 560C for 30 seconds, extension 680C for 30 seconds, and final extension 680C. The 5 min program was set to 30 cycles of Amplicon virtualized using agarose gel electrophoresis.

Agarose Gel Electrophoresis

Agarose gel powder (0.3 g) was dissolved in 25 ml of 1XTBE buffer (1X:89 mM tribasic, 19 mM boric acid, and 2 mM EDTA) and placed in a microwave to boil at boiling temperature for 3 minutes. The solution was cooled to 50-60°C. Then 4µl of 10µl mg/ml ethidium bromide solution was added, vortexed, and poured into a gel dish containing a solid comb. Once the gel was transferred to a gel tank and covered with TBE buffer, it was left to stand for 20 minutes. A pipette was used to load the PCR samples (150µl) into running wells from negative to positive (Ezeamagu et al., 2018). The Gel was run to 100V UV irradiation for 15 minutes (TMW-20 transilluminator in collaboration with Alpha Innotech, USA, Malek et al., 2015). Data were analyzed using descriptive statistics and interpreted using graphs.

Results

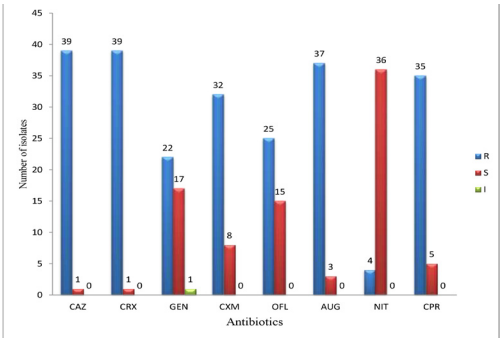
In this study, 80 bacteria were obtained, of which 9, 24, 2, 2, 3, and 40 isolates were recovered from the blood, urine, throat, sputum, wound, and pharmaceutical effluent, respectively. The percentages of bacteria from the pharmaceutical effluent were 45%, 35%, 7.5%, 7.5%, and 5% for *P. aeruginosa*, *S. enterica, E. cloacae*, *E. hormaechei,* and *A. caviae*, respectively. Similarly, the proportions of E. coli, K. pneumoniae,

S. enterica, and P. aeruginosa were 40 %, 25 %, 22.5 %, and 12.5 %, respectively. In this study, *Pseudomonas aeruginosa* and *Salmonella enteric* were recovered from the two settings (clinical and environmental).

A total of eight antibiotics were used in this study which includes ceftazidime (CAZ), cefuroxime (CRX), gentamicin (GEN), cefixime (CMX), ofloxacin (OFL), amoxicillin/clavulanate (AUG), nitrofurantoin (NIT,) and ciprofloxacin(CPR). The results showed that most clinical isolates were highly resistant to CAZ, CRX, AUG, CPR, CMX, and GEN (Figure 1). The environmental isolates also showed high resistance to CAZ, CRX, AUG, CMX, and CPR (Figure 2).

Figure 1

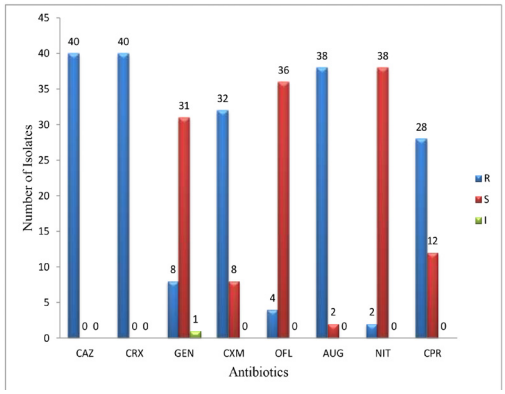
Clinical isolates sensitivity profile



Key: Ceftazidime (CAZ), Cefuroxime (CRX), Gentamicin (GEN), Cefixime (CXM), Ofloxacin (OFL), Augmentin (AUG), Nitrofurantoin (NIT), Ciprofloxacin(CPR) Resistant(R), Susceptible (S), Intermediate (I)

Figure 2

Environmental isolates sensitivity profile

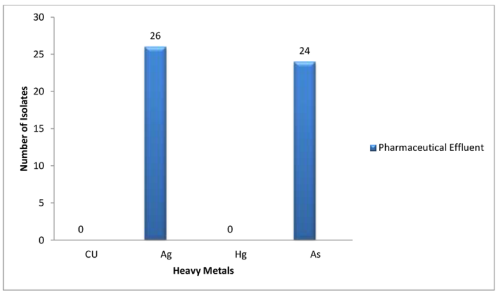


Key: Ceftazidime (CAZ), Cefuroxime (CRX), Gentamicin (GEN), Cefixime (CXM), Ofloxacin (OFL), Augmentin (AUG), Nitrofurantoin (NIT), Ciprofloxacin (CPR) Resistant(R), Susceptible (S), Intermediate (I)

Four (4) heavy metals, Silver (Ag), Arsenic (As), Copper (Cu), and Mercury (Hg), were screened in the bacterial isolates. The results indicated that Ag and As were detected in the isolates (Figure 3). Figures 4 and 5 show electropherograms for detecting Silver (Ag) and Arsenic (As) in the environmental isolates.

Figure 3

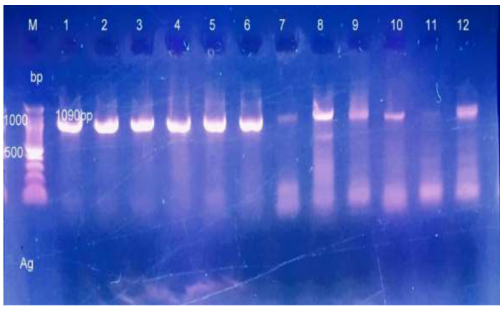
Heavy metals resistance profile of the isolates



Key: Silver (Ag), Arsenic (As), Copper (Cu), Mercury (Hg)

Figure 4

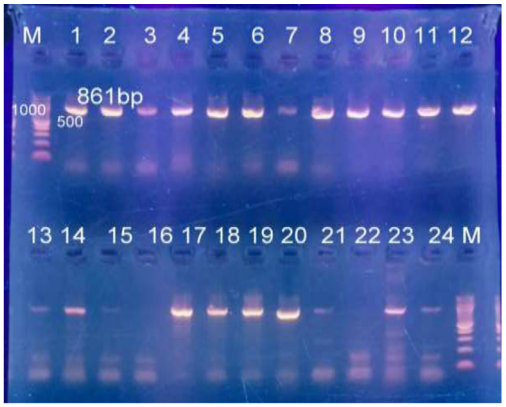
Electropherogram of the heavy metal gene for Detection of Silver (Ag) in the isolates



M: molecular weight marker (100bp) positive isolates:1, 2, 3, 4, 5, 6, 8, 9, 10, and 12. Negative controls: 11

Figure 5

Electropherogram of heavy metal genes for detecting arsenic (As) in the isolates



M: molecular weight marker (100bp). Positive isolates: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 17, 18, 19, 20, 21, 23, and 24. Negative controls: 16

Discussion

The presence of potential pathogens such as *E. coli, Salmonella, and Klebsiella* indicates that they are widespread in the environment. In this study, the clinical isolates showed higher rates of resistance than the environmental isolates. This may be due to the frequent use of broad-spectrum antibiotics during clinical sessions, leading to the development of antibiotic-resistant strains. High antibiotic resistance against CAZ, CRX, AUG, CXM, and CPR was also observed in environmental isolates (Figure 2). The high level of resistance observed in this study was not surprising, as similar levels of resistance have been previously reported (Li et al., (2012). This is attributed to the presence of antibiotic residues. An

environment with selective pressure of metals can contribute to the development of bacterial resistance.

Self-prescription is a crucial issue in the context of antibiotic abuse. It has been reported that bacteria acquire resistance through the horizontal gene transfer of mobile genetic elements. Frequent antibiotic use promotes the selection of existing antibiotic-resistance mechanisms (Gillings & Stokes, 2012). Self-medication can lead to ineffective antibiotics and may contribute significantly to the bacterial resistance profile (Ezemago et al., 2018).

Anthropogenic activities (industrial and agricultural) release heavy metals, which affect soil and water quality (Wei & Yang, 2010; Wuana & Okieimen, 2011; Su et al., 2014). Heavy metal scans can withstand decades of use in the environment and are capable of withstanding long-term and permanent exposure and extensive co-selection pressure (Baker-Austin et al., 2006). Several studies have been conducted on the co-selection of antibiotic resistance genes (ARG) and metal resistance genes (MRG) in various metal-contaminated environments. These studies identified the enrichment and dissemination of such genes in different environments, including human aquatic environments (Cesare et al., 2016; Fang et al., 2016; Henriques et al., 2016; Ju et al., 2016; Zhao et al., 2018).With the overarching global health problems associated with heavy metal resistance, it is important to understand the distribution and diversity of metals, environmental resistance genes, and their hosts.

Metals are often dispersed in the soil through agricultural work; they are very stable and resistant to deterioration. Trace amounts of heavy metals have also been reported to cause the emergence and spread of antimicrobial resistance (AR) strains by co-selection, cross-resistance, co-regulation, or biofilm induction (Yu et al., 2017). Low doses of antibiotics or toxic compounds in the environment can induce bacteria resistance to antibiotics and heavy metals. Reduction of cell permeability, target site modification, efflux inflation regulation, acquisition of neutralizing enzymes with neutralizing action are all different mechanisms that can activate multiple resistances of bacteria to more than two antibacterial agents (Fard et al., 2012). The co-selective potential of metals and their positive roles in the spread of antimicrobial resistance genes across different ecosystems should be carefully studied.

Four heavy metals (silver, arsenic, copper, and mercury) were screened in this study. No heavy metals were detected in the clinical isolates. Wales et al. (2015) found that heavy metal resistance is consistently associated with antibiotic resistance. However, the results of the present study were different. This may be because of the low concentrations of heavy metals that exist in the clinical environment to confer resistance.

However, environmental isolates contained both Ag and As. This

is because bacterial resistance to heavy metals is often, but not always, conferred by certain bacteria, such as plasmids or transposons. Recent progress in understanding the mechanisms of heavy-metal resistance has shown a similar mechanism for specific resistance. Metals are found in a wide variety of bacteria and their related genera. The resistance mechanisms can be applied to various heavy metals. This is in agreement with Yanga et al. (2020), who reported metal-resistant genes in *E coli* and *Salmonella* in poultry farms and retail meat. This suggests that heavy metal tolerance genes exist and are ubiquitous in bacteria in the environment and food. This is due to the widespread use of chemicals in animal husbandry. An environment where there is agriculture, engineering, and pharmacy can also pose selection pressure for heavy metal resistance.

Metal tolerance is common in many microorganisms. They come into contact with metals in their habitats. Pal et al. (2015) demonstrated that resistance genes, biocides, and metals are abundant in the environment. In addition, metalworking industrial activities are considered to be one of the primary sources of heavy metal pollution. Nonetheless, drug resistance associated with heavy metal resistance has been observed in wastewater samples (Varma et al., 1976) and contaminated sites (Sinegani et al., 2017). Clinical sources (Baquero, 2001) are often associated with antibiotic resistance (Wales & Davies., 2015). Moreover, the incidence of heavy-metal resistance is similar to or higher than drug resistance (Altug et al., 2009). This is because microbes have evolved multiple resistance mechanisms to survive in the presence of toxic heavy metals in the environment.

Conclusion

This study concluded that heavy metal resistance genes are present among bacteria in the environment and may cause serious health issues (drug resistance). When these genes are transmitted to pathogenic organisms that affect humans and animals, the association between bacterial drug resistance and heavy metal acquisition may greatly influence the treatment outcomes of infected patients. Pharmaceutical effluent has become a tool for the diffusion of heavy metal-resistance genes in the community. Therefore, control measures, such as the effective evaluation of waste management plans, should be implemented to reduce the dissemination of these metals in the environment**.**

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