Journal of Basic Pharmacology and Toxicology
Vol. 3 No. 2 (2019), 1-5
Original Research Articles

Antibiotic and heavy metal tolerance in bacteria isolated from drinking water sources in Nsukka metropolis, Enugu state, Nigeria

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Resistance to antimicrobial agents and heavy metals is inevitable as a result of natural consequence of bacterial cell adaptation to exposure to antimicrobials and heavy metals in the environment. Heavy use of bioactive substances in the medical and agricultural sectors has led to increasing resistance to those substances. This study was carried out to demonstrate antibiotic and heavy metal tolerance in bacteria isolated from drinking water supplies in Nsukka, Southeast, Nigeria. Fresh water samples were collected from different sources in different locations in Nsukka metropolis from where bacterial isolates were obtained. The isolates were assessed for their antibiotic and heavy metal tolerance by the agar disc diffusion assay. Determination of the maximum tolerable concentrations (MTC) of the metal salts was carried out by the broth dilution method. Six bacterial isolates obtained and identified tentatively based on their cultural, morphological and biochemical characteristics were *Escherichia coli*, *Citrobacter* spp., *Bacillus cereus*, *Micrococcus varians, Proteus mirabilis and Salmonella* spp. The antibiotic resistance assay revealed that *Salmonella* spp. and *Proteus mirabilis* were resistant to all antibiotics tested and fifty percent of all the isolates were sensitive to sulphafurazole and furazolidone. It was observed that *Bacillus cereus* had MTC values for Pb^{2+} (800 µg/mL), Cu^{2+} (700 µg/mL) and Cr^{6+} (200 µg/mL) while *Citrobacter* spp. had the least MTC value for Cr^{6+}. There was a significant difference ($p \leq 0.05$) in the effect of heavy metal concentration on the sensitive isolates. We, therefore, conclude that the water environment in Nsukka, Southeast Nigeria is a potential reservoir of antibiotic and heavy metal tolerance determinants.

1 Introduction

Antibiotic products and heavy metals have become agents of environmental pollution as these agents affect water quality and soil health. This is attributed to industrialization and heavy use of agricultural products with high metal content in current agricultural practices [1]. Owing to the recalcitrance or indestructibility of heavy metals occasioned by their inability to undergo chemical or biological degradation, they are difficult to eliminate from polluted sites [1]. Naturally, the environment comprises heavy metals, but careless use by humans has changed their biochemical balance and geochemical cycles. This leads to heavy release of too many heavy metals into environments such as the water bodies and soil [2]. Heavy metals inundate the environment through metallurgical activities, disposal of wastes, corrosion of metals, petroleum exploration and agriculture, among other anthropogenic activities. Essential metals such as Zn, Fe, Pb, Ni, Cd, Cr, Cu, Co function in osmoregulation and stabilization of molecules through electrostatic interactions and as cofactors in various enzyme-catalysed reactions [3]. However, at high concentrations, these metals become toxic to cells. Heavy metals also affect both the biochemical activities and growth of

organisms, thereby leading to decrease in biomass. High levels of metals can be found naturally in some locations that are not polluted by human activity and the metal levels at these sites can pose a potential threat to humans [4].

Metal tolerance reflects the capacity of an organism to survive at a high metal concentration or accumulate them without dying [5]. Heavy metal tolerance is a phenomenon which describes the ability of bacteria and other organisms to accommodate or tolerate the effect of heavy metals on them with respect to their concentrations. Some bacteria are very sensitive to low metal concentrations while others are sensitive to high concentrations. Incidentally, products such as heavy metals, sterilants, disinfectants and antibiotics used in industries make room for the environmental pressure that selects for organisms which are better genetically endowed to survive and multiply [6].

Antibiotic resistance is a phenomenon used to describe a situation where a drug fails or loses its capacity to inhibit bacterial growth [7]. However, this ability to resist antibiotics can be acquired through mutation or by transfer of antibiotic-resistance plasmids [8]. Multidrug resistance (MDR) can also be due to the clustering of multiple antibiotic resistance genes in which one gene codes for resistance of a single antibiotic on (R) plasmids [9]. Antimicrobial resistance in bacteria has become a problem of concern in recent times; for example, the cost of medical care is now assuming an alarming proportion with the heavy use of current antibiotics against resistant pathogens [10]. An earlier study revealed a direct relationship between human activities (e.g. pollution) and spread; persistence of resistance genes and bacteria resistant to antibiotics within the resident bacterial communities [11]. Furthermore, a direct relationship between antibiotic and metal resistance has been widely documented owing to the tendency that genes coding for resistance to both heavy metals and antibiotics may be clustering together on the same region of plasmids and therefore can be transferred together in the environment by one or more processes of horizontal gene transfer (8, 11]. Moreover, bacterial transposons and plasmids can carry multiple genes that encode antibiotic and metal resistance [12]. Survival of microorganisms in polluted environments is dependent on genetic adaptation, physiological adaptation and cell morphological alterations, as well as environmental modifications of metal speciation, among other factors [2]. The current phenomenon of multiple antibiotic/metal resistance among bacterial populations increases public health risk. The objective of the present study was, therefore, to isolate and identify some bacterial species from drinking water supply samples and assess their tolerance levels to some heavy metals and antibiotics.

2 Materials and methods

2.1 Collection of samples

Water samples were aseptically collected in sterile 20 ml bijou bottles from several locations within Nsukka metropolis. Sources of the samples included bore-holes and pipe-borne water which were immediately transported to the laboratory for the study.

2.2 Isolation and identification of bacteria

The water samples which were first serially diluted (10-fold) were then plated out on sterile nutrient agar and incubated for 24 - 48 hr at 37 ^{o}C. A number of morphologically different colonies were randomly selected and sequentially sub-cultured for purification on the same medium. The isolates were characterized and identified tentatively using standard bacteriological methods [13].

2.3 Preparation of heavy metal salt-impregnated paper discs

Whatman number 4 filter paper was cut into discs of uniform size (6mm, diameter) using a perforator and were sterilized in the autoclave at 121 ^{o}C for 15 min. The sterile discs were soaked

in each of the heavy metal salt solutions of different concentrations (500, 200, 100, 50, 20 µg/mL) for 30 min and the excess salt solution was drained off. The salts of Cu^{2+}, Cr^{6+}, Pb^{2+} and Zn^{2+} were used in the form of CuSO4, $K_2Cr_2O_7$, $Pb(NO_3)_2$ and $ZnCl_2$, respectively.

2.4 Heavy metal tolerance test

Heavy metal tolerance assay was carried out by the disc diffusion method. The heavy metal-impregnated discs of different concentrations were placed on nutrient agar inoculated with overnight cultures of the test bacteria. The plates were incubated at 37 ^{o}C for 24 hr. After incubation, tolerance or sensitivity to a heavy metal was determined by the absence or presence of clear zone around the colonies on the plates. Isolates that showed no clear zones around them were employed in the maximum tolerable concentration assay of the tested heavy metal.

2.5 Determination of maximum tolerable concentration (MTC) of heavy metal

The maximum tolerable concentrations of the heavy metals under study were determined for the bacterial isolates using Tris-minimal salts (TMS) agar medium [14]. The medium was composed of (g/L): KCl (1.49), D-glucose (10), NH_4Cl (1.07), Tris-HCl (6.06), Na_2SO_4 (0.43), $CaCl_2.2H_2O$ (0.03), NaCl (4.68), $MgCl_2.2H_2O$ (0.2), pH 7. The tested concentrations were increased to 550-800 µg/mL for Cu^{2+}, 600-850 µg/mL for Pb^{2+}, 1200-1400 for Zn^{2+}. The concentration of Cr^{6+} was not increased in the MTC assay since the MTC of Cr^{6+} for all the heavy metal-tolerant isolates were already gotten from the heavy metal tolerance test preceding MTC determination. Isolates that exhibited relatively higher sensitivities were not employed in the determination of MTC. The highest concentration of heavy metal that allowed for the growth of the bacterial isolates after incubation for 48 hr at 37 ^{o}C was designated the maximum tolerable concentration (MTC) of that particular heavy metal.

The results obtained on TMS agar were confirmed in TMS broth by inoculating 0.1 mL of overnight broth culture (OD_{620}= 0.8) of each isolate in 10 mL sterile TMS broth supplemented with respective concentrations of the metals under study. A negative control (culture medium supplemented with the same concentrations of metals without inoculation) and blank (culture medium neither with inoculation with bacteria nor heavy metal supplementation) were set up. After incubation for 48 hr at 37 ^{o}C, bacterial growth was measured spectrophotometrically (UNICO 2100 UV) at 620 nm.

2.6 Antibiotic susceptibility test

Susceptibility of the bacteria to the different antibiotics used was performed by the agar-disc diffusion method [15] using antibiotic-impregnated discs (multi discs). The antibiotics used in the present study were oxytetracycline (50 µg), chlortetracycline (50 µg), chloramphenicol (50 µg), furazolidone (100 µg), sulphafurazole (500 µg), penicillin G (5 µg) and streptomycin (25 µg).

The antibiotic-impregnated discs were placed over freshly prepared Mueller Hinton agar seeded with overnight incubated bacterial isolates under study. Plates were incubated at 37 °C for 24 hr. The mean zones of inhibition were obtained by measuring in millimeters. The isolates were classified as resistant or sensitive by the mean diameter of inhibition zone given in standard antibiotic disc chart [16].

2.7 Statistical analysis

Results obtained were reported as the mean ± standard deviation of triplicate determinations. All data were subjected to one-way analysis of variance (ANOVA) using the Statistical Package for The Social Sciences (SPSS), version 20.0. Significant difference between means was established at p value of ≤ 0.05.

3 Results and discussions

3.1 Bacteria isolated

A total of six bacteria, namely, *Bacillus cereus, Citrobacter* spp., *Micrococcus varians, Escherichia coli, Proteus mirabilis and Salmonella* spp. (Table 1) were isolated from the different water samples and were identified tentatively on the basis of their cultural, morphological and biochemical characteristics. Some of these isolates have been reported [17] as drinking water isolates.

3.2 Heavy metal tolerance by the isolates

Isolates that were resistant to various concentrations of the different heavy metals were *Bacillus cereus, Citrobacter* spp, *Proteus mirabilis* and *Salmonella* spp. The above organisms have been reported by several researchers [1, 18] as having metal-tolerant attributes. Heavy metal tolerance was found in both Gram positive and Gram negative bacteria. Devika *et al*. [18] noted the same trend in a similar study in which they isolated both Gram negative and Gram positive multiple antibiotic and heavy metal tolerant bacteria from equatorial Indian ocean. This result revealed that drinking water sources are potential reservoirs for heavy metal-tolerant bacteria.

3.3 Maximum tolerable concentrations (MTC) of isolates to heavy metals

Isolates that were resistant from the heavy metal tolerance assay were subjected to MTC determination. The MTC profile of isolates is shown inTable 2.

Heavy metal tolerance is a common phenomenon in polluted environments. The MTC results revealed that *Bacillus cereus* had MTC value of 800 µg/mL for Pb^{2+}, 700 µg/mL for $Cu^{2+}$and 200 µg/mL for Cr^{6+} while *Salmonella* sp had MTC value of 1300 µg/mL for Zn^{2+}. Singh *et al*. [19] recorded lower MTC value (100 µg/mL) for Zn^{2+} among three heavy metal-tolerant *Bacillus* species isolated from Kanpur, India. *Citrobacter* spp. had MTC (100 µg/mL) for $Cr^{6+}$and Pb^{2+} (650 µg/mL). *Proteus mirabilis* had MTC (700 µg/mL) for Cu^{2+}. In this study, all the isolates showed tolerance in the order of metal concentrations of Cr^{6+}> Cu^{2+}> Pb^{2+}> Zn^{2+}. The above trend varies slightly with Devika *et al*. [18] that had Pb^{2+}> Cu^{2+} instead. However, Ahemad and Malik [1] in a study on prevalence of heavy metal and antibiotic resistance among bacterial isolates noted a similar trend of heavy metal resistance in the order Cu^{2+}> Pb^{2+} among bacteria belonging to the genera *Bacillus, Pseudomonas* and *Staphylococcus*. Wani *et al*. [20] recorded a higher tolerance to Cr^{6+} by three *Bacillus* isolates PSB1, PSB7 and PSB10 which tolerated Cr^{6+} ($K_2Cr_2O_7$) 550, 400 and 550 µg mL^{-1}, respectively. Their variance with the present study can be attributed to the report of Hassan *et al*. [21], who noted that the differences that exist in the toxicity profile of bacterial isolates could be as a result of the physiological characteristics, nature of bacteria and the conditions surrounding each bacterial cell isolation. *Salmonella* spp and *Proteus mirabilis* also demonstrated tolerance to the heavy metals and antibiotics (Table 2 and Table 3). This could be as a result of the fact that the genes coding for both heavy metal and antibiotics tolerance are located on the same region on bacterial operons and are hence likely transferred together in the environment [22]. As can be noted from the present study (Table 3), *Salmonella* spp. and *Proteus mirabilis* were the most tolerant in the antibiotic tolerance assay while *Bacillus cereus* was most tolerant to the varying concentrations of heavy metals except Zn^{2+} (Table 2).All the heavy metal-tolerant isolates shown in Table 2 have also been reported by several researchers [23, 24, 18] as exhibiting high heavy metal tolerance. These metals, have also been reported widely by several authors [1, 24] as being toxic to microbial cells. *Escherichia coli* was sensitive to various concentrations of the heavy metals. Ahemad and Malik [1] noted a similar attribute in a sensitive *Escherichia coli* strain which they used as the control for heavy metal and antibiotic resistance experiment.

Property	Characteristic					
Gram reaction	+	+	-	-	-	-
Catalase	+	+	+	+	+	+
Oxidase	-	+	-	+	-	-
H2S production	-	-	-	-	+	+
Starch hydrolysis	+	-	-	-	-	-
Methyl red	+	-	+	-	+	-
Urease	-	+	-	+	+	-
Citrate utilization	-	+	-	+	+	-
Voges Proskauer	-	+	-	-	-	-
Motility	+	-	+	+	+	+
Spore formation	+	-	-	-	-	-
Probable identity	Bacillus cereus	Micrococcus varians	E. coli	Citrobacter spp	Proteus mirabilis	Salmonella spp

Table 1. ***Biochemical characteristics of bacterial isolates*** +: Positive; -: negative

Tolerant strains				
Metal ion	*Bacillus cereus*	Citrobacter spp.	*Proteus mirabilis*	Salmonella spp.
Cu2+	750	600	650	600
Pb2+	800	650	700	700
Cr6+	200	100	150	150
Zn2+	1200	1000	950	1300

Table 2. ***Maximum tolerable concentrations (MTC) (µg/mL) of resistant strains***

Antibiotic	Conc . (µg/disc)	Bacillus cereus	Micrococcus varians	Escherichia coli	Citrobacter spp.	Proteus mirabilis	Salmonella spp.
Oxytetracycline	50	I	S	R	R	R	R
Chlortetracycline	50	R	I	R	R	R	R
Chloramphenicol	50	R	R	R	R	R	R
Furazolidone	100	R	S	R	R	R	R
Sulphafurazole	500	S	R	S	S	R	R
Penicillin G	5	R	R	R	R	R	R
Streptomycin	25	R	S	I	I	R	R

Table 3. ***Antibiotic tolerance patterns of isolates*** T: tolerant; S: susceptible; I: Intermediate

3.4 Antibiotic tolerance of isolates

The antibiotic tolerance/susceptibility profile of the isolates is shown in Table 3. The isolates exhibited varying degrees of tolerance and susceptibility towards the antibiotics at various concentrations. All the isolates showed resistance to penicillin G at the concentrations used. Penicillin is among the first antibiotics produced and as such, organisms may have developed resistance to the natural antibiotics faster because they might have been pre-exposed to these compounds in nature. McDonnell and Trenois [25] recorded resistance to penicillin among forty coliform isolates tested. Odeyemi [26] in a similar study reported the highest resistance to penicillin. Furthermore, penicillin is active against Gram-positive bacteria. Meanwhile, the majority (66.7%) of the isolates were Gram negative (Table 1). This might also explain the high penicillin tolerance by all the Gram negative isolates in this study. Also, all the Gram negative isolates were tolerant to five antibiotics including penicillin probably because of heavy use of these antibiotics which are fundamentally intended against them as frequent drinking water enteric Gram negative isolates. Corroborative evidence of this trend was clearly documented [22].

In the present study, fifty percent of the isolates were susceptible to furazolidone and sulphafurazole. Furazolidone antibiotics are used heavily against diarrhoea and cholera-causing bacteria such as *Escherichia coli* and *Vibrio cholera.* Sulphafurazole is also a broad-spectrum bacteriostatic agent. *Proteus mirabilis* and *Salmonella* spp. showed high tolerance to all the antibiotics. Poonia *et al*. [27] reported a similar trend in which *Escherichia, Citrobacter,*and *Proteus*isolated from natural water sources in East Sikkim were shown to be resistant to the antibiotics against which they were tested. Multidrug-resistant strain of *Salmonella typhymurium* has also been identified in cattle in England and other locations [28].

4 Conclusion

In the present study, the bacterial isolates were relatively resistant to some antibiotics and heavy metals employed. Furthermore, it was observed that most of the antibiotic-resistant bacteria were also metal-tolerant. This observation suggests that the genes that code for antibiotic and heavy metal tolerance are horizontally transferred together in the environment.

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