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Effects of Heavy Metals on Bacterial Growth, Biochemical Properties and Antimicrobial Susceptibility

Faith Ngwewa[1,2,3,*], George Kasali[4], Ernatus M. Mkupasi[3], Abdul A. S. Katakweba[5]

[1]Inter- University Council for East Africa, P.O Box 7110, Kampla, Uganda; Sponsor
[2]Southern African Centre for Infectious Disease Surveillance (SACIDS), P.O Box 3297, Sokoine University of Agriculture, Morogoro, Tanzania; Sponsor
[3]Department of Veterinary Medicine and Public Health, Sokoine University of Agriculture, P. O. Box 3015, Chuo Kikuu, Morogoro, Tanzania; Registered Department at SUA
[4]Department of Biological Sciences, Copperbelt University, P.O Box 21692, Kitwe, Zambia
[5]Institute of Pest Management, Sokoine University of Agriculture, P.O.BOX 3110, Chuo Kikuu, Morogoro, Tanzania
*Corresponding author: faithngwewa7@gmail.com

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Abstract Heavy metals are metallic elements that occur naturally and have a high atomic weight and a density which is five times greater than the density of water. Several studies have demonstrated that metal contamination in natural environments could have an important role in the development and spread of antimicrobial resistance. This is of particular concern, considering that heavy metals usually occur at higher levels than pharmaceutically produced antimicrobials. Also, the bio-accumulative and non-biodegradable nature of heavy metals may result in long- term antimicrobial resistance selection pressure. Therefore, this study assessed the effects of Copper, Cobalt, Cadmium, Zinc and Lead salts on growth, biochemical properties and antimicrobial susceptibility of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* to commonly used antimicrobials. The selected bacteria were exposed to varying concentrations of the heavy metal salts ranging from 1 ppm to 1000 ppm after which growth was measured using a spectrophotometer. The effect on biochemical reactions and antimicrobial susceptibility were also tested by the use of conventional biochemical tests and Kirby-Bauer disk diffusion methods respectively. Different heavy metals affected the growth of each individual microorganism differently. Despite that, the growth of *E. coli*, *P. aeruginosa* and *S. aureus* was observed to be inversely proportional to the concentrations of the heavy metals used. Following exposure to heavy metals, *E. coli* and *S. aureus* showed no changes in their biochemical properties but *P. aeruginosa* gave a positive urease result contrary to the control. *E. coli* developed resistance to Levofloxacin, Meropenem and Tetracycline while *S. aureus* to Azithromycin and Gentamicin and *P. aeruginosa* developed resistance only to Meropenem. These results confirm the adverse effects of heavy metals on bacterial growth and reveals that heavy metals can cause changes in some biochemical properties of bacteria that produce any of these properties under stressful growing conditions. This research also supports the findings that heavy metals enhance the development of antimicrobial resistance in otherwise antimicrobial sensitive strains of bacteria. This study therefore, gives insight into the likely state of the proliferation of heavy metal induced antimicrobial resistance in heavy metal contaminated areas.

Keywords: *heavy metals, antimicrobial resistance, resistance mechanisms, toxicity, biochemical reactions, bacterial growth*

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1. Introduction

Heavy metals are metallic elements that are naturally occurring and have a high atomic weight and a density which is five times greater than the density of water [1]. They include but are not limited to Cadmium, Copper, Lead, Zinc, Cobalt, Mercury, Nickel and Chromium [2]. These exist in the environment through natural weathering processes or chemical reactions such as redox reactions, acid/ base reactions and alteration of pH levels occurring in the soil and water [3,4]. Heavy metals are also added through anthropogenic sources like mining, agriculture (both crop and livestock farming), waste water discharge from industries and households and these are more bioavailable than the naturally occurring ones hence presenting a serious worldwide problem [5].

In environmental ecosystems, there is interaction between heavy metal contaminants and the microorganisms that reside there. Small quantities of these metals such as Magnesium (Mg), Nickel (Ni), Chromium (Cr3+), Copper

(Cu), Calcium (Ca), Cobalt (Co), Manganese (Mn), Sodium (Na) and Zinc (Zn) are essential for metabolism and redox functions but can be toxic at higher concentrations [6]. Others such as Aluminium (Al), Lead (Pb), Cadmium (Cd), Gold (Au), Mercury (Hg), and Silver (Ag) usually have no biological role and hence can be toxic to microorganisms and other living organisms [7,8,9]. In biological systems, heavy metals interact with cellular components such as the cell membranes, deoxyribonucleic acid (DNA), nuclear proteins and some enzymes involved in metabolism, detoxification, and damage repair [10]. These interactions sometimes lead to apoptosis or cell death [10,11,12]. This has eventually led to the use of metal compounds as antimicrobial agents in a number of medical devices such as catheters and other medical products [13].

However, bacteria tend to be highly sensitive to metal pollution and evolve a variety of resistance mechanisms that are intrinsic or acquired either by chromosomal mutations or through transferable genetic materials [14]. These resistance mechanisms include modification and inactivation of therapeutic agents, overexpression of efflux pumps, alteration of bacterial receptors and use of the impermeable cell envelope adaptive response that promotes survival in harsh environments [15,16]. They are very similar to antimicrobial resistance mechanisms, consequently, causing great concern about heavy metals selecting indirectly for antimicrobial resistance by co-selection. This indirect selection process occurs as a result of coupling of the resistance mechanisms against antimicrobial agents and heavy metals. They can be coupled physiologically (cross-resistance) or genetically (co-resistance) [14].

Several studies have also demonstrated that metal contamination in natural environments could have an important role in the development and spread of antimicrobial resistance (AMR) [17,18]. This is of particular concern, considering that heavy metals usually occur at higher levels than pharmaceutically produced antimicrobials [19] and as a result, the bio-accumulative and non-biodegradable nature of heavy metals may result in long- term AMR selection pressure [20,21].

This in- vitro study therefore seeks to analyze the effect of different concentrations of Copper, Cobalt, Cadmium, Zinc and Lead selected from a list of metals deemed to have increasing recognition as antimicrobial agents [22] on microbial growth, biochemical properties and antimicrobial susceptibility in *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923. These microorganisms are control strains of some of World Health Organization's (WHO) antimicrobial resistant priority pathogens [23]. American Type Culture Collection (ATCC) were used as all the biochemical properties are known and any changes in these properties could easily be attributed to the presence of heavy metals in the in-vitro growth environment. Therefore, this study gives predictive insight into the microbial diversity and the proliferation of antimicrobial resistance in heavy metal contaminated areas.

2. Materials and Methods

Control strains of *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 25923 were obtained from the storage vials at the Tropical Diseases Research Center (TDRC) in Ndola, Zambia and screened using basic microbiological identification techniques for confirmation of the bacteria. Four to five identical colonies of each confirmed microorganism were then picked from the culture plates (Blood Agar and MacConkey Agar) and inoculated into Normal saline adjusting the turbidity to that of 0.5 McFarland standards making the microbial inoculum solution.

Five heavy metal salts; Cobalt (ii) nitrate hexahydrate [$Co(NO_3)_2.6H_2O$], Copper (ii) acetate monohydrate [$Cu(CH_3COO)_2.H_2O$], Lead (ii) acetate trihydrate [$Pb(CH_3COO)_2.3H_2O$], Zinc acetate dihydrate [$Zn(CH_3COO)_2.2H_2O$] and Cadmium acetate dihydrate [$Cd(CH_3COO)_2.2H_2O$] were used to make 1000 ppm concentrations. This was done by dissolving a calculated amount of each metal salt into 1000 ml of sterile distilled water. The amount of metal salt was determined by;

$$\text{Amount of metal salt} = \frac{\text{molecular weight of metal salt}}{\text{molar mass of target metal}} \quad (1)$$

Therefore, 4.9385 g of $Co(NO_3)_2.6H_2O$, 3.1418 g of $Cu(CH_3COO)_2.H_2O$, 1.8307 g of $Pb(CH_3COO)_2.3H_2O$, 3.3572 g of $Zn(CH_3COO)_2.2H_2O$ and 2.3710 g of $Cd(CH_3COO)_2.2H_2O$ where dissolved in 1000 ml of sterile distilled water each to make 1000 ppm concentrations which were considered as the stock solutions. From the heavy metal stock solutions, serial dilutions were carried out to make 100 ppm, 10 ppm and 1 ppm concentrations [24].

5 ml of each of the four concentrations of each metal salt was pipetted into sterile falcon tubes to which 5 ml peptone water (source of nutrients) was added in order to ensure equal volume of nutrients and heavy metals for all microorganisms. For the control (denoted by PW + NS), 5 ml of normal saline was added to the 5 ml of peptone water in the falcon tubes. This protocol was an altered version of the one used by Rao *et al.* [25]. All culture media was prepared and dispensed according to the manufacturer's instructions (Oxoid, UK) and sterility and quality control tests were performed before use.

2.1. Exposure of *E. coli, P. aeruginosa* and *S. aureus* to Different Heavy Metal Concentrations

A loopful of the inoculum was inoculated into each of the tubes containing the mixture of heavy metals and nutrients and the initial absorbance (optical density) was measured using a spectrophotometer at a wavelength of 600 nm. The tubes were then incubated at 37 °C for 24 h after which the final absorbance was measured [25]. A loopful of the inoculum from the incubated tubes was then obtained using a sterilized wire loop and streaked onto MacConkey agar for *E. coli* and *P. aeruginosa* and Blood agar for *S. aureus* to investigate viability of the microbes after 24 h exposure to heavy metals.

2.2. Biochemical Tests

After the 24 h incubation, routinely used conventional biochemical tests for identification of clinical bacteria were employed [26].

For *E. coli* and *P. aeruginosa*, a pure isolated colony of each organism was aseptically picked from each MacConkey agar plate and inoculated into Triple Sugar Iron (TSI), Lysine Iron Agar (LIA), Sulphur Indole and Motility (SIM), Urease and Citrate media. All culture tubes were then incubated at 37 °C for 24 h after which the results were read and recorded. Oxidase reagent was prepared according to the manufacturer's instructions and used to perform the oxidase test on the exposed *E. coli* and *P. aeruginosa* [26].

For *S. aureus*, a pure isolated colony was aseptically picked from each Blood Agar plate and inoculated onto Mannitol Salt Agar (MSA) and incubated at 37°C for 24 h. Catalase and coagulase tests were also performed [26].

2.3. Antimicrobial Susceptibility Tests

Antimicrobial susceptibility testing (AST) was done using the Kirby-Bauer disc diffusion method [27] and interpretation of the results was based on Clinical Laboratory Standards Institute (CLSI) guidelines [28]. Commonly administered antimicrobial agents (Oxoid, UK) were used. These were selected from resistance prone antimicrobial classes which include β Lactams, Aminoglycosides, Quinolones, Tetracyclines and Macrolides. Therefore, the list of antimicrobials used included Ampicillin (10 µg), Ceftazidime (30 µg), Meropenem (10 µg), Levofloxacin (5 µg) and Tetracycline (30 µg), Gentamicin (10 µg), and Azithromycin (15 µg) (Oxoid, UK). Direct colony suspension technique was used by suspending 4-5 colonies of *E. coli*, *P. aeruginosa* and *S. aureus* in 5 mL 0.85% (w/v) Normal saline each and adjusting the visual opacity of the inoculum to that of 0.5 McFarland Standard (1.5 X 10^8 CFU/ml). A sterile cotton swab was dipped into the colony suspension and then evenly lawned onto the surface of Mueller Hinton (MH) agar (HiMedia) plates. Antibiotic paper discs were dispensed onto the MH agar plates as stated in Table 1. The plates were allowed to dry for 20 minutes before being incubated at 37 °C for 18-24 h, after which the zone diameters were measured and interpreted using standard break points by the use of the CLSI guidelines [28] as resistant, intermediate or susceptible.

Table 1. Specific Antibiotics and Their Concentrations Used for AST on Each Microorganism

Organism	Antibiotic	Concentration (µg)
E. coli	Ampicillin	10
	Ceftazidime	30
	Levofloxacin	5
	Meropenem	10
	Tetracycline	30
P. aeruginosa	Ceftazidime	30
	Gentamicin	10
	Levofloxacin	5
	Meropenem	10
S. aureus	Ampicillin	10
	Azithromycin	15
	Gentamicin	10
	Levofloxacin	5
	Tetracycline	30

3. Results and Discussion

3.1. Effects of the Heavy Metals on Growth of *E. coli, P. aeruginosa* and *S. aureus*

There was increased optical density (absorbance) in all tubes just like the control (PW + NS). However, the growth of *E. coli*, *P. aeruginosa* and *S. aureus* was observed to be inversely proportional to the concentrations of the heavy metals used. This was drawn from the lower differences between the final and initial absorbance in organisms that were exposed to heavy metals in comparison to the control as shown in Table 2 below.

Table 2. Growth of Microorganisms After 24 h Exposure to Heavy Metals

Metal	Conc (ppm)	*E. coli*	*P. aeruginosa*	*S. aureus*
		Diff. in Abs	Diff. in Abs	Diff. in Abs
	Control	**0.757**	**0.516**	**0.841**
Cu	1	0.598	0.392	0.614
	10	0.377	0.250	0.655
	100	0.089	0.202	0.238
	1000	0.002	0.060	0.009
Co	1	0.552	0.288	0.550
	10	0.518	0.247	0.457
	100	0.008	0.016	0.054
	1000	0.008	0.005	0.011
Cd	1	0.548	0.305	0.440
	10	0.294	0.202	0.360
	100	0.044	0.128	0.252
	1000	0.004	0.054	0.107
Zn	1	0.508	0.454	0.532
	10	0.464	0.287	0.460
	100	0.179	0.189	0.360
	1000	0.004	0.002	0.052
Pb	1	0.489	0.288	0.402
	10	0.453	0.246	0.385
	100	0.444	0.219	0.373
	1000	0.42	0.201	0.348

These results confirm the toxic effects of the heavy metals on microorganisms as reported by other researchers [29,30]. Heavy metals create a stressful environment for the growth of microorganisms when present in the broth. This was observed even in the tubes that had no growth on the culture plates but showed a slight increase in absorbance. This entails that the microorganisms begun to grow but could not survive because the heavy metals present in the broth made the environment toxic.

Different heavy metals affected the growth of each individual microorganism differently which led to some concentrations of the heavy metals completely inhibiting the growth of the microorganisms as shown in Figure 1.

The growth of *E. coli* was decreased by all the metals. Cobalt however was the most toxic metal for *E. coli* as it completely inhibited growth at 100 ppm and its toxicity can be attributed to its ability to produce hyperoxide radicals [31]. Copper, Cadmium and Zinc all completely

inhibited the growth of *E. coli* at 1000 ppm whereas Lead did not completely inhibit growth. Bacteria tends to sediment Lead as a phosphate salt using the intra- and extracellular binding of Lead ions together with membrane transport pumps as a way of escaping the toxicity of free Lead ions [32]. This could explain the behavior of *E. coli* and the other microorganisms in the broth culture containing Lead. Otherwise, this supports the findings by other researchers [31] that Lead is not extraordinarily toxic to microorganisms.

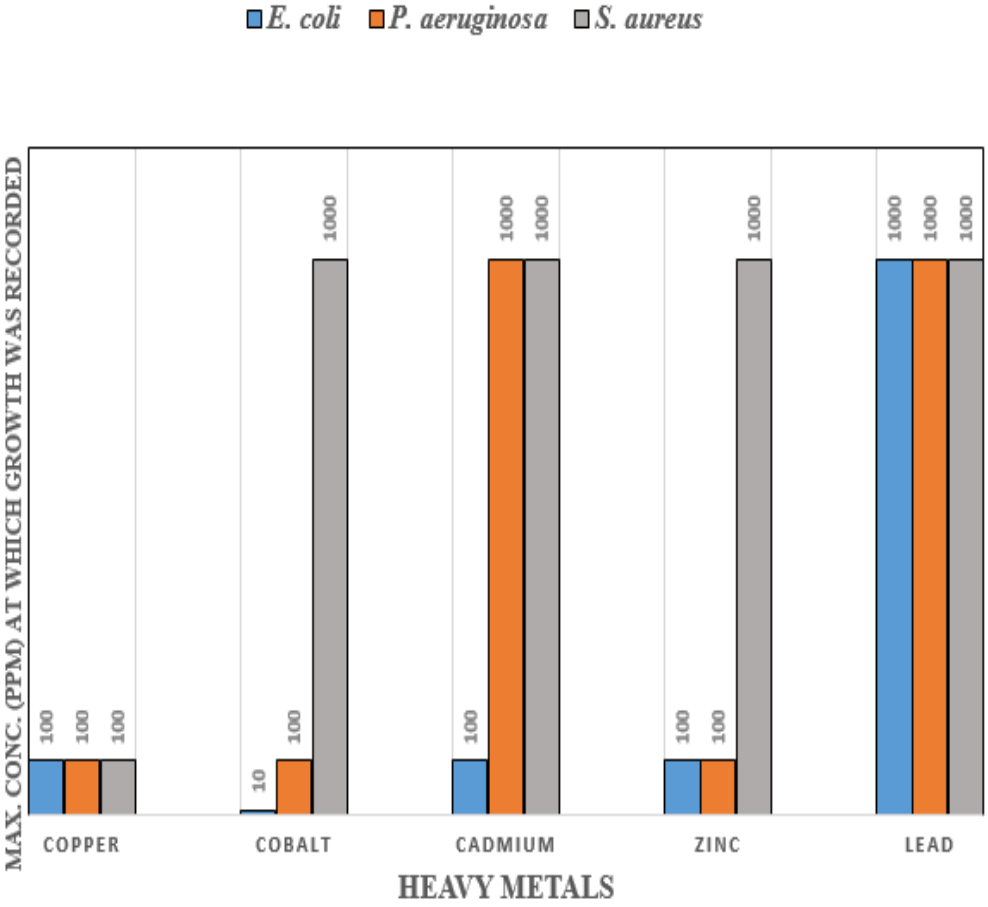

Figure 1. Effect of the different heavy metals on growth of the individual microorganism

For *P. aeruginosa*, Copper, Cobalt and Zinc were the most toxic metals and they completely inhibited growth at 1000 ppm. Copper and Cobalt are known to induce toxicity by the use of hyperoxide radicals while Zinc utilizes "thiol-binding and protein denaturation as ways of affecting microorganisms [14,31]. Lead and Cadmium also decreased the growth of *P. aeruginosa* but did not completely inhibit it. Many researchers have reported *P. aeruginosa* to be Cadmium-resistant when isolated from different environments. The tolerance mechanism *P. aeruginosa* utilizes against Cadmium is efflux pumps found in the P-type ATPase transport system. This is drawn from research findings that revealed that the *CadA* system for Cadmium efflux is the mechanism used for Cadmium tolerance and is the most prominent metal system [15,33].

S. aureus on the other hand was only completely inhibited by 1000 ppm Copper. The radical character of Copper makes it very toxic. It is assumed that radicals and unstable products of enzymatic reactions, disrupt enzymatic hydrolysis and redox reactions eventually leading to cell death [31]. This is the mode of action that Copper used to inhibit the growth of *S. aureus* at a concentration of 1000 ppm. Cobalt, Cadmium, Zinc and Lead did not completely inhibit the growth of *S. aureus* at any concentration because this organism is known to employ different resistance mechanisms to evade heavy metal toxicity. In this instance, blocking membranes were used in conferring resistance to Zinc while detoxification of Cadmium, Lead and Copper was done by ATPases which bind metals according to affinity of individual structures [34]. *S. aureus* has generally been known to evolve signal regulators that enable it to adapt to variable habitats by modulating bacterial cellular responses and expressing virulence genes. It also utilizes biofilm formation to protect itself in response to stressful conditions [35] which could explain how *S. aureus* grew in almost all the concentrations of heavy metals.

3.2. Effects of the Heavy Metals on Biochemical Properties of *E. coli*, *P. aeruginosa* and *S. aureus*

The heavy metals under study had no effect on the biochemical properties of *E coli* and *S. aureus*. This was depicted by the biochemical tests which showed no difference from the control characterization. However, despite there being no difference in biochemical reactions between *P. aeruginosa* control and heavy metal exposed *P. aeruginosa* in TSI, LIA, Citrate and SIM media, different

concentrations of Copper, Cobalt, Cadmium and Zinc induced a positive urease result, contrary to that of the control as shown in Figure 2 and Figure 3 below.

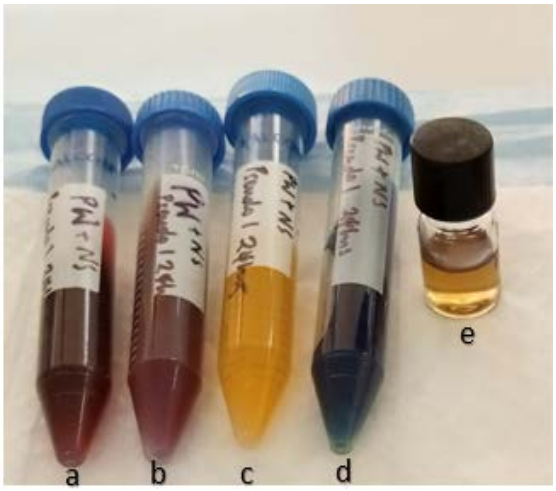

Figure 2. Biochemical reactions of *P. aeruginosa* control in TSI (a), LIA (b), Urease (c), Citrate (d) and SIM (e)

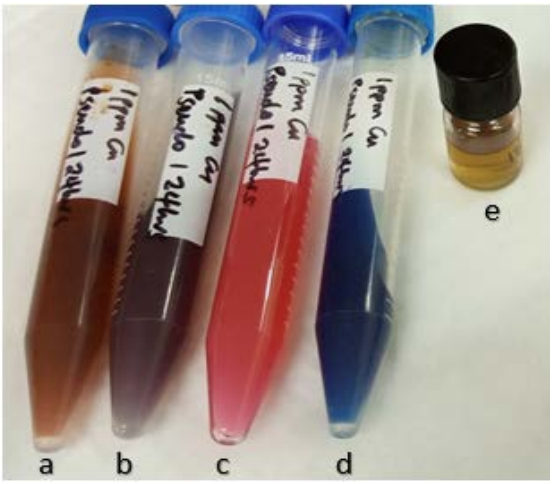

Figure 3. Biochemical reactions of heavy metal exposed *P. aeruginosa* in TSI (a), LIA (b), Urease (c), Citrate (d) and SIM (e)

An *in vitro* study had confirmed measurements of urease activity of *P. aeruginosa* by Isotope Ratio Mass Spectrometry by the use of similar techniques as prior studies of *M. tuberculosis* [36]. Also, a proof of concept study to detect urease producing bacteria in the lungs selected Cystic Fibrosis patients with confirmed *P. aeruginosa* colonization because urease enzyme is a widely expressed virulence factor of many bacterial and fungal pathogens, including *P. aeruginosa* [37]. These studies reveal the ability of *P. aeruginosa* to express urease as a virulence factor therefore entailing that 1 ppm Cu, 10 and 100 ppm Co, 100 and 1000 ppm Cd and 10 ppm Zn induced the expression of urease enzyme as a virulence factor in *P. aeruginosa*. However, this needs to be studied in detail in order to confirm this assumption and to ascertain the exact mechanism of action of these concentrations of different metals that leads to the production of this virulence factor.

On the other hand, the positive urease test in *P. aeruginosa* could have been as a result of the utilization of peptones or other proteins and not necessarily urease production. This is because one of the limitations of urea agar base is that prolonged incubation causes a rise in pH due to protein hydrolysis and the release of excessive amino acid residues causing a positive urease result [38]. This change in one of the biochemical properties due to exposure to heavy metals can lead to incorrect identification of causative agents which in turn can consequently lead to misdiagnosis and presents a potential threat to health of people in heavy metal polluted areas.

3.3. Effects of the Heavy Metals on Antimicrobial Susceptibility of *E. coli, P. aeruginosa* and *S. aureus*

The ultimate goal of antimicrobials is high efficacy at low dosage without the evolution of resistance. But following exposure to heavy metals, *E. coli* developed resistance to Levofloxacin, Meropenem and Tetracycline, *S. aureus* developed resistance to Azithromycin and Gentamicin while *P. aeruginosa* only developed resistance to Meropenem. Some of the control organisms used showed resistance to some of the antimicrobials they were exposed to. This could have resulted from the continuous thawing and freezing of the microorganisms; therefore, development of resistance was interpreted with reference to the control. The antimicrobial susceptibility tests of the bacterial cultures revealed that the *E. coli* and *S. aureus* bacterial isolates were multi drug resistant with multiple antibiotic resistance (MAR) indexes of 0.6 and 0.4 respectively.

This trend could be attributed to the production of enzymes which are capable of inactivating or modifying specific antimicrobial agents and can alter bacterial cell membranes and antibiotic target sites [39]. The resistance of the organisms to the antimicrobial agents confirms the correlation between metal resistance and antimicrobial resistance [40]. This is drawn from the likelihood that resistance genes to both antimicrobials and heavy metals could be closely located on the same plasmid in bacteria and are thus more likely to be transferred together in the environment [41].

With regards to *E. coli*, Copper, Cobalt, Cadmium and Zinc induced resistance to Levofloxacin and Meropenem and Cobalt induced resistance to Tetracycline. Resistance to Levofloxacin could have been caused by genetic changes in the *gyrA* gene which is involved in transcription and translation and encodes DNA gyrase, which is the target of Fluroquinolones. This happens via mutagenesis as a result of exposure to sub-lethal concentrations of heavy metals [42]. Exposure to heavy metals is also known to induce genetic changes that are associated with *dacA* gene which encodes D-alanyl-D-alanine carboxypeptidase and thereby playing an important role in Beta-lactam resistance [43]. This explains how heavy metals induced resistance to Meropenem in *E. coli*. The Cobalt present in the broth could have activated the expression of Tetracycline-responsive repressor, *TetR* which tightly regulates the expression of *tetA* mRNA which is a common Tetracycline efflux pump thereby conferring resistance to Tetracycline [44]. However, lower concentrations of the heavy metals produced a synergic effect with Tetracycline which entails that they could have

blocked the expression of *TetR*, a phenomenon that needs further investigation.

For *P. aeruginosa*, the question of heavy metal and antibiotic resistance is of particular concern since this organism is used in bioremediation where selection of antimicrobial resistance after exposure to heavy metals is undesirable. Regardless, *P. aeruginosa* exposed to Copper, Cadmium, Zinc and Lead showed resistance to Meropenem which is a Carbapenem. The cross-resistance recorded between the heavy metals and Meropenem in *P. aeruginosa* can be attributed to co-regulation of the Carbapenem influx and heavy metal efflux. This is known to cause a change in *CzcR-CzcS*, a two-component sensor protein thereby regulating heavy metal efflux pump expression [45]. *P. aeruginosa* also produced pyoverdine, a siderophore shown in Fig 4 which is produced as a means of survival under stressful conditions and has several known functions that contribute to virulence and biofilm formation which are essential for pathogenesis in mammalian infections [46]. This entails that the presence of heavy metals increases virulence in *P. aeruginosa*. Biofilm formation which is typical of *P. aeruginosa* also contributes to the microorganism being able to evade the effects of the Meropenem [47].

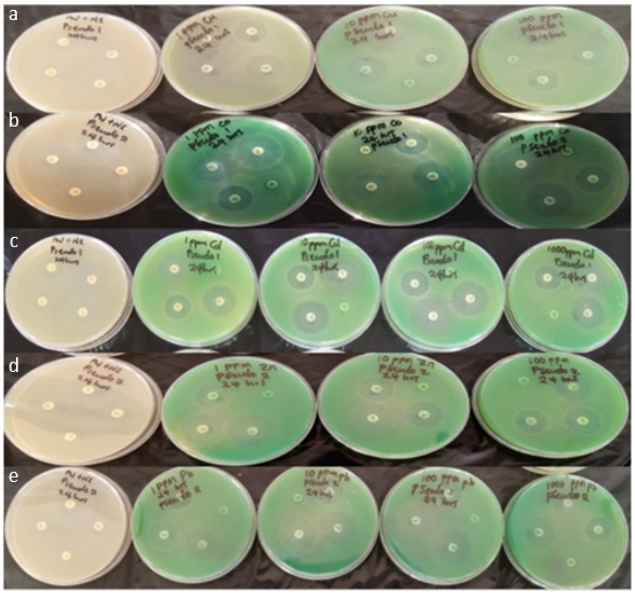

Figure 4. AST results for *P. aeruginosa* control against heavy metal exposed *P. aeruginosa* *First column shows AST results for *P. aeruginosa* control (PW+NS) against exposure to Copper (row a), Cobalt (row b), Cadmium (row c), Zinc (row d) and Lead (row e)

In the case of *S. aureus*, many studies have shown positive correlation between heavy metal (Cobalt and Zinc) tolerance and AMR. *S. aureus* isolated from livestock was described to be harboring plasmids carrying resistance genes for Copper and Cadmium (*copA*, *cadDX* and *mco*) and for multiple antimicrobials including Macrolides, Lincosamides, Streptogramin B, Tetracyclines, Aminoglycosides and Trimethoprim (*erm(T)*, *tet(L)*, *aadD* and *dfrK*) [48]. The link between Zinc usage in animal feeds and the occurrence of methicillin resistant *S. aureus* (MRSA) is explained by the physical presence of the Zinc resistance gene, *czrC*, on the Methicillin resistance-encoding *SCCmec* element [49,50]. These provide evidence of co-resistance which could be the same mechanism that Zinc and Copper used to confer resistance to Azithromycin, a Macrolide and Gentamicin, an Aminoglycoside.

4. Conclusion

This study confirms that bacterial growth decreases with increase in heavy metal concentration. It also revealed that individual microorganisms have different mechanisms of evading the toxicity of different heavy metals. As a result, each microorganism was affected differently by each heavy metal. The study also showed that heavy metals do not affect the biochemical properties of microorganisms. However, changes may occur in instances where the property is produced under stressful growing conditions as was the case with *P. aeruginosa* which produced urease after exposure to heavy metals. The findings of this research also entail that Copper, Cobalt, Cadmium, Zinc and Lead are all potential co-selectors for antimicrobial resistance thereby proving that heavy metal contamination enhances the development of antimicrobial resistance in otherwise antimicrobial sensitive strains of bacteria and may contribute to multiple drug resistant bacteria. This therefore, gives insight into the likely state of the proliferation of heavy metal induced antimicrobial resistance in heavy metal contaminated areas.

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Statement of Competing Interests

The authors have no competing interests.

List of Abbreviations

TDRC: Tropical Diseases Research Centre

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