

Original Research Article

Antibacterial Susceptibility of Heavy Metals Tolerant Bacteria Isolated From NILEST Tannery Effluent

ABSTRACT

Background: The emergence of multiple antibiotics resistance among bacterial population poses a potential threat to human health. The co-existence of metal/antibiotic resistance in bacterial strains suggests the role of heavy metals as a factor which can also contribute to drug resistance phenomenon since heavy metals pollution results in selective pressure that leads to the development of multiple drug resistance among bacterial populations probably through horizontal gene transfer.

Aim: This study was aimed to isolate, identify, and determine the antibiogram profile of heavy metals tolerant bacteria from NILEST tannery effluent using standard microbiological methods.

Methodology: Tannery effluent was cultured on heavy metals incorporated nutrient agar. The resultant isolates were purified by sub-culturing on fresh heavy metals incorporated nutrient agar and the antibiogram profile was determined using agar diffusion method.

Results: The bacteria isolated include *Bacillus cereus* (18.75%), *Escherichia coli* (12.50%), *Klebsiella aerogenes* (18.75%), *Klebsiella pneumoniae* (6.25%), *Pseudomonas aeruginosa* (12.50%), *Proteus mirabilis* (6.25%), and *Staphylococcus aureus* (25.00%) with *Staphylococcus aureus* being the most prevalent. Of the total of nine antibiotics tested, gentamicin was highly resisted followed by ampicillin and amoxicillin.

Conclusions: The identified heavy metals tolerant bacteria strains could be useful for bio-remediation of heavy metal contaminated wastewater and soil environments even though they are potential threat to successful chemotherapy if incriminated in infections.

Keywords: Antibiotic resistance, Heavy metals, Tannery effluents, Public health, Bio-remediation.

1. INTRODUCTION

Among the pollutants into the ecosystems are heavy metals such as mercury, cadmium, chromium, lead, copper, zinc, arsenic, and other chemicals which affect aquatic life including microbial communities [1]. Introduction of heavy metals in various forms in the environment produces considerable modifications of microbial communities and their activities [2].

Microorganisms have evolved mechanisms to detoxify heavy metals, and some even use them for respiration. To survive under metal stressed conditions, bacteria have evolved mechanisms to tolerate the uptake of heavy metal ions. These several mechanisms include the efflux of metal ions outside the cell, accumulation, complexation, and reduction of the metal ions to a less toxic state [3]. These microbial interactions with metals may have several implications for the environment. The microbes may play an important role in the biogeochemical cycling of toxic metals as well as remediating metal contaminated environments.

Toxic metals in the environment lead to selective pressure among the microbial communities resulting in the development of metal resistant populations [4]. There is evidence of a correlation between tolerance to heavy metals and antibiotic resistance, a global problem threatening the treatment of infections in plants, animals, and humans [5]. Resistance genes to both antibiotics and heavy metals may be located closely together on the same plasmid in bacteria and are more likely to be transferred together in the environment. Metal tolerance and antibiotic resistance in bacteria have been shown to increase proportionally along industrial contamination gradients [6]. It has been shown that bacterial isolates resistant to vanadium show increased resistance to quinolones (Ciprofloxacin and Norfloxacin), which are crucial in the management of salmonella infections. The genes can be transferred to indigenous

populations of microorganisms occurring frequently in the environments thereby enhancing the spread of antibiotic resistance [7].

The significant increase of Multiple Antibiotic Resistant (MAR) bacteria are observed in various aquatic systems and human infections caused by these bacteria and could be difficult to treat with available drugs [8]. The resistance development may be due to nonspecific mechanism with gene regulation of plasmids and chromosomes, which may be heritable or transferable due to the presence of resistance (R-factor) factor [9].

Microbes have adapted to tolerate the presence of metals or can even use them to grow. Thus, a number of interactions between microbes and metals have important environmental and health implications. Some implications are useful, such as the use of bacteria to clean up metal-contaminated sites. Other implications are not as beneficial, as the presence of metal tolerance mechanisms may contribute to the increase in antibiotic resistance. Overall, it is most important to remember that what we put into the environment can have many effects, not just on humans, but also on the environment and on the microbial community on which all other life depends.

The objective of this study therefore is to isolate and biochemically identify heavy metals tolerant bacterial isolates in NILEST tannery effluent and determine their antibiogram profile.

2. MATERIALS AND METHODS

2.1 Collection of Sample

Tannery effluent was collected from NILEST Tannery. Liquid (effluent) sample was collected in sterile sample bottles and were transported to Microbiology Laboratory of the Nigerian Institute of Leather and Science Technology Samaru, Zaria for microbiological analysis. The inoculation of sample was done within one hour of collection.

2.2 Preparation and Inoculation of Sample

A ten-fold serial dilution was carried out on the effluent from 10^{-1} to 10^{-5} and a 10^{-3} dilution was inoculated into heavy metals incorporated nutrient agar media.

2.3 Identification of Heavy Metals Tolerant Bacteria

Heavy metal incorporated nutrient agar media was used for selective isolation of heavy metals (Pb^{2+} , Cr^{6+} , Hg^{2+} , Zn^{2+}) tolerant bacteria. The media was prepared with the concentration of 5mg/L of each of the heavy metal incorporated into nutrient agar [10]. A dilution of 10^{-3} prepared sample was inoculated onto the heavy metals incorporated media plates and was incubated for 24-48 hours at $37^{\circ}C$. This procedure was repeated for all heavy metals tested. The agar plates were observed for growth within 24-48 hours. The distinct colonies observed were sub-cultured on the same media for purification. Gram staining as well as appropriate biochemical tests were carried out according to standard procedures [11,12]. The isolates were identified by comparing their morphological and biochemical characteristics with those of known taxa as described by Bergey's Manual for Determinative Bacteriology [13]. A confirmatory identification was done using the bioMérieux's API® identification kit.

2.4 Determination of Antibiotics Susceptibility

Nine antibiotics belonging to three classes were used in this test. The selection of the antibiotics was based on the frequency of use in Nigeria. The main classes of the antibiotics used include Beta-lactams/Penicillins (Ampicillin 25µg, Amoxicillin 30µg, Cephalexin 30µg), Aminoglycosides (Kanamycin 30µg, Gentamicin 10µg, Streptomycin 30µg), and Glycopeptides (Vancomycin 30µg, Teichoplanin 30µg, Telavancin 30µg).

The antibiotics susceptibility test was carried out using standard agar disc diffusion method. Sterile Mueller-Hinton agar plate surface was spread evenly with a standardized McFarland 0.5 culture of the bacteria using a sterile swab. After 10-15 minutes the different antibiotics discs were placed on the medium using a sterile forceps then incubated at 37°C for 24 hours. Zones of inhibition were measured in millimetres (mm) using callipers and transparent meter rule. Strains were classified as Resistant (R), Intermediate (I) and Sensitive (S) according to the criteria recommended by the National Committee for Clinical Laboratory Standards [14]. All antibiotics sensitivity tests were performed in triplicate and average zones of inhibition calculated.

2.5 Determination of Multi Drug Resistance (MDR)

Multi Drug Resistance is defined as resistance to at least one of the tested antibiotics in at least three of the antibiotics classes tested [15]. The Multi Drug Resistance (MDR) character of the isolates was identified by observing the resistance pattern of the isolates to the antibiotics.

2.6 MAR Index Study

The MAR Index of an isolate was determined using the formula:

$$\text{MAR Index} = \frac{a}{b}$$

Where 'a' represents the number of antibiotics to which the isolate was resistant to and 'b' represents the number of antibiotics to which the isolate was subjected to [15]. The MAR indexes of the isolates were calculated and noted.

3. RESULTS

Sixteen (16) heavy metals tolerant bacteria were isolated from the tannery sample. *Bacillus cereus* (18.75%), *Escherichia coli* (12.50%), *Klebsiella aerogenes* (18.75%), *Klebsiella pneumoniae* (6.25%), *Pseudomonas aeruginosa* (12.50%), *Proteus mirabilis* (6.25%), and *Staphylococcus aureus* (25.00%) were identified with *Staphylococcus aureus* being the most prevalent (Table 1 & Fig. 1).

120 **Table 1: Biochemical characterization of heavy metals tolerant bacteria from NILEST tannery effluent**

ISOLATE CODE	GRAM	SHAPE	IND O	MR	VP	CIT	CAT	COA	MOT	URE	OXI	BIL. SOL.	MAN	GLU	LAC	SUC	H ₂ S	CO ₂	Probable organism
IS01	-	R	-	-	+	+	+	-	-	+	-	-	+	+	+	+	-	+	<i>K. aerogenes</i>
IS02	+	R	-	+	+	-	+	-	+	-	-	-	-	+	-	+	-	+	<i>Bacillus cereus</i>
IS03	+	CC	-	+	+	+	+	+	-	+	-	-	++	+	-	-	-	-	<i>S. aureus</i>
IS04	-	R	-	-	-	+	+	-	+	-	+	-	++	+	+	-	-	+	<i>Ps. aeruginosa</i>
IS05	-	R	+	+	-	-	-	-	+	-	-	-	+	+	+	+	-	+	<i>E. coli</i>
IS06	-	R	+	+	-	-	-	-	+	-	-	-	+	+	+	+	-	+	<i>E. coli</i>
IS07	-	R	-	-	+	+	+	-	-	+	-	-	+	+	+	+	-	+	<i>K. aerogenes</i>
IS08	-	R	-	-	-	+	+	-	+	-	+	-	++	+	+	-	-	+	<i>Ps. aeruginosa</i>
IS09	+	CC	-	+	+	+	+	+	-	+	-	-	++	+	-	-	-	-	<i>S. aureus</i>
IS10	-	R	+	+	-	+	+	-	+	+	-	-	-	+	+	-	+	-	<i>Proteus mirabilis</i>
IS11	+	CC	-	+	+	+	+	+	-	+	-	-	++	+	-	-	-	-	<i>S. aureus</i>
IS12	-	R	-	+	+	-	+	-	+	-	-	-	-	+	-	+	-	+	<i>Bacillus cereus</i>
IS13	-	R	-	-	+	+	+	-	-	+	-	-	+	+	+	+	-	+	<i>K. aerogenes</i>
IS14	+	CC	-	+	+	+	+	+	-	+	-	-	++	+	-	-	-	-	<i>S. aureus</i>
IS15	-	R	-	-	-	+	+	-	-	+	-	-	+	+	+	+	-	+	<i>K. pneumoniae</i>
IS16	+	R	-	+	+	-	+	-	+	-	-	-	-	+	-	+	-	+	<i>Bacillus cereus</i>

121 **KEY:** INDO=Indole, MR= Methyl Red, VP= Voges Proskeuar, CIT= Citrate, COA= Coagulase, MOT= Motility, OXI= Oxidase, URE= Urease,
 122 MAN= Mannitol, GLU= Glucose, LAC= Lactose, SUC= Sucrose, (+) = Positive, (-) = Negative, (++) = Mannitol Fermentation, BIL. SOL. =
 123 Bile Solubility, R= Rod Shape, CC=Coccal in Clustered Shape

Table 2: Antibiotics susceptibility profile of heavy metals tolerant bacteria isolated

Antibiotic class	Antibiotics	Number of isolate (%)		
		Sensitive	Intermediate	Resistant
β-lactam/Penicillins	Ampicillin	05(31.25)	02(12.50)	09(56.25)
	Amoxicillin	04(25.00)	03(18.75)	09(56.25)
	Cephalexin	04(25.00)	06(37.50)	06(37.50)
Aminoglycosides	Gentamicin	04(25.00)	02(12.50)	10(62.50)
	Kanamycin	11(68.75)	01(06.25)	04(25.00)
	Streptomycin	09(56.25)	02(12.50)	05(31.25)
Glycopeptides	Vancomycin	09(56.25)	0(0.00)	07(43.75)
	Teichoplanin	12(75.00)	01(06.25)	03(18.75)
	Telavacin	08(50.00)	02(12.50)	06(37.50)

Table 3: Multiple Antibiotics Resistance (MAR) Index analysis of bacterial isolates

Isolate number	Bacteria species	Antibiotics	NAR	MAR INDEX
IS01	<i>K. aerogenes</i>	AMX, CEP, GEN, VAN, TEI,	05	0.56
IS02	<i>Bacillus cereus</i>	AMP, AMX, GEN, TEL,	04	0.44
IS03	<i>S. aureus</i>	AMP, AMX, KAN, VAN,	04	0.44
IS04	<i>Ps. aeruginosa</i>	AMP, AMX, STR, TEL,	04	0.44
IS05	<i>E. coli</i>	AMP, AMX, KAN, VAN,	04	0.44
IS06	<i>E. coli</i>	AMX, CEP, STR, VAN,	04	0.44
IS07	<i>K. aerogenes</i>	AMP, CEP GEN, TEL,	04	0.44
IS08	<i>Ps. aeruginosa</i>	AMP, CEP, GEN, STR, VAN,	05	0.56
IS09	<i>S. aureus</i>	AMP, GEN, STR, TEL,	04	0.44
IS10	<i>Proteus mirabilis</i>	AMX, GEN, VAN, TEL,	04	0.44
IS11	<i>S. aureus</i>	AMX, GEN, STR,	03	0.33
IS12	<i>Bacillus cereus</i>	CEP, GEN, VAN,	03	0.44
IS13	<i>K. aerogenes</i>	CEP, GEN,	02	0.22
IS14	<i>S. aureus</i>	AMP, AMX, TEI, TEL,	04	0.44
IS15	<i>K. pneumoniae</i>	AMP, GEN, KAN,	03	0.33
IS16	<i>Bacillus cereus</i>	KAN, TEI,	02	0.22

KEY: AMP= Ampicillin, AMX= Amoxicillin, CEP= Cephalexin, STR= Streptomycin, GEN= Gentamycin, KAN= Kanamycin, VAN= Vancomycin, TEI= Teichoplanin, TEL= Telavancin, NAR= Number of Antibiotics Resisted, MAR= Multiple Antibiotics Resistance.

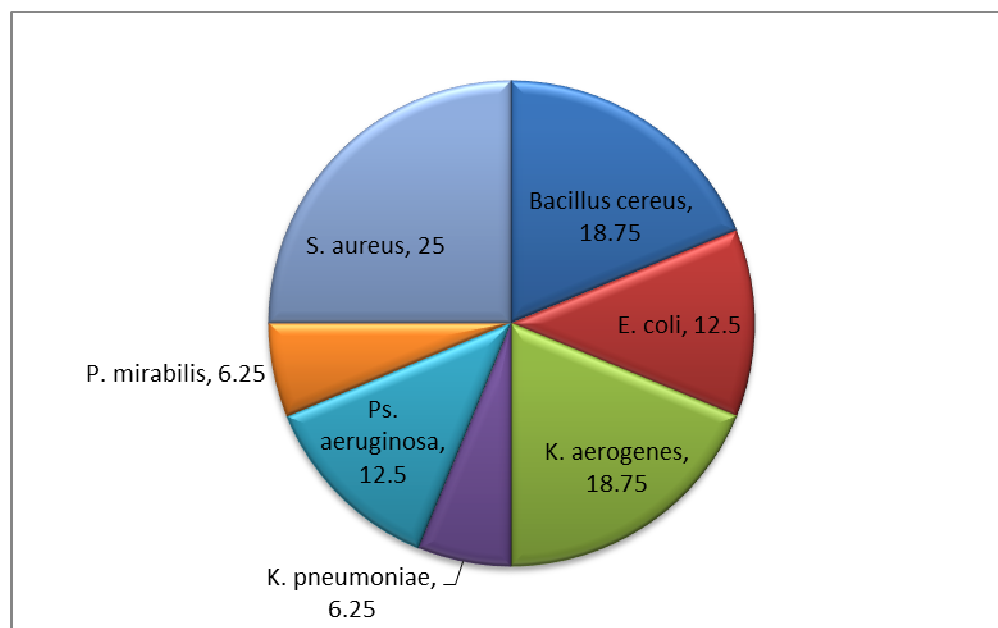


Figure 1: Percentage prevalence of bacteria isolated

4. DISCUSSION

In this study, based on the biochemical characterisation of the isolates *Bacillus cereus* (18.75%), *Escherichia coli* (12.50%), *Klebsiella aerogenes* (18.75%), *Klebsiella pneumoniae* (6.25%), *Pseudomonas aeruginosa* (12.50%), *Proteus mirabilis* (6.25%), and *Staphylococcus aureus* (25.00%) were identified. The presence of heavy metals tolerant bacteria is an indication that the effluent is indeed polluted by the metals examined in this study leading to selective pressure by which the tolerant bacteria have device means of circumventing the deleterious activity of the heavy metals. This result is similar to that of Raja *et al.* [10] who isolated similar heavy metals resistant enteric bacteria from sewage.

The high percentage resistance to gentamicin (62.50%), amoxicillin, and ampicillin with resistance level of 56.25% each observed in this study may be attributed to the indiscriminate use of these drugs by patients and in animal treatments and feeds. However, the resistance genes to these antibiotics may also have been acquired through horizontal transfer of plasmids from a donor bacterium to a recipient [9] or from mutation due to selective pressure of heavy metals on these bacteria. There is evidence of correlation between heavy metal tolerance and antibiotic resistance in this study since antibiotics such as Telavancin and Teichoplanin which are 'rarely' available over the counter in Nigeria which should reduce their misuse and abuse have shown some alarming levels of ineffectiveness against these heavy metals tolerant bacteria. The selective pressure by the heavy metals on the bacteria may have conferred resistance to the bacteria against the antibiotics. Many studies have found significant correlation between heavy metal and antibiotic resistance. Such studies which have specifically examined the association between heavy metals and antibiotics resistance in wastewater, fresh and drinking water include those of Odjadjare *et al.* [16], Raja *et al.* [10] and Boga *et al.* [17] who confirm resistance of *Pseudomonas*, *Acinetobacter*, *Proteus* *etc.* to several heavy metals and antibiotics. Henrietta *et al.* [18] also observed association between chromium and ampicillin resistance and tolerance in zinc and chromium with resistance to streptomycin and tetracycline.

The results of this study have shown that most of the isolates were resistant to at least one antibiotic across the three classes of antibiotics tested which qualify them as multiple drug resistant strains of bacteria. Multiple antibiotic resistance (MAR) in bacteria is most commonly associated with the presence

of plasmids which contain one or more resistance genes, each encoding a single antibiotic resistance phenotype [19].

The multiple antibiotic resistance (MAR) indices of the isolated bacterial strains were all greater than 0.2 which indicate high risk source of contamination of antibiotics [19,20]. In this case, the high pressure of heavy metals on the isolates may have been responsible for the high MAR indices. This is in line with Belliveau *et al.* [21] who stated that organisms undergo selective pressures in the presence of toxic compounds and develops resistance. The most common resistance is to metals and antibiotics which can be as a result of bio-essentiality or of abuse of metals and/or antibiotics. Both resistances are carried on the same plasmid and are transferable among organisms through conjugation or transduction [22].

The high incidence of resistance in bacteria isolated from sites away from human influence is possible due to the production of antibiotics by native bacteria and fungi which gives the bacteria a competitive advantage [23]. Usually essential metal tolerance genes are chromosome borne and toxic metals tolerance system are plasmid mediated [24]. Isolates observed in this study that show tolerance to metals tested and also multi-drug resistance indicate that these characteristics may have been plasmid mediated.

4. CONCLUSION

The selection of heavy metals tolerant bacteria was reflected in the high degree of resistance to antibiotics tested. Multidrug resistant strains are abundant in this effluent and this may pose a potential health hazard and requires intervention as the MAR indices of all the isolates are far greater than 0.2. The resistance pattern in different isolates is more or less similar which implies that different bacterial strains carry similar antibiotic resistance determinants.

RECOMMENDATIONS

Multiple antibiotic resistant bacteria are a public health issue. Therefore we hereby advocate for regular and consistent monitoring of tannery effluents with a view to preventing the dissemination of these pathogens into the environment where they may become clinically important in causing diseases that may be difficult to treat.

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