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Identification and Characterization of Heavy Metal Resistant Bacteria from the Klip River

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***Abstract*—**Pollution of the Klip River has caused microorganisms inhabiting it to develop protective survival mechanisms. This study isolated and characterized the heavy metal resistant bacteria in the Klip River. Water and sediment samples were collected from six sites along the course of the river. The pH, turbidity, salinity, temperature and dissolved oxygen were measured in-situ. The concentrations of six heavy metals (Cd, Cu, Fe, Ni, Pb and Zn) of the water samples were determined by atomic absorption spectroscopy. Biochemical and antibiotic profiles of the isolates were assessed using the API 20E® and Kirby Bauer Method. Growth studies were carried out using spectrophotometric methods. The isolates were identified using 16SrDNA sequencing. The uppermost part of the Klip River with the lowest pH had the highest levels of heavy metals. Turbidity, salinity and specific conductivity increased measurably at Site 4 (Henley on Klip Weir). MIC tests showed that 16 isolates exhibited high iron and lead resistance. Antibiotic susceptibility tests revealed that the isolates exhibited multi-tolerances to drugs such as Tetracycline, Ampicillin, and Amoxicillin.

***Keywords*—**Klip River, heavy metals, resistance.

I. Introduction

THE Klip River Catchment is located in Gauteng Province, south of Johannesburg, South Africa. It lies between longitude 27°45′ and 28°05′ E and latitude of 26°10′ and 26°25′ S at an altitude of 1750 m above mean sea level [1]. The source of the Klip River is in Roodekrans and from there it gradually descends into the Vaal River. Formerly a significant source of water for industrial and domestic purposes, the Klip River System has become polluted from industrial, mining, agricultural and domestic activities [2]. The upper catchment is largely characterized by mine dumps. Most of the polluted water arising from mines accumulates in the Klip River via tributaries that enter the wetland on its northern bank. Much of the industrial waste from the Witwatersrand Escarpment flows into the Klip River [2].

The mining of certain minerals including gold, copper and nickel is associated with Acid Mine Drainage (AMD). Effluents produced by mining industries contain large amounts of poisonous substances such as cyanides and heavy metals [3]. AMD arises when sulfide bearing material is exposed to oxygen and water [3]. It is a major source of heavy metal pollution in the Klip River since the river drains the southern portion of the Witwatersrand Escarpment where gold tailings are common [2].

The number of informal settlements along the Klip River Catchment is increasing. These communities use water directly from the river for domestic purposes such as drinking, washing clothes etc. Poor water quality of the river can consequently have detrimental effects on these communities [4].

Microorganisms populating contaminated surface water rapidly adapt and are sensitive to low concentrations of heavy metals. These microorganisms have developed coping mechanisms in order to survive in environments with high toxic levels of drugs and metals. Therefore they can be used as bio-indicators to detect heavy metal pollution in the environment [5]. The survival of these organisms relies on the intrinsic biochemical, structural, and physiological properties and genetic adaptations [6]. There are generally four basic mechanisms used in heavy metal resistance and include: (i) exclusion of toxic heavy metal ions from the cell by the alteration of membrane transport systems involved in initial cellular accumulation, (ii) extra- and intra-cellular sequestration of metal binding components similar to metallothioneins. (iii) cation/anion efflux systems that are encoded by resistance genes, and (iv) enzymatic detoxification of heavy metals from toxic to less toxic forms [7], [8]. Isolation and characterization of heavy metal resistant microorganisms has been studied extensively in different environments and water bodies [9], [10]. However, there are no reports on heavy metal resistant microorganisms in the Klip River.

Antibiotics are effective tools for controlling pathogenic bacteria. However, bacteria have evolved and have developed antibiotic resistance to several antibiotics and this poses a serious problem that overcomes the advantage of using antibiotics as chemotherapeutic agents. At present resistance to nearly all the clinically useful antibiotics are evident and there is speculation that the situation might be pushed back to that resembling the pre-antibiotic era [11]. Antibiotic resistant microorganisms are normally selected in environments contaminated with antibiotics and they are also found in natural environments in the presence of some non-antibiotic substances, especially heavy metals (mercury, arsenic, lead, cadmium etc.). Genes conferring antibiotic and heavy metal resistance are generally located on the same plasmid. Therefore, environments polluted with heavy metals also contain several bacteria that are resistant to both antibiotics

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and heavy metals [11].

The objectives of this study were: (i) to isolate indigenous heavy metal resistant bacteria from the Klip River, (ii) to determine the isolates' Minimum Inhibitory Concentration (MIC) values with respect to the following metals: cadmium, chromium, lead, zinc, iron, nickel and copper, (iii) to assess the biochemical and antibiotic profiles of the isolates, (iv) to characterize the physiological characteristics of the isolates, and (v) to identify the isolates by sequencing the gene encoding 16SrDNA.

II. Methods

A. Study Area

Five sites were selected along the Klip River (Site 1- near the source, site 2 - before Lenasia, site 3- Lenasia, site 4- Henley on Klip Weir, site 5- confluence of the Klip and Vaal river, site 6 (reference site) –the Vaal Barrage. The coordinates of the sites are shown in Table I.

TABLE I
COORDINATES OF SITES

Site no. and location	Geographical Coordinates	
	Latitude N	Longitude E
1 (Source- Roodekraans)	26°08.428′	27°49.280′
2 Before Lenasia	26°10.558′	27°49.037′
3 (Lenasia)	26°17.668′	27°05.650′
4 (Henley on Klip Weir)	26°32.428′	27°03.8445′
5 (Confluence of the Klip and Vaal River)	26°39.879′	27°56.303′
6 (Vaal Barrage)KR48	26°46.068′	27°40.498′+

B. Water Sample Collection and in situ Physico-Chemical Analysis

Water samples for heavy metal analysis were collected in pre-conditioned polyethylene bottles using the grab method at depths of 20-30 cm from the river surface. The samples were acidified with 1% nitric acid to keep the metal ions in a dissolved state. Water samples for bacteriological analysis were collected in a similar manner in autoclaved glass bottles and were stored on ice.

At each site the dissolved oxygen (DO), temperature, conductivity, salinity and turbidity were measured *in situ* with a multiparameter meter (HANNA Instrument Model 9828, Ann Arbor, MI). The pH at each site was measured with a HANNA Instrument Model 9025.

C. Sediment Sample Collection

Sediment cores were obtained from depths of 10 cm by using a sediment corer constructed from a graduated PVC pipe that was 4.8 cm in diameter. The corer had a removable cap on one end. The PVC pipe was pressed into the sediment to a depth of 10 cm and the cap was placed underneath the pipe. The different samples were transferred to pre-sterilized wide mouth glass jars and kept on ice until they were transported to the laboratory for analysis.

D. Heavy Metal Analysis of Water Samples

The concentration of the following heavy metals (Cd, Cu, Fe, Ni, Pb and Zn) was quantified using a flame atomic absorption spectrophotometer (Shimadzu-AA700, Kyoto, Japan). The metals' standards were prepared from stock solutions of 1000 mg/L by successive dilutions. All the water samples were aspirated for a minimum time of 5 sec before a reading was obtained [12]. All measurements were performed in triplicate.

E. Enumeration and Isolation of Bacteria

Water and core samples (0.1 ml) were plated and enumerated on Luria Bertani (LB) agar plates supplemented with 5 mg/L of each the following metals: chromium, zinc, copper, cadmium, lead, iron and nickel, respectively, by the standard pour plate method [13]. Plates were incubated at 30°C for 72 hr and colonies were selected on the basis of their morphology. The colonies were further purified by the streak method on their respective heavy metal constituted LB agar. Each bacterial culture was inoculated in nutrient broth, incubated overnight and glycerol stocks were prepared and frozen at -80°C [14].

F. Colony Morphology

Purified strains were grown on solidified LB agar plates and colony morphology was recorded according to the following characteristics (chromogenesis, size, shape, margin, elevation, opacity and surface texture [15].

G. Cellular Morphology

The microorganisms were Gram stained [16] and observed under the microscope (oil immersion, 100X). The Gram-reaction, shape, and morphology of the microorganisms were recorded.

H. Minimum Inhibitory Concentrations

Stock solutions (1 M) of cadmium chloride, potassium dichromate, lead chloride, iron sulphate, zinc chloride, copper sulphate, nickel chloride and cadmium chloride were prepared with deionized water and sterilized by autoclaving at 121°C for 15 min. The minimum inhibitory concentration (MIC) of the selected isolates were determined against increasing concentrations of Cd, Cu, Cr, Fe, Ni, Pb and Zn on LB agar plates until no growth was observed. [13]. Starting with an initial concentration of 0.2 mM, further MIC tests were carried out with concentrations of 0.4 mM, 0.6 mM, 0.8 mM, 1 mM, 1.2 mM, 1.5 mM, 2 mM, 3 mM and 4 mM. Cultures that showed growth at a particular concentration were transferred to the next higher concentration. The MIC tests were determined at 30°C for 10 days [13].

I. Determination of Antibiotic Sensitivity

Overnight cultures of 16 out of 48 bacterial isolates exhibiting high MICs were tested for antibiotic sensitivity towards nine different antibiotics [17]. Antibiotics used in this study included: Ampicillin (10 µg/ml), Amoxicillin (10 µg/ml), Cephalothin acid (30 µg/ml), Cotrimoxazole (25 µg/ml), Neomycin (30 µg/ml), Streptomycin (10 µg/ml), Tetracycline (30 µg/ml), Tobramycin (10 µg/ml) and Vancomycin (30 µg/ml).

For each antibiotic, 100 µl of a culture was transferred to a

Muller-Hinton agar plate and spread evenly with a sterile swab. After 10-15 min the different antibiotic discs were placed on the medium then incubated at 37°C for 24 hr. Zones of inhibition, where applicable, were measured in millimeters (mm). Strains were considered to be susceptible when the inhibition zone was 12 mm or more in diameter [13]. All antibiotic tests were performed in triplicate.

J. Biochemical Characterization

Biochemical phenotype profiles for the selected heavy metal resistant isolates were generated by using the API 20E® test strips. The isolates were tested for the presence of the following enzymes: beta-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, deaminase and gelatinase. Additional biochemical tests included citrate utilization, urea hydrolysis, indole production and acetoin production. API 20E® test strips also included fermentation or oxidation tests for the following carbohydrates: glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose and nitrate reduction. Tests were performed according to the manufacturers' instructions (Biomerieux ™, Marcy l'Etoile, France). Additional tests such as the catalase test, oxidase test and growth on MacConkey agar were also carried out.

K. Determination of Optimal Growth Conditions

The optimum pH and temperature conditions for growth of each of the 16 isolates were determined. For pH, three milliliters of LB broth was dispensed into different test tubes and the pH adjusted from 5 to 10 by using either 1M HCl or 1M NaOH. A 100 µl of overnight culture of each isolate was dispensed into the test tubes and incubated at 37°C for 24 hr. The tests were carried out in triplicate. The optical density (OD) of each culture was obtained at 600 nm with a UV spectrophotometer (Nanocolour UV/VIS Spectrophotometer Mahery-Nagel, (Düren, Germany) [19].

The optimal growth temperature was assessed as follows: three milliliters of LB broth was placed in different test tubes. A 100 µl of overnight culture of the pure culture of each isolate was dispensed into each of the test tubes. The tubes were incubated at four different temperatures i.e. 25°C, 30°C, 37°C and 40°C for 24 hr, respectively. The OD was measured at 600 nm using a UV/VIS Spectrophotometer (Nanocolour UV/VIS Spectrophotometer Mahery-Nagel (Düren, Germany) [19].

L. DNA Extraction

Genomic DNA was extracted from the 16 isolates by using the ZR Fungal/Bacterial DNA Extraction Kit (ZYMO Research, Wilmington, DE) according to the manufacturer's protocol. This method combines physical, chemical and silica gel column procedures. The quantity and quality of DNA was assessed by measuring the absorbance of a 2 µl sample at 260 and 280 nm using Nanodrop 2000c Spectrophotometer. The DNA concentration was calculated using standard $1A_{260}$= 50 µg/ml [20]. The DNA quality was assessed by electrophoresis on a 1.0% (w/v) agarose gel and staining with ethidium bromide.

M. 16SrDNA Amplification

The 16SrDNA fragments were amplified using the universal primer combination 27F (5'- AGA GTT TGA TCC TGG CTC AG-3') and 1,429R (5'-GGT TAC CTT GTT ACG ACT T-3'). The primers were synthesized by Inqaba Biotechnologies Industry, Pretoria, South Africa. Amplification was performed in a 50 µl reaction mixture containing 2x PCR Mastermix (Emerald Amp R MAX HS Master Mix, Japan), 22 µl of PCR quality water, 1 µl of each forward and reverse primer (0.2 µM) and 1 µl DNA template. PCR was performed in a T100 Bio-Rad Thermocycler (Bio-Rad, Hercules, CA). Thermal cycling conditions were as follows: initial denaturation at 94°C for 5 min followed by 35 cycles consisting of denaturation 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 5 min. The amplicons were analysed in a Bio-Rad electrophoresis system for 1 hr at 90 V in 1xTBE buffer. The images of the gels were captured in a Bio-Rad Gel Doc™ EZ Imager (Bio-Rad, CA) using ImageLab™ Software version 5.0. Each gel contained 5 µl of KAPA Universal Ladder (KAPA Biosystems, Boston, MA) in the first well. The obtained PCR products were purified using the GeneJet™ PCR Purification Kit (Fermentas, Hanover, Germany) according to the manufacturer's protocol. The PCR products were then sent for sequencing at Inqaba Biotechnologies Industry. The 16SrDNA sequences were aligned and compared with other 16SrDNA genes in the Genbank by using the NCBI Basic Local Alignment Search Tools, BLASTn program [21]. Based on the scoring index, the most similar sequences were aligned to those of other bacterial 16SrDNA regions using MAFFT Multiple Sequence Alignment Software Version 7 [22].

III. Results

A. Physico-Chemical Analysis of Water Samples

The values for the physico-chemical profiles of the six sampling sites are shown in Table II. These values were compared with those provided in the Klip River Instream Guidelines [23]. The pH of the sites ranged from 5.9 to 7.9 and were within the compliance range (<6.0 > 9.0) except for site 1, where a slightly lower pH of 5.9 was recorded. The Dissolved Oxygen (DO) values of 4.23 and 4.32 mg/L at sites 3 (Lenasia) and 5 (confluence of Klip river and Vaal River), respectively, were below the required range of >5 mg/L. The turbidity, conductivity and salinity almost doubled at site 4, Henley on Klip Weir), and a slight decrease was recorded at sites 5 (Confluence of the Klip and Vaal River) and 6 (Vaal Barrage). The concentrations of heavy metals were generally very high at site 1 compared to those of the other sites. The lead, iron and cadmium levels were higher than those of most sites with lead being 9.57 mg/L. With the exception of site 5, nickel was not detected in the other sites.

TABLE II
PHYSICOCHEMICAL PARAMETERS OF WATER SAMPLES COLLECTED FROM THE KLIP RIVER

Sample	1	2	3	4	5	6	Non-compliance Limit
pH	5.9	6.7	7.08	7.9	6.44	8.3	<6.0;>9.0
Temp. (°C)	20.3	21.1	19.1	19.1	16.7	20.1	No range
DO (mg/L)	NT	7.36	4.23	5.93	4.32	5.51	<5
Turbidity (tds ppm)	NT	187	250	476	471	456	>55
Conductivity (µS/m)	313	374	501	927	800	782	>150 (ms/m)
Salinity (ppt)	NT	0.18	0.24	0.47	0.47	0.45	-
Heavy metal concentrations (mg/L)							
Fe	2.23	3.31	0.23	0.43	0.34	0.25	>1.5
Ni	bdl	bdl	bdl	bdl	0.18	bdl	>0.1
Pb	9.52	0.77	0.73	1.42	1.03	0.90	>0.05
Cu	0.86	0.32	0.31	0.34	0.34	0.32	>1
Zn	0.43	0.12	0.09	0.14	0.08	0.05	>0.08
Cd	1.00	bdl	bdl	bdl	bdl	bdl	>3

NT= Not Tested; bdl=below detectable

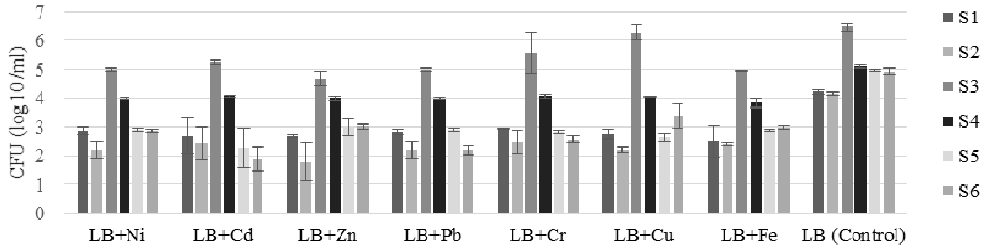


Fig. 1 Microbial CFUs per volume for water samples

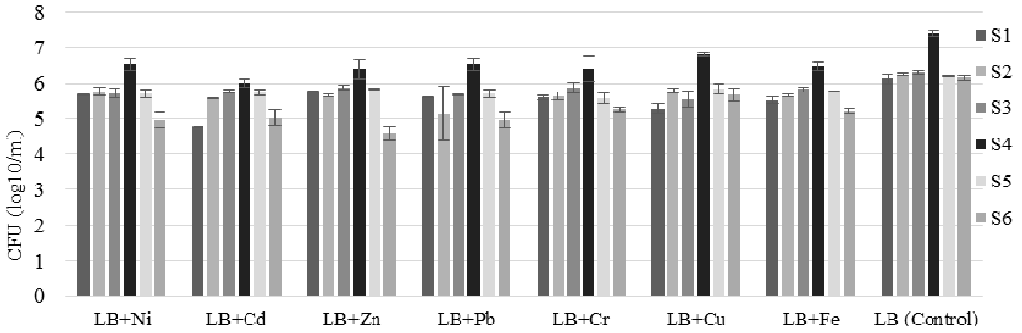


Fig. 2 Microbial CFUs per weight for sediment samples

B. Enumeration of Heavy Metal Resistant Microorganisms

The number of colony forming units (CFUs) on LB agar supplemented with 5 mg/L of each heavy metal (Cd, Cr, Cu, Fe, Ni, Pb and Zn) for the water samples is shown in Fig 1. The CFUs for the sediment samples are shown in Fig. 2. The number of CFU's for the water samples were considerably lower compared to those obtained for the sediment samples. The water sample at Site 3 recorded the highest CFUs for all the heavy metals whereas site 2 showed the lowest CFUs. For the sediment samples the highest CFUs for all the heavy metals was recorded at site 4, whereas the Vaal Barrage exhibited slightly lower CFUs for all the heavy metals. The controls for all the sites for both the water and sediment samples had higher CFUs than those of the heavy metals constituted agar plates.

C. Morphological and Physiological Characteristics of Isolates

A total of 48 bacterial isolates were obtained from the water and sediment samples and were designated as KR01-KR48. The isolates that exhibited MIC values greater than 1 mM for any of the metals under study were considered for further study. On this basis, 16 of these isolates were selected for further analysis. The morphological and physiological characteristics of the isolates are shown in Table III.

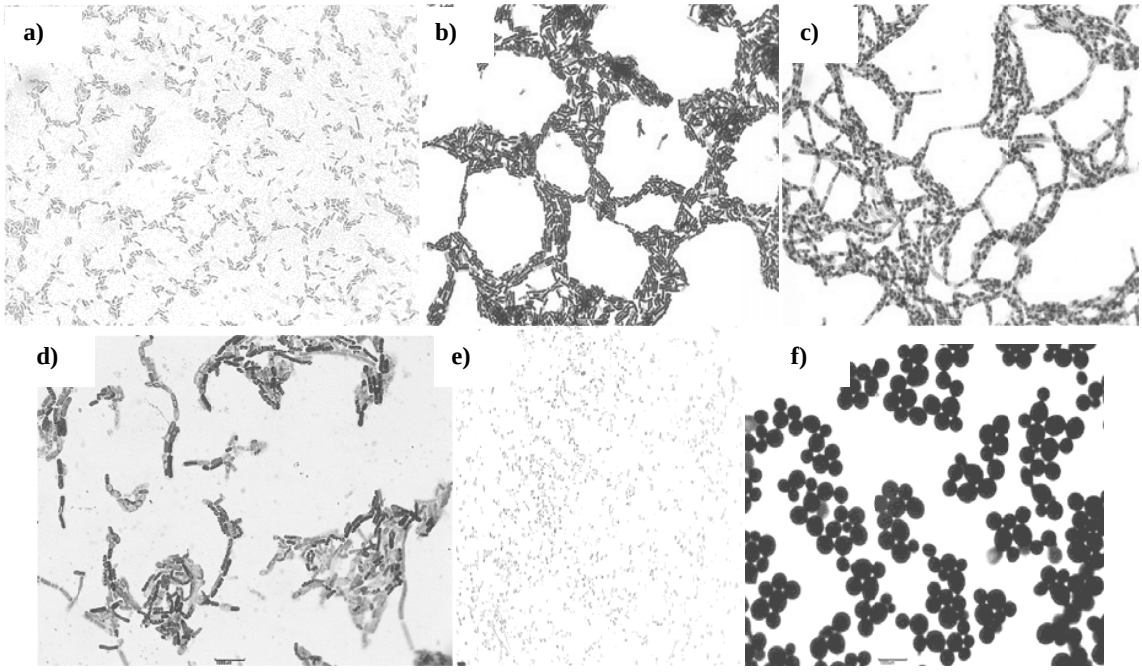


Fig. 3 Light microscope images (oil immersion x100) of bacterial strains a) KR01 b) KR02 c) KR03 d) KR04 e) KR29 f) KR44

TABLE III
MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF HEAVY METAL RESISTANT ISOLATES FROM THE KLIP RIVER

	Colony Colour	Gram reaction	Cell morphology	Optimum pH	Optimum temp.(°C)
KR01	cream	-	rod	7	30
KR02	white	+	rod	7	37
KR03	white	+	-	7	37
KR04	cream	+	rod	5	37
KR06	white	+	rod	5	40
KR07	cream	-	rod	8	30
KR08	white	-	rod	8	37
KR17	cream	-	rod	7	25
KR18	cream	-	rod	7	25
KR19	cream	-	rod	7	25
KR22	cream	-	rod	8	30
KR23	cream	-	rod	7	30
KR25	white	+	rod	7	37
KR29	white	-	rod	7	30
KR44	pink	+	-	7	37
KR48	yellow	+	-	7	37

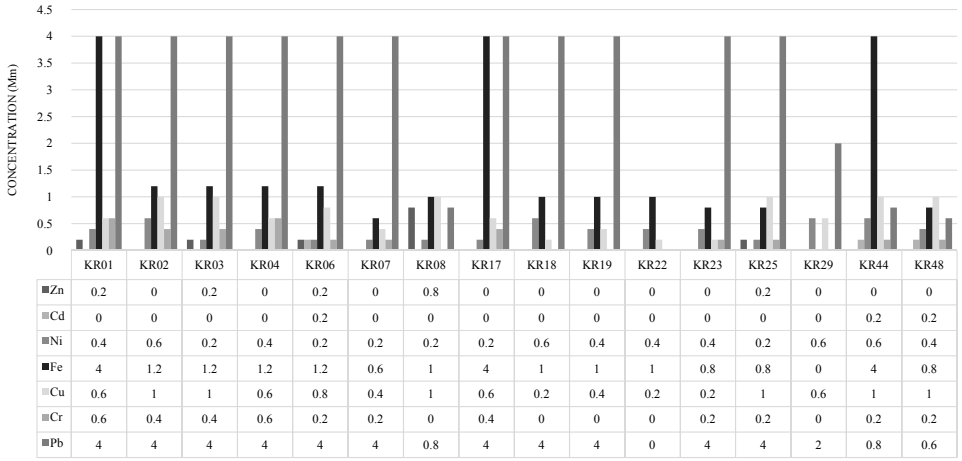
Thirteen (87.5%) of the 16 of the isolates were rod shaped e.g. KR01 (Fig. 3 (a)), KR02 (Fig. 3 (b)), KR04 (Fig. 4) and KR29 (Fig. 3 (e)). Nine (56%) of 16 the isolates were Gram negative, except for KR02, KR03, KR04, KR06, KR25, KR44 and KR48. Sixty-nine percent of the isolates exhibited optimum growth at pH 7, except for KR04 and KR06 which grew in acidic conditions (pH 5) and KR07 and KR08 which grew under slightly alkaline conditions (pH 8). The optimum temperatures for 75% of the isolates was in the range of 30-37°C except for KR06 which was 40°C, while KR17 and KR19 had optimum temperatures of 25°C (Table III).

D.Minimum Inhibitory Concentrations

The Cd, Cr, Cu, Fe, Ni, Pb and Zn concentrations used during screening ranged from 0.2 - 4 mM. The MIC values for the 16 isolates are shown in Fig. 4. It was observed that 100% and 94% of the isolates were resistant to iron and lead, respectively. Lead was toxic to isolate KR22 as shown by an MIC value of 0 mM. Cadmium was toxic to 88% of the isolates. KR44 and KR48 exhibited very low MIC values of 0.2 mM. Chromium was moderately toxic to the isolates with the highest MIC value of 0.6 mM being recorded for KR01 and KR04. KR01 and KR17 had MIC value of 4 mM for both lead and iron, while KR02, KR06, KR07, KR18 and KR23 showed an MIC value of 4 mM for lead and finally KR44 exhibited an MIC value of 4 mM for iron. Zinc was toxic to 94% of the isolates with the exception of KR08 which showed moderate zinc resistance with an MIC value of 0.8 mM.

E. Biochemical Characteristics of Isolates

The isolates that were analysed exhibited a range of biochemical phenotypes when tested with the API 20E® test strips. The test strips were designed for the rapid identification of enteric bacteria. However several tests on the strip are based on the traditional biochemical tests [18]. The strips were used in an ecological context in order to replace the traditional methods and rapidly construct a phenotypic profile of the isolates. The biochemical profiles of the isolates are shown in Table IV. Isolate KR17 showed positive results for most of the biochemical tests, while KR44 displayed negative results for all the tests except for catalase. Most of the isolates did not ferment glucose.



	KR01	KR02	KR03	KR04	KR06	KR07	KR08	KR17	KR18	KR19	KR22	KR23	KR25	KR29	KR44	KR48
Zn	0.2	0	0.2	0	0.2	0	0.8	0	0	0	0	0	0.2	0	0	0
Cd	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0.2	0.2
Ni	0.4	0.6	0.2	0.4	0.2	0.2	0.2	0.2	0.6	0.4	0.4	0.4	0.2	0.6	0.6	0.4
Fe	4	1.2	1.2	1.2	1.2	0.6	1	4	1	1	1	0.8	0.8	0	4	0.8
Cu	0.6	1	1	0.6	0.8	0.4	1	0.6	0.2	0.4	0.2	0.2	1	0.6	1	1
Cr	0.6	0.4	0.4	0.6	0.2	0.2	0	0.4	0	0	0	0.2	0.2	0	0.2	0.2
Pb	4	4	4	4	4	4	0.8	4	4	4	0	4	4	2	0.8	0.6

Fig. 4 MICs for heavy metal resistant isolates form the Klip River

TABLE IV
BIOCHEMICAL CHARACTERISTICS OF HEAVY METAL RESISTANT ISOLATES FROM THE KLIP RIVER

	KR 01	KR 02	KR 03	KR 04	KR 06	KR 07	KR 08	KR 17	KR 18	KR 19	KR 22	KR 23	KR 25	KR 29	KR 44	KR 48
CAT	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
OX	+	+	NT	-	+	-	-	-	-	+	-	+	+	-	NT	-
McC	+	-	-	-	-	-	-	+	+	+	+	+	-	-	-	-
ONPG	+	-	-	+	+	-	-	+	-	+	-	+	-	+	-	-
ADH	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-
LDC	+	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
ODC	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
CIT	-	+	-	-	-	+	+	-	-	-	-	+	-	-	-	-
H_2S	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-	-
URE	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-
TDA	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-
IND	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-
VP	+	-	-	-	-	+	+	+	-	+	-	+	-	-	-	-
GEL	+	+	+	+	+	-	-	+	+	+	+	+	+	-	-	+
Carbohydrate fermentation or oxidation																
GLU	+	-	-	-	-	-	+	+	-	+	+	-	-	+	-	-
MAN	+	-	-	-	-	-	-	+	-	+	-	-	-	+	-	-
INO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
SOR	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-
RHA	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+
SAC	+	-	-	-	-	-	-	+	-	+	+	-	-	+	-	-
MEL	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
AMY	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
ARA	+	-	-	-	-	-	+	+	-	+	-	-	-	+	-	-
NO_2	+	-	-	+	-	-	-	+	-	+	+	+	-	+	-	-

Tests: Cat, Catalase test; OX, Oxidase test; McC; growth on MacConkey; ONPG, β-galactosidase activity; ADH, Arginine Dihydrolase; LDC, Lysine Decarboxylase; ODC, Ornithine Decarboxylase; CIT, Citrate Utilization; H2S, Hydrogen Sulfide Production; URE, Urease; TDA, Tryptophan Deaminase; IND, Indole Production; VP, Acetoin Production (Voges-Proskaur); GEL, Gelatinase; GLU, Glucose; MAN, Mannitol; INO, Inositol; SOR, Sorbitol; RHA, Rhamnose; SAC, Sucrose; MEL, Melibiose; AMY, Amygdalin; ARA, Arabinose; NO2, Nitrate Reduction to Nitrite: + positive result; - negative result

F. Antibiotic Susceptibility Tests

Vancomycin was effective against all Gram positive bacteria as shown in Table V. Cephalothinic acid was effective against 69% of the isolates. However, KR04, KR06, KR25, KR29 and KR48 were resistant to this drug. The following isolates were resistant to streptomycin: KR01, KR06, KR17, KR18, KR22 and KR25. Several isolates showed resistance towards the β-lactam antibiotics, except for KR04, KR18, KR25 and KR48. Tetracycline was effective against 94% of the isolates with the exception of KR17 which was resistant to this antibiotic. The following isolates showed resistance to the sulphonamide, Cotrimoxazole: KR01, KR02, KR07, KR08, KR17 and KR44. Eighty-eight percent of the isolates were susceptible to the drug Tobramycin, with the exception of KR22 and KR48. KR17 showed resistance to all antibiotics except for Tobramycin, while KR04 in contrast was susceptible to all 9 antibiotics.

G. Sequence Results Analysis

PCR amplification of the 16SrDNA genes produced

fragments of approximately 1500 base pairs in size. Identification of the strains isolated in this study using comparative analysis of the 16SrDNA sequences that were aligned with previously obtained sequences in the NCBI database is shown in Table VI.

IV. Discussion

A. Physico-Chemical Properties of Water Samples

The pH of a water sample is an important variable when assessing the concentrations of heavy metals in solution since it affects their removal as hydroxides in water [24]. With the exception of nickel, all the metals (Cd, Cu, Fe, Pb and Zn) remained in solution at site 1. The pH of this site was 5.9. The low pH at this site could be attributed to mining and industrial activities [4], since the source is close to industrial areas such as Chamdor. The concentrations of heavy metals decreased along and course of the Kip River as the pH of the water samples generally increased downstream from site 1. The decreasing concentrations of the heavy metals with an increasing pH may be due to the formation of metal hydroxides which are precipitated out of solution, consequently decreasing the amount of heavy metal concentration in the water [24]. Neutralization of the water along the course of the Klip River is accredited to prolonged exposure to the Malmani Dolomites and the presence of wetlands in the area [25]. The dissolved oxygen (DO) at sites 3 and 5 were considerably lower than 5 mg/l, the limit set by the Klip River Instream Guidelines [23]. DO can be used to evaluate the ecological health of a river. Unpolluted water bodies are normally characterized by elevated dissolved oxygen values, whereas low DOs indicate higher pollution levels [9]. The low DO at site 3 could perhaps be attributed to the close proximity of this site to an informal settlement in Lenasia. Pollution of the river may be due to runoff from the informal settlement (personal observation). Evidence for this comes from the high microbial load at this site as shown by the high CFUs (Fig. 1). The DO was also low at site 5. This could be due to the relatively high CFUs (Fig. 1) and the high salt content as shown by the salinity value of 0.47 ppt (Table II). Water with high concentrations of dissolved minerals such as salt can reduce the amount of DO concentration in water [26] and this could have been a contributing factor to the decrease in DO at site 5.

One interesting aspect of this study was the almost doubling of the turbidity, salinity and conductivity at site 4. This is perhaps due to AMD. AMD is a major contributor to high levels of salinity, conductivity and Total Dissolved Salts (TDS) [27]. Site 4 is situated after the Klip River's confluence with the Rietspruit which is greatly influenced by AMD. It is known that AMD is discharged into the Elsburgspruit, which flows into the Natalspsruit and Rietspruit before joining the Klip River [1], [27].

B. Enumeration of Bacteria

The presence of heavy metal resistant bacteria (MRB) in both the water and sediment samples is an indication that the Klip River is indeed polluted by the heavy metals examined in this study (Fig. 1). However, nickel and cadmium were undetectable in some of the sites. The heterotrophic plate counts (HPC) in the controls (plates without heavy metals) for both the water and sediments samples were relatively higher than those obtained in the presence of heavy metals (Figs. 1, 2). This suggests that growth of some of the microorganisms were inhibited by the presence of heavy metals. The highest HPC at site 3 (Lenasia) was possibly due to its proximity to an informal settlement in Lenasia. The heterotrophic plate counts were highest in the sediment at site 4 (Henley on Klip Weir). This could be an indication of the extent to which the sediment at this site is polluted, possibly due to AMD [1], [27].

TABLE V
ANTIBIOTIC SENSITIVITY PROFILES OF HEAVY METAL RESISTANT BACTERIAL ISOLATES FROM THE KLIP RIVER

	Antibiotic Disc								
	Neomycin	Vancomycin	Cephalothin acid	Streptomycin	Ampicillin	Amoxicillin	Tetracycline	Cotrimoxazole	Tobramycin
KR01	19(S)	9(R)	NZ	7(R)	NZ	NZ	18(S)	NZ	15(S)
KR02	21(S)	18(S)	8(R)	24(S)	NZ	NZ	18(S)	NZ	18(S)
KR03	18(S)	16(S)	NZ	21(S)	NZ	NZ	22(S)	13(S)	16(S)
KR04	22(S)	20(S)	31(S)	26(S)	27(S)	29(S)	29(S)	24(S)	24(S)
KR06	20(S)	20(S)	34(S)	11(R)	11(R)	NZ	29(S)	24(S)	24(S)
KR07	18(S)	NZ	NZ	13(S)	NZ	NZ	19(S)	NZ	18(S)
KR08	16(S)	NZ	NZ	18(S)	NZ	NZ	18(S)	12(R)	17(S)
KR17	7(R)	NZ	NZ	NZ	NZ	NZ	NZ	NZ	13(S)
KR18	18(S)	14(S)	NZ	8(R)	17(S)	13(S)	17(S)	23(S)	17(S)
KR19	17(S)	NZ	NZ	16(S)	NZ	NZ	24(S)	17(S)	16(S)
KR22	NZ	NZ	NZ	NZ	NZ	NZ	15(S)	20(S)	11(R)
KR23	20(S)	7(R)	NZ	16(S)	NZ	NZ	23(S)	28(S)	21(S)
KR25	17(S)	18(S)	27(S)	NZ	27(S)	30(S)	21(S)	22(S)	18(S)
KR29	15(S)	NZ	13(S)	16(S)	14(S)	12(R)	19(S)	22(S)	13(S)
KR44	22(S)	16(S)	11(R)	24(S)	8(R)	8(R)	22(S)	NZ	13(S)
KR48	16(S)	21(S)	40(S)	18(S)	35(S)	29(S)	28(S)	23(S)	8(R)

Letters in parenthesis indicates the sensitivity of the isolate to the antibiotic; diameter (mm)
R=Resistant; S=Sensitive; NZ=No Zone

TABLE VI
COMPARATIVE ANALYSIS OF 16SRDNA SEQUENCES OF HEAVY METAL RESISTANT ISOLATES FROM THE KLIP RIVER USING HIGHLY MATCHED SPECIES AVAILABLE IN NCBI

	Sequence Length	Accession no.	Highly matched bacteria/ accession no.	%Similarity	Confidence level
KR01	1142	KJ935907	*Aeromonas hydrophila* strain M-1/HQ609947.1	99	Species
KR02	1155	KJ935908	*Bacillus* sp. hb91/KF8638801	99	Species
KR03			*Unidentified*		
KR04	1157	KJ935909	*Bacillus megaterium* strain 1AR1-AN28	98	Genus
KR06	1146	KJ935910	*Bacillus subtilis* strain P38/JQ669676.1	99	Species
KR07	1153	KJ935911	*Pseudomonas* F15/KF573430.1	97	Genus
KR08	1171		*Acinetobacter oleivorans* DR1/NR102814.1	84	No match
KR17	1136	KJ935912	*Proteus penneri* T202/KC764983.1	98	Genus
KR18	1152	KJ935913	*Shewanella* enriched culture clone AP-Enrich 0/JX82848.1	99	Species
KR19	1145	KJ935914	*Aeromonas* sp. IW-211/KF556692.1	98	Genus
KR22	1142	KJ935915	*Proteus* sp. W15 Dec34/JN106439.1	99	Species
KR23	1135	KJ935916	*Pseudomonas* sp. THG/KF532133.1	99	Species
KR25	1123	KJ935917	*Lysinibacillus* sp. C22 KF720925.1	99	Species
KR29	1139	KJ935918	*E.coli strain S5-6/* KC202264.1	98	Genus
KR44	1141	KJ935919	*Bacillus licheniformis* D43/KC441778.1	99	Species
KR48	1164	KJ935920	*Arthrobacter* SMP5	98	Genus

C. Morphological and Physiological Characterization of Bacterial Isolates

Most of the isolates grew optimally at pH of 7 suggesting that they are neutrophilic. This characteristic could be attributed to the fact that they were isolated from an environment which was more or less neutral as shown by the pH values (Table II). Microorganisms tend to respond to external pH changes by using certain mechanisms to maintain a constant internal environment. For neutrophilic bacteria, it has been suggested that they appear to exchange potassium for protons using an antiport transport system [28]. Acidophilic bacteria (0 - 5.5) use a variety of measures to maintain a neutral internal pH. For example, the transportation of cations such as potassium into the cells decreases the movement of H^+ into the cell [28]. Another mechanism involves proton transporters that pump. H^+ out of the cell and highly impermeable membranes [28].

D. Minimum Inhibitory Concentrations

The 16 isolates showed wide variability in the MIC for the different metals except for Zn, Cd and Cr to a lesser extent (Table IV). This suggests that the latter metals are relatively toxic to the bacterial isolates. Each isolate also differed in their MIC values for the different metals. Of all the metals, the isolates were most resistant to Pb and Fe and were able to grow in concentrations of up to 4 mM. The high levels of lead and iron in the Klip River (Table II) appears to have given the indigenous microbial strains some level of tolerance to these metals. Micro-organisms have developed a variety of protective mechanisms to thrive in very high levels of lead and iron. Among the various adaptative mechanisms adopted by lead resistant microorganisms include: P -type ATPase mediated efflux of lead [29] and metallothioneins (BmtA). It is known that metallothioneins play a crucial role in the immobilization of lead within the cell. In *Bacillus megaterium*, lead was sequestered by proteins which were almost similar to metallothioneins [30]. Lead resistance in *Proteus penneri* GM10 is due to the gene *SmtA* which encodes metal binding metallothioneins. Other methods of lead resistance include its sequestration by exopolysaccharides (EPS) [31], cell surface adsorption and biosorption involving ion exchange, and adsorption and diffusion through cells and membranes [32]. *Bacillus subtilis* has been shown to biosorb high amounts of lead ions, up to 97.68% under acidic conditions [33]. Heavy metal resistant microorganisms with resistance mechanisms may serve as potential biotechnological agents for bioremediation of lead contaminated sites [34]. Further research with some of the heavy metal resistant bacteria isolated in his study may be necessary to verify this potential.

E. Antibiotic Susceptibility Tests

Antibiotic resistance of the 16 isolates used in this study may be due to the presence of antibiotics in the Klip River. Antibiotic resistant microorganisms are normally selected in environments contaminated with antibiotics and they are also found in natural environments in the presence of some non-antibiotic substances, especially heavy metals (mercury, arsenic, lead, cadmium etc.) [11].

Streptomycin [35], Amoxicillin [36], Ampicillin [37] and Tetracyline [38] are broad spectrum antibiotics which inhibits both Gram- positive and negative bacteria. However, some isolates identified in this study were resistant to these drugs. Isolate KR17 which was identified as *Proteus penneri* showed antibiotic resistance to all the antibiotics except Tobramycin. In a previous study *Proteus penneri* was tested against 71 antibiotics and found to be naturally resistant to several antibiotics such as penicillin G, oxacillin, all tested macrolides, streptogramins, lincosamides, rifampicin, glycopeptides, rifampicin and fusidic acid [39]. Tobramycin is active against Gram-negative bacteria especially *Pseudomonas* spp, *Enterobacteriaceae* and *Acinetobacter* spp. [40]. In this study KR22 (*Proteus* sp.) and KR48 (*Arthrobacter* spp.) were resistant to Tobramycin, and to all the β-lactam antibiotics.

Aeromonas spp. were shown to be resistant to Cotrimoxazole [41]; however, both of the *Aeromonas* sp (KR01 and KR19) in this study were resistant to this drug. Vancomycin is effective against Gram positive bacteria such as *Streptococci, Corynebacteria, Clostridia, Bacillus* and *Listeria* species [42]. In the current study all the Gram positive bacteria were susceptible to Vancomycin (Tables III and V)

F. Comparative Analysis of 16SrDNA Sequences

A comparative analysis of the 16SrDNA sequences of the 14 isolates identified 8 bacterial genera (Table VI) in this study. Nine (57%) of the 14 identified isolates belong to the phylum *Gamma proteobacteria*. The isolates were closely related to *Aeromonas, Pseudomonas, Proteus, Shewanella* and *Escherichia*. Members of the γ-*proteobacteria* are all Gram negative, rod shaped bacteria and have been isolated from various heavy metal polluted habitats [13], [43], [44]. Isolate KR01 was identified as *Aeromonas hydrophila* while KR19 was similar to *Aeromonas* sp IW-211. Members of the genus *Aeromonas* are Gram-negative, rod shaped [45], catalase positive and facultative anaerobic bacteria [46]. These morphological and biochemical characteristics corresponded with those of KR01 (Fig 3 (a)) and KR19 (Tables III, IV) in this study. *Aeromonas* spp. isolated was previously isolated from water and sediment contaminated with heavy metals and the isolates showed both, heavy metal and antibiotic resistant characteristics [43]. KR07 was identified as *Pseudomonas* F15 while KR23 was identified as *Pseudomonas* sp. THG-T1. *Pseudomonas* are Gram negative aerobic bacteria that are known to be resistant to heavy metals and antibiotics [13], [47]. KR18 was identified as *Shewanella* sp. which is a ubiquitous organism isolated from food, sewage, and both from fresh and salt water. Earlier it was termed *Pseudomonas putrefaciens* or *Shewanella putrefaciens* [48]. Several reports have arisen stating that this organism causes human infections such as cellulitis, abscesses, bacteremia, and wound infection [48]. It is oxidase and catalase-positive non-fermenter Gram-negative rod that produces hydrogen sulfide (Table IV) [48]. *Shewanella* species were shown to mobilize copper [49]. KR29 was identified as *E. coli* (Table VI). It is rod shaped, Gram-negative, able to ferment glucose, and was susceptible to almost all the antibiotics except for Vancomycin and Amoxicillin. *E. coli* is an accurate indicator of faecal contamination in drinking water and other matrices [50]. Several strains of *E. coli* were isolated from wastewater of El-Mahah in Egypt and were analysed for heavy metal resistance. One particular isolate showed multiple resistance to Cu, Co, Ni, Zn, Cr, Cd and Pb [44]. Different MICs of several heavy metals were determined for *E. coli*: silver, gold, chromium and palladium proved to be the most toxic metals to *E. coli*.

Only five (36%) of the isolates were grouped with low-G+C Gram-positive bacteria in the *Firmicutes* and were most closely related to *Bacillus* and *Lysinibacillus*. *Firmicutes* are Gram positive bacteria and have been known for formation of endospores, namely; *Bacilli, Clostridia* and *Negativicutes*. This enables the bacteria to withstand a variety of environmental challenges, such as heat, solvents, UV irradiation and lysozyme [51]. The Gram positive isolate KR02 was identified as *Bacillus* sp. In a previous study, *Bacillus* sp. was shown to have exceptional metal resistance against lead and arsenate [52]. KR04 was identified as *Bacillus megaterium*. This is a rod-like, Gram-positive, spore forming bacteria. This is one of the largest known bacteria that occur in pairs and chains (Fig. 3 (d)) with an optimum growth temperature of 37°C. The optimum temperature for sporulation for *B. megaterium* is 40°C [53]. KR06 was identified as *Bacillus subtilis*. This was confirmed by its rugose colony appearance and Gram positive staining. *Bacillus subtilis* was isolated from agricultural and industrial areas in Mauritius, and was shown to be resistant to Cu, Ag, P, Zn and Hg [54]. KR25 was recognized as *Lysinibacillus* sp, which is a potential bioremediation agent. Several isolates have been obtained from heavy metal polluted environments; for instance *L. sphaericus* strain OT4b.31 is a native Columbian strain widely applied in the bioremediation of heavy metals [55]. In this study, KR44 could possibly be *Bacillus licheniformis* which has sporulated (Fig. 3 (f)). The bacterial isolate was not metabolically active in this state as elucidated by its metabolic profile (Table IV).

One (7) of the 14 isolates belonged to the high-G+C Gram-positive bacteria in the *Actinobacteria* and was most closely related to *Arthrobacter*. KR48 was ascertained to be *Arthrobacte*r sp. that is known to display heavy metal resistance. For example, *Arthrobacter* sp. isolated from heavily polluted industrial areas showed remarkable resistance to nickel (up to 20mM) [56].

All the sixteen isolates were resistant to lead except for KR22 (*Proteus* sp.). Lead resistant bacteria exhibiting multiple drug resistance is a disturbing signal for medical microbiologists since lead pollution can lead to the occurrence of bacterial pathogens resistant to all known antibacterial drugs (superbugs) [34].

V. Conclusion

There was wide variation in the heavy metal concentration in the different sites. Sampling for heavy metals at one site may not provide a true reflection of the heavy metal pollutants in the Klip River. The concentration of iron and lead was highest at the source of the river perhaps due to the industrial and mining activity in the area. The high number of metal resistant bacteria is an indicator of the extent to which the Klip River is polluted. This situation can be rectified by bioremediative methods. The heavy metal resistant bacterial isolates obtained from this study are autochthonous to the Klip River. Their uniqueness and characteristics could be used as potential bioremediation agents to remove heavy metals from the environment. Further studies are necessary to evaluate the heavy metal removal abilities of these isolates. Investigations into the presence of heavy metal resistance genes in these isolates may lead to the development of biosensors. The summation of all the information attained from this study will prove to be valuable when setting up bioremediation projects of the Klip River.

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