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Full Length Research Paper

Molecular characterization of heavy metal resistant *Proteus species*

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Heavy metals are silent killer of mankind and the cause of environmental pollution. The ability of some microorganisms to resist heavy metals makes them useful in bioremediation. The aim of this study was to molecularly characterize heavy metal-resistant *Proteus* species isolated from the soil of a cement factory. *Proteus* species were tested for resistance to lead, chromium, copper and iron at concentrations 0.00, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/L. Minimum inhibitory concentration (MIC) was determined at mg/mL. Plasmid profiling was done. Genomic DNA was extracted using DNA Kit by Zymo Research USA. The concentration of genomic DNA was determined using NanoDrop Spectrophotometer. Twenty-five microlitre was used for polymerase chain reaction. Amplicons were electrophoresed and sequenced. Nucleotide sequences were blasted at the NCBI website. *Proteus* species showed resistance to the test heavy metals. MIC was determined for lead, copper, partly for iron and not for chromium. Plasmid profiling showed that six *Proteus* species harbor high molecular weight plasmids. Concentration of genomic DNA ranged between 1.88 and 2.03 ng/µl. Electrophoresis revealed 16S rRNA genes amplified at 1500 base pair. Blast analysis revealed that six was *Proteus mirabilis* and one was *Proteus terrae*. Phylogeny constructed and study revealed that these *Proteus* species may be useful as bioremediation agents.

Key words: *Proteus* species, resistance, heavy metal, 16S rRNA gene, bioremediation.

INTRODUCTION

Heavy metals are regarded as one of the environmental pollutants due to their toxic effects on plants, animals, human beings and even microorganisms. According to Bharti (2012), heavy metals such as arsenic, lead, cadmium, nickel, mercury, selenium, cobalt, antimony, vanadium, zinc, platinum, palladium and rhodium are highly toxic even in small amount. Heavy metal pollution of the environment and exposure to heavy metals such as mercury, cadmium and lead is a serious growing problem throughout the world. Human exposure to heavy metals has risen dramatically in the last few decades, as a result of an exponential increase in the use of heavy metals in industrial processes and products. Microorganisms that are able to survive well in high concentration of heavy metals are of great interest as bioremediation agents because they can achieve different transformation processes (Adamis et al., 2004).

Heavy metals are increasingly found in microbial

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habitats due to natural processes and anthropogenic activities. Hence, microorganisms have acquired a variety of mechanisms of adaptation and biotransformation responses to heavy metals which include intracellular and extracellular sequestration, compartmentalization, complex formation, synthesis of binding proteins such as metallothioneins, reduction of the heavy metal ions to a less toxic state and to use them as terminal electron acceptors in anaerobic respiration.

Gustav Hauser first described the genus *Proteus* in 1885. The genus *Proteus* belongs to the family Enterobacteriaceae and the tribe Proteeae together with the genera *Morganella* and *Providencia*. *Proteus* species are differentiated from other genera by their ability to swarm across agar surfaces of solid media. Currently, the genus is divided into *Proteus mirabilis*, *Proteus vulgaris*, *Proteus penneri*, *Proteus hauseri* and three unnamed genomospecies. *Proteus* bacteria are widely found in the natural environment, occurring in polluted water, soil and manure (Różalski et al., 2012). It is an age-long fact that microorganisms are detrimental and beneficial. *Proteus* bacteria are no exception in that they have these two sides of the coin. In medical microbiology, the genus *Proteus* exhibits varied clinical significance in humans and animals as opportunistic pathogens. This is one side of the coin but the other side of the coin is different because of its beneficial effects in the natural environments. Environmental microbiologists have explored the innate ability of *Proteus* bacteria, most especially *Proteus mirabilis* in natural environments and discovered that they exhibit more positive aspects of their existence in natural environments.

There were reports on *Proteus* species as bioremediators of heavy metals, hydrocarbons, pesticides, insecticides, herbicides, aromatic compounds and azo dyes in contaminated environments. Hassen et al. (1998) isolated many Gram-negative bacteria from wastewater in Tunisia with *Proteus* mirabilis as the dominating strain. He reported that the *Proteus mirabilis* were highly resistant to several heavy metals such as copper, chromium, lead, iron cobalt, cadmium, zinc, silver and mercury. Ibrahim et al. (2013) worked on soil samples collected from the rhizosphere of legumes planted on crude-oil contaminated soil in Kaduna, Nigeria and reported that *Proteus mirabilis* and *Proteus vulgaris* were the most active crude oil degraders among the several isolated species. *Proteus* species isolated from the rhizosphere of rice in West Bengal, India, used hexachlorocyclohexane (HCH) pesticide (Das et al., 1995) and phorate insecticide (Das and Mukherjee, 2000; Das et al., 2003) as a source of carbon and energy, and the addition of these chemicals to soil promoted the growth of bacteria. Correa and Steen (1995) found *Proteus mirabilis* strain to be the fastest degrader of a commonly used herbicides called propanil among the natural microflora inhabiting a pristine lake in northeast Georgia, USA. *Proteus* mirabilis strain identified in wastewater samples from Casablanca City, Morocco, exhibited resistance to naphthalene and anthracene (Filali et al., 2000). Olukanni et al. (2010) isolated *P. mirabilis* from municipal dump site soil near Lagos, Nigeria. The isolated *P. mirabilis* was used to degrade a Reactive Blue 13 azo dye to phyto-non-toxic products. The aim of this study was to investigate the ability of *Proteus* species isolated from the soil of a cement factory to resist heavy metals namely lead, chromium, copper and iron.

MATERIALS AND METHODS

List of chemicals and reagents

All chemicals and reagents used were of analytical grade. Chemicals used were manufactured by Merck Specialties Pvt. Ltd., Mumbai, India. The MacConkey and nutrient agar used were manufactured by HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Sample collection

Forty (40) soil samples were collected at the depth of 0-30 cm with the aid of soil auger from the control site and Dangote cement factory located at Tse-Kucha Gboko, Benue State. The soil samples were collected from the mining, waste disposal and industrial sites of Dangote cement factory.

Isolation of *Proteus* species from soil samples

Streak plate technique was used for isolating *Proteus* species from the soil samples. Ten (10) grams of soil sample was added to 90 ml sterile diluent. A loopful from the suspension was streaked on MacConkey and blood agar plates. Plates were incubated at 30°C for 24 h. After incubation, distinct colonies were randomly picked, sub-cultured severally to obtain pure cultures and then preserved on agar slants in the refrigerator.

Heavy metals resistance assay for *Proteus* species

Heavy metal resistance assay was carried out on *Proteus* species according to the method described by Mgbemena et al. (2012). Heavy metals used for this assay were lead (Pb), chromium (Cr), copper (Cu) and iron (Fe) at concentrations of 0.00, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/L respectively. The concentration 0.00 mg/L containing no heavy metal served as the control. Nutrient agar medium was supplemented with different salts of heavy metals namely: Lead nitrate Pb $(NO_3)_2$, potassium dichromate $K_2Cr_2O_7$, copper sulphate pentahydrate $CuSO_4.5H_2O$ and iron sulphate heptahydrate $FeSO_4.7H_2O$. Pure isolates were spot inoculated on the heavy metal supplemented medium. The petri- dishes were incubated at 30°C for 48 h. After the period of incubation, the plates were examined for growth and results recorded.

Determination of minimum inhibitory concentration (MIC) of heavy metals on *Proteus* species

Determination of the minimum inhibitory concentration (MIC) of heavy metals on *Proteus* species was carried out according to the method described by Amalesh et al. |(2012). Heavy metals used for MIC were lead (Pb), chromium (Cr), copper (Cu) and iron (Fe) at concentrations of 0.00, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/mL

Table 1. Lead resistance assay on *Proteus* species.

Isolate	Concentration mg/L						
	0.00	0.25	0.50	0.75	1.00	1.25	1.50
Proteus mirabilis (MSS7)	+++	+++	+++	+++	+++	+++	++
Proteus mirabilis (ISS10)	+++	+++	+++	+++	+++	++	+
Proteus terrae (WSS7)	+++	+++	+++	+++	+++	++	++
Proteus mirabilis (WSS14)	+++	+++	+++	+++	+++	+++	++
Proteus mirabilis (WSS16)	+++	+++	+++	+++	+++	++	++
Proteus mirabilis (CSS10)	+++	+++	+++	+++	+++	+++	++
Proteus mirabilis (CSS11)	+++	+++	+++	+++	+++	+++	++

MSS = Mining Site Soil, ISS = Industrial Site Soil, WSS = Waste Site Soil, CSS = Control Site Soil, + = Scanty Growth, ++ = Moderate Growth, +++ = Exuberant Growth.
Source: Authors.

respectively. Control experiment was 0.00 mg/mL with no heavy metal. Pure isolates were spot inoculated on nutrient agar supplemented with different salts of lead nitrate $Pb(NO_3)_2$, potassium dichromate $K_2Cr_2O_7$, copper sulphate pentahydrate $CuSO_4.5H_2O$ and iron sulphate heptahydrate $FeSO_4.7H_2O$ of heavy metals. The Petri- dishes were incubated at 30°C for 48 h. After the period of incubation, the plates were examined for growth and results were recorded.

Plasmid extraction and profiling of *Proteus* species

Plasmid extraction was carried out by alkaline lysis method described by Sumathy and Lekha (2017). The plasmid DNAs were run in Tris-Boric EDTA (TBE) 0.8% agarose gel stained with ethidium bromide and was visualized in UV Transilluminator.

Extraction of genomic DNA, PCR and 16S rRNA gene sequencing

Extraction of genomic DNA was carried out according to the method described by Macherey-Nagel (2009). Each pure culture of *Proteus* species grown overnight in 1.5 ml Muller Hinton broth inside microcentrifuge tubes were centrifuged at 14000 rpm for 5 min to obtain pellets. Quick-DNA™ Universal Kit by Zymo Research USA was used for extraction according to the manufacturer's instructions. The concentration of extracted genomic DNA was checked using NanoDrop 2000 Spectrophotometer at 260/280 absorbance. Twenty-five (25) µl volume was used for polymerase chain reaction (PCR). The components of each reaction mixture were: Master mix 4 µl, forward primer 2 µl, reverse primer 2 µl, DNA template 2 µl and DNase free water 15 µl. The two universal primers used have the following sequence (27F:5′-AGAGTTTGATCCTGGCTCAG-3′) and (1492R: 5′-GGTTACCTTGTTACGACTT-3′). Amplification was done for 16S rRNA gene in the thermal cycler as follows: Initial denaturation at 95°C for 5 min, 30 cycles of denaturation, annealing and extension at 94, 52 and 72°C for 30, 30 and 85 s, respectively, followed by a final extension at 72°C for 10 min and kept at a hold temperature of 4°C. The PCR products were run in Tris-Boric EDTA (TBE) 2.0% agarose gel stained with ethidium bromide and amplicons were visualized in UV Transilluminator. Amplicons were purified for sequencing using Zymo PCR cleanup Kit according to the manufacturer recommendations. Both strands of the purified DNAs were sequenced using Applied Biosystems Seqstudio Genetic Analyzer at the Macrogen Laboratory in Maryland USA with the following address: 1330 Piccard Drive, Suite 205, Rockville, MD 20850. Forward and reverse nucleotide sequences of each 16S rRNA gene were aligned and edited using Geneious Sequence Alignment Editor and were subjected to blast (Basic Local Alignment Search Tool) at NCBI website to find the best match of sequences producing significant alignments in order to christen each of the isolates. Seven (7) 16S rRNA genes of the *Proteus* species were submitted at the website of NCBI (National Centre for Biotechnology Information) Genbank. They were accepted and accession numbers were issued for them.

Construction of phylogeny

Nucleotides sequences of other strains of *Proteus* and related genera were pulled from website of NCBI and together with each of the seven nucleotide sequences of *Proteus* strains in this study; phylogeny was constructed using Molecular Evolutionary Genetic Analysis (MEGA) 7.0 version.

RESULTS AND DISCUSSION

Resistance assay of *Proteus* species to lead, chromium, copper and iron is presented in Tables 1 to 4. The growth of all organisms at concentrations of 0.25 to 1.50 mg/L of heavy metals indicated resistance. Scanty growth was depicted by + while moderate growth was indicated by ++ and exuberant growth was indicated by +++.

The minimum inhibitory concentration (MIC) of lead, chromium, copper and iron on *Proteus* species is shown in Table 5. In order to determine the concentration of each heavy metal that hinders the growth of *Proteus* species, MIC was carried at higher concentration of 0.00, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/mL. MIC was determined for all *Proteus* species grown on lead and copper supplemented agar while MIC was only determined for three (*Proteus mirabilis* [MSS7], *Proteus terrae* [WSS7] and *Proteus mirabilis* [WSS16]) out of seven *Proteus* species incubated in iron supplemented agar. MIC was not determined for all *Proteus* species inoculated on chromium metal supplemented agar.

Plasmid electrophoresis profile of *Proteus* species is presented in Figure 1. The DNA ladder in the first well

Table 2. Chromium resistance assay on *Proteus* species.

Isolate	Concentration mg/L						
	0.00	0.25	0.50	0.75	1.00	1.25	1.50
Proteus mirabilis (MSS7)	+++	+++	+++	+++	+++	+++	+++
Proteus mirabilis (ISS10)	+++	+++	+++	+++	+++	+++	+++
Proteus terrae (WSS7)	+++	+++	+++	+++	+++	+++	+++
Proteus mirabilis (WSS14)	+++	+++	+++	+++	+++	+++	+++
Proteus mirabilis (WSS16)	+++	+++	+++	+++	+++	+++	+++
Proteus mirabilis (CSS10)	+++	+++	+++	+++	+++	+++	+++
Proteus mirabilis (CSS11)	+++	+++	+++	+++	+++	+++	+++

MSS = Mining Site Soil, ISS = Industrial Site Soil, WSS = Waste Site Soil, CSS = Control Site Soil, + = Scanty Growth, ++ = Moderate Growth, +++ = Exuberant Growth.
Source: Authors.

Table 3. Copper resistance assay on *Proteus* species.

Isolate	Concentration mg/L						
	0.00	0.25	0.50	0.75	1.00	1.25	1.50
Proteus mirabilis (MSS7)	+++	+++	+++	++	++	+	+
Proteus mirabilis (ISS10)	+++	+++	+++	++	++	+	+
Proteus terrae (WSS7)	+++	+++	+++	++	++	+	+
Proteus mirabilis (WSS14)	+++	+++	+++	++	++	+	+
Proteus mirabilis (WSS16)	+++	+++	++	++	+	+	+
Proteus mirabilis (CSS10)	+++	+++	+++	+++	+++	+++	++
Proteus mirabilis (CSS11)	+++	+++	+++	+++	+++	++	+

MSS = Mining Site Soil, ISS = Industrial Site Soil, WSS = Waste Site Soil, CSS = Control Site Soil, + = Scanty Growth, ++ = Moderate Growth, +++ = Exuberant Growth.
Source: Authors.

Table 4. Iron resistance assay on *Proteus* species.

Isolate	Concentration mg/L						
	0.00	0.25	0.50	0.75	1.00	1.25	1.50
Proteus mirabilis (MSS7)	+++	+++	+++	+++	+++	++	+
Proteus mirabilis (ISS10)	+++	+++	+++	+++	+++	+++	+++
Proteus terrae (WSS7)	+++	+++	+++	++	++	+	+
Proteus mirabilis (WSS14)	+++	+++	+++	+++	+++	+++	+++
Proteus mirabilis (WSS16)	+++	+++	+++	+++	++	++	++
Proteus mirabilis (CSS10)	+++	+++	+++	+++	+++	+++	+++
Proteus mirabilis (CSS11)	+++	+++	+++	+++	+++	+++	+++

MSS = Mining Site Soil, ISS = Industrial Site Soil, WSS = Waste Site Soil, CSS = Control Site Soil, + = Scanty Growth, ++ = Moderate Growth, +++ = Exuberant Growth.
Source: Authors

shows standard bands. The molecular weight of DNA ladder used are 23130, 9416, 6557, 4361, 2322 and 2027 kilobase pair (kpb). The plasmids found in the heavy metal resistant *Proteus* species at ng/µl concentration were in the region of high molecular weight plasmids (23130 kbp) of the DNA ladder. Plasmids habour resistant markers. The presence of these high molecular weight plasmids in *Proteus* species may be responsible for their heavy metal resistance.

Gel electrophoresis of amplified 16S rRNA genes of

Table 5. Minimum Inhibitory concentrations (mg/mL) on *Proteus species* for different heavy metals.

Isolate	Lead	Chromium	Copper	Iron
Proteus mirabilis (MSS7)	1.50	ND	0.75	1.25
Proteus mirabilis (ISS10)	1.25	ND	0.75	ND
Proteus terrae (WSS7)	1.25	ND	0.75	0.75
Proteus mirabilis (WSS14)	1.50	ND	0.75	ND
Proteus mirabilis (WSS16)	1.25	ND	0.50	1.00
Proteus mirabilis (CSS10)	1.50	ND	1.50	ND
Proteus mirabilis (CSS11)	1.50	ND	1.25	ND

MSS = Mining Site Soil, ISS = Industrial Site Soil, WSS = Waste Site Soil, CSS = Control Site Soil, ND- Not Determined.
Source: Authors.

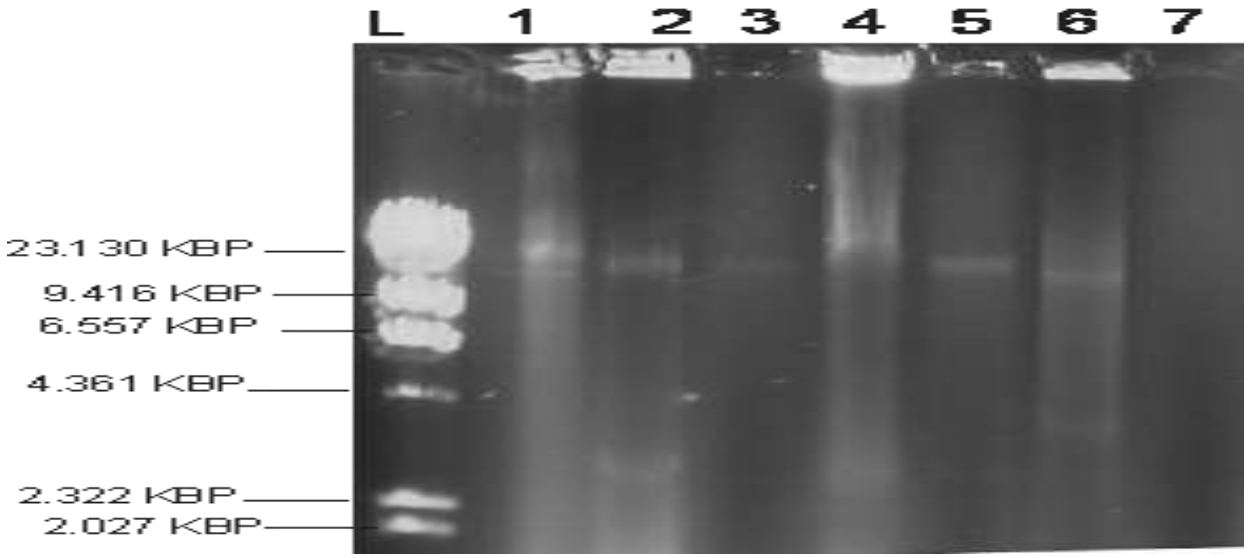

Figure 1. Gel Electrophoresis showing Plasmid Profile of *Proteus* species**.** L = Ladder, 1 = MSS7, 2 = ISS10, 3 = WSS7, 4= WSS14, 5 = WSS16, 6 = CSS10, 7 = CSS11.
Source: Authors

heavy metal resistant *Proteus* species is presented in Figure 2. The DNA ladder containing standard bands was loaded in the first well of the agarose gel. Control sample (nuclease free water) was loaded in well no 6. The highest molecular weight of DNA ladder used was 1500 base-pair (bp) and the lowest molecular weight of the ladder was 100 base-pair. Well no 1, 2, 3, 4, 5, 7 and 8 contained amplified 16S rRNA genes of *Proteus* species. The amplified 16S rRNA aligned at the corresponding region 1500 bp of the DNA ladder. This is because the molecular weight of 16S rRNA gene is 1500 bp.

Table 6 presents the molecular characterization of the *Proteus* species. The generic and specific names of the organisms were identified. The strain number and the accession number given for each organism by the national center for biotechnology information (ncbi) are also presented.

Figure 3 presents the phylogenetic tree showing genetic relatedness between other genera and *Proteus*. The linkage distance of 0.050 depicts that 5% percentage base substitution occurred in the nucleotide sequences of various organisms in the phylogeny. The base substitution brought about evolution of new organisms from parental organisms. The *Proteus* species in bold letters are isolates from this research. Phylogeny was constructed using nucleotides sequences of 16S rRNA genes of *Proteus* species in this study and related genera pulled from ncbi website. The software used for the construction was Molecular Evolutionary Genetic Analysis (MEGA) 7.0 version.

The results of heavy metal resistance assay for *Proteus mirabilis* and *P. terrae* revealed that *Proteus mirabilis* isolated from mininig, industrial and waste site soil of a cement factory and control site soil showed resistance to the four heavy metals (Pb, Cr, Cu and Fe) in this study (Table 1 to 4). A similar research by Adekanmbi et al.

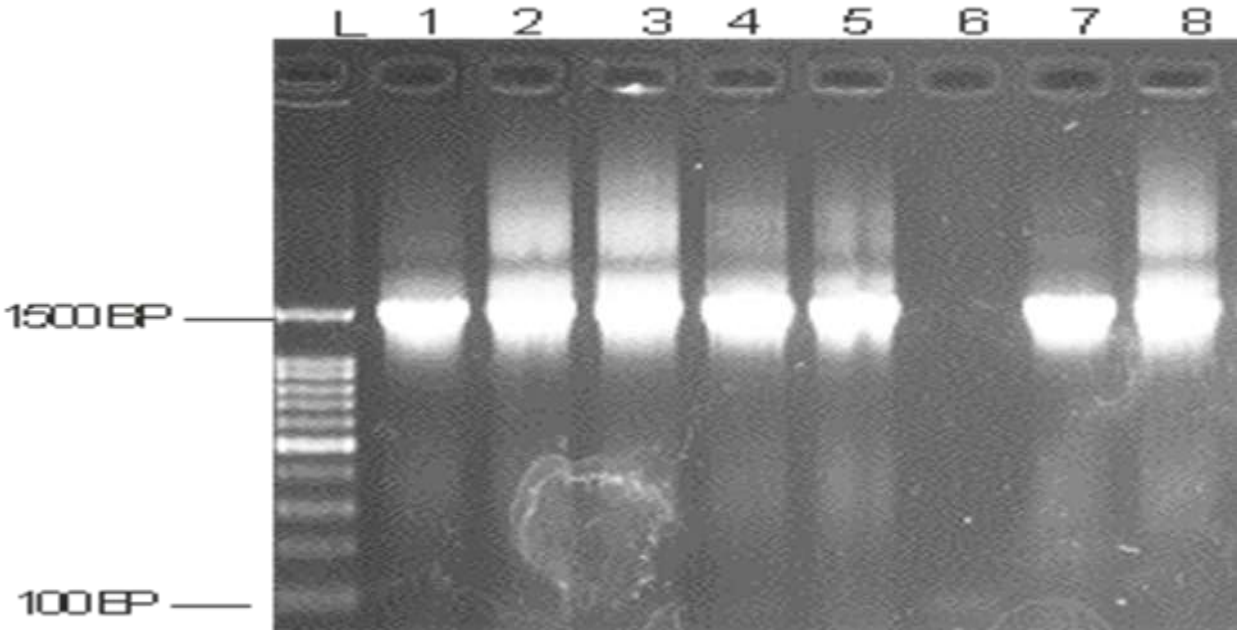

Figure 2. Gel Electrophoresis showing amplified 16S rRNA genes of *Proteus* species. Key: L = Ladder, 1= MSS7, 2= ISS10, 3= WSS7, 4= WSS14, 5= WSS16, 6= Control sample, 7= CSS10, 8=CSS11.
Source: Authors.

Table 6. Molecular characterization of the *Proteus* species.

Isolate	Organism identified	Blast best match	%Similarity	Strain number	Accession number
MSS7	*P. mirabilis*	*Proteus mirabilis* strain ALK418	99	ADY 13	MH084801
ISS10	*P. mirabilis*	*Proteus mirabilis* strain ALK418	99	ADY23	MH084809
WSS7	*P. terrae*	*Proteus terrae* strain N5/687	86	ADY17	MH119106
WSS14	*P. mirabilis*	*Proteus mirabilis* strain JCM 1669	99	ADY 19	MH084805
WSS16	*P. mirabilis*	*Proteus mirabilis* strain ATCC 29906	97	ADY 20	MH084806
CSS10	*P. mirabilis*	*Proteus mirabilis* strain JCM 1669	99	ADY 30	MH084814
CSS11	*P. mirabilis*	*Proteus mirabilis* strain JCM 1669	99	ADY 28	MH084813

MSS = Mining Site Soil, ISS = Industrial Site Soil, WSS = Waste Site Soil, CSS = Control Site Soil, BLAST = Basic Local Alignment Search Tool.
Source: Authors.

(2019) reported that *Proteus mirabilis* isolated from printeries wastewaters demonstrated resistance to lead, cadmium, chromium, copper, silver and zinc. Nwagwu et al. (2017) reported that *Proteus mirabilis* isolated from Panteka stream showed resistance to lead, iron, cadmium, zinc and nickel. Also *Proteus mirabilis* isolated from industrial wastewater displayed resistance to zinc (Owolabi and Hekeu, 2015). Mgbemena et al. (2012) found that a strain of *Proteus mirabilis* isolated from Otamiri River showed resistance to lead, iron and zinc. *Proteus terrae* isolated from waste site soil of a cement factory in this study demonstrated resistance to lead, chromium, copper and iron. Based on literatures at our disposal, the heavy metal resistance ability of *Proteus terrae* has not been determined prior to this time and this is likely to be the first research reporting the heavy metal resistance ability of this organism.

Badar et al. (2000) opined that contamination with a specific metal is known to increase the level of resistance of the bacterial community to that metal. Bacteria adapt to metal stress in their environment and respond to it by developing several resistances or coping mechanisms to its toxicity (Adekanmbi et al., 2019). The 100% resistance (growth) of *Proteus* species to the heavy metals at various concentrations (mg/L) for resistance assay (Tables 1 to 4) suggests that the microbes have adapted to, tolerate and grow in the presence of these heavy metals in their metal-stressed natural soil habitats. Plasmid profiling (Figure 1) of the *Proteus* species revealed that the *Proteus terrae* and five out of the six

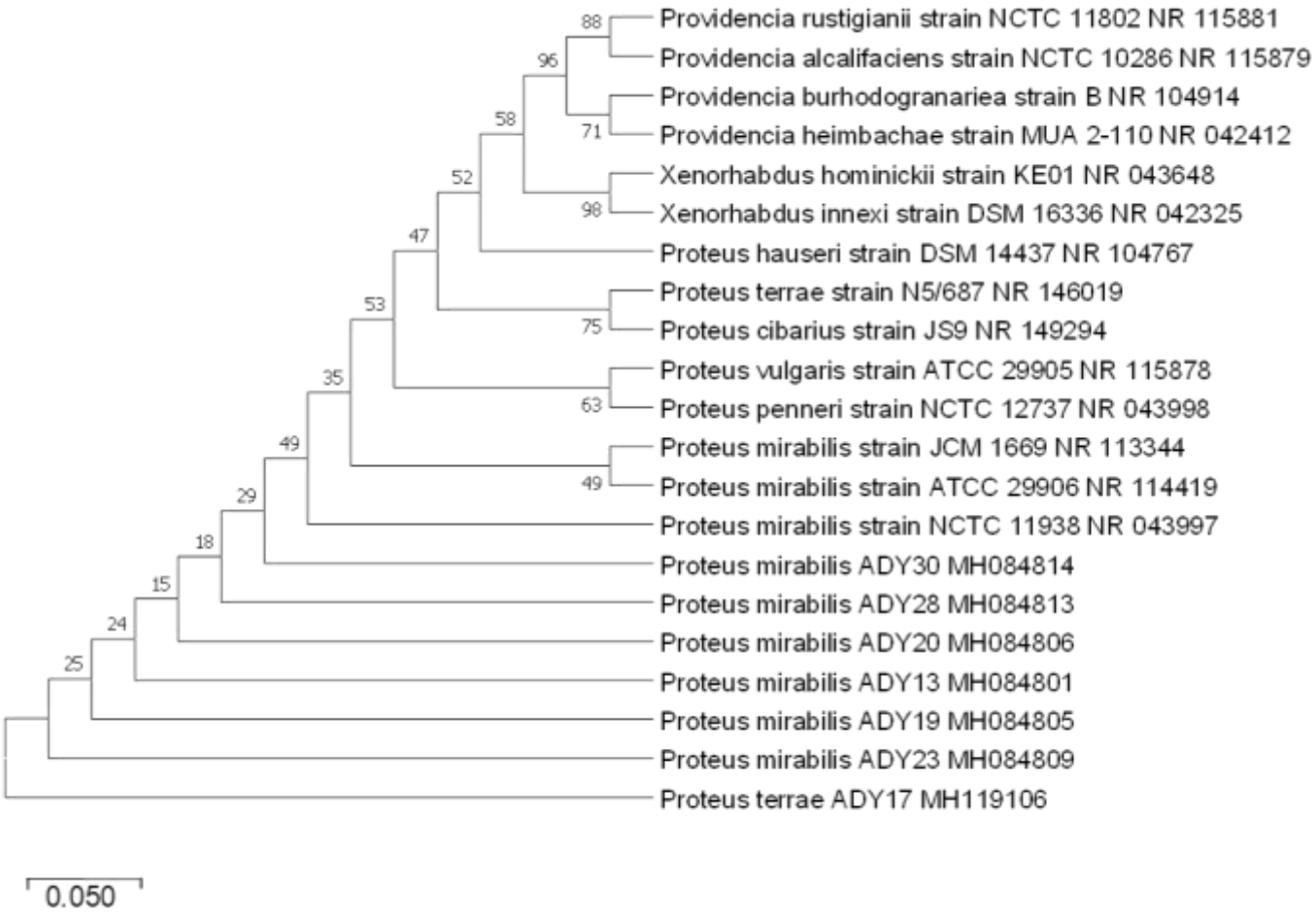

Figure 3. Dendrogram showing the phylogenetic relationship of different strains of *Proteus* species and related genera. The organisms with letters ADY in their strain number are the *Proteus species* isolated in this study.
Source: Authors.

Proteus mirabilis habour high molecular weight plasmid (23000 bp). The resistance determinants to the test metals are likely located on the plasmids. Abou-Shanab et al. (2007) reported that genes encoding heavy metal resistance in bacteria can be located within bacterial plasmids, chromosomes, or on transposons. Plasmid genes may code for proteins involved in metal reduction, binding, sequestration, complex formation and for specific transport systems e.g. efflux pumps (Ghosh et al., 2000). Plasmid-borne resistance to copper has been reported in several species of bacteria and documented by several authors (Hansen et al., 2016; Buberg et al., 2020). Also chromosomal resistance to copper has also been reported in species of bacteria (Jones et al., 2005). This might be responsible for the resistance to copper and other metals by *Proteus mirabilis* strain ADY28 in this study, even without the possession of the plasmid-borne resistance determinants.

In addition, Adekanmbi et al. (2019) reported the detection of plasmid-borne chrA gene responsible for chromate resistance in *Proteus mirabilis* PW3a from printeries wastewater. The possession of plasmids encoding chromate resistance has also been reported in certain species of *Alcaligenes*, *Bacillus*, *Escherichia coli*, *Pseudomonas* and *Salmonella* by several authors (Ghosh et al., 2000; Verma et al., 2002; Kamala-Kannan and Lee, 2008: Das et al., 2014). Adekanmbi et al. (2019), detected chrB gene which regulates the chrA transporter in *Proteus mirabilis* PW4c and *Providencia vermicola* PWAP3 and this corroborated the report on the possession of the chr operon on the plasmids of Gram negative bacteria (Verma et al., 2009). The CBA-transport systems responsible for export of metal ions, xenobiotics and drugs are found in Gram negative bacteria. This system safeguards cytoplasm of Gram negative cells through translocation of metals and other toxicants across their outer membrane (Adekanmbi et al., 2019). The pbr proteins are a group of proteins encoded in the widely studied metal-resistant *Cupriavidus metallidurans* CH34, and they include pbrT, pbrA, pbrB, pbrC, pbrD and pbrR. The pbrA is a PIB-type ATPase in *Cupriavidus metallidurans*, and is the main lead efflux

transporter (Borremans et al., 2001). The pbr proteins might be present in *Proteus* species in this study due to the appreciable resistance shown to lead.

In order to determine the minimum inhibitory concentration (MIC) of each heavy metal on *Proteus* species, the concentration of the heavy metals was increased from mg/L to mg/mL (Table 5). Despite this geometric increase, MIC was wholly determined for lead and copper, partly for iron but not for chromium at various concentrations of 0.00, 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50 mg/mL. The resistance shown to lead by *Proteus terrae* strain ADY17 and all variants of *Proteus mirabilis* (strains ADY13, ADY19, ADY20, ADY23, ADY28 and ADY30) up to 1.00 mg/mL and even at 1.25 mg/mL is highly commendable because lead is known to be a very poisonous heavy metal with high toxic effect on bacteria as reported by Eghomwanre et al. (2016). The minimum inhibitory concentration of lead on *Proteus mirabilis* (ISS10), *Proteus terrae* (WSS7) and *Proteus mirabilis* (WSS16) was 1.25 mg/mL and that of *Proteus mirabilis* (MSS7), *Proteus mirabilis* (WSS14), *Proteus* mirabilis (CSS10) and *Proteus* mirabilis (CSS11) was 1.50 mg/mL. It is noteworthy that these organisms survived high concentrations of lead and could be very advantageous as remediating agents in lead polluted soil and water. Furthermore, the growth of all these variants of *Proteus* at concentrations of 0.25 to 1.50 mg/mL of chromium depicts they are highly resistant to chromium which is an advantage in bioremediation of chromium in polluted environment. The very high resistance shown by *Proteus mirabilis* (CSS11) and (CSS10) strains ADY28 and ADY30 to copper up 1.00 and 1.25 mg/mL is of interest and indication of the effectiveness of these two strains as potential bioremediation agents in copper polluted environment. Minimum inhibitory concentration of copper was determined for other *Proteus* species. The minimum inhibitory concentration of copper on *Proteus mirabilis* (MSS7), *Proteus mirabilis* (1SS10) *Proteus terrae* (WSS7) and *Proteus mirabilis* (WSS16) was 0.75 mg/mL and that of *Proteus mirabilis* (WSS14), was 0.50 mg/mL. Eghomwanre et al. (2016) reported the toxic effect of copper on bacterial isolates from soil and sediment. Nonetheless, all these organisms thrived well in the presence of copper and could be exploited for remediative advantage in copper polluted areas. *Proteus terrae* strain ADY17 had the least MIC for iron compared to all other variants of *Proteus mirabilis.* This indicates that *Proteus mirabilis* had higher resistance to iron compared to *Proteus terrae* and this might be due higher need of iron for metabolic activities by *Proteus mirabilis than P*. *terrae*. In addition, it has been reported that *Proteus mirabilis* strains are able to produce siderophores which translocate iron from outside across the cell membrane into these organisms (Prescott et al., 2008). It is worthwhile to point out that *Proteus mirabilis* (ISS10), *Proteus mirabilis* (WSS14), *Proteus* mirabilis (CSS10) and *Proteus* mirabilis (CSS11) survived iron concentrations from 0.00 to 1.50 mg/mL. This finding corroborates the report of Mgbemena et al. (2012) and Nwagwu et al. (2017). It can be inferred that these strains: ADY23 ADY19 ADY30 and ADY28 of *Proteus* can be used to get rid of excess iron in both human and environmental systems. In summary, the MIC results revealed that copper had highest toxic effect on the growth of *Proteus* species, followed by lead, then iron and chromium had no toxic effect at these concentrations.

Conclusion

On a general note, *Proteus* species are known for their detrimental activities such as being opportunistic pathogens in humans and animals, indicator of fecal pollution, marine food-borne pathogens etc. Contrariwise, this study reveals that *Proteus* species are also beneficial to humans in our natural and industrial environment. *Proteus species* investigated for heavy metal resistance in this study, turned out to be potential bioremediation agents for the test heavy metals [lead (Pb), chromium (Cr), copper(Co) and iron(Fe)]. These organisms should be explored more to harness their full potential in bioremediation of toxic heavy metals in polluted environment.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Antibiotic and heavy metal resistance genes in hospital effluents and streams in Benin

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Poor effluent management is known to release antibiotic resistance genes and heavy metal resistance genes into streams. The objective of this study was to investigate the occurrence of antibiotics and heavy metals in hospital effluents and streams in Benin. The extraction of genomic DNA from multidrug-resistant bacterial strains isolated from stream and hospital effluents samples was performed according to the recommendations of the Quick-DNA TM miniprep kit (Zymo Research Corp, United States). Real-time PCR was used to identify twelve antibiotic and six heavy metal resistance genes. The results showed that *sulII* (77.77%), *sulI* (67.67%), and bla_{TEM-1} (44.44%) were the resistance genes to antibiotics, the most detected in gram-negative bacilli isolated from hospital effluents. Two genes, *tetA* (33.33%) and *ermB* (20%), were found in gram-positive cocci. *zntA* (57.57%), *czcA* (24.24%), and *copA* (22.22%) are the genes encoding resistance to heavy metals, most found in gram-negative bacilli, but *zntA* (20%) and *czcA* (10%) were both found in *Staphylococcus aureus* isolates. Concerning streams, *sulII* (38.23%), *sulI* (26.47%), and bla_{TEM-1} (23.53%) were detected in gram-negative bacilli. *czcA* (38.23%), zntA (35.29%), and copA (11.76%) are the genes encoding heavy metal resistance found in gram-negative stream bacilli. These results highlight the need for measures to be taken to ensure the integrity of natural resources and thereby preserve human, animal and environmental health.

Key words: Antibiotic and heavy metal resistance genes, hospital effluents and streams, Benin.

INTRODUCTION

Water is one of the most precious resources on earth. For humans, it is one of the basic needs used for food and other ancillary needs. It is therefore agreed that its availability in quantity and quality is essential for life on earth. Among these resources, streams represent one of the most important resources after the oceans. It is widely used for many human activities, such as fishing, agriculture, transportation, and many others. However, it is subject to numerous contaminations. This contamination can come from various sources related to

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human activities, such as industrial, agricultural, domestic discharges and hospital effluents (Adelowo et al., 2018; Kayembe et al., 2018; Zhou and Wang, 2019; Laffite et al., 2020; Yeh et al., 2020; Duan et al., 2021). The latter is one of the most important sources of contamination. Indeed, the contamination of streams can be of various natures that are chemical, microbiological, and sometimes radioactive (Pietryczuk et al., 2018; Carles et al., 2019; Mandaric et al., 2019; Yeh et al., 2020). Given that hospitals are environments par excellence where we witness the presence of large numbers of microbes of various kinds (bacteria, parasites, fungi, and viruses), these hospital effluents therefore contribute strongly to the microbiological contamination of streams (Verburg et al., 2019; Suzuki et al., 2020). In addition to microbes, the most important of which are bacteria, hospital effluents also contain antibiotic residues and resistance genes. Among the priority antibiotic-resistant pathogens, carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa*, carbapenem-resistant and ESBL-producing Enterobacteriaceae are at the top of the WHO list and represent the greatest threat to human health (Sheu et al., 2019). In addition, Colistin regained global interest as a consequence of the rising prevalence of multidrug-resistant Gram-negative Enterobacteriaceae. In parallel, colistin resistant bacteria emerged in response to the unregulated and increased use of this antibiotic, which is a last resort drug due to failure of carbapenems, has possibly contributed to the development and spread of resistance to colistin among Enterobacteriaceae (Gogry and Siddiqui, 2019; Gogry et al., 2021, 2022). Indeed, many studies have shown the presence of these priority resistant pathogens in stream and hospital effluents (Adelowo et al., 2018; Bartley et al., 2019; Posada-Perlaza et al., 2019; Niestępski et al., 2020; Suzuki et al., 2020). Even if other activities, such as livestock farming and domestic water, can bring these elements into watercourses, hospital effluents are suspected to be one of the main sources (Adelowo et al., 2018).

Bacteria are known to be the most prevalent in the hospital environment and in these effluents (Giannakis et al., 2017; Mittelman and Jones, 2018). These bacteria are most often multidrug-resistant to antibiotics due to the presence of antibiotics in the environment and the strong transmission of resistance genes (Giannakis et al., 2017; Mittelman and Jones, 2018; Laffite et al., 2020). Additionally, the presence of heavy metals in the medical environment exerts pressure favoring the selection of opportunistic pathogens resistant to antibiotics. Heavy metal resistance (HMR) associated with antibiotic resistance (AR) in hospital effluents makes them potentially dangerous (Chen et al., 2019; Dahanayake et al., 2019; Turner et al., 2020). Moreover, several studies have shown the presence of antibiotic and heavy metal resistance genes (ARGs and HMRGs) in wastewater, sewage sludge, river water, and Black sea (Sabatino et al., 2020; Hubeny et al., 2021; Martin et al., 2021). Other studies have established the correlation between these two types of resistance (Di Cesare et al., 2016; Yuan et al., 2018; Ohore et al., 2019). This is why it is advisable to have a water treatment and purification system in every hospital or city. Even if industrialized countries have these types of systems, this is not the case in developing countries. In developing countries, the metabolites of products used in hospitals or their byproducts, accompanied by a bacterial load (ARGs and HMRGs), are potentially found in hospital effluents treated in situ or collected by urban sewage systems, which are themselves connected to a water treatment plant and discharge the treated effluent into the natural environment (Laffite et al., 2016). In Benin, National Hospital and University Center of Cotonou is the only one hospital with a purification and treatment system for hospital effluents but this system remains moderately efficient (Todedji et al., 2020). Some studies have looked at the microbiological quality of hospital effluents and streams and the isolated bacterial strains have been characterized (Deguenon et al., 2022; Gbotche et al., 2023).

However, none of these studies have evaluated the presence of ARGs and HMRGs in the genome of pathogenic in these different matrices. The present study was undertaken to address this lack of data through. This nationwide study was therefore conducted to determine the current status of ARGs and HMRGs in hospital effluents and waterways in Benin.

MATERIALS AND METHODS

Stream samples were collected from the main streams in Benin. In northern Benin, the Kota and Tanougou waterfalls, the Koumagou, Malanville, Okpara, Sota, Mékrou and Pendjari rivers were sampled. In southern, the rivers of Ganvié, Grand-Popo, Tori, and Porto-Novo, and the lakes of Bopa, Adjohoun, Tokpa, and Zangnannando were sampled (Figure 1). Hospital effluent samples were collected in the National Hospital and University Center (CNHU) of Benin, in the five Departmental Hospital Centers of Benin (CHD Porto-Novo, CHD Borgou-Alibori, CHD Donga and CHD Atacora), in 7 main Zonal Hospitals in the country (HZ Malanville, HZ Dassa-Zoumè, HZ Zou-Collines, HZ Pobè, HZ Aplahoue, HZ Lokossa, HZ Calavi, and HZ Menontin), and in four confessional hospitals in the country (L'ordre de Malte de Djougou, Boko, CS Savè, La croix de Zinvié, and Grand-Popo hospital) (Figure 1).

Two samples were taken from each stream while four samples were collected per site for hospitals effluents. The samples were collected in sterile 1-liter bottles and transported to the laboratoryin a cooler equipped with an accumulator. To target departments with high antibiotic consumption such as intensive care, emergency, pediatrics and maternity departments, collectors were chosen. In total, 32 stream samples and 72 hospital effluent samples were collected from different locations as indicated in Figure 1.

The identification and antimicrobial susceptibility test of multiresistant bacterial strains isolated from these stream and hospitals effluent samples were previously performed and described in the work of Deguenon et al. (2022) and Gbotche et al. (2023).

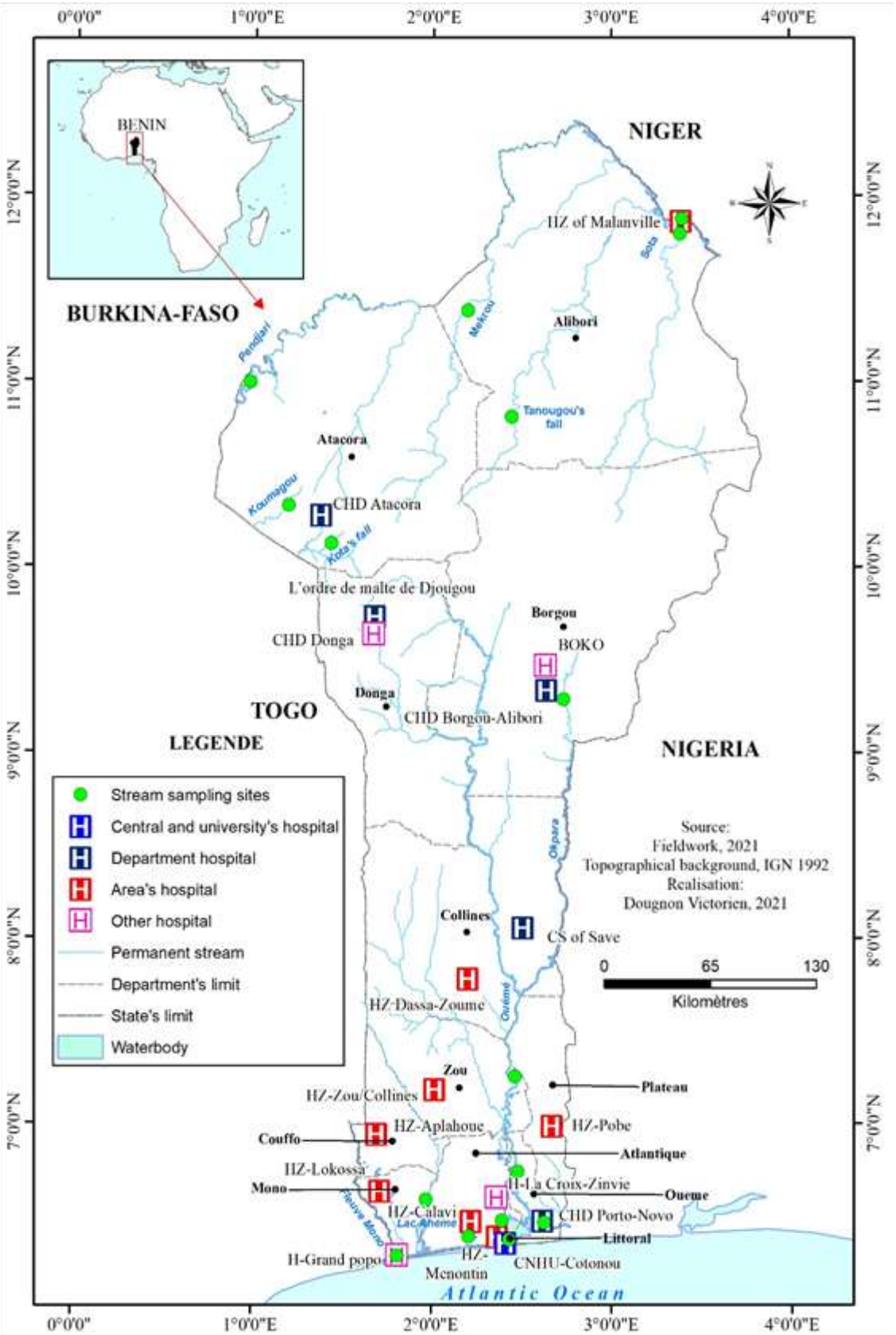

Figure 1. Geographic repartition of collect site
Source: Authors

Table 1. List of the genes detected in this study.

	Gene function	Forward sequence	Reverse sequence	Annealing temperature (°C)	References
ZntA	Resistance to Zinc/cadmium/lead	GCTCGGGTCTGGCATTGAAG	TTGCAGCATCGGCGCGCAGGGTA	60.8	Aleem et al., 2021; Raza et al., 2021
copA	Resistance to Copper	GGTGCTGATCATCGCCTG	GGGCGTCGTTGATACCGT	58.0	De la Iglesia et al., 2010
czcA	Resistance to Cobalt/Zinc/Cadmium	GGSGCGMTSGAYTTCGGC	GCCATYGGNYGGAACAT	57.6	Kaci et al., 2014
czcC	Resistance to Cobalt/Zinc/Cadmium	AGCCGYCAGTATCCGGATCTGAC	GTGGTCGCCGCCTGATAGGT	63.6	Roosa et al., 2014
czcD	Resistance to Cobalt/Zinc/Cadmium	TCATCGCCGGTGCGATCATCAT	TGTCATTCACGACATGAACC	55.2	Roosa et al., 2014
pbrT	Resistance to Lead	AGCGCGCCCAGGAGCGCAGCGTCTT	GGCTCGAAGCCGTCGAGRTA	63.6	Roosa et al., 2014
tetW	Resistance to Tetracycline	ACGGCAGCGCAAAGAGAA	CGGGTCAGTATCCGCAAGTT	59.1	This study
tetA	Resistance to Tetracycline	GCTACATCCTGCTTGCCTTC	CATAGATCGCCGTGAAGAGG	57.1	Ng et al., 2001
tetQ	Resistance to Tetracycline	TTATACTTCCTCCGGCATCG	ATCGGTTCGAGAATGTCCAC	56.1	Smith et al., 2004
tetX	Resistance to Tetracycline	CAATAATTGGTGGTGGACCC	TTCTTACCTTGGACATCCCG	56.3	Ng et al., 2001
tetG	Resistance to Tetracycline	GCTCGGTGGTATCTCTGCTC	AGCAACAGAATCGGGAACAC	57.5	Ng et al., 2001
catII	Resistance to Chloramphenicol	GATTGACCTGAATACCTGGAA	CCATCACATACTGCATGATG	52.2	Yoo et al., 2003
cmlA	Resistance to Chloramphenicol	ACGGCATACTCGGATCCATG	CTTAACGGGGAGTAGCAGCT	58.0	This study
sulI	Resistance to Sulfonamide	CGCACCGGAAACATCGCTGCAC	TGAAGTTCCGCCGCAAGGCTCG	65.0	Pei et al., 2006
sulII	Resistance to Sulfonamide	TCCGGTGGAGGCCGGTATCTGG	CGGGAATGCCATCTGCCTTGAG	57.5	Pei et al., 2006
ermG	Resistance to Erythromycine	GTGAGGTAACTCGTAATAAGCTG	CCTCTGCCATTAACAGCAATG	57.1	Koike et al., 2010
ermB	Resistance to Sulfonamide	GGATTCTACAAGCGTACCTTGGA	AATCGAGACTTGAGTGTGCAAGAG	61.1	Flórez et al., 2014
bla_{Tem-1}	Resistance to Betalactam	TCGGGGAAATGTGCG	GGAATAAGGGCGACA	50.8	De Gheldre et al., 2003

Source: Authors

Genomic DNA was extracted from the identified multiresistant drug-resistant bacterial strains using a Quick-DNA TM miniprep kit (Zymo Research Corp, United Stat) according to the manufacturer's instructions. In all, 12 ARGs (*cml*A, *cat*II, bla_{TEM-1}, *sul*I, *sul*II, *tet*A, *tet*Q, *tet*X, *tet*G, *tet*W, *erm*G, ermB) and six HMRGs (*znt*A, *pbr*T, *czc*A, *czc*C, *czc*D, *cop*A) were researched. *cml*A, *cat*I, bla_{TEM-1}, *sulI,* and *sul*II were researched in gram-negative bacilli, and *cml*A, *catII, tet*A, *tet*Q, *tet*X, *tet*G, *tet*W, *erm*G and *erm*B were researched in gram-positive cocci. Real-time PCR was run using a LineGene9600 Plus Fluorescent Quantitative Detection System (Hangzhou Bioer Technology, China) with the following program: 95°C for 60 s, 40 cycles consisting of (i) 95°C for 15 s, (ii) annealing temperature for 15 s, and a melting stage consisting of (i) 95°C for 15 s, (ii) melting temperature for 60 s and (iii) 95°C for 15 s. Cycle thresholds (CT) were reported, and positive samples were isolated with CT below 30. Primer sequences and annealing temperatures are displayed in (Table 1). Positive controls for antibiotic resistance genes were clinical isolates that carry those genes and the detection was done by standard PCR. For heavy metal genes, to none positive controls were used, but the experiments were ruled twice to confirm the true positive. All negative controls were RNA/DNA free water.

RESULTS

The distribution of ARGs and HMRGs detected in gram-negative bacilli and gram-positive cocci bacteria is as follows. As shown in Figure 2, two ARGs were found in gram-positive cocci bacteria strains, namely, *tet*A and *erm*B. *tet*A was found in 33.33% of the *Staphylococcus aureus* strains isolated from hospital effluents. While *erm*B was detected in 66.66% of the *Enterococcus* strains isolated from hospital effluents. As for the HMRGs detected in gram-positive cocci bacteria, three genes were detected: *znt*A, *czcA,* and *cop*A (Figure 2). *znt*A and *czc*A were found in 33.33% and 16.66% of the *S. aureus* strains isolated from hospital effluents, respectively. While, *cop*A was detected in 33.33% of coagulase-negative *Staphylococcus* (CNS) strains isolated from waterways (Figure 2).

As shown in Table 2, four ARGs (*cml*A, bla_{TEM-1}, *sulI,* and *sul*II) and three HMRGs (*znt*A, *czcA,* and *cop*A) were detected in gram-negative bacilli strains. *cml*A was found in 20% of the *E. coli* and *Klebsiella* spp. strains; 14.28% of the *Pseudomonas* spp. strains; and 10.52% of the non-enterobacteria strains, all of which were obtained from hospital effluents. In streams, only 8.33% and 7.69% of *Klebsiella spp.* and non-enterobacteria strains, respectively, carry the *cml*A gene (Table 2). bla_{TEM-1} was detected in 60% of *E. coli* strains, 54% of *Acinetobacter spp*.

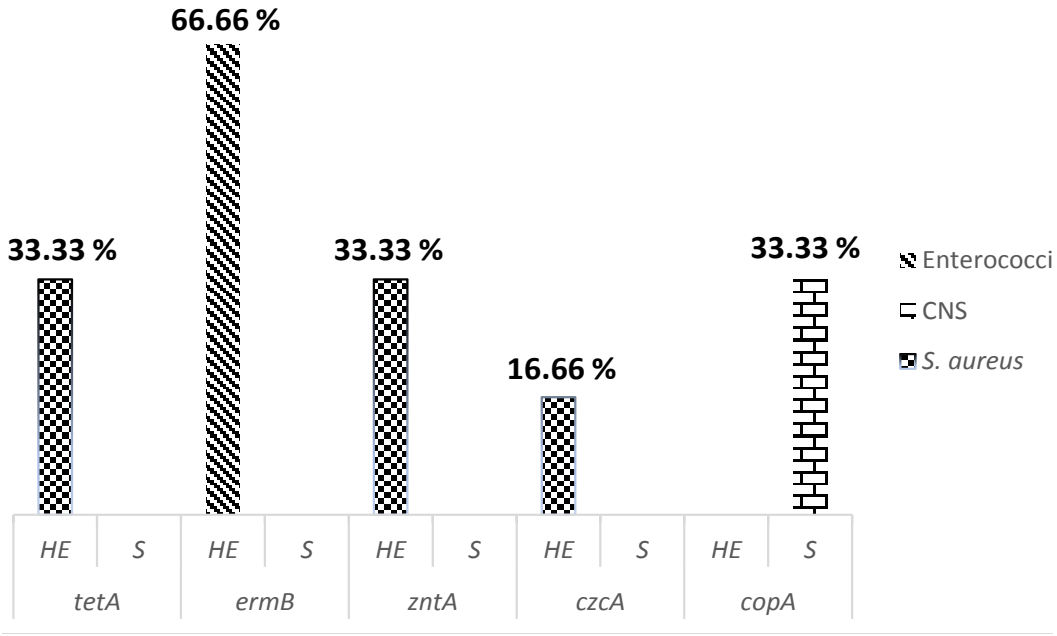

Figure 2. Distribution of antibiotic and heavy metal resistance genes detected in gram-positive cocci bacteria from hospital effluents and streams. HE: Hospital effluents; S: Streams; CNS: Coagulase Negative Staphylococcus; *S. aureus*: *Staphylococcus aureus*
Source: Authors

of *Klebsiella spp*. strains, and 35.71% of *Pseudomonas* spp. strains all isolated from hospital effluents. While the same gene was found in 100% of *E. coli* strains, 33.33% of *Enterobacter spp.* strains, and 25% of *Klebsiella spp.* strains, all isolated from streams. The *sul*I and *sul*II genes were detected in strains of *E. coli*, *Klebsiella spp*. non-enterobacteria and *Pseudomonas* spp*.,* all isolated both in hospital effluents and in streams (Table 2). Regarding the HMRGs, only the *znt*A, *czcA,* and *cop*A genes were detected in the gram-negative bacilli. *znt*A was found in 100% of *E. coli* strains, 74% of *Acinetobacter spp*. strains, and 57.14% of *Pseudomonas* spp*.* strains. All isolates were from hospital effluents. The same gene was detected in 100% of *E. coli* strains, 50% of *Klebsiella spp.,* and *Pseudomonas spp*. strains, all isolated from streams. *czc*A was found in the only strain of *Yersinia enterolitica*, in 40% of strains of *E. coli* and *Klebsiella spp*., 34% of strains of *Acinetobacter spp*. All isolated in the streams. As for the *cop*A gene, it was found in strains of *E. coli*, *Klebsiella spp*. isolated both in hospital effluents and in streams (Table 2).

DISCUSSION

The problem of liquid effluent management remains a concern in developing countries like Benin. The objective of this study was to assess the presence of antibiotic and metal resistance gens in Benin hospital effluents and streams. The detection of resistance genes in the extracted DNA of the different bacterial strains isolated showed the presence as well as of ARGs (*tetA*, bla_{TEM-1}, ermB, sulI, sulII) and HMRGs (*zntA*, *czcA*, *copA*). These genes are found both in hospital effluents (tetA, bla_{TEM-1}, *ermB*, *sulI*, *sulI*I, *zntA*, *czcA*, *copA*) and in waterways (cmlA, bla_{TEM-1}, *sulI*, *sulII*, *zntA*, *czcA*, *copA*). In hospital effluents, the resistance genes were found in strains of *Staphylococcus aureus*, coagulase-negative staphylococci, *Acinetobacter spp.*, *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas spp.* and *Yersinia enterolitica*. Bacterial strains of *Acinetobacter spp.*, *Yersinia spp.*, *Klebsiella spp.*, *Staphylococcus aureus*, and *Pseudomonas spp.* isolated from wastewater at a sewage treatment plant in western Massachusetts, USA, contained resistance genes to β-lactams, sulfonamides, tetracyclines, zinc, and copper (Martin et al., 2021). Sewage treatment plants receive wastewater from the entire community, including hospital effluents. Therefore, we can say that the results obtained in the present study are in line with those of (Martin et al., 2021). Several studies have shown the presence of antibiotic resistance genes in hospital effluents (Hara et al., 2018; Paul et al., 2018; Yousfi et al., 2019; Fadare and Okoh, 2021), but very few have focused on the presence of HMRGs in effluents. This study provides new scientific data on the presence of heavy metals in hospital effluents. This can be explained by the different human activities practiced and the important flow of humans in hospitals (kitchen,

Table 2. Distribution of antibiotic and heavy metal resistance genes in gram-negative bacilli from hospital effluents and streams.

Bacterial strains		*cmlA*	*bla*$_{TEM-1}$	*sulI*	*sulII*	*zntA*	*czcA*	*copA*
Acinetobacter spp	HE: n=50	4 (8%)	27 (54%)	34 (68%)	42 (84%)	37 (74%)	17 (34%)	13 (26%)
	S: n=0	-	-	-	-	-	-	-
Escherichia coli	HE: n=5	1 (20%)	3 (60%)	5 (100%)	5 (100%)	5 (100%)	2 (40%)	3 (60%)
	S: n=1	0 (0%)	1 (100%)	0 (0%)	1 (100%)	1 (100%)	0 (0%)	1 (100%)
Klebsiella spp	HE: n=10	2 (20%)	5 (50%)	8 (80%)	6 (60%)	3 (30%)	4 (40%)	3 (30%)
	S: n=12	1 (8.33%)	3 (25%)	5 (41.66%)	4 (33.33%)	6 (50%)	5 (41.66%)	2 (16.66%)
No-Enterobacteria	HE: n=19	2 (10.52%)	3 (15.78%)	10 (52.63%)	15 (78.94%)	3 (15.78%)	0	0
	S: n=13	1 (7.69%)	2 (15.38%)	3 (23.07%)	4 (30.76%)	2 (15.38%)	6 (46.15%)	1 (7.69)
Pseudomonas spp	HE: n=14	2 (14.28%)	5 (35.71%)	9 (64.28%)	8 (57.14%)	8 (57.14%)	0	3 (21.42%)
	S: n=4	0 (0%)	0 (0%)	1 (25%)	1 (25%)	2 (50%)	2 (50%)	0 (0%)
Yersinia enterolitica	HE: n=1	0	1 (100%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)	0
	S: n=0	-	-	-	-	-	-	-
Enterobacter spp	HE: n=0	-	-	-	-	-	-	-
	S: n=3	0 (0%)	1 (33.33%)	0 (0%)	2 (66.66%)	0 (0%)	0 (0%)	0 (0%)
Salmonella spp	HE : n=0	-	-	-	-	-	-	-
	S: n=1	0 (0%)	1 (100%)	0 (0%)	1 (100%)	1 (100%)	0 (0%)	0
Total	HE: n=99	11 (11.11%)	44 (44.44%)	67 (67.67%)	77 (77.77%)	57 (57.57%)	24 (24.24%)	22 (22.22%)
	S: n=34	11 (11.11%)	8 (23.53%)	9 (26.47%)	13 (38.23%)	12 (35.29%)	13 (38.23%)	4 (11.76%)

HE: Hospital effluents, S: Stream, n: effective.
Source: Authors

medical care, discharge of heavy metal residues through urine, and feces). These results also support the fact that there is a correlation between the presence of antibiotic and heavy metal resistance genes (Di Cesare et al., 2016). Furthermore, it noted a low presence of *bla*$_{TEM-1}$ gene, while it is known that penicillin and cephalosporins are widely used in the country, as indicated by the studies of Dougnon et al. (2020). It would therefore be interesting to update the scientific data on the consumption of antibiotic molecules in Benin. However, it should be noted that the *bla*$_{TEM-1}$ genes represent only one of the many genes coding for cephalosporin resistance. It should also be noted that other origins may contribute to the presence of these genes in rivers, including migratory birds in which the same genes have been noted (Yuan et al., 2018). In Poland, and more precisely in the Warmia and Mazury regions, the resistance genes *bla*$_{TEM-1}$, sulI, and sulII were detected in river, wastewater and sewage sludge samples (Hubeny et al., 2021). These antibiotic resistance genes were correlated with heavy metals found in variousconcentrations in the same samples (Hubeny et al., 2021). These results are consistent with what have been obtained in this with those in this study, where the presence of *bla*$_{TEM1}$, *sulI, sulII*, and heavy metal resistance genes *zntA, czcA*, and *copA*) has been detected. Similar results were obtained by Sabatino et al. (2020) in samples from the Black Sea, where an abundance of *tetA, sulII* and *czcA* genes were detected. Hubeny et al. (2021) has reported that wastewater and sewage sludge are discharged into the river. This supports our argument that antibiotic and heavy metal resistance genes are transferred from hospital effluents and community wastewater to Benin's streams. Al Salah et al. (2021) have shown in their studies that the co-occurrence of heavy metals, antibiotic resistant bacteria (ARB), and ARGs in hospital effluent spreading in riverine receiving systems and the assessment of the associated risks are topics of scientific interest and are still little studied in developing countries

under tropical conditions.

All these results show the involvement of hospital effluents in the contamination of rivers. It is therefore important that other studies showing the flow of this dissemination should be carried out to identify the treatment and purification sites of hospital effluents before their discharge into streams. This will contribute to the conservation of water resources and help prevent the spread of antimicrobials through a One Health approach.

Conclusion

Antibiotic and heavy metal resistance genes are environmental pollutants that contribute significantly to the emergence of multidrug resistance. In the present study, the presence of multidrug-resistant bacteria in hospital effluents was linked to the main streams of Benin. Similar antibiotic resistance genes were found in hospital effluents and streams. These results indicate that hospital effluents are a potential source of dissemination of these hazardous contaminants into water sources. However, it is urgent that these results be used as a basis for monitoring both hospital effluents and streams and for setting up treatment and purification systems for these waters. DNA sequencing to characterize resistance genes and phylogenetic analysis will help to understand and track the flow of antibiotics and metal resistance genes between hospital effluents and streams. However, in the present study, we were not able to carry out these techniques due to the unavailability of the necessary equipment in Benin.

CONFLICT OF INTERESTS

The authors have not declared any conflicts of interests.

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