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Research Article

ANTIMICROBIAL SUSCEPTIBILITY OF SOIL BACTERIA ISOLATED FROM RUSTING METAL DUMP SITE, MUNICIPAL WASTE DUMP SITE AND MECHANIC/ENGINE OIL CONTAMINATED SOIL SAMPLES IN PORT HARCOURT

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A B S T R A C T

High rate of both adult and child mortality from waste related diseases have increased tremendously over the past five decades. Soil samples were taken from three different waste dump sites in Port Harcourt and analysed for the presence of viable bacteria as follows: rustingmetal contaminated soil, municipal waste dump soil and mechanic workshop/engine oil contaminated soil respectively. The pH, temperature and colour of samples were recorded. Bacterial count and antimicrobial susceptibility tests for isolated organisms were carried out using standard microbiological procedures. The bacteria isolated from waste dumping sites included *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Bacillus subtilis, Enterobacter amnigenus, Enterobacter gerognes and Salmonella bongery.* Bacterial count of microorganisms from the dumpsite ranged between 4.24 x 10^4 and 5.62 x 10^4 cfu/g. Soils from the dump sites had a temperature range of 26°C to 27°C and pH range of 6.8 –7.4 respectively. Silver nanoparticles synthesized in the laboratory had the most effective antimicrobial activity against the isolates followed by Erythromycin, Gentamicin and the Cephalosporins. Isolates showed high resistance to Augmentin® and Cloxacillin. Ahigh prevalence of enterobacteria was observed probably due to the dumping of faecal matter contaminated waste at the municipal waste dump.

INTRODUCTION

The Niger Delta oil rich region of Nigeria has Port Harcourt as the capital city and houses over thirty multinational oil companies [1]. On-shore and offshore exploration of oil by these companies has undoubtedly increased environmental hazards over the last five decades with improper management practices and protocols grossly responsible for untold pollution of aquatic life [2]. Uncontrolled pollution goes with increase in water – borne diseases such as cholera, diarrhoea, typhoid fever and dysentery [3-5]. Waste dumps house decaying organic and inorganic material which encourages the proliferation of harmful and disease causing rodents and reptiles. The harmfulrodents can invariably cause Lassa fever in man. Solid waste is defined by theUnited States Environmental Protection Agency (USEPA-2006) as non – air and sewage emission created within and disposed of by a municipality, including household garbage, commercial refuse, industrial and demolition debris, dead animals and abandoned iron (vehicles) [6].

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Soil bacteria associated with waste dumps have been extensively studied and Lactobacillus, Bacillus, Streptococci, Staphylococci, Escherichia and Pseudomonas species are well documented [7-10]. Some of these soil bacteria are known to be pathogenic in nature and some could be virulent. The rusting metal dumps are mostly known to house the class of highly virulent species of bacteria including Bacillus and Clostridium species [11]. In the Niger Delta region of Nigeria the practice of waste disposal, control and management clearly falls short of the ideal with very obvious dire consequences such as outbreak of serious epidemics. There is a nonchalant attitude not only by companies operating in the area, but also by the general populace towards waste disposal. Lack of proper legislation and/or non-implementation of existing legislation on waste management and control has resulted in an ever increasing level of pollution of the oil rich region [12-14]. The current investigation, therefore, aimed to determine the susceptibility to antibiotics and silver nanoparticles of bacteria isolated from waste dump sites in Port Harcourt, Nigeria.

METHODS

Collection of samples

Soil samples were collected aseptically from different locations in Port Harcourt metropolis. These include a rusted metal dump site at the mile 3 area of the city, municipal waste dump site and engine-oil contaminated soil at Alakahia area of Port Harcourt. The age of dumping at these sites was above 5 years

Microbial Analysis of Soil Samples

Ten grams (10g) of each soil sample was thoroughly shaken in 10 ml of distilled water. A 1.0 ml aliquot was used to prepare a ten-fold serial dilution to yield different inoculum concentrations such as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} up to 10^{-5} dilution. From the 10^{-4} dilution of each soil sample was taken a 0.1ml volume which was aseptically transferred onto freshly prepared nutrient agar plates and spread with a sterile bent glass rod. The inoculated plates were then inverted and incubated at 37^{o}C for 24 to 48 hours. The resultant colonies were counted and the average counts of duplicate cultures were recorded and used to obtain the total viable aerobic heterotrophic bacteria in the sample. The dilutions were also plated out on MacConkey agar, Mannitol salt agar and Cetrimide agar which had been prepared according to manufacturer's instructions using the pour plate method. The inoculated media were incubated at 37^{o}C for 24hours and examined for growth thereafter.

Sterilization of media and glass apparatus

Glass apparatus including bijou bottles were sterilized using the oven at 180^{o}C for three hours. Inoculating wire loop was sterilised by red hot flame. Hockey sticks were sterilized by dipping in absolute ethanol followed by flaming. Other fragile materials were wrapped in aluminium foil and subsequently autoclaved at 121^{o}C for 15 min. All solid media used were sterilized at a temperature of 121^{o}C for 15 min, using the autoclave while liquid media used were sterilized at 121^{o}C for 15 min. Double distilled water were used as diluents where necessary.

Characterization and identification of bacterial isolates

Bacterial isolate identification was based on morphology and biochemical characteristics [15]. Subsequently characterization protocol of Buchanan and Gibbons 1999 were adapted [16]. Further tests were performed on isolates as shown below.

Colonial morphology

The colonial size, shape, colour, elevation and marginal appearance of bacterial species were assessed on nutrient agar plates after predetermined incubation periods.

Gram stain

Freshly cultured (24 hours old) bacterial isolates were smeared on a clean slide and heat fixed followed by staining with crystal violet for 60 seconds. The dye was fixed with Lugol`s iodine for 30 seconds after draining the overflow. Tap water was carefully run over the slides, decolourized with absolute ethanol and subsequently washed with tap water. Safranin was used to counter stain the slides for 30 seconds which were then rinsed, air dried and examined under oil immersion at 1000 x magnification lens for Gram reaction and morphological identification.

Triple sugar iron agar test (TSI)

Acid and sometimes acid and gas (Carbon dioxide or hydrogen) are products of fermentation of sugars. The ability of an organism to ferment several sugars can be shown by incorporating the sugars into basal medium (Peptone water) and subsequently testing for acid and gas production. A sterile wire loop was used to inoculate the TSI agar plate and acid production was investigated using phenol red indicator as presence of gases were indicated by the presence of bubbles on the medium.

Urease test

This test is carried out using a differential medium that tests the ability of an organism to produce an exo enzyme, called Urease. The enzyme hydrolyses urea to ammonia and carbon dioxide. The procedure involves streaking the surface of the Urea agar slant with a portion of an isolated colony and incubating at 35^{o}C for 24 hours. Development of an intense magenta to bright pink colour in 15 minutes to 24 hours is a positive indication.

Citrate Utilization

Nutrient substrate that has ammonium salts as the only source of nitrogen with citrate being the only source of carbon is the Simmons citrate medium. The citrate agar slant was streaked with light inoculums of the isolates and incubated at 35-37^{o}C for 48 hours. An intense Prussian blue colouration on the slant surface gives a positive indication.

Coagulase test

Coagulase is an enzyme that clots blood plasma and also a virulence factor of *S. aureus*. The formation of clot around an infection caused by these bacteria likely protects it from phagocytosis. This is used to distinguish *Staphylococcus aureus* from coagulase negative Staphylococci. A 1 in 10 dilution of plasma was collected and 0.5 ml was placed into 2 test tubes, 0.5 ml of a 24 hour culture was added into one of the test tubes and both tubes were incubated at 37^{o}C. On examination after 24 hours, the clumping of cells was an indication of a positive result.

Indole Production

The amino acid tryptophan is capable of being hydrolysed by some microbes to give indoles. The indole formed could react with 4-dimethyl amino benzaldehyde to form dark red dye stuff. The isolates were grown in tryptone broth for 2 days at 35^{o}C, 2 ml chloroform was added to the culture medium and mixed.Kovac's reagent (2 ml) was then added to the mixture which was shaken and allowed to stand for 20 minutes. A frank – red colour on the reagent indicates the presence of indole.

Oxidase test

The presence of cytochrome oxidase in micro-organism is detected using the oxidase test. To an overnight broth culture of isolates were addedbacterial oxidase test strips. The strips were examined for colour change after being withdrawn and left for 15 minutes. A colour change from yellow to dark purple indicates the presence of oxidase. The strips were impregnated with 1 % tetramethyl – p – phenyldiamine solution.

Antibiotic assay

Molten Mueller Hinton agar was prepared by weighing 38 g into 100 ml of double-distilled water and subsequently sterilized at 121^{o}C for 20 minutes in an autoclave. The above preparation was allowed to cool and then poured into sterile petri dishes. Plates were left to set as the surface was dried in an oven at 45^{o}C. Mueller Hinton agar plates were subsequently seeded with viable test strains of about 24 hours old at a concentration of 10^5 cells/ml and 0.1 ml aliquot test organism suspension was placed onto the agar plates and spread aseptically with the aid of hockey stick. The plates were allowed to dry for 1 hour at ambient temperature. Antibiotic Multi – disks containing Cloxacillin, Ceftriaxone, Ceftazidime, Cefuroxime, Gentamicin, Erythromycin, Ofloxacin, Augmentin and Silver nanoparticle (0.05 mg/ml) were placed onto the inoculated plates using disc diffusion methods. The plates were incubated at 35^{o}Cfor 2 hours. After incubation period, the culture plates were examined forzones of inhibition or areas of no growth. Bacterial strains resistant to the antimicrobial agents did not show any clear zone of inhibition

RESULTS

Table 1 Physical Characteristics of Soil Samples

S/N.	Sample	Colour	Temperature (^{o}C)	pH
1	Rusted Metal Dump Site (Soil)	Reddish Brown	27	6.8
2	Mechanic Workshop/Engine-oil Contaminated Soil	Black	26	7.4
3	Municipal Waste Dump Site (Soil)	Brown	26	6.7

Table 2 Total Viable Cell Count of Mesophilic Isolates from Waste Dumps on MacConkey Agar

S/N.	Waste Dump Sample	Dilution 10^0	Dilution 10^2	Dilution 10^4
1	Rusted Metal Dump Site (Soil)	Numerous	$4.24x10^4$	$5.68x10^4$
2	Mechanic Workshop/Engine-oil Contaminated Soil	Numerous	$1.60x10^4$	$2.4x10^4$
3	Municipal Waste Dump Site (Soil)	Numerous	$5.62x10^4$	$3.0x10^4$

Table 3 Total Viable Cell Count of Mesophilic Isolates from Waste Dumps on Nutrient Agar

S/N.	Waste Dump Sample	Dilution 10^0	Dilution 10^2	Dilution 10^4
1	Rusted Metal Dump Site (Soil)	Numerous	$5.82x10^4$	$4.02x10^4$
2	Mechanic Workshop/Engine-oil Contaminated Soil	Numerous	$1.84x10^4$	$1.90x10^4$
3	Municipal Waste Dump Site (Soil)	Numerous	$5.60x10^4$	$2.52x10^4$

Table 4 Total Viable Cell Count of Mesophilic Isolates from Waste Dumps on Mannitol Salt and Cetrimide Agar

S/N.	Waste Dump Sample	Dilution 10^0	Dilution 10^2	Dilution 10^4
1	Rusted Metal Dump Site (Soil)	NIL	NIL	NIL
2	Mechanic Workshop/Engine-oil Contaminated Soil	NIL	NIL	NIL
3	Municipal Waste Dump Site (Soil)	NIL	NIL	NIL

Organoleptic and physical parameters of soil samples from waste dumps showed that soil samples from rusted metal dump, municipal waste dump and mechanic workshop/engine oil contaminated soil appear reddish brown, brown and black in colour respectively (Table 1). These soil samples had a pH of 6.8, 6.7 and 7.4 respectively with a temperature range of 26^{o}C – 27^{o}C. The least bioload ofheterophilic bacteria of 1.60 x 10^4 cfu/g was recorded for mechanic/engine oil contaminated soil sample while rusted metal dump site and municipal waste dump soil sample gave the highest heterophilic bacterial count of 4.24 x 10^4 and 5.62 x 10^4 cfu/g respectively. Observation showed that manitol salt agar and Cetrimide agar did not support any bacterial growth. Total *Staphylococcus aureus, Klebsiella pneumonia and Bacillus subtilis* count isolated using nutrient agar medium were 5.82 x 10^4, 8.0 x 10^6 and 4.5 x10^6 cfu/g respectively. Possible identitiesof the microbial isolates were justifiedusing morphological properties of colonial growth on solid media and Gram reaction of the waste dump soil isolates as shown in tables 5 – 7 respectively. Investigation showed that bacteria associated with the rusted metal dump waste soil sample were *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia* and *Bacillus subtilis* (Table 5). From municipal waste dump soil sample, seven (7) isolates namely *Escherichia coli, Staphylococcus aureus, Klebsiella pneumonia, Bacillus subtilis , Enterobacteria aminigenus, Enterobacter aerogenes and Salmonella bongory* were obtained (Table 6). A total of 4 isolates from rusted metal dump site was recorded, out of which 100% were susceptible to Erythromycin and Silver nanoparticles, 50% were susceptible to Ceftazidine, Ceftriazone and Ofloxacin, 25% were susceptible to Cloxacillin and Cefalexin while 75% were susceptible to gentamicin and none showed susceptibility to Augmentin (Table 8).

DISCUSSION

Physico-chemical analysis showed that the soil sample from rusted metal dump and municipal waste dump site was acidic in nature while the samples from mechanic workshop/engine oil contaminated area were within the alkaline pH. It was not surprising that rusted metal dump housed heterotrophic bacterial isolates such as virulent *Bacillus subtilis* [17], *Staphylococcus aureus and Klebsiella pneumonia* though organisms like clostridium species were not isolated despite the fact that the above is a possible habitat for spore forming Clostridium species.The enteric bacterialload in municipal waste dump site was higher than those of rusted metal dump and engine oil contaminated waste dump soil. Probable explanation here could be the uncontrolled dumping of decaying house-hold food and organic wastes as well as faecal matter in the municipal dump site which are sources of pathogenic bacteria such as *E.coli* and Salmonella species. The interaction between scavenging vectors which feed on dump site and house hold effects such as foods, water and fruits can also spread disease causing germs. High level of resistance was observed to Augmentin®, Cloxacillin and some Cephalosporins studied. The reason for this resistance could be the indiscriminate use and abuseof antimicrobial agents in both human and veterinary practices. From this investigation isolates from mechanic workshop/engine oil contaminated soil and rusting metal dump soil showed the highest level of drug resistance because they are mostly spore formers which have been reported to possess resistance plasmids.

Table 5 Colonial Morphology and Biochemical Characterizationof Bacterial Isolates from Rusted Metal Dump site

CoM	CM	GR	IR	CR	OR	CoR	UR	TSIR	M	Probable Identity
Shiny circular colonies with entire margins and are slightly raised	Rods	-	+	-	-	-	-	-	M	*Escherichia coli*
Circular, pinhead colonies, convex with entire margins (Golden brown colonies)	Cocci in clusters	+	-	-	-	+	-	-	N	*Staphylococcus aureus*
Creamy, mucoid, circular and convex	Rods	-	-	+	-	-	+	+	N	*Klebsiella pneumonia*
Spore forming dry colonies, flat irregular with lobate margins	Rods	+	-	+	V	-	-	+	M	*Bacillus subtilis*

KEY: CoM- Colonial Morphology; CM- Cell Morphology; GR - Gram Reaction; IR - Indole Reaction; CR- Citrate Reaction; OR – Oxidase Reaction; CoR - Coagulase Reaction; UR - Urease Reaction; TSIR- Triple Sugar Iron Reaction; M - Motility Test; + (Positive) ;– (Negative); V – Variable ; N- Non motile; M - Motile

Table 6 Colonial Morphology and Biochemical characterization of Bacterial Isolates from Municipal Waste Dump (Soil)

CoM	CM	GR	IR	CR	OR	CoR	UR	TSIR	M	Probable Identity
Pink, large, smooth, round and raised elevation	Rods	-	-	+	-	-	-	+	M	*Enterobacter amnigenus*
Pink, medium, smooth, round and raised elevation	Rods	-	-	+	-	-	+	+	M	*Enterobacter gerognes*
Black, small, smooth, round and convex	Rods	-	-	+	-	-	-	+	M	*Salmonella bongery*
Shiny circular colonies with entire margins and are slightly raised	Rods	-	+	-	-	-	-	-	M	*Escherichia coli*
Circular, pinhead colonies, convex with entire margins (Golden brown colonies)	Cocci in clusters	+	-	-	-	+	-	-	N	*Staphylococcus aureus*
Creamy, mucoid, circular and convex	Rods	-	-	+	-	-	+	+	N	*Klebsiella pneumonia*
Spore forming dry colonies, flat irregular with lobate margins	Rods	+	-	+	V	-	-	+	M	*Bacillus subtilis*

KEY: CoM- Colonial Morphology; CM- Cell Morphology; GR - Gram Reaction; IR - Indole Reaction
CR- Citrate Reaction; OR – Oxidase Reaction; CoR - Coagulase Reaction; UR - Urease Reaction
TSIR- Triple Sugar Iron Reaction; M - Motility Test; + (Positive) ; – (Negative); V – Variable N- Non motile; M – Motile

Table 7 Colonial Morphology and Biochemical Characterization of Bacterial Isolates from Engine Oil/Mechanic Workshop Contaminated (Soil)

CoM	CM	GR	IR	CR	OR	CoR	UR	TSIR	M	Probable Identity
Circular pink head colonies, convex with entire margins golden-brown colonies	Cocci in clusters	+	-	-	-	+	-	-	N	*Staphylococcus aureus*
Creamy, mucoid circular and convex	Rods	-	-	+	-	-	+	+	N	*Klebsiella pneumonia*
Spore forming dry colonies, flat, irregular with lobate margins	Rods	+	-	+	V	-	-	-	M	*Bacillus subtilis*

Table 8 Antibiotic Susceptibility Test shown byInhibition zone diameter of Bacterial Isolates from rusted metal dump (Soil) site

Probable Identity	AU	CLX	CEFT	CFT	CEFX	GN	ERY	OFX	AgNPs
Escherichia coli	R	R	24	22	R	25	18	25	21
Staphylococcus aureus	R	17	R	R	R	19	14	R	20
Klebsiella pneumonia	R	R	22	28	23	18	16	25	20
Bacillus subtilis	R	R	R	R	R	R	14	R	18

IZD ≤12 (mm) ≡ Resistance
> 12 (mm) ≡ Sensitive
AU - Augmentin; CLX - Cloxacilin; CEFT - Ceftazidine; CFT - Ceftriazone; CEFX - Cefixim;
GN - Gentamicine; ERY - Erythromicin; OFX - Ofloxacin ;AgNPs - Silver nanoparticles.

Table 9 Antibiotic Susceptibility Test shown by Inhibition Zone Diameter for Bacterial Isolates from Municipal Waste dump (Soil)

Probable Identity	AU	CLx	CEFT	CFT	CEFX	GN	ERY	OFX	AgNPs
Enterobacter amnigenus	14	R	20	18	16	R	17	18	24
Enterobacter gerogenes	20	R	18	20	14	17	16	20	25
Salmonella bongony	22	14	R	16	18	18	R	18	20
Escherichia coli	R	R	20	18	R	20	18	21	24
Staphylococcus aureus	R	18	R	R	R	22	16	R	26
Klebsiella pneumonia	R	R	20	24	20	18	14	20	22
Bacillus subtilis	R	R	R	R	R	14	R	16	25

Table 10 Antibiotic Susceptibility test shown by Inhibition Zone Diameter for Bacterial Isolates from Mechanic Workshop/ Engine Oil Contaminated (Soil)

Probable Identity	AU	CLX	CEFT	CFT	CEFX	GN	ERY	OFX	AgNPs
Staphylococcus aureus	R	20	R	R	R	20	16	R	22
Klebsiella pneumonia	R	R	22	25	22	18	14	20	25
Bacillus subtilis	R	R	R	R	R	14	18	R	24

CONCLUSION

Bacterial isolates from rusting metal dump and mechanic workshop/engine oil contaminated soil are related and belong to the class of sporulating bacteria which are less susceptible to antimicrobial agents.

The isolates from municipal waste dump are mainly members of the enterobacteriaceae which are very susceptible to the antimicrobial agents under study. Since these organisms canbe spread to the general population by vectors and human scavengers and pose threats to health, the need for improved

waste management and control measures cannot be overemphasized.

Conflict of interest

The authors declare no conflict of interest.

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