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Isolation, Characterization, Antibacterial Resistance and Plasmid Profile of Microorganism in Palm-oil Factory and Hospital Soils

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ABSTRACT

This research work targeted the isolation of soil bacteria with a view of establishing the antibiotic resistance status and plasmid profile. To investigate the effect of soil contamination as a factor in the horizontal transfer of resistant genes in the soil microbiome. Microorganisms were isolated from soils sampled from palm oil factory, hospital and uncontaminated soil from Achievers University using Nutrient agar and blood agar. Plate count was performed on the plates using the colony counter. Anaerobic organisms were isolated using the anaerobic incubator. The isolates were characterized and identified using structural, biochemical and morphological features. The isolates were identified to specie level with the aid of Bergey's Manual of Systematic Bacteriology (BMSB, 1994). Pure cultures were tested for susceptibility tests to standard antibiotics using the Kirby Bauer disk diffusion method. The multiple antibiotic resistance index was determine using the method of Krumperman, (1983). Bacteria with MARi above 0. 2 were considered to be highly resistant and such organisms were subjected to Plasmid profiling. Results showed that five organisms were isolated from both soil samples which are: Bacillus subtilis, Acetinobacter, Klebsiella, Pseudomonas Spp, Flavobaterium. Only two organisms; Flavibacterium and Klebsiella isolated from Palm oil and Hospital soil respectively had plasmid borne resistance. The result of this research shows that the soil isolates from palm oil factory had a visible plasmid presupposing the fact that antibacterial resistance factor can be transmitted to other bacteria while the other isolates have chromosomal borne resistance factor.

Keywords: Palm oil factory soil, Hospital soil, antibiotic discs, resistance, plasmid, chromosome.

1.0 Introduction

It is increasingly being recognized that the transfer of conjugative plasmids across species boundaries plays a vital role in the adaptability of bacterial populations in soil. There are specific driving forces and constraints of plasmid transfer within bacterial communities in soils. Microbiota of the soil is greatly important for life on our planet, including its role in the cycling of carbon, nitrogen and other nutrients (Jansson and Hofmockel, 2018).

Palm oil mill effluent (POME) is one of the major wastes from the palm oil industry and it has the most

problematic environmental pollution potential among the palm oil mill wastes. It is the residual liquid waste product obtained after extraction of oil from the fruits of the oil palm (Orji *et al.,* 2006). Hospital wastes" refers to all waste, biological or non-biological from hospitals, that is discarded and not intended for further use (USEPA, 1989)

Antimicrobial resistance is one of the biggest problems in human and animal medicine at present. Since a high percentage of antibiotics are discharged from the human or animal body without degradation, this means that different habitats, from the human body to river water or soils, are polluted with antibiotics (Martínez, 2017). There is limited knowledge of antimicrobial concentrations that might exert selection for resistant bacteria in the environment (Bengtsson-Palme and Larsson, 2016).

The concentrations of antibiotics in soils usually are low in most ecosystems, but even low concentrations may trigger specific bacterial responses, and analysis of such responses is a topic of interest (Martínez, 2017). Even though the usage of antibiotics is considered one of the most important risk for the development of antimicrobial resistance, the emergence of the resistance in clinical environment can also be based on the theory about a pre-existing pool of antibiotic resistance genes in natural environmental reservoirs and a transferability of these genes (Nesme and Simonet, 2015).

There is the possibility that the soil structure and content could contribute to the horizontal spread of bacterial resistance to antimicrobial and it is against this backdrop that this research work intends to investigate the antimicrobial resistance in bacteria isolated from soil exposed to different contaminants especially hospital and palm oil contaminants with an intent of identifying the effects of soil content on antibiotic resistance factors in bacteria.

2.0 Materials and Methods

2.1 Sampling

Soil samples were obtained from two different locations in Owo metropolis; Federal medical center and Palm oil factory. Owo is located in Ondo state of Nigeria. It is reported to have a geographical coordinate of 7.1989° North, 5.5932° East. Owo is part of the Yoruba tribe of Ondo state in Nigeria. Samples for analysis were collected in sterile containers and in the process, special care was taken to avoid contamination of samples. For each sample of soil collected the ground was dug with a trowel and the soil samples were collected with a foil paper and transported to the laboratory for microbiological analysis.

2.2 Physico- Chemical Analysis of Soil Sample

The soil samples were analyzed for pH and moisture content

2.2.1 pH Determination

Five (5) grams of soil sample from the various site were dissolved in 40 mL of distilled water thoroughly shaken to ensure homogeneity of the mixture. The electrode was then dipped inside the mixture to determine the pH, the determination was repeated for five times to get the actual pH value of the various soil sample as the obtained values were recorded.

2.2.2 Determination of Moisture Content

A portion of sample (5gram) was weighed into previously weighed crucible and transferred to the oven at 105˚C for 1hrs; the sample was allowed to cool in a desiccator.

Subsequent weighing was done for 30 minutes interval for three (3) consecutive reading until a constant weight was obtained. The percentage moisture content was then calculated with the following formula:

$$\% \, Moisture = \frac{W2 - W1 \;\; X \;\; 100}{W - W0}$$

Where:

W_0 =Weight of empty crucible

W_1 = Weight of crucible + Weight of sample

W_2 = Weight of crucible + Weight of dried sample

2.3. Isolation and Characterization

2.3.1. Serial Dilution

A 1 ml aliquot from the stock samples was transferred into 9 ml of prepared sterile distilled water in the first tube with sterile pipette and mixed thoroughly. From the first dilution, 1ml from the sample water was transferred aseptically into 9 mL of prepared sterile distilled water in the first labelled test tube with sterile pipette and mixed thoroughly. 1ml was then transferred from the first tube to the second tube and repeated until the third test tube was inoculated. The number of dilution factors was chosen depending on how heavy the microbial load is likely to be based on the physiochemical analysis such as the turbidity and the total dissolved solid (Cheesebrough, 2000). Both Nutrient agar and blood agar were used in the isolation procedure

2.3.2. Total Plate Count

An aliquot of 0.1 mL from the suitable dilution (10-5 and 10-6 dilutions) was inoculated on different agar using pour plate method and incubated at 37°C for 24 hours. Colonies were observed and counted using the colony counter.

2.3.3. Determination of Bacterial Load

Total Plate Count of Bacteria in each sample was determine using a colony counter, and calculated as colony forming units per ml (cfu/ml) with the formula:

$$Cfu/ml = \frac{No\ of\ colonies \quad X \quad dilution\ factor}{Volume\ of\ inocula}$$

2.4. Characterization and Identification of Isolates

2.4.1. Colonial Identification of Isolates

Bacterial isolates were examined for size, opacity, shape, edge, surface appearance and the elevation of the colonies.

2.4.2. Microscopic Identification of Isolates

Isolates were subjected to Gram staining and the stained colonies were view under the microscope to establish the Gram status.

2.4.3. Morphological and Biochemical Identification of Isolates

The isolates were subjected to biochemical test such as oxidase (Win *et al.,* 2006), urease (Bailey and Scott, 1974), Catalase (Facklam and Elliott, 1995), Indole (Macfaddin, 2000), citrate utilization (Jawetz *et al*., 1989), motility (Aygan and Arikan, 2007), coagulase (Holt *et al*., 1994). and sugar fermentation using standard methods described by Barrow and Feltham (1999).

2.5. Antibiotic Susceptibility

Antibiotic susceptibility were determined by the agar diffusion technique on Mueller-Hinton agar (Kirby-Bauer *et al.,* 1996; NCCLS modified disc diffusion technique 2008) using 8 antibiotic discs (Biotec Lab. United Kingdom) corresponding to the drugs most commonly used in the treatment of human and animal infections caused by bacteria; OFX=Tarivid,PEF=Reflacine,CPX=Ciproflox,AUG=Augmentin,GEN=Gentamycin,STREPT=Streptomycin, CEP=Ceporex, NA=Nalidixic Acid, SXT=Septrin, AMP=Amplicin .

2.6. Multi Drug Resistance Index (MAR) or Isolates

The MAR index for the isolates was determined according to the procedure described by Krumperman (1983). The indices were determined by dividing the number of antibiotics to which organisms were resistant to (a) by the number of antibiotics tested (b) Resistant to three (3) or more antibiotics is taken as MAR.

2.7. Genetic Amplification of Isolates to Determine the Presence of Plasmids.

Sterile Luria –Bertani (LB) medium was inoculated with a single bacterial colony and incubated at 35˚C for 24hrs to form a good growth to saturation. Exactly 1.5ml of cells was centrifuge for 1minutes at 8000rpm. Pellets were resuspended in 400µl Glucose/Tris/EDTA(GTE) solution and allowed to stand for 5 minutes at room temperature. Two hundred (200) µl of NaOH/SDS (Sodium hydroxide/Sodium deodecyl solution), was added and mixed well then placed on ice for 5 minutes. One hundred and fifty (150) µl of potassium acetate solution, was added and vortexed briefly and placed on ice for 5 minutes. This was centrifuged at 10,000 rmp for 5 minutes and supernatant transferred to a new tube. Eight hundred (800) µl of 95% ethanol was added and minutes and supernatant decanted. The ethanol was air- dried and pellet re- suspend in 50µl of Tris/EDTA (TE) buffer (Roderick *et al.,* 1992).

3.0. Results and Discussion

3.1. Physicochemical analysis result of soil samples.

The result of the physicochemical analysis carried out on the soil samples are presented in table 1. The pH of soil sampled from the palm oil factory was 6.74 while that of the hospital environment was 5.92

Table 1: Physiochemical Analysis of Soil Samples.

Samples	pH	Moisture content
Hospital soil	5.92	85.26g
Palm oil factory soil	6.74	84.20g

3.2. Isolation and Identification of Soil Organisms.

Four organisms were isolated from the hospital soil sample while only one organisms were isolated from the palm oil factory soil. The Gram reaction of the isolates showed that only Klebsiella was Gram positive. The other isolates were Gram negative. They were all catalase positive, citrate positive while some of them could ferment some of the sugars tested. So the characterization and biochemical test carried out indicate the organisms to be Klebsiella, Pseudomonas spp, Bacillus spp and Acinetobacter. Only Flavobacterium was isolated from palm oil factory soil (Table 2).

3.3. Antibiotic Susceptibility of Isolates

The antibiotic susceptibility test of the isolated showed that the bacteria were resistant to most of the antibiotics used (Table 3). Bacillus was susceptible to Gentamycin and ciprofloxacin with zones of inhibition of 25 mm each but was resistant to all other antibiotics used therefor having a percentage resistance of 80% resistance. Klebsiella was sensitive to gentamycin and Ceporex with a zone of inhibition of 23mm and 21 mm respectively but had intermediate resistance to perfloxacin (70%). Pseudomonas was susceptible to augmemntin, (25 mm) and gemtamycin (21 mm) (80%) but resistant to all other antibiotics used in this study. Acinetobacter was susceptible to Reflacin (25 mm) and gentamycin (25 mm) but resistant to other antibiotics used (80%). Flavobacterium was resistant to all the antibiotics used in this work except Ampicillin with a zone of inhibition of 21 mm (90%).

3.4. Multiple Antibiotics Resistance Index (MATi)

The multiple antibiotic resistance index of all isolate was calculated and it was observed that all

Isolate	Gram status	shape	SUGAR FERMENTATION						Biochemical test							Motiliy	Suspected organism
			Lactose	glucose	fructose	galactose	maltose	sucrose	Oxidase	coagulas	catalase	Indole	urease	VP	Citrate		
Ah1	+	Rod	+	+			+	+	-		+	-	+	+	+	-	Klebsiella spp
Ah2	-	Short rod	-	-	-	-	-	-	+	-	+	-	-	-	+	+	Pseudomonas
Ah3	-	Rod	+	+	+	-	+	+			+	+	-	+	+	+	Bacilus
Ah4	-	cocci	+	+		+	+	-	-	-	+			-	+	-	Acinetobacter
PO1	-	Rod	+	+	+	-	+	+	+		+	+	-	-	+	-	Flavibacterium

the isolates had MARi greater than 0.3 which implies that the isolates have high MARi. Bacillus had MARi of 0.6. Klebsiella had MARi of 0.9. Pseudomonas, Acinetobacter and flavobacterium had MARi of 0.4, 0.9 respectively (Table 4).

3.5. DNA Amplification for the Identification of Plasmids

The isolates were subjected to DNA amplification to identify plasmids bearing the resistance factors. The result of the amplification showed that isolate 5 (Falvobacterium) had only one high molecular weight plasmid with less than 10 bps similarly bacillus has one plasmid with less than 10kbp (Fig 1).

Table 2. Identification of Isolates from Soil Samples

Table 3. Antibiotic Resistance of Isolates

Organisms	OFX	PEF	CPX	AUG	GEN	STREP	CEP	NA	SXT	AMP
Bacillus	0R	0R	25S	0R	25S	4R	0R	0R	0R	0R
Klebsiella	0R	12I	0R	0R	23S	0R	21S	0R	0R	0R
Pseudomonas	0R	0R	0R	25S	21S	0R	4R	0R	0R	5R
Acinetobacter	0R	25S	0R	0R	25S	0R	0R	0R	0R	13I
Flavobacterium	0R	4R	0R	0R	0R	0R	0R	0R	0R	21S

Legend; S--- Susceptible

R—Resistant

I----Intermediate

KEY:
OFX=Tarivid,PEF=Reflacine,CPX=Ciproflox,AUG=Augmentin,GEN=Gentamycin,STREPT=Streptomycin, CEP=Ceporex, NA=Nalidixic Acid, SXT=Septrin, AMP=Amplicin .

Table 4. Multiple Antibiotic Resistance Index (MARi) of Isolates

	No of Antibiotics		
Bacterial isolate	Resistant (a)	Tested (b)	MAR indices (a/b)
Bacillus	6	10	0.6
Klebsiella	7	10	0.9
Pseudomonas	4	10	0.4
Acinetobacter	7	10	0.9
Flavobacterium	9	10	0.9

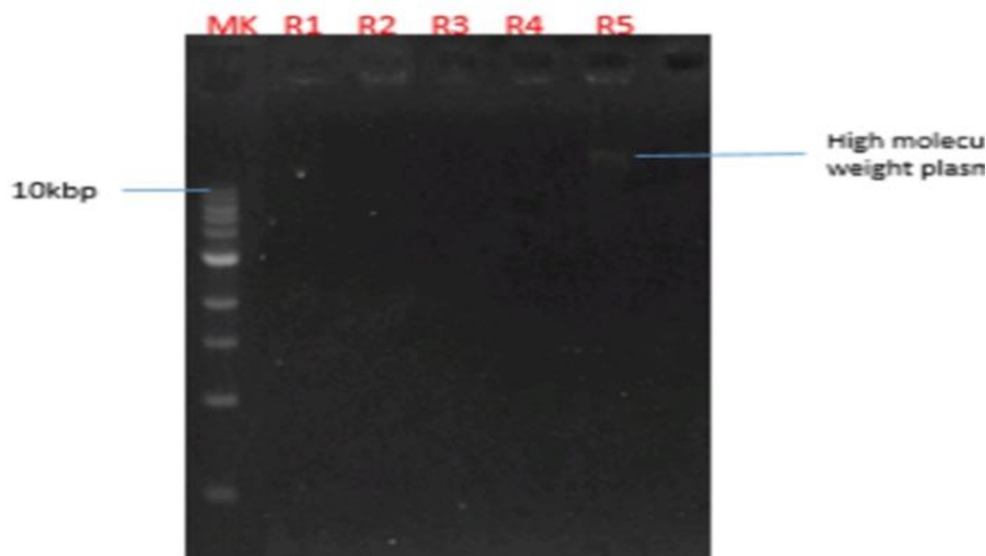

Figure 1: Agarose gel electrophoresis of plasmid DNA from MAR bacteria isolated from soil samples

4.0. Discussion.

Soil pH has been showed to be a primary determinant of microbial community composition and diversity (Chu *et al.,* 2010). The optimum pH for microbial growth is neutral (pH 6-8) (Maier and Pepper, 2009). The difference in the pH values of the two soil samples indicate the difference in the geochemical properties of the two soil samples. The pH of the palm oils soil is within the range of the optimum pH for microbial growth while the hospital soil is more acidic. This could be as a result of the types of waste disposed at the hospital environment. Caltivelli, (1990) listed disposed hospital waste to include biological and non-biological wastes such as toxic chemicals, cytotoxic

drugs, flammable and radioactive wastes. These materials could affect the pH of the soil and make it more acidic. Whereas the palm oil contaminated soil has a pH within the optimum range from microbial growth. The composition of the pail oil soil which are fatty acids and supports the growth of bacteria, unfortunately, the nutritional content of the POME soil does not encourage the growth of many aerobic bacteria.

The scanty microbial load in the oil palm soil is not surprising, Orji *et al.* (2006) in their work showed that soils where palm oil mill effluents were freshly discharged had very scanty microbial population and diversity. This could be because soil organisms die off or move away from the contaminated environment. Hospital environment on the other hand is beehive of activity with abundant biological (such as blood and blood products, animal carcasses and pharmaceutical wastes) and non-biological wastes discarded after use. The type and diversity of organisms found in the hospital soil sample agrees with the work of Caltivelli, (1990) and Ekhaise and Omavwoya (2008) who revealed the presence of Pseudomonas aeruginosa, Klebsiella and other organisms.

The pervasive distribution of antibiotic determinants in bacteria is responsible for the failure in the efficacy of antibiotics (Brown and Wright, 2016). Antimicrobial resistance is one of the biggest problems in human and animal medicine at present. Since a high percentage of antibiotics are discharged from the human or animal body without degradation, this means that different habitats, from the human body to river water or soils, are polluted with antibiotics (Martínez, 2017).

It is essential to know that the hospital is not the only source of antimicrobials present in the environment community sewage (Kümmerer, 2008; Schuster *et al.,* 2008). The concentrations of antibiotics in soils usually are low in most ecosystems, but even low concentrations may trigger specific bacterial responses, and analysis of such responses is a topic of interest (Martínez, 2017). Routine investigations of environmental bacteria are important in providing predictive information on the development of AR in the environment (Zhang, *et al.,* 2018) The high level of resistance recorded in this work is a cause for concern. All the isolates were resistant to most of the antibiotics tested (Table 3). Walsh and Duffy, (2013) discovered that all soil bacteria isolated during his research work had over 80% resistance to all antibiotics he used. WHO (2020) decried the increasing rate of bacterial resistance to antibiotics. These bacteria may infect humans and animals, and the infections they cause are harder to treat than those caused by non-resistant bacteria. The result obtained in this work is consistent with the report of some authors who observed the low resistance to aminoglycosides and quinolones (Olayinka, *et al.,* 2000; Oduyebo, *et al.,* 2008).as observed in the efficacy of gentamycin and Reflacine in table 3. The high level of resistance to cephlosporins (Ceporex and Ciproflox) underscore the emergence beta lactamases. High level resistance to cephalosporin has been widely reported by several authors (Lambert, 2002; Okeke, *et al.,* 2005; Lautenbach *et al.,* 2010)

The multiple antibiotic resistance index is a method of differentiating bacteria from different sources using antibiotics that are commonly used for human therapy. MAR index is a useful marker to ascertain the danger of pollution that could be life-threatening (Kathleen, 2016). The monitoring of both antibiotic consumption and multiple antibiotic resistances (MAR) especially in nosocomial infections is critically necessary to setting up of effective containment programs and audit of such programs (Kamat, 2008). The high MARi of the isolates indicate previous exposure to the antibiotics. In order words, isolates are from high risk sources of antibiotic resistance. The menace of antimicrobial resistance is particularly worrisome in developing countries like ours where there is a high burden of infectious disease with concomitant high rate of poverty which constrains the access to newer, more effective and conversely more expensive antimicrobial agents (Okeke *et al.,* 2005).

The observation that only one isolate from the hospital environment had plasmid borne resistance factor is worrisome. Hospital environment is known

to harbor high level of antimicrobial resistance especially in nosocomial infect. This could be attributed to the sampling site and method of sampling. The other isolates lack plasmid as no visible band was observed. Consequently, the resistance factors may be chromosomal which enabled their persistence in the polluted soil.

Conclusion

Findings in this work have implications for the choice of antibiotics for empiric management of infections, continuous surveillance of antibiotic susceptibility patters and effective hospital infection control. Bacterial strains resistant to most classes of antibiotics will continue to emerge unless inappropriate uses of drugs are curtailed and continuous education of infection control practices maintained. This study also shows that there was a high level of multi-drug resistance in soil bacteria to a wide variety of antibiotics and was not dependent on the soil contaminants.

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