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Isolation and Characterization of Antibiotic-Resistant Bacteria from Pesticide-Contaminated Agricultural Soils in Edo State, Nigeria.

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Abstract
Antibiotic-resistant bacteria from pesticide-contaminated soils in Benin City, Nigeria, were isolated and characterized using standard microbiological methods. The heterotrophic bacterial count ranged from 3.20 x 10^4 ± 0.63 cfu/g to 1.26 x 10^5 ± 0.17 cfu/g. The pesticide-degrading bacterial count ranged from 1.40 x 10^4 ± 0.07 cfu/g to 5.85 x 10^4 ± 0.53 cfu/g. The different bacterial species isolated were: Enterobacter aerogenes, Arthrobacter sp., Bacillus pumilus, Micrococcus roseus, Pseudomonas aeruginosa and Micrococcus loteus. In assessing the antibiotic susceptibility pattern of the isolates before curing, E. aerogenes was resistant to 6 (60 %) and susceptible to 4 (40 %) of the antibiotics. P. aeruginosa was resistant to 9 (90 %) and susceptible to 1 (10 %) of the antibiotics. Arthrobacter sp. was resistant to 9 (90 %) and susceptible to 1 (10 %) of the antibiotics, while B. pumilus was resistant to 7 (70 %) and susceptible to 3 (30 %) of the antibiotics. M. roseus was resistant to 6 (60 %) and susceptible to 4 (40 %) of the antibiotics, while M. loteus was resistant to 8 (80 %) and susceptible to 2 (20 %) of the antibiotics. After curing, E. aerogenes was resistant to 3 (30 %) and susceptible to 7 (70 %) of the antibiotics. P. aeruginosa was resistant to 4 (40 %) and susceptible to 6 (60 %) of the antibiotics. Arthrobacter sp. was resistant to 6 (60 %) and susceptible to 4 (40 %) of the antibiotics, while B. pumilus was resistant to 5 (50 %) and susceptible to 5 (50 %) of the antibiotics. M. roseus was resistant to 3 (30 %) and susceptible to 7 (70 %) of the antibiotics, while M. loteus was resistant to 5 (50 %) and susceptible to 5 (50 %) of the antibiotics. The isolates were resistant to more than one antibiotic, hence they were multi-drug resistant.

Keywords: Bacteria, Antibiotics, Pesticide, Contamination, Plasmids, Agricultural soils

Introduction
Pesticides are used in many situations such as livestock farming, cropping, horticulture, forestry, home gardening, homes, hospitals, kitchens, road-sides, recreational and industrial areas. Substances which are used to control, destroy and repel pests in order to minimize their detrimental effects are pesticides (1). Several tonnes of pesticides are applied annually in modern agriculture to increase food production by controlling harmful effects caused by pest organisms, including insects, microorganisms as well as grasses growing in between economical crops (2). However, less than 5 % of these chemicals are estimated to reach the target organisms (3; 4). Most of the pesticides therefore reach the non-target parts of the agricultural ecosystems. The quality of soils, groundwater, continental and coastal water as well as the air, is therefore compromised by pesticide contamination (5). Modern pesticides are almost all completely new synthetic chemicals, previously unknown in nature. They are designed to be biologically active, and while a remarkable degree of selectivity has been achieved in some materials, as in the case of sensitive herbicides and insecticides, it is not surprising that pesticides may produce undesirable effects particularly if they are used especially at high concentrations (3). Reliance on synthetic chemicals to control pests has given rise to a number of problems, which may affect the food chain and consequently impacting negatively on biological diversity (6). Some of the side effects of these chemicals include the health hazards, undesirable side effects and environmental pollution. Recently, National Agency for Food and Drug Administration and Control (NAFDAC) announced the ban of the use of 30 chemicals in Nigeria in line with the new European Union legislation on pesticide use. It has been established that farmers in Nigeria have poorly adopted much of the technical knowledge on pest management acquired from scientific research.
The major factors responsible for inefficient application of pesticides are financial constraints, poor techniques, inappropriate equipment for applying the pesticides, ill timing of application, inadequate understanding of how to use and lack of concern for the consequences of careless use of pesticides (7). Pesticides accumulate in soil, sediments (8), and water (9; 10), where they are responsible for the contamination and deterioration of soil and

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groundwater, particularly in the close vicinities of agricultural fields (11; 12). In soil, pesticides influence not only the population of various groups of soil microbes but also their biochemical activities like nitrification, ammonification, decomposition of organic matter and nitrogen fixation (13). As a biologically active chemical is applied to ecological systems, it is inevitable that the system will alter in response to the interference (14). However, there are some microorganisms that can survive in pesticide-contaminated soil, since pesticides stimulate the growth of certain microorganisms and exert toxic effects, inhibiting the growth of others.

Previous studies have shown that long term and short term stresses such as high temperature, extremes of pH or chemical pollution often result in altered metabolism, species diversity and plasmid incidence of soil bacterial populations. Some microbial strains possess genetic determinants that confer the resistance (13). In bacteria, these determinants are often found on plasmids, which have facilitated their study at the molecular level. Bacteria isolated from toxic chemical wastes more frequently contain plasmid DNA and demonstrate antimicrobial resistance than do bacterial isolates from domestic sewage-impacted waters or from uncontaminated open ocean sites. A higher incidence of plasmids was found among *Pseudomonas* sp in an industrially polluted river (18 %) than in a non-polluted upstream area (7 %). If the number of plasmids is found to have increased at a given site, an investigation of the responsible stress factor can be initiated (14). Similarly, monitoring of antibiotic-resistant bacteria in soil can be used as an indicator of industrial and urban pollution.

The research was therefore focused on the isolation and characterization of antibiotic-resistant bacteria from pesticide-contaminated soil, as their presence could be used as indicator of pesticide contamination.

Materials and Methods

Sampling Area

Soils samples including sandy, clayey and loamy soils were obtained from Presco PLC, 6.34 $^{\circ}$N and 5.63°E and Okada 6.20°N and 5.38°E, Edo State, Nigeria. Edo state has the lowland rainforest towards the south and the guinea savanna in the north, as well a mixture of soil types (sandy, clayey and loamy soils) with high rainfall in the southern part, where the study was conducted.

Sample collection

Collection of samples was carried out using an auger of 2.5 cm in diameter, at a depth of 0 – 10 cm, and placed in sterile polyethylene bags for microbiological and physico-chemical analyses in the laboratory.

Preparation of culture media

Nutrient agar and mineral salt agar were used for the isolation of bacteria and pesticide-degrading bacteria respectively. All media were prepared according to manufacturer's instruction.

Isolation and Enumeration of Bacteria

Ten grams of each soil type was mixed with 90 ml of sterilized water in a sterile 250 ml beaker. Serial dilution was carried out to obtain different dilutions. One milliliter of the appropriate dilution was plated on nutrient agar using pour plate technique, for the isolation of bacteria. The nutrient agar was amended with nystatin to prevent fungal growth. The plates were incubated for 24 – 48 hrs at room temperature. Enumeration was carried out and results obtained as colony-forming unit per gram (cfu/g). Single isolated colonies of bacteria were picked and streaked on fresh nutrient agar medium and incubated at 37 $^{\circ}$C for 24 hrs. Slants were prepared and stored at 4 $^{\circ}$C.

Isolation and Enumeration of Pesticide-degrading microorganisms

Aliquot of the appropriate dilution was plated in mineral salt agar enriched with 1 % DDT. The modified mineral salt agar was amended with nystatin to prevent the growth of fungi, in order to isolate pesticide-degrading bacteria. Plates were incubated at room temperature (28 ± 2 $^{\circ}$C) for 24 – 48 hrs. After incubation, the number of discrete colonies were counted and recorded in colony forming unit per gram.

Characterization and identification of bacterial isolates

Distinct colonies were picked and identified using morphological and biochemical characterization according to Jolt *et al*[15], Cheesbrough[16], Oyeleke and Manga`[17].

Physico-chemical analysis

Physico-chemical parameters were analyzed according to the methods in APHA[18]. Soil classification was done based on percentage composition of sand, silt and clay, using the United States Department of Agriculture soil textural classification.

Antimicrobial Susceptibility Testing:

Antibiotic susceptibility tests were performed by Bauer-Kirby (19) disc-diffusion technique. The results were expressed as susceptible/resistant according to criteria developed by National Committee for Clinical Laboratory Standards (20) and Manual of Antimicrobial susceptibility testing guidelines (21; 16; 22; 23), The following antibiotic discs were ampiclox (30µg), zinnacef (20µg), amoxicillin (30µg), ciprofloxacin (l0µg), streptomycin (30µg), augmentin (10µg), sparfloxacin (10µg), erythromycin (l0µg), septrin (30µg), chloramphenicol (25ug),

cikatrin (10µg), perfloxacin (l0µg), and ofloxacin (30µg). The antibiotics resistance was interpreted by diameter of inhibition zones around the antibiotic discs. Multidrug resistance was defined as ≥3 of the antimicrobial agents tested (24).

Statistical analysis

Statistical analysis was carried out to determine whether there was significant difference in the results obtained for the various soil types, clayey, sandy and loamy soils.

Results and Discussion

In this research, antibiotic-resistant bacteria from pesticide-contaminated soils in Benin City, Nigeria, were isolated and characterized using standard microbiological methods. The total viable bacterial count of the pesticide-contaminated soil were in the order of 10^3 to 10^5 cfu/g of soil. This ranged from $3.20 \times 10^4 \pm 0.63$ cfu/g to $1.26 \times 10^5 \pm 0.17$ cfu/g (Table 1). The pesticide-degrading bacterial count ranged from $1.40 \times 10^4 \pm 0.07$ cfu/g to $5.85 \times 10^4 \pm 0.53$ cfu/g (Table 2). The results reveal that the bacterial count of uncontaminated soil samples among the three types of soil were higher than the bacterial counts of pesticide-contaminated soils. The microbial count in the loamy soil was comparably higher than clay and sandy soils. The higher microbial count in the loamy soil than the clayey and sandy soils could be due to greater bioavailability of the pesticides in the clay and sand. Six different bacterial species were isolated (Table 3). The isolates were: *E. aerogenes, Arthrobacter* sp., *B. pumilus, M. roseus, P. aeruginosa and M. loteus.* The results of this study were in agreement with earlier reports, Singh *et al*[25] and Lee[26] . Singh *et al*[25] reported that *E. aerogenes* used chloropyrifos pesticide as a source of carbon and phosphorus. Some organophosphorus insecticides such as diazinon, chloropyrifos, ethion, parathion, malathion and gusathion are susceptible to microbial hydrolysis and serve as carbon sources for the growth of pure and mixed cultures of *Flavobacterium* sp., *P. aeruginosa,* and *Arthrobacter* sp. (27). The higher microbial count in the uncontaminated soil compared to pesticide-contaminated soil could be due to the toxic residues of pesticide in the soil which affect the soil microbial activity and reduce the action of bacteria in the soil. Pesticides that stay long in the soil usually affect soil bacteria resulting to chemical degradation of the soil (28). Table 3 also shows the occurrence of the isolates among the different soil types. It was observed that all the isolates were present in the different soil types. This is in agreement with the reports of Suett[29].

Table 1: *Total heterotrophic bacterial count (THBC) (cfu/g)

Samples	**THBC (cfu/g)**	**Control (cfu/g)**
Sandy	$3.20 \times 10^4 \pm 0.63^b$	$1.16 \times 10^5 \pm 0.05^a$
Loamy	$1.26 \times 10^5 \pm 0.17^a$	$2.93 \times 10^5 \pm 0.12^a$
Clay	$4.10 \times 10^4 \pm 0.28^b$	$2.90 \times 10^5 \pm 0.45^b$

*Values are means ± standard error

Means with the same letter are not significantly different ($P \leq 0.05$)

Table 2: *Pesticide-degrading bacterial count (PDBC) in samples (cfu/g)

Samples	**PDBC (cfu/g)**	**Control (cfu/g)**
Sandy	$1.40 \times 10^4 \pm 0.07^b$	0^c
Loamy	$5.85 \times 10^4 \pm 0.53^a$	$1.20 \times 10^4 \pm 0.07^b$
Clayey	$1.85 \times 10^4 \pm 0.17^b$	0^c

*Values are means ± standard error

Means with the same letter are not significantly different ($P \leq 0.05$)

Table 3: Occurrence of Bacterial Isolates in the samples

Samples	**Bacterial Isolates**					
	Enterobacter aerogenes	*Pseudomonas aeruginosa*	*Arthrobacter* sp	*Micrococcus luteus*	*Micrococcus roseus*	*Bacillus pumilus*
Sandy	+	+	+	+	+	+
Loamy	+	+	+	+	+	+
Clayey	+	+	+	+	+	+

Table 4: Antibiotic susceptibility pattern of Gram positive isolates before curing

Gram +ve	**PEF**	**CN**	**APX**	**Z**	**AM**	**R**	**CPX**	**S**	**SXT**	**E**
Arthrobacter sp	R	R	R	R	R	R	S	R	R	R
Micrococcus luteus	R	R	R	R	R	R	S	R	R	S
Micrococcus roseus	S	S	R	R	R	R	S	R	R	S
Bacillus pumilus	R	S	R	R	R	R	S	R	R	S

Key: PEF = Pefloxacin, CN = Cikatrin, APX = Ampiclox, Z = Zinnacef, AM = Amoxicillin, R = Rocephin, CPX = Ciprofloxacin, S = Streptomycin, SXT = Septrin, E = Erythromycin, R = Resistant, S = Susceptible, Gram +ve = Gram positive.

From the study, the degree of susceptibilities shown by the isolates to the antibiotics indicated the efficacy the antibiotics (30; 31). The susceptibility of a chemotherapeutic agent is usually expressed on the basis of the higher zones of inhibition (31; 32). Susceptibility assessment measures the ability of the different antibiotics to inhibit bacterial growth. In assessing the antibiotic susceptibility profiles of the isolates before curing, *E. aerogenes* was resistant to 6 (60 %) and susceptible to 4 (40 %) of the antibiotics. *P. aeruginosa* was resistant to 9 (90 %) and susceptible to 1 (10 %) of the antibiotics. *Arthrobacter* sp. was resistant to 9 (90 %) and susceptible to 1 (10 %) of the antibiotics, while *B. pumilus* was resistant to 7 (70 %) and susceptible to 3 (30 %) of the antibiotics. *M. roseus* was resistant to 6 (60 %) and susceptible to 4 (40 %) of the antibiotics, while *M. loteus* was resistant to 8 (80 %) and susceptible to 2 (20 %) of the antibiotics. After curing, *E. aerogenes* was resistant to 3 (30 %) and susceptible to 7 (70 %) of the antibiotics. *P. aeruginosa* was resistant to 4 (40 %) and susceptible to 6 (60 %) of the antibiotics. *Arthrobacter* sp. was resistant to 6 (60 %) and susceptible to 4 (40 %) of the antibiotics, while *B. pumilus* was resistant to 5 (50 %) and susceptible to 5 (50 %) of the antibiotics. *M. roseus* was resistant to 3 (30 %) and susceptible to 7 (70 %) of the antibiotics, while *M. loteus* was resistant to 5 (50 %) and susceptible to 5 (50 %) of the antibiotics. The isolates were resistant to more than one antibiotic; hence they were multi-drug resistant (33).

The antibiotic susceptibility tests carried out (tables 4 to 7) revealed that the pesticides conferred antimicrobial resistance on the isolates; hence the isolates were more resistant to the antibiotics before curing than after curing. The plasmid curing results in this study also revealed that antibiotic resistant gene in the bacterial isolates was encoded in the plasmid. This result is in agreement with those of Singh[25]. It has been suggested that the development of the resistant population in a contaminated soil can result from gene transfer (30).

Table 5: Antibiotic sensitivity pattern of Gram negative (Gram -ve) isolates before curing

Gram -ve	**SXT**	**CH**	**SP**	**CPX**	**AM**	**AU**	**CN**	**PEF**	**OFX**	**S**
Enterobacter aerogenes	R	R	R	S	R	R	S	S	S	R
Pseudomonas aeruginosa	R	R	R	R	R	R	R	R	S	R

Key: CH = Chloramphenicol, SP = Sparfloxacin, PEF = Pefloxacin, Cikatrin, AM = Amoxicillin, AU = Augmentin, OFX = Ofloxacin, CPX = Ciprofloxacin, S = Streptomycin, SXT = Septrin, R = Resistant, S = Susceptible, Gram -ve = Gram negative.

Table 6: Antibiotic susceptibility pattern of Gram positive isolates after curing

Gram +ve	**PEF**	**CN**	**APX**	**Z**	**AM**	**R**	**CPX**	**S**	**SXT**	**E**
Arthrobacter sp	R	S	R	R	S	R	S	R	R	S
Micrococcus luteus	S	S	R	R	R	S	S	R	R	S
Micrococcus roseus	S	S	R	S	S	S	S	R	R	S
Bacillus pumilus	S	S	R	R	S	R	S	R	R	S

Key: PEF = Pefloxacin, CN = Cikatrin, APX = Ampiclox, Z = Zinnacef, AM = Amoxicillin, R = Rocephin, CPX = Ciprofloxacin, S = Streptomycin, SXT = Septrin, E = Erythromycin, R = Resistant, S = Susceptible, Gram +ve = Gram positive.

Table 7: Antibiotic sensitivity pattern of Gram negative (Gram -ve) isolates after curing

Gram -ve	**SXT**	**CH**	**SP**	**CPX**	**AM**	**AU**	**CN**	**PEF**	**OFX**	**S**
Enterobacter aerogenes	R	R	S	S	S	S	S	S	S	R
Pseudomonas aeruginosa	R	R	R	S	S	S	S	S	S	R

Key: CH = Chloramphenicol, SP = Sparfloxacin, PEF = Pefloxacin, Cikatrin, AM = Amoxicillin, AU = Augmentin, OFX = Ofloxacin, CPX = Ciprofloxacin, S = Streptomycin, SXT = Septrin, R = Resistant, S = Susceptible, Gram -ve = Gram negative.

Table 8: Physico-chemical properties of soil samples

Parameter	**Sand**	**Clay**	**Loam**
pH	6.30	7.80	5.80
Conductivity (µs/cm)	34.70	81.50	72.30
Moisture (%)	15.99	23.17	19.10
Organic carbon (%)	3.82	4.97	6.60
Nitrogen (%)	0.53	0.74	1.04
Phosphate (mg/kg)	6.41	9.93	12.25
Sand (%)	92.50	67.60	71.60
Silt (%)	3.80	8.40	23.80
Clay (%)	3.70	24.00	4.60

Soil physico-chemical properties affect the density of and diversity of microbes in the soil, as well as determining the abundance of indigenous microorganisms. They are essential for microbial functioning and ensures active microbial population in the soil. The results of the physico-chemical analysis are shown in table 8. The lowest pH (5.8) was observed in loam while the highest (7.8) was observed in clay. Here the highest value of organic carbon (6.60 %) and total nitrogen contents (1.04 %) were recorded in loamy soil, whereas the lowest organic carbon (3.82 %) and nitrogen (0.53 %) were recorded in sandy soil. These differences were documented previously by Silver *et al*[34]. This may explain why the highest heterotrophic bacterial count and pesticide-degrading bacterial count were observed in the loamy soil than in the sandy and clayey soils, since the organic carbon and nitrogen content were highest in the loam than in the other soil types. In previous studies, Matus *et al*[35], observed that soil organic carbon tends to be associated with the fine fraction of soils and it was significantly greater three times in clay-rich soils than

in coarser soils. Fine texture soil shows more stable aggregates, which in turn may act as a media of greater amount of carbon and total nitrogen contents (36).

Conclusion: The present study reports the identification and characteriazation of antibiotic-resistant bacteria in pesticide-contaminated soils from agricultural areas. Utilization of xenobiotic compounds by soil microorganisms is a crucial phenomenon by which these compounds are removed from the environment, thus preventing environmental pollution. Results from this study suggest that the isolated bacteria could be used for bioremediation of pesticide-contaminated soil.

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