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Preliminary Investigation on the Resistance of Some Environmental Bacteria in Yola Metropolis, Adamawa State, Nigeria, to Biocides and Antibiotics

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Abstract

Background: Bacteria are capable of developing resistance against the effect of antibacterial agents used in eliminating them from their typical environment. This should be monitored to ensure an economic practice while eliminating or preventing bacteria in an environment.
Objectives: The aim of this study was to isolate and identify environmental bacteria and investigate their ability to resist antibacterial effects of biocides and antibiotics.
Methods: Environmental samples were collected and different bacterial isolates were obtained and characterized. Antimicrobial susceptibility testing was carried out on 6 of the obtained isolates.
Results: Six different bacterial species were isolated and characterized from the environmental samples, including *Staphylococcus aureus* (32%), *Klebsiella* spp. (20%), *Salmonella* spp. (16%), *Proteus* spp. (15%), *Staphylococcus* spp. (11%), and *Serratia* spp. (6%). The most effective biocides were the Tiscol disinfectant and Dettol antiseptics while the most effective antibiotic was Tarivid. However, all the tested isolates showed different levels of resistance to all the antibacterials.
Conclusions: Biocide and antibiotic resistant bacteria species were isolated from the environment and most of them showed some resistance to administered antibacterials; *Staphylococcus aureus* was the most resistant organism to antibiotics while Proteus spp. was the most resistant to the 3 biocides. The 3 biocides had different rates of inhibition, with Tiscol disinfectant and Dettol antiseptics having greater effectiveness against all the isolates.

Keywords: Bacteria, Resistance, Biocides, Antibiotics

1. Background

Our typical environment accommodates wastes from domestic and industrial activities. This leads to changes in quality of water, soil, and air, consequently affecting floral and fauna (1). The types and number of microorganisms in an environment are influenced by environmental characteristics and the substances that are introduced in the environment (1, 2). These substances may either inhibit or stimulate the growth of the microorganisms (2).

Bacteria could degrade dead animals and plants to valuable nutrients in the soil. Some species of these microorganisms could remove harmful pollutants from the environment in a process called bioremediation (3). However, bacteria are also pathogenic (4). To survive, bacteria in the environment respond to a variety of physical and chemical variables. These, among others, include low or high dissolved oxygen concentration, redox potential, pH etc. (5). This behaviour has posed a major challenge with reference to human health and environment as it enhances the appearance and spreading of antibiotic resistant bacteria among other pathogens (6).

Antibiotics are secondary metabolites produced by a variety of microorganisms and are used as antimicrobial chemotherapeutic agents (7). Among all interventions in medicine, antibiotics have been outstandingly potent and life-saving as they have significantly reduced the death rate from all types of bacterial infections (4, 8). In the

United States, antibiotics reduced death rate from bacterial infections by almost 80% within just a few years of their availability (4). Therefore, without effective antibiotics, intensive treatment of bacterial infections will be hindered, which may lead to major failure in health care and medicine (9).

Antimicrobial biocide is the term used for chemical agents (disinfectants and antiseptics) used to kill bacteria, viruses, and moulds. These are used in a wide range of domestic and public utility items, including soaps, cosmetics, and cleaning products. Most specifically, they are used extensively in clinical settings as disinfectants and surgical scrubs to improve hygiene (10). Some examples of antimicrobial biocides, based on the chemical structure, include alcohol, formaldehyde, anilides, biguanides, chlorhexidine, chlorine releasing agents, iodine releasing agents, phenols, quaternary ammonium compounds (QACs), glutaraldehydes, etc. There is growing concern that the widespread use of antimicrobial biocides may select for antibiotic resistance (11).

Antibacterial agents have several routes of entry in the environment, such as sewage from the community or hospitals through manure and water bodies (12). The accumulation and persistence of these antibacterial agents and the organism's survival adaptation selects resistant microorganisms, turning the environment to a gigantic reservoir for antibiotic resistant genes that feed on the constant and increasing environmental pollution (13).

Bacteria initiate mechanisms to resist biocides and these include impermeability of the cell envelope, existence of active efflux pumps, ability to form biofilms, and enzymatic transformation of biocides. Different groups or forms of bacteria however vary in their intrinsic resistance to biocides, with bacterial spores being the most resistant, followed by Mycobacterium spp., then gram negative organisms; with gram positive bacteria generally being the least resistant (14). Typically for antibiotics, the development of mutation or acquisition of resistant determinants or mobile DNA coding i.e. transposon and plasmids, for resistant elements i.e. enzyme, transporter, or modification of target sites, are the mechanisms associated with resistance (8, 14). The objective of this study was to investigate the ability of some environmental bacteria to resist antibacterial effects of some common biocides and antibiotics.

2. Methods

2.1. Study Area and Period

The study was conducted in Yola, Adamawa State, Nigeria. Samples were collected from 5 different locations in the city. The locations included a hostel in the Federal University in Adamawa state, a specialist hospital, which is one of the biggest health centres visited by people from within and nearby regions of the state, surface water from River Benue, a very large river that cuts across the country, and a dump site in Yola, which is located along a federal road. The study was carried out from February to April, 2016.

2.2. Antibiotics and Biocides Used

Three biocides namely, Tiscol antiseptic (dichloroxylenol 1.2% (w/v)), and Tiscol disinfectant (dichloroxylenol 3.6% (w/v)) of TISCO Industries Limited, Akure, Ondo State, Nigeria and Dettol antiseptic (chloroxylenol 4.8% (w/v)) of Reckitt Benckiser Nigeria Limited, Ogun State, Nigeria were used. Antibiotics used against gram positive bacteria were gentamycin (10 μg), pefloxacin (10 μg), ampiclox (30 μg), zinnacep (20 μg), amoxicillin (30 μg), receptrin (25 μg), ciprofloxacin (10 μg), streptomycin (30 μg), septrin (30 μg), and erythromycin (10 μg) while the antibiotics against gram negative bacteria were ofloxacin (10 μg), reflacin (10 μg), ciprofloxacin (10 μg), augmentin (30 μg), gentamycin (10 μg), streptomycin (30 μg), Sefalexin (10 μg), nalidixic acid (30 μg), septrin (30 μg), and ampicillin (30 μg).

2.3. Sample Collection

Samples were collected from 5 areas in Adamawa State, Nigeria; including surface water of River Benue, wastewater around block D of Oba Adetona hostel, MAUTECH, Yola, waste water from the Specialist Hospital, Yola, soil from the waste disposal site of the hospital, and soil from Jimeta bypass dump site, Shinko, Jimeta. Soil samples were collected in clean polyethylene bags while water samples were collected in sterilized universal bottle. Samples were taken immediately to the laboratory for analysis.

2.4. Isolation and Characterization

Each of the samples were diluted serially in different test tubes except for the surface water samples and labelled. Culturing was done using both the streak and pour plate techniques on MacConkey, nutrient and blood agar. For the diluted samples, 10^{-2} dilution was used for the inoculation. Six pure cultures were obtained after several sub culturing and stored in the refrigerator at 4°C. Isolates were characterized using the Gram staining method and some biochemical tests such as coagulase, catalase, indole, citrate, methyl red, Voges-Proskauer, oxidase, and triple sugar iron (15, 16).

2.5. Inoculums Preparation

The bacterial inoculums were prepared using the method described by Wiegand et al. (15) whereby the colony suspension method with Mueller-Hinton broth and turbid solutions were spectrophotometrically standardized for the absorbance range of 0.08 to 0.10 at 625 nm, which is equal to 1×10^8 CFU/mL, i.e. the McFarland standard turbidity.

2.6. Biocide Dilution

The 3 biocides were diluted to give 25% (v/v), 20% (v/v), 15% (v/v), 10% (v/v), and 5% (v/v) concentrations for each of the biocides.

2.7. Determination of Bacteria Resistance to Biocides Using the Agar Well Diffusion Technique

Few drops of the prepared inoculum was cultured on sterile Mueller-Hinton agar plates for the different isolates and a sterile cork borer was used to make wells of about 6 mm in diameter on the plates. Various biocides were added to the wells on separate plates, respectively, and incubated at 37°C for 18 to 24 hours. Six wells were made on each of the plates. The inhibition zones diameter (IZD) produced by various antimicrobials against the test organisms was measured. No inhibition indicated that the test organism was susceptible to the biocide (16).

2.8. Determination of Minimum Inhibitory Concentration (MIC) of the Biocides Using the Agar Well Diffusion

The prepared inoculum for 3 of the isolates that were more susceptible to the biocides was introduced to plates by spreading a few drops of each inoculum on prepared sterile Mueller-Hinton agar plates. Different concentrations of the various biocides obtained from the dilution above was introduced to wells made on each of the agar plates, such that for each isolate, 3 plates for the 3 biocides were obtained, respectively. The plates were then incubated at 37°C for 24 hours. The minimum Inhibitory Concentration (MIC) is the lowest concentration of biocide that inhibits the growth of the organism (15, 16).

2.9. Determination of Antimicrobial Resistance of Biocide-Resistant Bacteria Using the Disk Diffusion Method (Kirby-Bauer)

Mueller-Hinton agar plates containing pure culture of bacteria, resistant to the biocides above, were impregnated with different commercial antibiotic disks using sterile forceps and incubated at 37°C for 24 hours. Antibiotics, to which organisms are sensitive, form a zone of inhibition around it. While those, to which organisms are resistant, do not form any zone of inhibition (16).

3. Results

3.1. Isolation and Identification of Isolates

Thirty isolates were obtained from the samples obtained from 5 locations, and these were examined. Six pure cultures were identified based on their cultural/colonial characteristics, gram reaction, and biochemical reactions, as presented in Table 1. The relative occurrences of the isolates from the samples were as follows, *Staphylococcus aureus* (32%) and *Klebsiella* spp. (20%), *Salmonella* spp. (16%), *Proteus* spp. (15%), *Staphylococcus* spp. (11%), and *Serratia* spp. (6%), (Table 2); the occurrence of these 6 with respect to the 5 sampling locations is shown in Table 3.

3.2. Bacterial Resistance to Biocides Using the Agar Well Diffusion

The resistance and susceptibility of the 6 isolates to Tiscol antiseptic, Tiscol disinfectant, and Dettol antiseptic is given in Table 4. *Proteus* spp. was resistant to all the 3 biocides. Tiscol antiseptic was effective only against *Salmonella* spp. and *Staphylococcus* spp., Tiscol disinfectant had the highest zone of inhibition of 23 mm against *S. aureus*. Dettol antiseptic had the lowest zone of inhibition of 11 mm against *Klebsiella* spp.

3.3. Minimum Inhibitory Concentration (MIC) of the Biocides Against the Test Organisms

Highest MIC of 25% was obtained for the 3 biocides tested against the organisms while the lowest MIC of 15% was obtained against *Klebsiella* spp. and *Staphylococcus* spp. using the Tiscol disinfectant (Table 5).

3.4. Resistance to Antibiotics by Biocide-Resistant Bacteria

Staphylococcus aureus was resistant to all the antibiotics on the disk, as shown in Table 6. All the gram negative bacteria were resistant to ampicillin, ceporex, and nalidixic acid. The test organisms were all susceptible to ofloxacin (Table 7). Also, only *Salmonella* spp. and *Klebsiella* spp. were susceptible to ciprofloxacin and gentamycin.

4. Discussion

Resistance of environmental bacteria to biocides and antibiotics is an emergent health concern. The environment plays important roles in the development of antibiotic resistance (17-19). Inappropriate or unprofessional use of these antimicrobials is often associated with an increased resistance of bacteria to these chemicals, especially in hospital settings (9). During the last 75 years pathogens have started to accumulate resistance genes on

Table 1. Biochemical Identification of Bacterial Isolates

Cell Shape	Gram Reaction	Coagulase	Catalase	Citrate	Methyl Red	Voges-Proskauer	Indole	Oxidase	Triple sugar Iron			Name of Bacteria
									B	G	H2S	
Rod	-ve	NT	NT	+	+	+	-	-	K	-	-	*Serratia marcescens*
Rod	-ve	NT	NT	+	+	-	-	-	A	+	+	*Salmonella* spp.
Rod	-ve	-	-	+	-	+	-	-	A	+	-	*Klebsiella* spp.
Rod	-ve			+	+	-	+	-	A	+	-	*Proteus* spp.
Cocci	+ve	+	+	NT	NT	NT	NT	NT	NT	NT	NT	*Staphylococcus aureus*
Cocci	+ve	-	+	NT	NT	NT	NT	NT	NT	NT	NT	*Staphylococcus* spp.

Abbreviations: A, Acid; B, Butt; G, Gas; H_2S, Hydrogen sulphide; K, Alkaline; NT, not tested; -ve, Gram negative; +ve, Gram positive; -, negative reaction; +, Positive reaction.

Table 2. Relative Occurrence of Bacterial Isolates

Isolate	Relative Occurrence
Staphylococcus aureus	9 (32%)
***Klebsiella* spp.**	6 (20%)
***Salmonella* spp.**	5 (16%)
***Proteus* spp.**	5 (15%)
***Staphylococcus* spp.**	3 (11%)
Serratia marcescens	2 (6%)
Total	30

Table 3. Occurrence of Bacteria in Sample Locations

Location	Bacterial
Surface water of River Benue	*Staphylococcus aureus*
	Salmonella spp.
	Staphylococcus spp.
Wastewater around block D of Oba Adetona hostel, MAUTECH, Yola	*Staphylococcus aureus*
	Salmonella spp.
	Proteus spp.
	Staphylococcus spp.
Wastewater from the Specialist Hospital, Yola	*Staphylococcus aureus*
	Klebsiella spp
	Salmonella spp.
	Serratia spp.
Soil at waste disposal site at the Specialist Hospital, Yola	*Staphylococcus aureus*
	Klebsiella spp.
	Proteus spp.,
	Staphylococcus spp.,
Soil from Jimeta by-pass dump site, Shinko, Jimeta.	*Staphylococcus aureus*
	Klebsiella spp.
	Salmonella spp.
	Proteus spp.
	Staphylococcus spp.

a larger scale and clear signs of exchange of antibiotic resistance genes have been observed between environmental bacteria and clinical pathogens (20). The result obtained in this study shows that the predominant isolates obtained were *Staphylococcus aureus* (32%), *Klebsellia* spp. (20%), *Salmonella* spp. (16%), *Proteus* spp. (15%), *Staphylococcus* spp. (11%), and *Serratia* spp. (6%). This result is consistent with that of Guimaraes et al. (21), for bacteria species common in the hospital environment.

In Table 3, *Proteus* spp. was resistant to the 3 biocides. Also, other gram negative bacteria showed less susceptibility to the three biocides when compared with the gram positive bacteria; this is similar to reports by Russell (22) and Randall et al. (23). The resistance of these gram negative bacteria could be attributed to the inability of these biocides agents to cross the outer membrane (8).

In Table 4, the highest MIC for the 3 biocides was 25% (v/v) and the lowest was 15%. At a very low concentration, the organisms were able to survive in the biocides. This was also observed in the study by Kaarina et al. (24).

From the antibiotic susceptibility testing carried out on the isolates resistant to at least one of the biocides, *S. aureus* was resistant to all the antibiotics. According to Rossolin et al. (25), among gram positive pathogens, a global pandemic of resistant *S. aureus* currently poses a big threat.

The chromosomally encoded multiple drug resistant efflux pump NorC of the MF superfamily was described in *S. aureus* and is also involved in quinolone resistance (26). All the isolates were multi-drug resistant, and all the gram negative bacteria were susceptible to the antibiotic Tarivid. Jeannette et al. (27) observed that low concentration of antibiotics in the environment may select for resistant bacteria. According to Reinthaler et al. (28), *Salmonella* spp. are one of the pathogenic microorganisms that hospitalized patients harbour in large numbers in their intestinal tract, which are antibiotic resistant. For cross-resistance to occur, the organism must possess a common mechanism of resistance to both types of antimicrobial agents, for example up-regulation of efflux pumps or changes in membrane permeability (14).

Table 4. Diameter of Zones of Inhibition (mm) Produced by the Biocides at 100% (v/v) Concentration Against the Six Isolates

Isolates	Tiscol Antiseptic	Tiscol Disinfectant	Dettol Antiseptic
Serratia marcescens	-	12	18
***Salmonella* spp.**	20	13	16
***Klebsiella* spp.**	-	11	20
***Proteus* spp.**	-	-	-
Staphylococcus aureus	-	23	20
***Staphylococcus* spp.**	20	13	10

Abbreviation: -, no zone of inhibition observed.

Table 5. Minimum Inhibitory Concentration (%) of the Three Biocides Against Test Organisms

Isolates	Tiscol Antiseptic	Tiscol Disinfectant	Dettol Antiseptic
Serratia marcescens	NT	25	20
***Salmonella* spp.**	25	20	25
***Klebsiella* spp.**	NT	15	25
***Staphylococcus* spp.**	20	15	20

Abbreviation: NT, not tested.

Table 6. Diameter of Zones of Inhibition (mm) Produced by Antibiotics

Isolates	APX, 30 μg	Z, 20 μg	AM, 30 μg	CPX, 10 μg	E, 10 μg	CN, 10 μg	R, 25 μg	S, 30 μg	PEF, 10 μg	SXT, 30 μg
S. aureus	R	R	R	R	R	R	R	R	R	R

[z] Abbreviations: AM, Amoxacillin; APX, Ampiclox; CN, Gentamycin; CPX, Ciprofloxacin; E, Erythromycin; PEF, Pefloxacin; R, Resistant (0 mm - 10 mm); R, Receptrin; S, Sensitive (11 mm); S, Streptomycin; SXT, Septrin; Z, Zinnacep.

Table 7. Diameter of Zones of Inhibition (mm) Produced by Antibiotics[z]

Isolates	PN, 30 μg	CEP, 10 μg	AU, 30 μg	CPX, 10 μg	NA, 30 μg	CN, 10 μg	OFX, 10 μg	ST, 30 μg	PEF, 10 μg	SXT, 30 μg
S. marcescens	R	R	R	R	R	R	S	S	R	S
***Salmonella* spp.**	R	R	S	S	R	S	S	R	S	R
***Klebsiella* spp.**	R	R	R	S	R	S	S	S	R	S
***Proteus* spp**	R	R	S	R	R	R	S	S	R	R

Abbreviations: AU, Augmentin; CEP, Sefalexin; CN, Gentamycin; CPX, Ciprofloxacin; NA, Nalidixic acid; OFX, Ofloxacin; PEF, Reflacine; PN, Ampicillin; R, Resistant (0 mm - 10 mm); S, Sensitive (11 mm); ST, Streptomycin; SXT, Septrin.

Evidence suggesting exposure of microorganisms to biocides at sub-lethal concentrations leads to increased antibiotic resistance, based primarily on results from in-vitro studies, with very few studies being undertaken in situ (8). Patients infected with antibiotic-resistant bacteria strains are likely to require hospitalization, sometimes for long periods (29).

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