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Antibiotic and Heavy Metal Tolerance of Bacterial Pathogens Isolated from Agricultural Soil

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Abstract: This study was conducted to investigate the antibiotic and heavy metal tolerance profile of bacterial pathogens isolated from farm land in Abakaliki. Soil samples were collected from different farm lands within Presco campus with sterile spatula container and were transported to Microbiology Laboratory Unit of Ebonyi State University, Abakaliki for bacteriological analyses Bacteria species isolated were characterized and identified using standard microbiological techniques. Bacteria isolated were *E. coli*, *Klebsiella* sp, *Staphylococcus* spp and *Shigella* spp. Antibiotic susceptibility studies was conducted using Kirby and Bauer method according to Clinical Laboratory Standards Institute (CLSI). The result of antibiotic studies showed that *Shigella* sp was resistant to ceftazidime, ampicillin, cefuroxime and amoxicillin/clavulanic acid. *Klebsiell a*sp was most susceptible to the antibiotic used. Metal tolerant studies were conducted using silver nitrate, copper II sulphate, zinc sulphate and lead acetate at varying concentrations of 0.5, 1.0, 3.0 and 5.0 mM, respectively. The result of the metal tolerance showed that all the bacterial isolates had growth at the metals concentration of 0.5 mM, but at higher concentration, there was no growth. Our results revealed that the isolates could be potential agents for the development of soil inoculants applicable in bioaugmentation of heavy metals polluted agricultural and industrial sites.

Key words: Antibiotic resistance · Bacteria · Abakaliki and Soil · Heavy metals

INTRODUCTION

Pollution of the natural environment has been increase in recent years as a result of increase in urbanization and industrialization. Pollution by heavy metals and other toxic substances have been found in increasing proportions worldwide and they are known to play major role in almost all metabolic process, growth and development of microorganisms [1].

Contamination of soils with heavy metals is becoming one of the most severe environmental and human health hazards. Soils contaminated by heavy metals and metalloids through emissions from the rapidly expanding industrial areas, mine tailings, disposal of high metal wastes, leaded gasoline and paints, land application of fertilizers, animal manures, sewage sludge, pesticides, wastewater irrigation, coal combustion residues, spillage of petrochemicals and atmospheric deposition [2, 3]. Metals play an essential role in the metabolic processes of the biota. Some of the heavy metals are essential and are required by the organisms as micro nutrients (cobalt, chromium, nickel, iron, manganese and zinc etc.) and are known as 'trace elements' [4].

However, Elevated levels of heavy metals not only decrease soil microbial action and crop production, but also threaten human health by biomagnification through the food chain [5]. However, at high levels both of the essential and non- essential metals become toxic to the organisms. These heavy metals influence the microbial population by affecting their growth, morphology, biochemical activities and ultimately resulting in decreased biomass and diversity [6]. Metals such as copper, cadmium, lead, zinc, nickel, mercury and chromium, when accumulated in the environment at high concentration may be toxic to plants, animals, humans and aquatic life [7].

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Furthermore, increasing concentration of metals beyond tolerance level have forced these organisms to adapt to various biological mechanisms such as efflux system, complexation, reduction of metal ions or utilization of the metal as a terminal election acceptor in anaerobic respiration to tolerate heavy metal accumulation to cope with this condition [8].

Antibiotic resistance has become a major health hazard due to its use and misuse. The use of antibiotics and antimicrobials in raising food animals has contributed significantly to the pool of antibiotic resistant organisms globally and antibiotic resistant bacteria are now found in large numbers in virtually every ecosystem on earth. Resistance to antibiotics is acquired by a change in the genetic makeup of a bacterium, which can occur by either a genetic mutation or by transfer of antibiotic resistance genes between bacteria in the environment. Heavy metals and other product such as antibiotics, sterilants, disinfectants creates selective pressure in the environment which leads to the mutations in microorganism that normally allows them better to survive and multiply [9].

Antibiotic resistance and metal tolerance by bacteria species are common phenomenon because metal exposure by bacteria selects for bacteria resistant to antibiotics [10], this is because multiple genes encoding for metal and antibiotic resistance are commonly found on the same plasmids or transposons. There are concerns about the possibility of metal contaminated sites in soils acting as favourable sites for drug resistant bacteria and thereby a reservoir for antibiotic resistant genes in both natural and clinical settings.

Therefore in the present study an attempt was made to determine antibiotic resistance and heavy metal tolerance of bacterial isolates from soils that are not affected by clinical waste, but subjected to probable heavy metal contamination.

MATERIALS AND METHODS

Collection of Soil Sample: The soil samples were collected from six selected sites from the city of Abakaliki. Soil samples from a depth of 15 to 20 cm from the surface were collected after removing the top layer. For each of the sampling sites, sub-samples of soil were collected from different locations, pooled together and homogenized so as to obtain representative sample. Samples were collected using a spade that is thoroughly cleaned and disinfected between sampling so as to prevent cross-contamination. The samples collected were transported to the Microbiology Laboratory unit of the Faculty of Biological Sciences, Ebonyi State University for analysis.

Isolation of Bacteria: Four (4) folds serial dilution of the samples was made using sterile distilled water in a test tube until a dilution of 10^{-4} was obtained. The surface of molted nutrient agar medium was flooded with 1ml of diluents each on a separate prepared nutrient agar plate and was incubated at 37°C for 18 – 24 hours. Physical colonies were counted and growth was sub-cultured to obtain a pure culture for further identification and characterization.

Identification and Characterization of the Isolates: After 18-24 hours of incubation, isolates on the media (pure colonies) obtained were further characterized and identified using standard Microbiological and Biochemical techniques such as morphological test, Gram staining technique, Indole test, Oxidase test, Catalase test, Coagulase test and sugar fermentation [11].

Antibiotic Susceptibility Test: The antibiotic susceptibility pattern of the bacteria isolates were determined by the modified Kirby and Bauer disk diffusion susceptibility test method as recommended by NCCLS (now CLSI). An overnight culture of the bacteria grown on nutrient broth was adjusted to 0.5 McFarland turbidity standards. The inoculums were aseptically swabbed on the surface of Nutrient Agar plates using sterile swab sticks. Commercially available antibiotic disc impregnated with the following antibiotics; Ampicillin(25µg), Amoxicillin-Clavulanic acid (25µg), Ceftazidime (30µg), Ciprofloxacin (5µg), Cefuroxime (30µg), Meropenem (10µg), Ofloxacin (5µg), Sulfamethoxazole (25µg) were asceptically placed on the Nutrient Agar plates. The plates were incubated at 37°C for 18-24 hours and the inhibition zone diameter produced by the antibiotic disc was measured using a meter rule and was recorded as recommended by CLSI [12].

Heavy Metal Tolerant Test: To examine the ability of the isolates to resist heavy metals, cells of overnight grown cultures were inoculated on a nutrient agar plates supplemented with different concentrations of 0.5, 1.0, 3.0 and 5.0 mM of heavy metals (Silver in silver nitrate, Lead in lead acetate, Zinc in zinc sulphate and Copper in coppersulphate). Cultures were incubated at 37°C for 24 hours and cell growth observed.

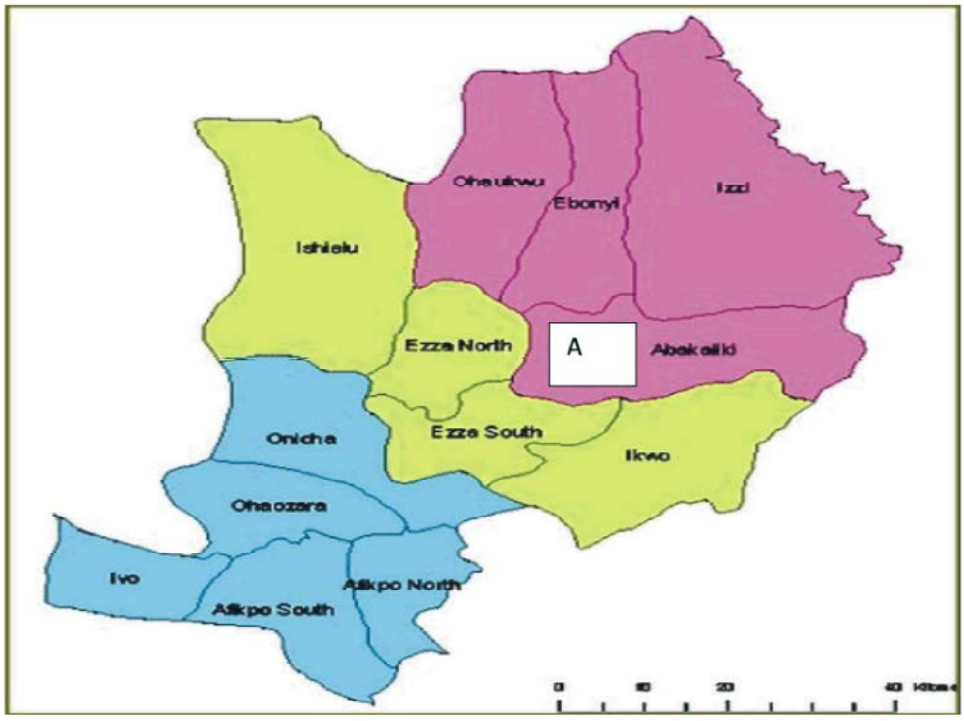

Fig. 1: Map of Ebonyi State showing the sampling locations;
A= Sample location

RESULTS AND DISCUSSION

Antimicrobial sensitivity of eight antibiotics including Ceftazidime, Ampicillin, Amoxicillin-Clavulanic acid, Ciprofloxacin, Cefuroxime, Meropenem, Ofloxacin and Sulfamethoxazole was assayed *in vitro* against four isolated bacteria: *Escherichia coli, Klebsiella* species*, Staphylococcus aureus and Shigella* species. The activity of the antibiotics studied showed that Meropenem was most potent against the organisms (*S. aureus*, *Shigella* spp, *Klebsiella* spp and *E. coli*), it was followed by the fluoroquinolones (ciprofloxacin and ofloxacin) which showed high inhibitory activity against all the tested isolates. Cefuroxime also showed inhibitory effect on all the organisms except *Shigella* species. However, all the other drugs showed sensitivity to two isolates (Figure 2).

Antibiotic resistant bacteria are generally higher in regions that are affected by pollution or agriculture. It was reported that in Norway, fields that were without antibiotic application for 10 years nevertheless had high levels of resistant organisms including resistance to chloramphenicol, tetracycline, ampicillin and streptomycin [13]. The study area was more or less unpolluted from clinical wastes. However, there are unaltered areas that might also contain high levels of antibiotic resistant bacteria as well, perhaps from natural production of antibiotics by soil bacteria

Our study revealed *Shigella* species as the most resistant among the isolates assayed. It was found to be resistant to five of the antibiotics including ceftazidime, ampicillin, amoxicillin-clavulanic acid, cefuroxime and sulfamethoxazole but showed sensitivity only to ciprofloxacin, ofloxacin and meropenem. All the other organisms were sensitive to almost all the antibiotics. In general, all the isolates were resistant to at least one antibiotic (Fig. 2).

The result of the study revealed similarity in resistance profile as obtained from previous studies [14]. Resistant organisms can be found naturally in the environment and most resistance is associated with man-made impacts of some type, either agricultural or direct human impact [15]. In general our study revealed moderate level of resistance which could be a possible reflection of less selection pressure for these human derived antibiotic residues in the soils of the study area.

The isolates were tested for their ability to grow in the presence of different concentration (0.5, 1.0, 3.0 and 5.0mM) of heavy metals as shown in Table 1. The results showed that all the isolates were able to tolerant heavy

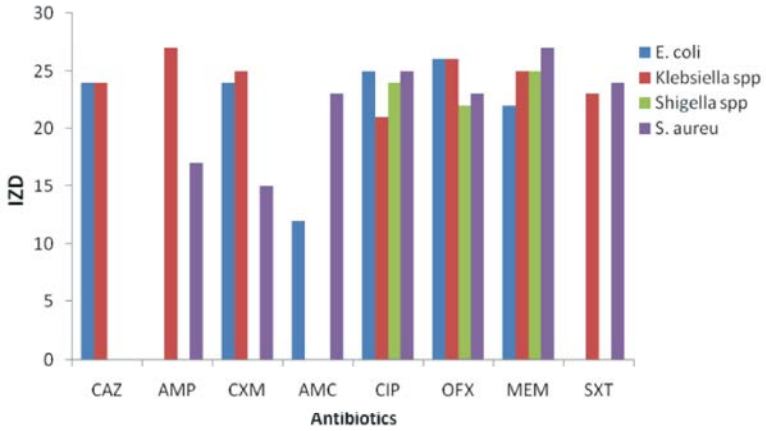

Fig. 2: Antibiotic Susceptibility Pattern of Bacterial Isolates
Key: IZD= inhibition zone diameter; CAZ=Ceftazidime, AMP=Ampicillin, AMC=Amoxicillin-Clavulanic acid, CIP=Ciprofloxacin, CXM=Cefuroxime, MEM=Meropenem, OFX=Ofloxacin, SXT=Sulfamethoxazole.

Table 1: Concentration of heavy metal at which bacterial isolates were able to grow (mM)

	Highest Concentration of heavy metal at which bacterial isolates were able to grow (mM)			
Bacteria isolates	Copper	Silver	Lead	Zinc
E. coli	0.5	0.5	0.5	0.5
Klebsiella spp	0.5	0.5	0.5	0.5
Shigella spp	0.5	0.5	0.5	0.5
S. aureus	0.5	0.5	0.5	0.5

metal at a lower concentration of 0.5mM. Differences in the bacterial resistance to different heavy metal have been attributed mainly due to the different concentration of different heavy metals. However, no isolate was able to grow in the presence of any of the metals at a concentration higher than 0.5mM (Table 1).

Sampling environments that contain elevated concentrations of heavy metals are potential source of toxic-metal-tolerant bacteria [16]. In the present study, bacterial strains obtained from the study area which basically are of clinical origin showed that the soil might have undergone a possible chemical contamination as well as exposure to domestic/clinical wastes. Also, the resistance of the isolates towards high concentration of metal may be due to unavailability of the metal to the bacteria, as there is a possibility of precipitation of metals in nutrient rich media such as nutrient agar, though specific studies were not conducted in this research to determine the same.

In addition, the inhibitory effects of higher concentrations of heavy metals to the isolates were most probably due to surface binding and disruption of membrane function [17]. For instance, the mechanism of resistance to lead might be due to an efflux by P-type ATPases and intracellular compounds complexation [18]. In fact, it has been found that many of the genes affected by metal stress are controlled by metallo-regulatory proteins known as Fur, MntR, PerR, ArsR and CueR [19]. Several bacterial species utilize intra- and extracellular binding mechanisms to avoid toxicity to Pb^{2+}. *Bacillus subtilis*for example, is able to accumulate lead ions in its cell wall [20] and *Bacillus subtilis* MTCC-1427 has been shown to have a high absorptive capacity for Pb^{2+}. Other mechanisms include lead ions efflux [18] and sequestration [21].

Tolerance to Cu^{2+} is due to ability of the isolates to accumulate copper ions in its cell wall thus preventing its entry into the cell [20]. However, at higher concentrations there is oxidation of lipid membranes, damage to nucleic acids [22] and generation of free radicals from hydrogen peroxide [23].

Some levels of resistance and the tolerance that was found among the isolates were probably due to the metal contamination in the soil [24]. Heavy metals and other toxicants have been suggested to play an important role in promoting antibiotic resistance [25]. Multiple genes encoding for metal and antibiotic resistance are usually found on the same plasmids and/or transposons, conferring co-resistance [25]. In certain cases, single enzymes play the role as efflux pumps for multiple metals and antibiotics, which is defined as cross-resistance [26]. Joint expression of antibiotic resistance and heavy metal resistance maynot be accidental. Nakahara *et al.* [27], have reported that the joint expressions of antibiotic resistance and metal tolerance may be caused by selection resulting from metals present in an environment. Many

reports have suggested that metal contamination in natural ecosystems could have key role in the maintenance and proliferation of antibiotic resistance [25]. This is of particular concern considering that anthropogenic levels of heavy metals are currently several orders of magnitude greater than levels of antibiotics [28]. Elevated frequencies of microbial resistance to various antibiotics have been observed in metal-contaminated freshwater streams [29], coastal areas [30] and metal contaminated ash settling basins [28].

The presence of bacteria capable of tolerating heavy metals from agricultural soil in Abakaliki was investigated. Our results revealed that the isolates could be potential agents for the development of soil inoculants applicable in bioaugmentation of heavy metals polluted agricultural and industrial sites.

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