Assessment of Antibiotic Resistance of Bacteria from a Lentic Freshwater Body in Iwo, Nigeria

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Authors' contributions

This work was carried out in collaboration between all authors. Author OEA carried out the water sampling, performed the statistical analysis, and read the draft. Author AAO managed the Laboratory analyses and wrote the first draft of the manuscript. Both Authors designed the study, managed the literature searches and read and approved the final manuscript.

Original Research article

ABSTRACT

Aims: Bacterial resistance to antibiotics has become a major public health concern, the aim was to study bacterial resistance to antibiotics in bacteria isolated from Aiba water sampled from 4 locations in 3 different months
Place and Duration of Study: Water samples were collected from Aiba reservoir Iwo and Isolation and characterization of bacteria was carried out at the Department of Biological Sciences, Bowen University, Iwo, Osun State between January 2013 and December 2013.
Methodology: Bacterial resistance to antibiotics was studied by collecting representative spatial and seasonal water samples from 4 locations and in 3 different months respectively. Isolation and identification of bacteria were carried out using standard isolation and biochemical methods. Antibiotic sensitivity testing was done for 8 antibiotics using the standard Kirby-Bauer disk diffusion method. Multivariate statistical analysis and plots were carried out using PAST software.
Results: One hundred percent (100%) of the isolates were resistant to cloxacillin followed by amoxicillin (87%), co-trimoxazole (78.3%) and augmentin (76%). Both gentamycin and ofloxacin recorded resistance of <10%. Thirty eight point five percent (38.5%) of the bacteria isolates were

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resistant to 4 antibiotics while only 11.5% exhibited mono-resistance.
Conclusion: The presence of high level multidrug resistance signifies a public health hazard.

1. INTRODUCTION

Antibiotic resistance is a phenomenon of increasing importance as demonstrated by the emergence of different international antimicrobial resistance surveillance programs. Since the first use of antimicrobial agents in the early 1940s, it has been known that bacteria carry mechanisms that allow them to resist antimicrobials. Bacterial resistance increased and became a worldwide human and veterinary health concern even with the introduction of the cephalosporins, fluoroquinolones among others [1].

Bacteria are capable of surviving in poor media such as water, floors and inanimate surfaces. Indicator bacteria have the ability to acquire and disseminate resistance that could be transmitted to pathogenic or zoonotic bacteria. Following antibiotic therapy, antibiotic residuals and resistant bacteria are shed into the environment via manure [2-3] which can be washed off via rain water into water bodies. Plasmids and other mobile genetic elements such as transposons, insertion sequence elements and integrons may play a role in facilitating transfer of antibiotic resistance genes to various bacterial communities in the environment [4-5]. Transport of these antibiotic resistance bacteria into groundwater and streams is not unlikely and it may be detrimental if the antibiotic resistance plasmids are ultimately mobilized to opportunistic human pathogens [6].

Aiba Reservoir being the second oldest impoundment of Osun River Basin was created for the provision of potable water with fisheries development as an ancillary benefit to Iwo and surrounding communities. In and around the reservoir are activities such as fishing, washing of domestic wares and automobiles, bathing and fetching of water for construction and domestic purposes [7].

These different contamination sources can lead to the introduction of multidrug resistant bacteria. Hence this paper was designed to study the antibiotic resistance patterns of bacteria isolated from the Aiba Reservoir water body.

2. MATERIALS AND METHODS

2.1 Sampling

Water samples from Aiba Reservoir were collected from four locations (A, B, C and D) within the reservoir (Fig. 1) and in three months (October, March and June) representing different seasonal periods. Water samples were collected from each point using a sterile 500ml polyethylene bottle. The water samples were transported to the laboratory within one hour of collection and kept in the refrigerator until ready for analyses [7].

2.2 Bacterial Isolation and Identification

Heterotrophic plate counts and total coliform counts were carried out according to standardized methods [8]. Bacterial culture and isolation was carried out using the serial dilution methods as described by Brown [9]. Different dilutions were pour plated on plate count agar for Total heterotrophic plate count and MacConkey agar for Total Coliform count, these were carried out in triplicates. Incubation was carried out at 37°C for 24h for the heterotrophic bacteria and at 37°C for 48h for the coliforms. Distinct colonies were picked and restreaked onto fresh agar plates until pure cultures were gotten. The pure cultures were kept on nutrient agar slants and kept at 4°C.

Isolates were characterized morphologically and biochemically. Gram staining, spore staining, colonial morphology, indole, Methyl red, Voges-Proskaeur, catalase, citrate, starch and casein hydrolysis were carried out to characterize the isolated bacteria [10]. Bergey's manual of systematic bacteriology [11] and the manual of identification of medical bacteria [12] were also used.

2.3 Antibiotic Sensitivity Testing of Isolates

Antibiotic susceptibility tests were done using the standard Kirby-Bauer disk diffusion methods [13]. One milliliter of an overnight actively growing broth culture adjusted to contain 1×10^6 cfu/ml of

each bacterial isolate was introduced into a Petri dish and 20 ml of molten Mueller Hinton agar added. Antibiotic sensitivity discs (Abtek Biologicals Ltd.) containing different antibiotics namely nitrofurantoin (200 µg), augmentin (30 µg), ofloxacin (5 µg), tetracycline (10 µg), gentamycin (10 µg), chloramphenicol (10 µg), ampicillin (25 µg), nalidixic acid (30 µg), and amoxicillin (10 µg), erythromycin (25 µg), cloxacillin (10 µg), co-trimoxazole (25 µg), and streptomycin (25 µg) were placed on the solidified agar surface. The plates were incubated overnight at 37°C. The relative susceptibility of each isolate to each antibiotic was shown by a clear zone of inhibition. Susceptible strains showed clear zones around the antibiotic discs [14-15].

2.4 Statistical Analyses

Multivariate analysis and plots were carried out using PAST software [16]. Principal Components Analysis (PCA) was determined using the correlation matrix and varimax rotation method; while PCA biplot was done to determine the relationship between bacterial isolates and sensitivity to antibiotics. Hierarchical Cluster Analysis (HCA) was done using Ward's method.

3. RESULTS AND DISCUSSION

Seventy-nine (79) species belonging to sixteen (16) genera were isolated from the water samples from Aiba Reservoir. This is as shown in Table 1. The *Bacillus* genus was the most isolated while *Acinetobacter, Edwardsiella, Listeria,* and *Proteus* were the least isolated with 2 species each.

Fig. 2 shows the percentage resistance of the isolated genera to the tested antibiotics. There was 100% resistance to cloxacillin, amoxixillin (87%), co-trimoxazole (78.3%) and Augmentin (76%). Ofloxacin and gentamycin had 0% and 7.7% resistance respectively.
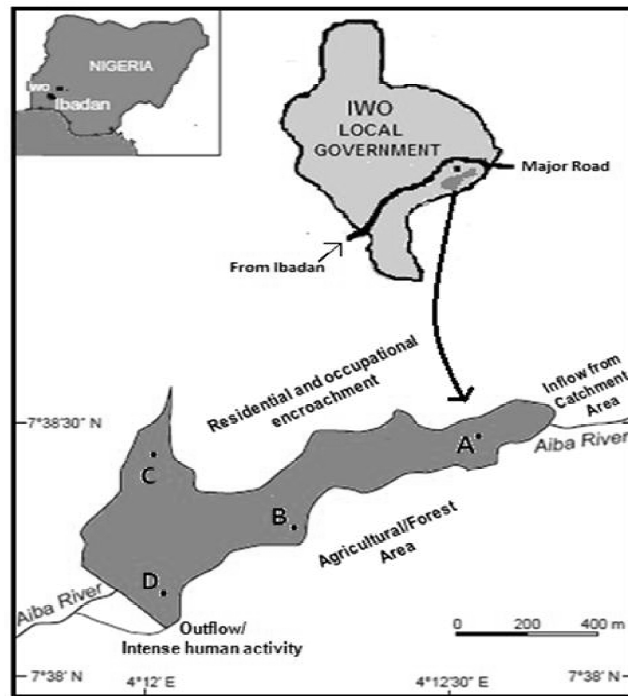

**Fig. 1. Map of Aiba Reservoir showing sampling locations**

Key: A: North-east, near source of inflow (upstream), little human activity (occasional washing/bathing); B: South-east, shares border with land used for agricultural activities, minimal direct human interference; C: North, rapid residential and occupational encroachment, food-vendor, carpentry, mechanic workshops, washing of vehicles and motorcycles; D: South, close to outflow (downstream), landing site for fishermen, high human activity-swimming, bathing, domestic washing, and removal of fish intestines

The percentage of mono- and multi resistant bacteria isolated form Aiba reservoir is shown in Fig. 3. Thirty-eight point five percent (38.5%) of the resistant bacteria were resistant to 4 antibiotics while 26.9% was resistant to between 5 and 8 antibiotics. Only 11.5% was mono-resistant. In all, 88.5% of the resistant bacteria were multi resistant as shown in Fig. 3.

Table 2 gives the patterns of multiple resistance in the isolated bacteria to the different antibiotics. Sixty-five point four percent (65.4%) of the isolates were resistant to both amoxicillin and augmentin while 53.9% was resistant to amoxicillin-augmentin and co-tromixazole simultaneously. The pattern/combination that

Table 1. Genera and number of isolated bacteria from Aiba Reservoir water

| S/N	Bacteria genera	Number isolated (per genus)
1	*Bacillus*	31
2	*Escherichia, Enterobacter*	5
3	*Aeromonas, Flavobacterium, Staphylococcus*	4
4	*Alcaligenes, Citrobacter, Corynebacterium, Klebsiella, Lactobacillus, Streptococcus*	3
5	*Acinetobacter, Edwardsiella, Listeria, Proteus*	2
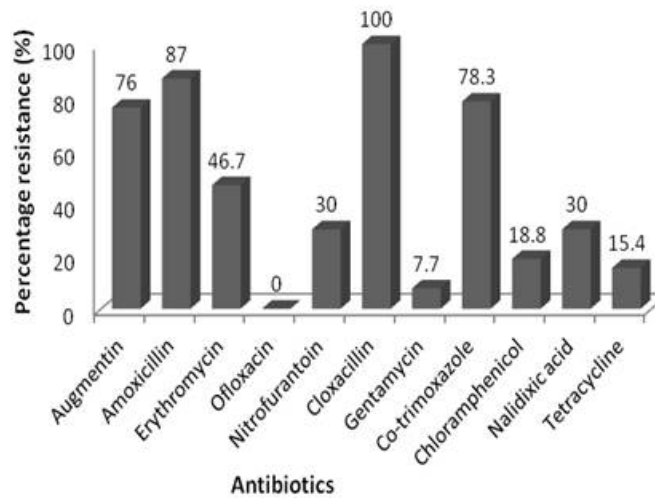
Fig. 2. Percentage resistance of isolated bacteria genera from Aiba Reservoir to different antibiotics
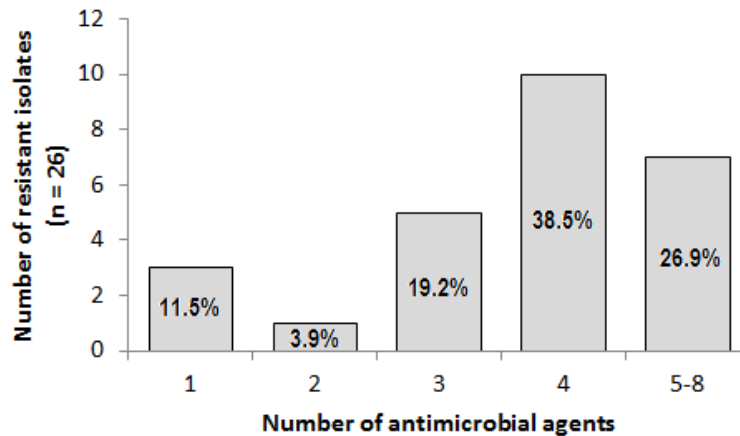
Fig. 3. Percentages of mono- or multi-resistant bacteria isolated from Aiba Reservoir water. (Percentage of resistant bacteria isolates is enclosed within each bar)

showed the lowest resistance pattern was amoxicillin, augmentin, ampicillin and cloxacillin.

Fig. 4 shows the Principal Component Analysis (PCA) biplots for Gram positive (a) and Gram negative(b) bacterial isolates resistant to the tested antibiotics. The PCA biplots show that the isolated bacteria were grouped into three and the antibiotics were grouped into two.
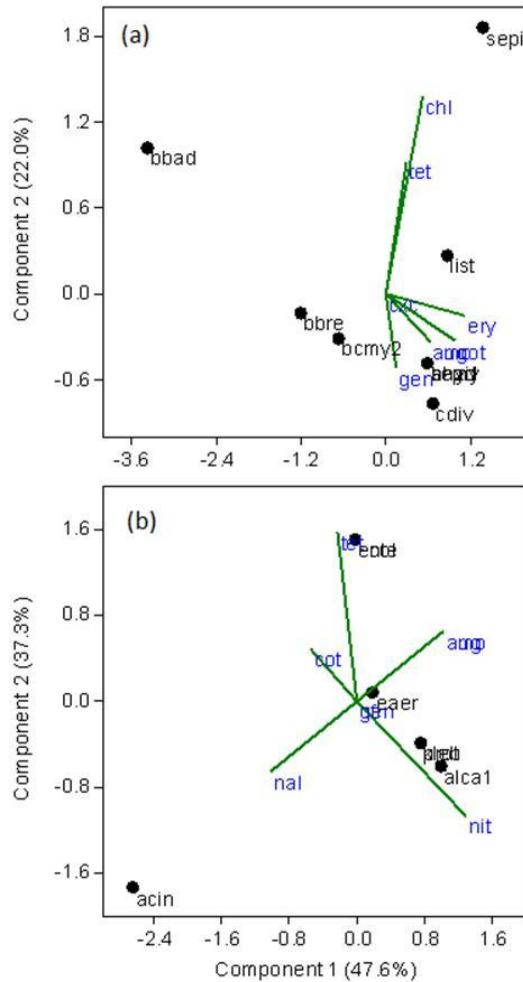
Fig. 4. Principal Component Analysis (PCA) biplots for aiba reservoir water (a) gram positive and (b) gram negative bacterial isolates resistant to tested antibiotics

The dendrograms of cluster analysis (Fig. 5) show that the resistance patterns of the *Bacillus brevis* and *Bacillus cereus* subsp *mycoides* was similar to *Bacillus badius*. They are members of a 60-phenon. The second group of *Staphylococcus* and *Listeria* species have a very similar resistance pattern (80-phenon) while the third

group have a 70-phenon. Resistance patterns of groups 2 and 3 were fairly similar (40-phenon) but there were no similarities between the resistance patterns of group 1 and the other two groups. The isolates in the group 2 were all resistant to chloramphenicol, tetracycline and gentamycin. It was also observed that the *Bacillus* group was resistant to the same group of antibiotics.

The high populations of viable heterotrophic bacteria encountered are typical of water bodies that receive organic pollutants. Similar high populations of heterotrophic bacteria have been reported [17]. It has been suggested that freshwater habitats harbour bacterial species [18].

Gastrointestinal microbial pathogens and many waterborne disease outbreaks are caused by intake of contaminated drinking water; in addition, drug resistant bacteria have been reported in surface water and groundwater [19]. This is a major public health concern as drug resistant bacteria could be transferred to humans via consumption of contaminated drinking water which then contributes to the spread and persistence of antibiotic resistance bacteria in the general population and environment. This study showed the presence of mono and multi drug resistant bacteria in the surface water of Aiba Reservoir, the major drinking water source in Iwo, Osun State.

Table 2. Patterns of multi-resistance in isolated bacteria

Antibiotic multi-resistance pattern	Resistant strains (%)
*amx-cot-aug-tet	7.7
amx-aug	65.4
amx-aug-cot	53.9
amx-tet	11.5
amx-aug-amp-cxc	3.9
amx-aug-cxc	34.6
cxc-cot	30.8
cot-cxc-gen	7.7

amx: amoxicillin, cot: co-trimoxazole, aug: augmentin, tet: tetracycline, cxc: cloxacillin, gen: gentamycin

Resistance patterns of isolated bacteria were cloxacillin (100%), amoxicillin (87%), cotrimoxazole (78.3%) and augmentin (76%). These drugs are sold easily across the counter and most antibiotics can be sold without a prescription. Amoxicillin is bacteriolytic and affects bacterial cell wall synthesis. It is a β-lactam antibiotic hence susceptible to β-

lactamase producing bacteria. It is often combined with clavulanic acid, a β-lactamase inhibitor and thus increases its effectiveness. This combined drug is sold as augmentin. It was observed in this study that resistance in amoxicillin was higher than that in augmentin. It was also observed that many of the isolates showed a multidrug resistance pattern. Sixty-five point four percent (65.4%) of the resistant bacteria were resistant to amoxicillin and augmentin simultaneously while 53.9% were resistant to amoxicillin-augmentin-co-trimoxazole. The highest multi-resistance pattern shown was resistance to 4 antibacterials by 38.5% of the isolates while 26.9% of the isolates were resistant to between 5 and 8 antibacterial agents. This should be a cause for concern by public health analysts. Quinolone resistance is important in human and veterinary medicine, since the situation becomes more serious when it is demonstrated that a bacteria resistant to one quinolone can be resistant to all antimicrobials of the same family [1]. In this study, all the isolates were susceptible to ofloxacin tested and only 7.7% of the isolates were resistant to gentamycin. It was observed in this study that most of the drugs to which resistance developed can be administered orally and are cheap, hence easily accessible. The injection form of gentamycin is most common hence the probable reason for very low resistance to it.

Bacillus was the most isolated organism in this study. They have the important feature of producing spores that are exceptionally resistant to unfavourable conditions. Although most *Bacillus* spp. are harmless, a few are pathogenic to humans and animals. *Bacillus cereus* causes food poisoning and bacteraemia in immuno-compromised patients as well as symptoms such as vomiting and diarrhea. *Bacillus anthracis* causes anthrax in humans and animals. Species are often detected in drinking water supplies, even supplies treated and disinfected by acceptable procedures. This is largely due to the resistance of spores to disinfection processes [20]. *Aeromonas* spp. are normal inhabitants of fresh water and occur in water, soil and many foods, particularly meat and milk. Some species cause infections in humans, including septicemia. Some also cause respiratory tract infections and gastrointestinal illnesses [21]. *Aeromonas hydrophila* produces cytotoxic enterotoxins contributing to maladies such as travelers' diarrhea and has shown an increase of antibiotic resistance [22]. *Citrobacter* rarely is associated with infections in humans. However, it

has been hypothesized that because of its low infection rate and virulence, *Citrobacter* could be a reservoir for antibiotic resistance genes [23].

Escherichia coli is present in large numbers in the normal intestinal flora of humans and animals where it generally causes no harm. However, in other parts of the body, *E. coli* can cause serious disease, such as urinary tract infections, bacteraemia and meningitis. Waterborne transmission of pathogenic *E. coli* has been well documented for recereational waters and contaminated drinking water [24]. Normal intestinal flora are a reservoir for resistance genes, the prevalence of resistance in commensal E. coli and some other bacteria is a useful indicator of antibiotic resistance in bacteria in the community.
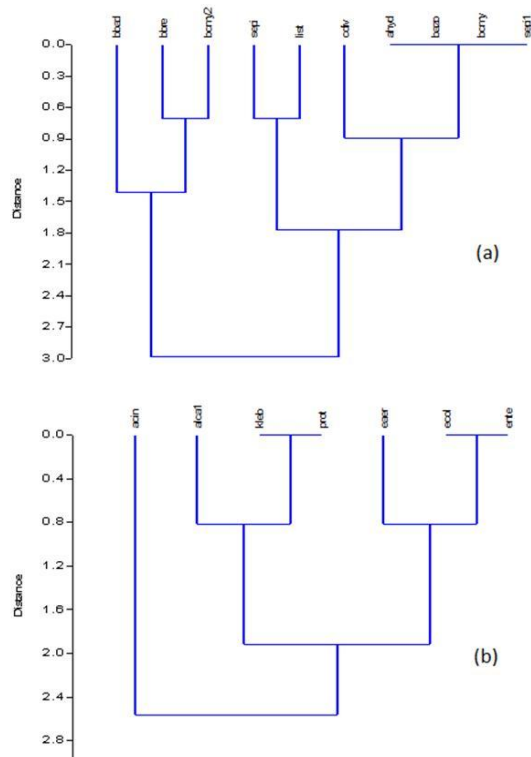
Fig. 5. Dendrogram of cluster analysis of resistance of (a) gram positive and (b) gram negative bacterial isolates from aiba reservoir water using ward's method

Commensal bacteria with reduced susceptibility to ceftriaxone have been isolated from drinking water and feed from dairy and poultry farms [25]. Yang and co-workers [25] concluded that ceftriaxone resistance was frequently associated with resistance to multiple antibiotics.

Chloramphenicol resistance was modest at 18.8% in this study. Chloramphenicol susceptible *Escherichia coli* was isolated from sewage but it was found that *E. coli* isolated from livestock was resistant to chloramphenicol [26]. It was observed that some soil and water environmental samples harboured significantly high numbers of drug-resistant bacteria [27]. Tetracycline resistance was the most prevalent at all sites and resistance frequencies ranged from 47% to 89% of total bacteria. Similar results have been reported from bacteria in the environment [28-29].

The PCA biplots show that the isolated bacteria were grouped into three and the antibiotics were grouped into two. It was also observed that the *Bacillus* group was resistant to the same group of antibiotics. The dendrograms of cluster analysis show that the resistance patterns of the *Bacillus brevis* and *Bacillus cereus* subsp *mycoides* was similar to *Bacillus badius*. They are members of a 60-phenon. The second groups of Staphylococcus and Listeria species have a very similar resistance pattern (80-phenon) while the third groups have a 70-phenon. Resistance patterns of groups 2 and 3 were fairly similar (40-phenon) but there were no similarities between the resistance patterns of group 1 and the other two groups. The isolates in the group 2 were all resistant to chloramphenicol, tetracycline and gentamycin. These three are antibiotics that work against protein synthesis. Majority of the other bacteria were resistant to antibiotics with the modes of actions of cell wall lyses.

The biplots and dendrograms also show that the resistance pattern of *Acinetobacter* was not similar to the remaining isolates. The group 2 isolates of *Alcaligenes*, *Klebsiella* and *Proteus* had resistance patterns that were very similar (80-phenon). *Klebsiella* and *Proteus* were totally similar (100-phenon). The 3rd group consists of *E. coli*, *Enterobacter* sp (100-phenon) and *Enterobacter aerogenes*. Groups 2 and 3 had resistance patterns that were fairly similar (40-phenon) but very different from that of *Acinetobacter.* Similarity in resistance patterns of the enterobacteria species could be due to horizontal gene transfer. This has been reported [30]. It is possible that many of the multi-drug resistant bacteria isolated in this study acquired their resistance via horizontal gene transfer which could be found on R-plasmids [30]. Application of manure/sludge is a major route of introduction of antibiotic resistant bacterial genes into water bodies. This has been reported [31-

32]. Another problem of resistant bacteria is the transference of resistance genes from harmless to pathogenic bacteria or to humans interacting with that aquatic environment.

4. CONCLUSION

In conclusion, the multiple drug resistance exhibited by the isolated organisms in this study is a threat to public health. Their presence signifies a public health hazard and could possibly lead to waterborne diseases.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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