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Distribution of Antibiotic-Resistant Bacteria Flora in Drinking Water Samples Sold in Calabar Metropolis

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ABSTRACT

The research study was aimed at investigating the distribution of antibiotic-resistant bacteria flora in drinking water samples sold in Calabar metropolis. Thirty (30) samples of drinking water (borehole, tap water, bottled water and sachet water) consumed within Calabar municipality and Calabar South local government area were randomly collected within different locations into sterile specimen bottles and were transported to the laboratory for prior examination and analysis. The study was completed within duration of six months. All the procedures were carried out using standard microbiological techniques. The results revealed the presence of *Pseudomonas spp, Shigella spp, Klebsiella spp, Staphylococcus aureus Micrococcus spp* and *Bacillus spp* in the analyzed water samples. *Escherichia coli* had the highest percentage of occurrence (26.32%) compared to another bacterial isolate that had; *Shigella* (17.54%), *Klebsiella* (15.79%), *Proteus* (12.28%), *Pseudomonas* (8.77%), *Staphylococcus aureus* (7.02%), *Bacillus* (7.02%) and *Micrococcus* (5.26%). The result of the antimicrobial susceptibility testing revealed that all the bacterial isolates from the analyzed water sample showed higher percentage resistance (100%) to amoxicillin and augmentin compared to other antibiotics tested against. Among the bacteria isolates from the analyzed water samples, *Staphylococcus aureus* showed the highest percentage resistance to ceftriaxone, amoxicillin, Cotrimoxazole, nitrofurantoin, augmentin, and tetracycline, while *Salmonella spp* and *Klebsiella spp* were sensitive to all the antibiotics tested against. However, the study has revealed the distribution and occurrence of multiple antibiotic resistance among bacteria isolates in drinking water samples sold in Calabar metropolis. Thus, there is the need for intensive surveillance of isolates during water processing and treatment to reduce or eliminate cases of waterborne infections, as well as to detect emerging antimicrobial resistance bacterial phenotypes especially in this our developing world.

INTRODUCTION

Water consumption is an important pathway for bacteria to infect humans, hence the presence of antimicrobial resistant bacteria in foods and water warrants particular attention (Okeke *et al*, 2000). Food and water contaminated by fecal material from the healthy human may also be an important source of antibiotic-resistant organisms those later cause human infections (Schoeder *et al,* 2004).

Antibiotics are the whole range of chemical substances that kill or inhibit the growth of bacteria (Brook *et al*, 2008), most are naturally produced by living organisms while others are produced synthetically (Brook *et al,* 2008). They are selectively toxic (affecting pathogenic microorganism more adversely than the host), and this may be as a result of the function of specific receptors required for drugs attachment or it may depend on the inhibition of biochemical events essential to the pathogen but not to the host (King *et al*, 2000).

Antibiotics when in this wastewater may be present at levels that can not only alter the ecology of the environment but also give rise to antibiotic resistance (Diab *et al,* 2008). Acquired resistance to antibiotics may arise by cellular mutation or by the acquisition of genetic elements in the form of plasmids or transposons (Diab *et al,* 2008). The occurrence of strongly selective environments such as water and food promotes, not only the growth of resistant bacteria but also leads to an increase in the frequency of resistant bacterial genes and genetic elements such as plasmids (Khachatourians *et al*, 2003). Wastewater when not effectively treated may contain pathogenic drug-resistant bacteria which constitute the most dangerous single risk factor for the dissemination of pathogenic and drug-resistant bacterial species in the environment (Cabrera *et al* 2004). These resistant bacterial species may be transmitted to humans and farm animals hereby causing the infection that cannot be treated with conventional antibiotics (Chitris, 2004). Hence, the waste water with its high content of multidrug resistant bacteria and the presence of enteric pathogens could pose a grave problem for the community receiving such waste waters (Summers *et al,* 2006), as often times in Nigeria, the untreated waste waters and urban sewage systems are released into rivers, lake and other surface waters which also serve as source of drinking water for local communities, poor homes and could also serve as sources of water for water treatment plants that provide both drinking water and water for domestic activities such as cooking in cities (Akubuenyi *et al,* 2011). Therefore, this forms the main rationale why this study is focused on investigating

the distribution of antibiotic-resistant bacterial flora in drinking water samples sold in Calabar metropolis.

MATERIALS AND METHODS

The study area

The study was carried out in Calabar, which is the capital of Cross River State, Nigeria. Odukpani and Akpabuyo Local Government Area bound it at the north, at east by the Republic of Cameroun, at the west by Akwa-Ibom State, and the south by the Atlantic Ocean. Calabar with an approximate population of about one million two hundred thousand (1,200,000) inhabitants (2006 census), is situated seventy-seven (77) kilometers up the Calabar river and over an area of about 604km^2.

Calabar has an average high temperature of 29ºC and an average low temperature of 25^{0}C. The precipitation of Calabar is about 51mm on the average during the dry season and 445mm on average during the wet season (weather base, 2011). Calabar is located approximately between longitude 80º19^{1}E, 800211E, latitude 400551N, and 40º58^{1}N. Calabar inhabited people are from three (3) ethnic groups, which are the Quas, Efuts and the Efiks.

Collection of samples

Water samples:

30 samples of drinking water (borehole and tap water, rainwater, bottled water and sachet water) consumed in Calabar south were randomly collected into sterile specimen bottles from water supplies in Calabar South at different locations and were immediately transported to the laboratory and stored at room temperature (20-22ºC) prior to examination analysis.

Media:

The media used in this study were Nutrient agar, Muller Hinton agar, Motility Indole Ornithine (MIO) (Hardydiagnostics, USA); Macconkey agar, Simmon Citrate Medium (Acumedia, USA). These media were prepared according to the manufacturer's instruction.

Chemical and reagents:

Chemical used in this study were of analytical grade. They include absolute alcohol, acetone, methanol (Sigma, USA) neutral red, methyl red indicators, phenol red indicator (Titan Biotech, India). Reagents used were oxidase strips, indole Kovacs and were products of Hardy Diagnostics, USA.

Sample preparations

Water samples:

1liter of the collected water sample was filtrated using cellular membrane filter of 0.45µm pores (millipores) and filters were transported onto Nutrient agar and MacConkey agar and incubated for 72hours at 37ºC.

Plaiting procedures:

0.1ml of desire dilutions 10^{-3} – 10^{-5} was spread plated in duplicate into the Nutrient agar and MacConkey agar plates supplemented with 50µg/ml of nystatin. Plates were incubated at 37ºC for 24 hours, after which bacterial counts were then recorded.

Purification of Isolates:

Following the enumeration of total heterotrophic bacteria, representatives of observed colonies were selected and sub-cultured repeatedly onto nutrient agar for purification. Purified isolates were stocked in nutrient agar slants for further studies.

Identification and characterization of isolates:

Purified bacterial isolates were characterized by gram staining and biochemical tests using the scheme in Bergey's manual of determinative bacteriology (Holt *el al.*, 1994; Cheesbrough, 2000).

Antibiotic susceptibility testing

The antibiotic susceptibility test was determined by the disc diffusion method as described by Bauer *et al.* (1996). Ten different commercially prepared antibiotic discs (Abtek Biological Ltd, Uk) were used and the concentration of each is as follows; amoxicillin (25µg), cotrimoxazole (25µg), nalidixic acid (30µg), nitrofurantoin (30µg), gentamycin (10µg),

ofloxacin (30µg), augmentin (30µg), tetracycline (30µg), doxycycline (30µg), trimethoprim/sulfamethoxazole (25µg).

After 18 hours incubation of the isolates in Mueller Hinton agar at 37^0C, the size of the zone of inhibition was measured and interpreted by comparing with 0.5 McFarland standard antibiotic sensitivity chart to determine their resistance patterns.

RESULTS

Biochemical characterization and identification of isolate

Bacteria isolates from the analyzed water samples were identified as; *Shigella spp, Pseudomonas spp, Proteus spp, Salmonella spp, Escherichia coli, Staphylococcus aureus, Klebsiella spp,* and *Micrococcus spp.*

Frequency and percentage occurrence of bacteria isolate from water samples

Table 1 presents the result of the frequency of occurrence of bacteria isolates from water samples.it showed that *Escherichia coli* had the highest frequency of occurrence (15), compared to *Klebsiella spp (9), Proteus spp (7). Salmonella spp (5), Shigella spp (5), Pseudomonas Spp (5), Staphylococcus spp (4) Bacillus spp (4) and Micrococcus spp (3)* from the analyzed water samples. Fig 1 presents the result of percentage occurrence of bacteria isolated from water samples. It showed that *Escherichia coli* had the highest percentage occurrence (26.32%), compared to *Shigella spp* (17.54%) *Klebsiella spp* (15.79%), *Proteus spp* (12.28%), *Pseudomonas spp* (8.77%), *Staphylococcus aureus* (7.02%), *Bacillus spp* (7.02%) and *Micrococcus spp* (5.26%).

Antibiotic susceptibility profile of bacteria isolates from analyzed water sample

Table 2 presents the result of antibiotic susceptibility profile of bacteria isolated from the analyzed water samples. It revealed that all the isolate showed a higher percentage resistance to amoxicillin (100%) and augmentin (100%) compared to other antibiotics tested against which had; Cotrimoxazole (44.44%), nitrofurantoin (44.44%), gentamycin (55.56%), tetracycline (44.44%) and ceftriaxone (77.78%) while the least antibiotic percentage resistance by the isolates was observed with nalidixic acid (22.22%), ofloxacin (22.22%) and ciprofloxacin (22.22%). Table 3 present the result of distribution and proportion of antibiotic

resistance pattern among isolates from the analyzed water samples. It showed that all the isolates were sensitive to ciprofloxacin.

Fig 2 presents the result of antibiotic resistance pattern in *Shigella Spp* from the analyzed water samples. It showed that the isolate had a higher percentage resistance to amoxicillin (100%), and was sensitive to ofloxacin as compared to other antibiotics tested against.

Fig 3 presents the result of antibiotic resistance pattern in *Pseudomonas Spp* from the analyzed water samples. It showed that the isolate had a higher percentage resistance to ceftriaxone (100%), amoxicillin (100%), Cotrimoxazole (100%), nitrofurantoin (100%) and least percentage resistance to ciprofloxacin (40%), as compared to other antibiotics tested against.

Fig 4 presents the result of antibiotic resistance pattern in *Proteus Spp* from the analyzed water samples. It showed that the isolate had a higher percentage resistance to ceftriaxone (100%), amoxicillin (100%), and augmentin (100%), while the least percentage resistance was observed with nalidixic acid (42.85%), ofloxacin (42.85%), tetracycline (42.85%) and ciprofloxacin (42.85%).

Fig 5 presents the result of antibiotic resistance pattern in *Salmonella Spp* from the analyzed water samples. It showed that the isolate had a higher percentage resistance to Cotrimoxazole (66.67%), augmentin (66.67%), nalidixic acid (66.67%), gentamycin (66.67%) and ciprofloxacin (66.67%), while all the isolate was sensitive to ofloxacin compared to other antibiotics they were tested against.

Fig 6 presents the result of antibiotic resistance pattern in *Escherichia coli* from the analyzed water samples. It showed that the isolate had a higher percentage resistance to amoxicillin (100%) and all where sensitive to Ofloxacin as compared to other isolate tested against.

Fig 7 presents the result of antibiotic resistance pattern in *Staphylococcus aureus* from the analyzed water samples. It showed that the isolate had a higher percentage resistance to ceftriaxone (100%), amoxicillin (100%), augmentin (100%), Cotrimoxazole (100%), nitrofurantoin (100%), and tetracycline (100%), while the least percentage resistance was observed with ofloxacin (50%) and ciprofloxacin (50%)

Fig 8 presents the result of antibiotic resistance pattern in *Klebsiella spp* from the analyzed water samples. It showed that the isolate had a higher percentage resistance to cotrimoxazole (66.67%), and were all-sensitive to ofloxacin as compared to other antibiotics tested against.

Fig 9 presents the result of antibiotic resistance pattern in *Bacillus spp* from the analyzed water samples. It showed that the isolate had a higher percentage resistance to ceftriaxone (100%), amoxicillin (100%) nitrofurantoin (100%), augmentin (100%) and tetracycline (100%), while the least percentage resistance was observed with cotriazone (50%), gentamycin (50%), nalidixic acid (50%) and ofloxacin (50%).

Fig 10 present the result of antibiotic resistance pattern in *Micrococcus spp* from the analyzed water samples. It showed that the isolate had a higher percentage resistance to ceftriaxone (100%), amoxicillin (100%), tetracycline (100%) while the least percentage resistance was observed with ofloxacin (33.33%) and cotrimoxazole (33.33%).

Table 1: Frequency of occurrence of bacteria isolate from water samples

Isolate	Frequency	% Occurrence
Escherichia coli	15	26.32
Salmonella spp	5	8.77
Shigella spp	5	8.77
Pseudomonas spp	5	8.77
Proteus spp	7	12.28
Staphylococcus aureus	4	7.02
Klebsiella spp	9	15.79
Bacillus spp	4	7.02
Micrococcus spp	3	5.26
Total	57	100

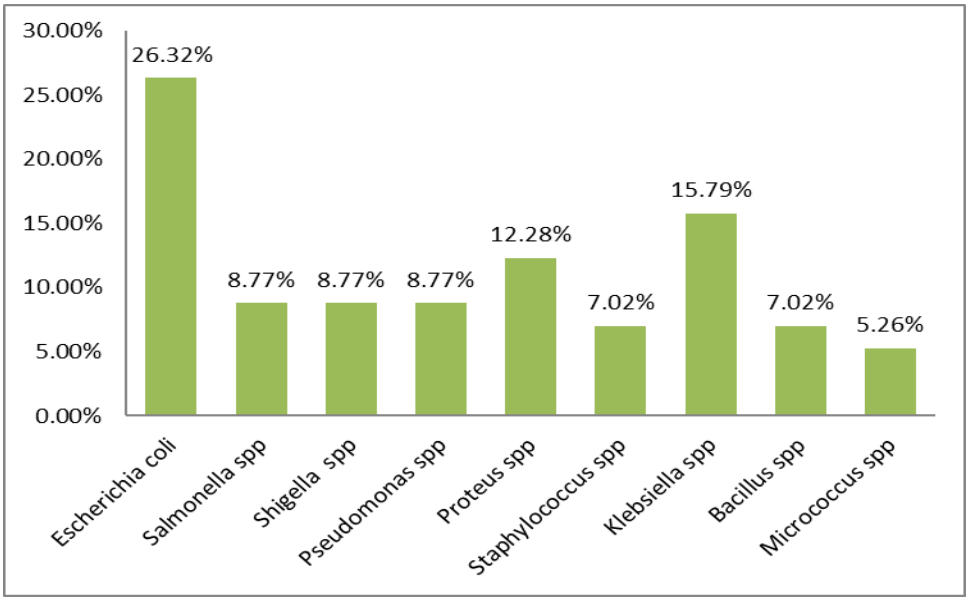

Fig. 1 Percentage occurrence of bacteria isolates from water samples

Table 2: Antibiotic susceptibility profile of bacteria isolates from water samples

Antibiotics tested	**Disc potency (µg/ml)**	***Shigella spp***	***Pseudomonas spp***	***Proteus spp***	***Salmonella spp***	***Escherichia coli***	***Staphylococcus***	***Klebsiella spp***	***Bacillus spp***	***Micrococcus spp***	**% resistance of all organism**
Amoxicillin	25	R	R	R	R	R	R	R	R	R	100
Cotrimoxazole	25	S	R	R	S	S	R	S	R	S	44.44
Nitrofurantoin	30	S	R	R	S	S	R	S	R	S	44.44
Gentamycin	10	S	R	S	R	S	R	S	R	R	55.56
Nalidixic acid	30	S	R	S	S	S	R	S	S	S	22.22
Ofloxacin	30	S	R	S	S	S	R	S	S	S	22.22
Augmentin	30	R	R	R	R	R	R	R	R	R	100
Tetracycline	30	S	R	R	S	S	R	S	R	S	44.44
Ciprofloxacin	10	S	S	S	S	S	R	S	R	S	22.22
Ceftriazone	30	S	R	R	R	S	R	R	R	R	77.78
% Resistance of single organisms		20	90	60	40	20	100	20	80	40	

Table 3: Distribution and proportion of antibiotic resistance among bacterial isolates from the analyzed water samples

		Total number and percentage resistance exhibited to antibiotics									
Isolates identified	**No.**	**Ce (%)**	**AMX (%)**	**COT (%)**	**NIT (%)**	**GEN (%)**	**NAL (%)**	**OFL (%)**	**AUG (%)**	**TET (%)**	**CIP (%)**
Shigella spp	5	3(60)	23(100%)	3(60)	3(60)	2(66.67)	2(66.67)	0(0)	3(60)	2(66.67)	2(40)
Pseudomonas spp	5	5(100)	5(100)	5(100)	5(100)	3(60)	3(60)	2(66.67)	4(80)	5(100)	2(40)
Proteus spp	7	7(100)	7(100)	4(57.14)	4(57.14)	4(57.14)	3(42.85)	3(42.85)	7(100)	3(42.85)	3(42.85)
Salmonella spp	5	3(60)	3(60)	2(66.67)	3(60)	2(66.67)	2(66.67)	0(0)	2(66.67)	3(60)	2(66.67)
Escherichia coli	15	10(66.67)	15(100)	9(60)	6(40)	10(66.67)	5(33.33)	0(0)	10(66.67)	7(46.67)	8(53.33)
Staphylococcus aureus	4	4(100)	4(100)	4(100)	4(100)	3(75)	3(75)	2(50)	4(100)	4(100)	2(50)
Klebsiella Spp	9	5(55.56)	5(55.56)	6(66.67)	5(55.56)	5(55.56)	4(44.44)	0(0)	5(55.56)	5(55.56)	5(55.56)
Bacillus Spp	4	4(100)	4(100)	2(50)	4(100)	2(50)	2(50)	2(50)	4(100)	4(100)	3(75)
Micrococcus spp	3	3(100)	3(100)	1(33.33%)	2(66.67%)	2(66.67%)	2(66.67%)	1(33.33%)	2(66.67%)	3(100%)	2(66.67%)
Total	57	44(77.19)	51(89.47)	36(63.16)	37(64.91)	33(57.89)	26(45.61)	10(17.54%)	41(71.92)	36(63.76)	29(50.87)

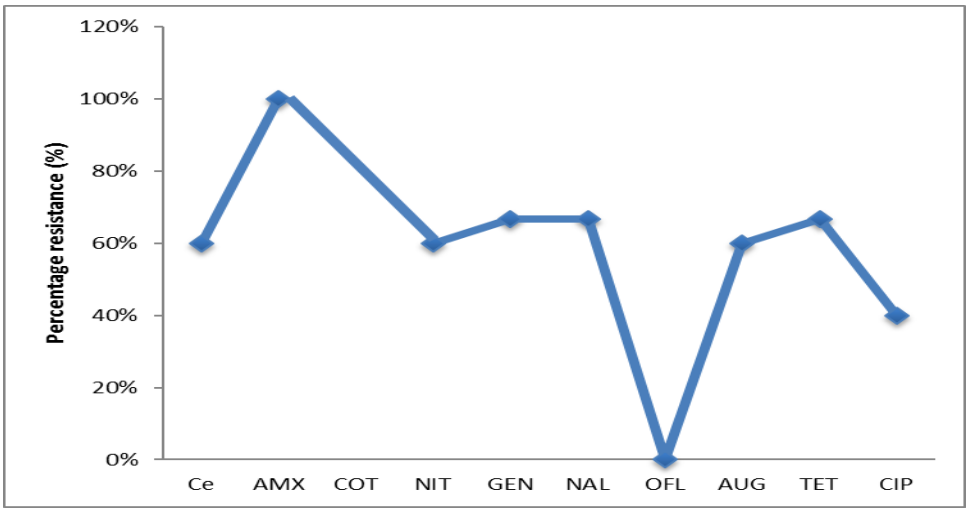

Fig. 2: Antibiotic resistance pattern in *Shigella spp* from water samples

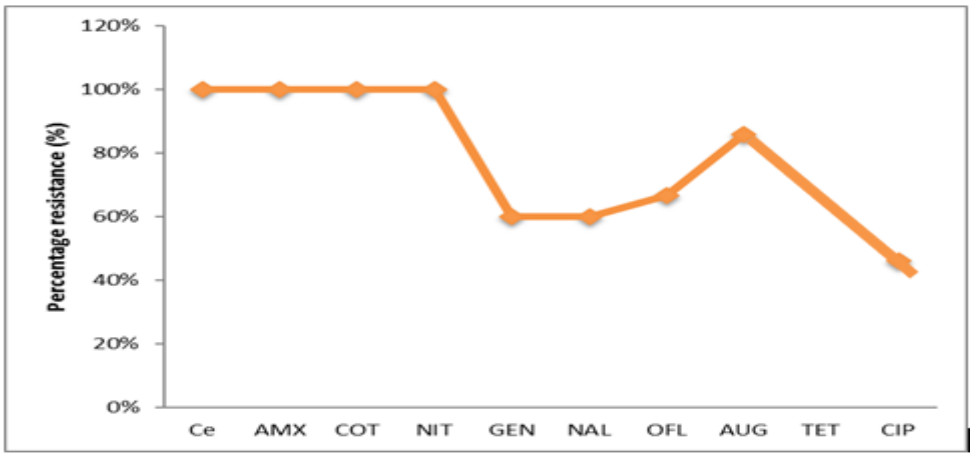

Fig. 3: Antibiotic resistance pattern in *Pseudomonas spp* from water samples

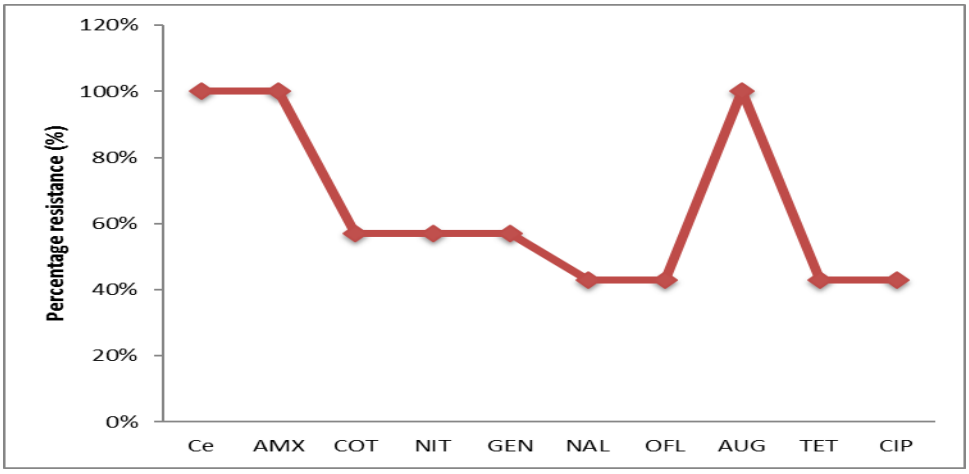

Fig. 4: Antibiotic resistance pattern in *Proteus spp* from water samples

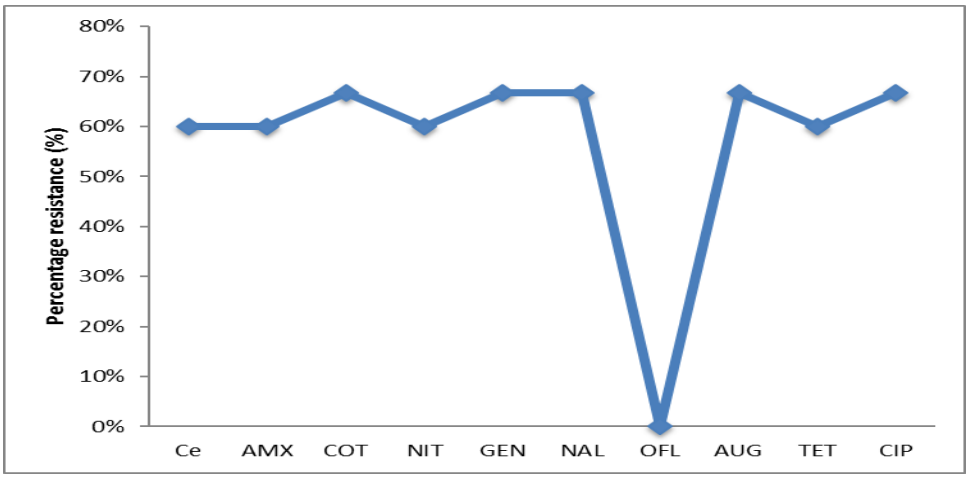

Fig. 5: Antibiotic resistance pattern in *Salmonella spp* from water samples

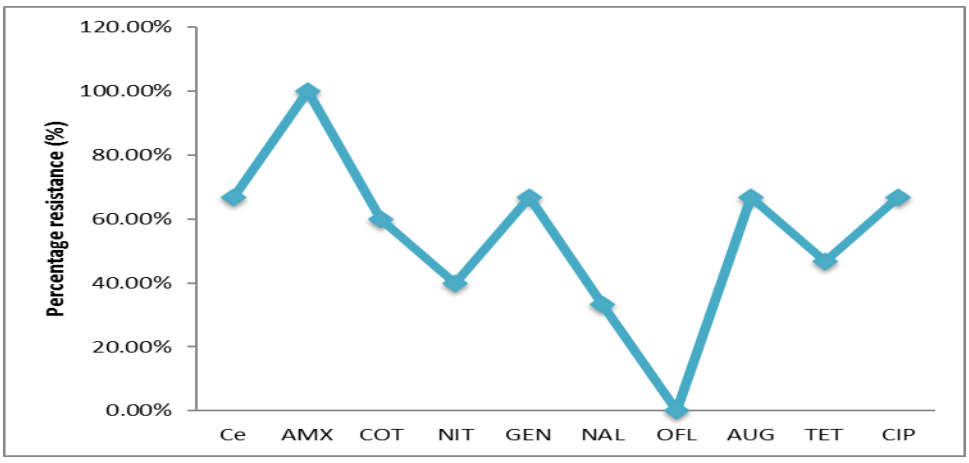

Fig. 6: Antibiotic resistance pattern in *Escherichia coli* from water samples

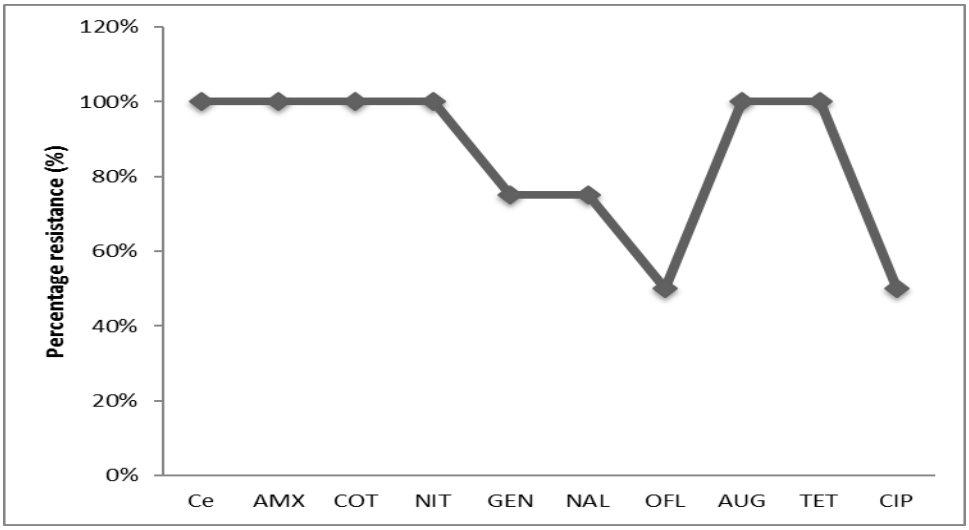

Fig. 7: Antibiotic resistance pattern of *Staphylococcus aureus* from water samples

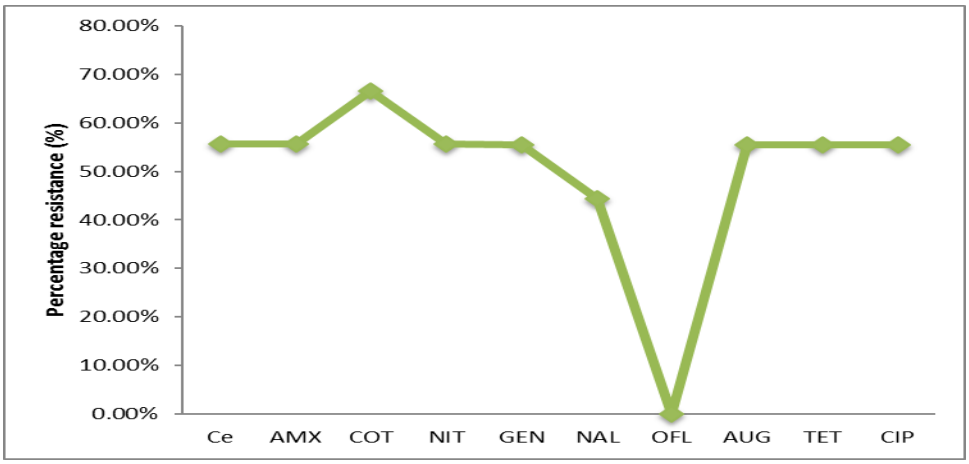

Fig. 8: Antibiotic resistance pattern of *Klebsiella spp* from water samples

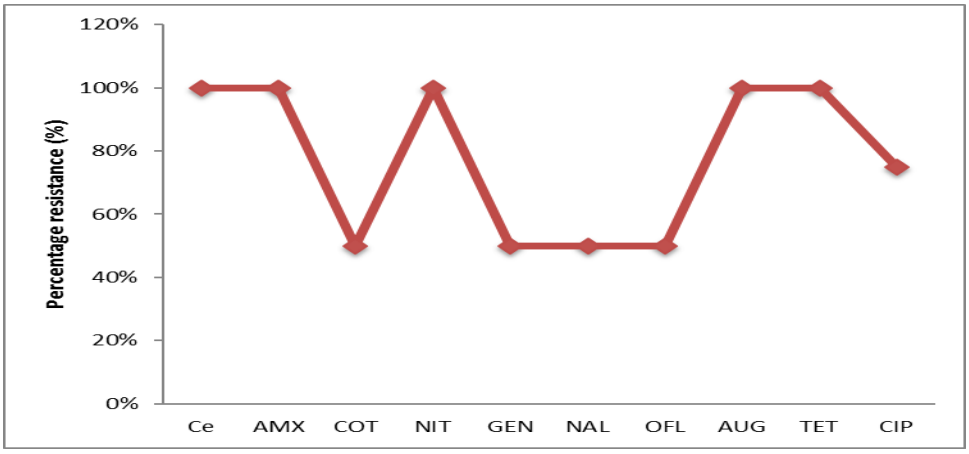

Fig. 9: Antibiotic resistance pattern in *Bacillus spp* from water samples

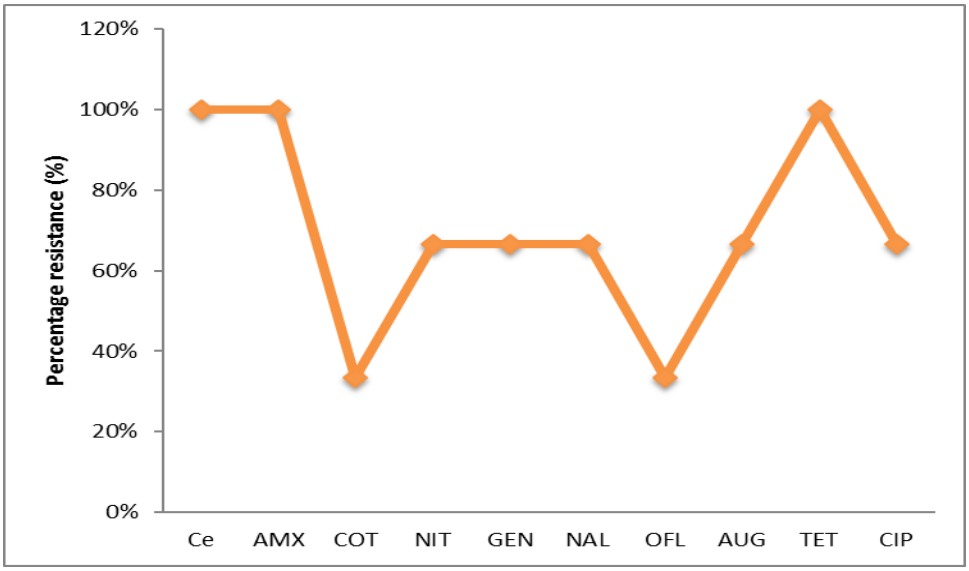

Fig. 10: Antibiotic resistance pattern in *Micrococcus Spp* from water samples

DISCUSSION

In this study, bacteria isolated from the analyzed drinking water samples were identified as *Escherichia coli, Pseudomonas Spp, Klebsiella spp, Salmonella spp, Bacillus spp, Staphylococcus aureus, Proteus spp, Shigella spp and Micrococcus spp.* This observation was not surprising as it corroborates with that of Oluyege *et al* 2009; Oladipo and Adejemobi, 2010; and Majolagbe *et al*, 2011, who all identified *Staphylococcus aureus, Bacillus, Marcescens, Streptococcus faecalis, Pseudomonas putida, Aeromonas hydrophila, Enterobacter aerogenes, Klebsiella spp, and Proteus spp* from ready-to-eat food and water samples in Ado-ekiti and Ogbomoso, Nigeria respectively. In addition, the similar study by Ghazi and Rajaa (2014) reported having isolated *Pseudomonas aeruginosa* in drinking water consumed in Basra. A study by Akubuenyi *et al.*, (2011) have also reported isolating *Pseudomonas spp, Streptococcus, Bacillus, Escherichia coli and Pseudomonas spp* from water samples. *Escherichia coli* was the most common bacterial isolate from the water samples with a percentage occurrence of 26.32% as compared to another isolate from the samples. This observation corroborates with that of Oluyege *et al.*, (2009), who reported a higher occurrence of *Escherichia coli* in ready-to-eat food and water samples analyzed. *Escherichia coli* when found in water and food supplies, is an indication of a recent fecal contamination and is a threat to public health (Moro *et al*, 2000; Mora *et al*, 2005). Their presence is a major health concern especially in cases of verotoxin producing *E. coli* (VTEC) sero group 0157, a major cause of hemorrhagic colitis (Moro *et al.*, 2000). Fecal

contamination of food and water cannot be prevented entirely, particularly in a setting where a hygienic standard of water treatment process is low and not monitored (Famurewa *et al.*, 2003). The frequency of isolation of *Klebsiella proteus and Shigella spp* was also reasonably high and supports individual studies and laboratory records that have however revealed that typhoid fever is endemic in Nigeria (Moro *et al*, 2001). The percentage occurrence of *Klebsiella spp, Shigella spp and Proteus* were a bit high, their contamination could be attributed to the poor hygiene practices of the food handlers before and after food processes upon returning from toilet, lack of disinfection of table tops before and after daily use by customers as well as poor water treatment process (Fang *et al,*. 2013) as this postulation is supported by the previous report on the isolation of *Salmonella spp, Klebsiella spp, Shigella spp, Staphylococcus aureus and Escherichia coli* from the hands of food and water vendors and food canteens in Nigeria (Famurewa *et al.*, 2003).

Antibiotics susceptibility profile of bacteria isolated from the water samples showed that all isolates had multiple resistance to the antibiotics they were tested against. This observation was in agreement with that of Gundogan *et al.*, (2006) who reported to have isolated *Klebsiella spp and Escherichia coli* with multiple antibiotic resistance from consumable water samples. Also, other studies by Lateef *et al.*, (2005); Majolagbe *et al.*, 2011, have reported isolating *E. coli, Staphylococcus aureus, Salmonella spp and Streptococcus spp* from water, food and clinical samples with multiple antibiotic resistance.

The relatively high level of resistance of the isolates to antimicrobial agents is a reflection of misuse or abuse of these agents in the environment (Abbar and Kaddar, 2001). Antibiotics prescriptions in hospitals are given without clear evidence of infection or adequate medical attention. Broad-spectrum antibiotics sometimes given in place of the narrow spectrum are drugs as a substitute for culture and sensitivity testing, with consequential risk being superinfections and the selection of drug-resistant mutants (Prescott *et al.*, 2005). In developing countries, drugs are available to the public and thus people may practice self-administration of antibiotics and further increase the prevalence of drug-resistant strains, also the long-standing practice of using low doses of antibiotics for a long period of time for growth promotion and arbitrary use of antibiotics in animal husbandry is a strong contributor to the development of antibiotic-resistant bacteria in the environment.

The ability of some of the isolates such as *Bacillus spp, Pseudomonas spp, Enterobacter spp, Salmonella spp, Proteus spp, E. coli and Staphylococcus aureus* to show 100% resistance to

some of the antibiotics such as triazole, amoxicillin, augmentin, tetracycline and Cotrimoxazole could possibly be as a result of selection pressure created by the indiscriminate use of antibiotics by human (Chui *et al* 2002; Threfall *et al*, 2000). The co-existence of resistance genes with mobile elements such as plasmids, transposons and integrons facilitates the rapid spread of antibiotic resistance genes among bacteria and this could possibly be the reason for the high percentage resistance (Sunde, 2005).

The total resistance (100%) shown by some of the isolates to ceftriaxone. (a cephalosporin and widely used as broad-spectrum antibiotic) which acts by inhibiting cell wall synthesis in growing or dividing cells (Kathleen and Arthur, 2000) is likely due to the presence of *β-Lactamase* which acts by cleaving β-Lactam ring of cell wall, thus inhibiting antibiotics like ceftriaxone (Warren 2006), while the resistance to other antibiotics by the isolates could as well be due to the fact that antibiotic resistance microorganisms may be associated with reduced penetration of antibiotic into the cell, or from active processes such as changes in the transport of those compounds into or from the bacteria cells (Hermansson *et al.*,2007).

Most of the bacterial isolates from the water samples showed significantly higher percentage resistance to the antibiotics tested. This observation was not surprising, as this could have possibly resulted from inappropriate or uncontrolled use of antibiotics in farming practices, indiscriminate use of antibiotics by humans, indiscriminate dumping of hospital wastes and antibiotic materials in the environment, indiscriminate use of manure as well as human excreta in the environment (Warburton *et al* 2002).

CONCLUSION

The study has revealed the distribution and occurrence of multiple antibiotic resistance among bacteria isolates in drinking water samples sold in Calabar Metropolis. The study thus emphasizes the need for intensive surveillance of isolates throughout water processing and treatment. To prevent water-borne infections, as well as detect emerging antimicrobial resistance bacterial phenotypes especially in this our developing world.

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