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ANTIBACTERIAL SUSCEPTIBILITY PATTERN OF BIOFILM PRODUCING BACTERIA ISOLATED FROM PACKAGED DRINKING WATER SOLD IN OWERRI METROPOLIS, IMO STATE, NIGERIA

Azuwike C. O.[1] Ekwerike, C. U.[1], Ajuruchi, V. C.[2] and Braide W.[3]*

[1]Biotechnology Department, Federal University of Technology, P.M.B 1526, Owerri, Imo State, Nigeria.
[2]Department of Biology, Federal University of Technology, P.M.B 1526, Owerri, Imo State, Nigeria.
[3]Department of Microbiology, Federal University of Technology, P.M.B 1526, Owerri, Imo State, Nigeria.

***Corresponding Author: Dr. Braide W.**

Department of Microbiology, Federal University of Technology, P.M.B 1526, Owerri, Imo State, Nigeria.

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ABSTRACT

Water is an essential natural resource required by all living organisms. Biofilm is as association of microorganism in which cells stick to each other on a surface encased within a matrix of extracellular polymeric substance produced by bacteria themselves. This study was done to isolate, characterize and show antibiotic susceptibility of biofilm producing bacteria in packaged drinking water sold in Owerri metropolis. One hundred (100) samples each was collected randomly from seven locations to make a total of seven hundred (700) samples. The sample were analysed using membrane filtration and standard plate count methods. The identities of the bacterial isolates were confirmed with standard bacteriological manual. Sample from *Eziobodo*, *Nazi*, FUTO campus and *Obinze* recorded higher bacterial population. Eleven genera of bacteria were isolated from the samples with their percentage occurrence as follows: *Staphylococcus aureus* (23.1%), *Bacillus cereus* (10.4%), *Klebsiella* sp (4.0%), *Pseudomonas aeruginosa* (10.5%), *Enterobacter* sp (3.3%) *and Enterococcus faecalis* (19.5%), *E. coli* (7.2%), *Corynebacterium* sp (4.7%), *Micrococcus leteus* (7.9%), *Micrococcus roseus* (5.8%) and *Shigella* sp (3.6%). The result of the antibiotic susceptibility test shows that all the biofilm producing bacteria in the water samples have 100% resistance to chloramphenicol, 99% to streptomycin, 98% to erythromycin and 90% to gentamycin. *Bacillus cereus*, *Corynebacterium* sp and *Enterococcus faecalis* were resistant to at least four antibiotics. The diseases caused by biofilm producing bacteria and their ability to resist antibiotics had been reported. Microbiological assessment of quality of drinking water should be done periodically by the regulatory agencies in compliance with internationally defined drinking water standards. The results of multiple antibiotic resistances obtained from this study challenges scientist on the need for more or development of new antibiotics to fight against the infections caused by these resistant strains.

KEYWORDS: Biofilm, bacteria, antibiotics, resistance.

INTRODUCTION

Water is an essential natural resource required by all living organisms. However among these organism, human beings tend to use water most for the purpose of drinking, personal, domestic and industrial purpose, recreational uses (Igbeneghu *et al.*, 2014). Water as one of the most important elements for all forms of life is indispensable in the maintenance of life on earth and essential for the composition and renewal of cells. Water represents 70% of human body mass and participates in the composition of tissues, digestion and transport most diverse substances throughout the body. Notwithstanding, human beings increasingly pollute the reserves that cause illness that can jeopardize the well-being of human population, animals and plants. (http://www.sabesp.com.br).

Nigeria like other developing nations is faced with problems of portable water supply for its estimated 160 million citizens (Adesiji, 2013). As a result of this, packaged drinking water has been used as alternative drinking water source (Oyedeji *et al.*, 2010).

Packaged drinking water is any water packaged in cans, plastic sachets, and nylon sachets for the main purpose of consumption. The increasing demand, sale and consumption of packaged drinking water in Owerri and its environs poses significant public health risks to her citizens especially individuals with compromised immune system (Mgbakor *et al.*, 2011) where most of the producers of packaged drinking water obtain their water from sources such as municipal piped water, borehole water and do not follow specified standards due

to lack of the appropriate drinking water technology (Oluyege *et al.*, 2014).

The quality of drinking water is also an important environmental determinant of health. Water in sachets is readily available and affordable but there are concerns about their purity. The integrity of the hygienic environment and the conditions where the majority of the water in sachets produced has also been questioned.

Biofilm is as association of microorganisms in which cells stick to each other on a surface encased within a matrix of extracellular polymeric substance produced by bacteria themselves. (Hall-stoodley, 2004). Biofilms are present everywhere in nature and can be found in facilities in the industries, hotels, waste water channels, bathrooms, laboratories, hospital settings etc., and commonly occur on hard surfaces submerged in or exposed to an aqueous solution. It can also be formed as floating mats on surface of liquid and its formation can occur on both living and non-living surfaces (Costerton *et al.*, 1999).

Literatures on biofilm producing microorganisms is well documented (Khan and Butt, 2015; Donlan, 2002; Lindsey and Holy, 2006; Moskowitz *et al*., 2004; Landry *et al*., 2006). The increasing reports on the pathogenicity of biofilm producing microorganisms portends imminent danger (Fux *et al*., 2005; Cunningham *et al*., 2008), especially to the third world countries where the facilities and technology to abate their prevalence is lacking.

In addition, the frequent resistance of biofilm producing bacteria isolated from portable drinking water to commercially available antibiotics has been attributed to mutation, adaptation to stress, resistant phenotype, stratified activity, nutrient gradient, oxidative stress, quorum sensing, failure of antibiotics penetration and heterogeneity (Khan and Butt, 2015; Driffield, *et al*., 2008; Conibear *et al*., 2009; Boaretti, 2003; Alhede *et al*., 2009; Gennip *et al*., 2009; Hoiby *et al*., 2010; Stewart and Costerton, 2001; Smith, 2005).

In Owerri metropolis, sachet water production has increased tremendously with over 1,000 registered and unregistered producers (personal communication, 2019) with majority producing under questionable hygienic environmental conditions.

This study reports on antibacterial susceptibility pattern of biofilm producing bacteria isolated from sachet drinking water sold in Owerri metropolis.

MATERIALS AND METHODS

Sources of Sample Collection

Sachet water samples were obtained from commercial vendors from different parts of Owerri metropolis such as autonomous communities in Eziobodo, Ihiagwa, Nekede, Obinze and Nazi and Federal University of Technology and Federal Polytechnique campuses. A total of 700 samples (100 samples from each location) were randomly collected and stored for three weeks after production.

Preparation of samples and inoculation

Two hundred milliliters (200 ml) of each water was emptied into a presterilized membrane filter machine. The filter paper was aseptically placed on freshly prepared surface dried media and incubated at ambient temperature for 48 hrs (Cheesbrough, 2002). Water sample were also diluted decimally and inoculated to obtain counts (Harrigan and McCance, 1990).

Colony counts, characterization and identification of microbial isolates

Colony counts obtained on the media were expressed as colony forming units per millilitre (CFU/ml) to obtain total population.

Microbial isolates were characterized based on cultural (colonial), microscopic and biochemical methods with reference to standard manuals (Cheesbrough, 2002). The identities of the isolates were cross-matched with reference to standard manuals for the identification of bacteria (Sneath *et al*., 1986).

Antibacterial susceptibility of Bacterial isolates

Test isolates were sub-cultured twice on nutrient agar and incubated at 37^0C for 24 hrs. Suspension equivalent to 0.5 McFarland standards was prepared and streaked on Mueller-Hinton Agar surface using a sterile swab stick and evenly distributed over the surface of the plate by a rotational streak at angles of 60 degrees. Oxoid antibiotic discs were placed on the surface of the inoculated plates at a distance of 40 mm to each other thereby obtaining a total of 5 discs per plate. The plates were incubated for 48 hrs at 37°C. Zone of inhibition was measured and recorded in millimeter with a transparent meter rule (Harrigan and McCance, 1990).

RESULTS

Table 1 shows the total bacteria counts from sachet water from three different media. Sachet water from Eziobodo, FUTO campus, Nekede, Obinze and Nazi showed higher counts on all the media. Colonial characteristics of bacteria isolated are shown in Table 2. Four gram positive bacteria namely, *Staphylococcus*, *Micrococcus*, *Enterococcus* and *Bacillus* and five gram negative bacteria namely, *Escherichia coli, Klebsiella*, *Shigella* and *Pseudomonas* species were isolated and identified.

Table 1: Enumeration of Bacteria Populations in Sachet Water.

Sample locations	Colony counts on Nutrient Agar (Cfu/ml)	Colony counts on Eosin Methylene Blue agar (Cfu/ml)	Colony counts on MacConkey Agar (Cfu/ml)
Eziobodo	2.3×10^2 - 1.28×10^3	1.0×10^1 - 1.00×10^3	6.3×10^1 - 1.18×10^3
Ihiagwa	1.2×10^2 - 6.9×10^2	2.1×10^1 - 5.3×10^2	3.3×10^1 - 1.10×10^3
FUTO Campus	9.0×10^2- 1.23×10^3	3.0×10^1 - 4.8×10^2	4.5×10^1 - 1.08×10^3
Nekede	3.9×10^2 - 1.65×10^3	2.3×10^1 – 2.8×10^2	4.3×10^1 - 1.19×10^3
NekPol Campus	3.1×10^2 - 8.9×10^2	2.3×10^1 – 6.9×10^2	2.9×10^1 - 1.38×10^3
Obinze	1.1×10^2 - 1.02×10^3	1.0×10^1 – 7.8×10^2	2.3×10^1 - 1.13×10^3
Nazi	7.1×10^2 - 1.11×10^3	7.3×10^1 - 1.28×10^3	2.3×10^1 - 1.00×10^3

FUTO, Federal University of Technology, Owerri; NekPol, Federal Polytechnic Nekede.

Table 2: Colonial Characteristics of Biofilm producing Bacteria isolates.

Colonial Characteristics	Motility Test	Spore Formation	Capsule Formation	Gram morphology/reaction	Probable Identity
Circular moist and shiny golden yellow colonies on Nutrient Agar and light yellow on Mannitol Salt Agar	-	-	-	Gram positive cocci predominantly in clusters, few in tetrads and pairs	*Staphylococcus* sp
Large slimy mucoid colonies on Eosin Methylene Blue Agar	+	-	+	Gram negative short thick rods in chains	*Klebsiella* sp
Small circular moist and shiny low convex cream colonies on Nutrient Agar	-	-	-	Gram positive cocci predominantly in chains and pairs	*Enterococcus* sp
Light pink mucoid moist and shiny colonies on MacConkey Agar	+	+	-	Gram negative single and short rods	*Shigella* sp
Serrated dull and dry flat cream colonies on Nutrient Agar				Large gram positive rods with central spores	*Bacillus* sp
Greenish metallic sheen on Eosin Methylene Blue Agar	+	-	-	Gram negative rods predominantly in single and pairs	*Escherichia coli*
Pink mucoid colonies on Eosin Methylene Blue Agar	+	-	-	Gram negative bacilli in singles and short chains	*Enterobacter* sp
Cream moist and slimy cream colonies on Nutrient Agar	+	+	-	Large gram positive rods with central spores in chains	*Bacillus* sp
Bluish green moist colonies on Nutrient Agar	+	-	-	Gram negative slightly curves rods	*Pseudomonas* sp
Dull and dry medusa head shape cream colonies	-	+	-	Gram positive rods in short chains	*Bacillus* sp
Small smooth moist and shiny low convex yellow colonies on Nutrient Agar	-	-	-	Cocci predominantly in tetrads and few in pairs and irregular	*Micrococcus* sp
Orange moist and shiny colonies on Nutrient Agar	-	-	-	Cocci predominantly in tetrads and few in pairs and irregular	*Micrococcus* sp

The isolates were further characterized and identified by few biochemical test and their ability to ferment some sugars as shown in Table 3. The percentage distribution of bacteria isolated from the water samples is shown in Table 4. *Staphylococcus aureus* and *Enterococcus faecalis* were predominant bacterial isolates while *Klebsiella* sp, *Shigella* sp, *Enterobacter* sp and *Escherichia coli* were among the least bacteria isolated. The distribution of the bacterial isolates across different sample locations is shown in Table 5, while the antibiotic sensitivity test results in Table 6 shows the effectiveness of the drugs against the test organisms except *Escherichia coli* that resisted erythromycin, amoxicillin, streptomycin and chloramphenicol.

Table 3: Biochemical and sugar Fermentation of Bacterial isolates.

Cat	Oxi	Coag	IN	MR	VP	Cit	Urs	NO_3	Glu	Suc	Lac	Mal	Xyl	Identity of isolates
+	-	-	-	-	+	-	+	+	+	+	+	+	-	*Staphylococcus aureus*
+	-	-	-	-	+	+	-	+	+	-	-	-	-	*Bacillus cereus*
+	+	-	-	+	-	+	+	+	+	-	-	-	+	*Pseudomonas aeruginosa*
-	-	-	-	+	-	+	-	-	+	+	+	-	-	*Enterococcus faecalis*
+	-	-	-	+	-	+	-	+	-	-	-	-	-	*Micrococcus luteus*
+	-	-	+	-	+	-	+	+	+	+	+	+	-	*Escherichia coli*
+	-	-	-	-	+	+	-	+	+	-	-	-	-	*Micrococcus roseus*
+	-	-	-	-	+	+	-	+	+	-	+	-	+	*Klebsiella* sp
+	-	-	-	+	-	-	-	+	+	+	+	-	+	*Enterobacter* sp

Cat, catalase; Oxi, oxidase; Coag, coagulase; IN, indole; MR, methyl red; VP, vogesproskaeur, Cit, citrate, Urs, urease production; No_3; nitrate reduction; Glu, glucose; Suc, sucrose; Lac, lactose; Mal, maltose; Xyl, xylose.

Table 4: Percentage occurrence of Bacterial Isolates.

Bacteria	% occurrence
Staphylococcus aureus	23.1
Micrococcus luteus	7.9
Bacillus cereus	10.4
Shigella sp	3.6
Pseudomonas aeruginosa	10.5
Enterococcus faecalis	19.5
Micrococcus roseus	5.8
Escherichia coli	7.2
Klebsiella sp	4.0
Enterobacter sp	3.3
Corynebacterium sp	4.7

Table 5: Distribution of Bacteria in the samples.

Samples	Bacteria
A	*Staphylococcus aureus, Enterococcus faecalis, Bacillus cereus, Pseudomonas aeruginosa*
B	*Staphylococcus aureus, Micrococcus luteus*
C	*Staphylococcus aureus, Enterococcus faecalis, Bacillus cereus, Pseudomonas aeruginosa*
D	*Staphylococcus aureus, Enterococcus faecalis, Micrococcus luteus, Pseudomonas aeruginosa,*
E	*Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa*
F	*Staphylococcus aureus, Enterococcus faecalis, E. coli Pseudomonas aeruginosa, Micrococcus roseus*
G	*Staphylococcus aureus, Enterococcus faecalis, Bacillus cereus, Pseudomonas aeruginosa*
H	*Staphylococcus aureus, Enterococcus faecalis, Micrococcus luteus,*
I	*Staphylococcus aureus, Enterococcus faecalis, Klebsiella* sp
J	*Staphylococcus aureus, Enterococcus faecalis, Micrococcus luteus, Micrococcus roseus*
K	*Staphylococcus aureus, Enterococcus faecalis, Micrococcus luteus*
L	*Staphylococcus aureus, Enterococcus faecalis, Micrococcus luteus, Pseudomonas aeruginosa*
M	*Staphylococcus aureus, Enterococcus faecalis, Bacillus cereus, Micrococcus luteus*
N	*Staphylococcus aureus, Enterococcus faecalis, Bacillus cereus, E. coli*
O	*Staphylococcus aureus, Enterococcus faecalis, Bacillus cereus, Pseudomonas aeruginosa, E. coli*

Table 6: Antibiotic susceptibility test of bacterial isolates (ZOI mm).

Bacterial isolates	Gen	Pef	Cot	CPX	Ery	AMX	Ofl	Str	Chl
Staphylococcus aureus	0	16	18	16	0	8	14	0	0
Pseudomonas aeruginosa	10	12	18	14	8	10	14	8	0
Bacillus cereus	0	10	14	14	0	0	12	0	0
Corynebacterium sp	0	18	16	14	0	0	14	0	0
Enterococcus faecalis	14	14	12	16	0	12	18	0	0
Klebsiella sp	0	12	12	8	8	12	16	0	0
Escherichia coli	0	14	12	14	0	0	18	0	0
Enterobacter sp	12	10	12	8	0	14	10	0	0

Gen, Gentamycin; Pef, Pefloxacin; Cot, Cotrimoxazole; Cpx, Ciprofloxacin; Ery, Erythromycin; Amx, Amoxicillin; Ofl, Ofloxacin; Str, Streptomycin; Chl, Chloramphenicol.

DISCUSSION

The results showed that packaged drinking water (pure water) is contaminated with heterotrophic bacteria such as *E. coli*, *S. aureus*, *P. aeruginosa*, *B. cereus*, *M. luteus*, *Corynebacterium* sp, *Ent. faecalis*, *M. roseus* and *Klebsiella* sp. The confirmed bacteria are notable soil and water borne organisms (Perry and Staley, 1997; Prescott *et al*., 2002). Their presence in the water indicates contamination that result from poor purification and treatment facilities in addition to inadequate hygiene practices (Prescott *et al*., 2002). Although, bacteria such as *Klebsiella* sp are natural inhabitants of many water environments and may multiply to high numbers in waters (Prescott *et al*., 2002; Pelczar, *et al.*, 2002) these pathogens may be naturally present in the environment but are generally not harmful to individuals with good immune systems but may be able to cause diseases in people with impaired immune defense mechanisms like the elderly and young, patients with burns or extensive wounds, people undergoing immunosuppressive therapy or with AIDs (Prescott *et al*., 2002). Water containing good amount and number of these organisms can produce infections of the throat, oral cavity, gastroenteritis, tonsillitis and mucous membranes. Notable examples are *Pseudomonas aeruginosa*, *Klebsiella pneumoniae, Staphylococcus aureus, Escherichia coli and Shigella dysenteriae* (Perry and Staley, 1997; Prescott *et al*., 2002; Pelczar, *et al.*, 2002).

An independent study conducted by Ekwunife *et al.* (2010) reported that the contamination of packaged drinking water may be associated with problems resulting from inadequate filtration and purification procedures, malfunctioning of equipment and poor quality control system.

Results obtained deduced that some sachet water companies failed to meet the WHO drinking water standard which affirmed that: "Drinking water should not contain any microorganism known to be pathogenic or any bacteria indicative of faecal pollution" (WHO, 1993). The result of the antibiotic susceptibility test also shows that all the biofilm producing bacteria found in the water samples have 100% resistance to chloramphenicol, 99% to streptomycin, 98% to erythromycin and 90% to gentamycin with *Bacillus cereus, Corynebacterium* sp and *Enterococcus faecalis* having the highest percentage of resistance.

Regular surveillance of drinking water producing companies should be stepped up by regulatory agencies to check quality, while equipment sanitation and turn around maintenance should be advocated by the monitoring agencies. In addition, zero tolerance of microorganisms in drinking water should be maintained by producers. To control the infection caused by these biofilms, the need for novel drug delivery approaches and enhanced therapeutic use of quorum sensing inhibitors is recommended.

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