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Antibiotic Susceptibility Patterns of Bacteria Isolated from Sachet-packaged Water Sold in Uyo Metropolis, Akwa Ibom State, Nigeria

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Authors' contributions

This work was carried out in collaboration among all authors. Author UEA designed the study, performed the statistical analysis, wrote the protocol. Author USU managed the analyses of the study and wrote the first draft of the manuscript. Authors MC and EEO managed the literature searches and final draft of the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was aimed at determining the antibiotic susceptibility patterns of bacteria isolated from sachet water sold in Uyo metropolis, Akwa Ibom State, Nigeria.
Study Design: Sachet water was randomly sampled in Uyo Metropolis.
Place and Duration of Study: Department of Microbiology, Akwa Ibom State University, Nigeria, between June and November 2018.
Methodology: Six different brands of sachets water sold and consumed in Uyo metropolis were studied for their physical and microbiological qualities. Thirty (30) sachets water from the six (6) different brands respectively, were serially diluted and cultured on Nutrient agar, Eosin Methylene Blue agar, MacConkey agar and Salmonella-Shigella agar, while Muller Hinton agar was used for sensitivity test. Suspensions of purified isolates were standardized with 0.5 McFarland turbidity standard and were subjected to antibiotics susceptibility testing using Agar Diffusion method.
Results: The bacterial counts obtained ranged from 2.0 x 10^1 cfu/ml to 1.34 x 10^2 cfu/ml. Species

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isolated from the samples analysed included: *Klebsiella* sp., *Escherichia* sp., *Staphylococcus* sp., *Salmonella* sp., *Pseudomonas* sp., *Citrobacter* sp., *Bacillus* sp. *Bacillus* sp. Was susceptible to all the antibiotics tested against it except streptomycin while *Staphylococcus* sp was resistant to gentamicine and ampiclox but susceptible to other antibiotics. All the gram negative isolates were susceptible to tarivia and peflacine but completely resistant to nalidixic acid. Klebsiella sp. was most resistant (70%) of all the isolates, these was closely followed bt *Escherichia* sp. and *Salmonella* sp. at 60% resistance. Some of the sachet water brands from bacteriological standpoints did not meet the World Health Organization Standard for portable water.

Conclusion: This study indicted sub-standard packaged waters as a vehicle for the spread of antibiotic resistant bacterial pathogens, and this poses a high risk to public health. Hence, routine monitoring of producers of sachet water should been enforced.

Keywords: Sachet water; antibiotics resistance; Uyo metropolis; water standards.

1. INTRODUCTION

The safety and quality of drinking water have become a public health concern all over world. In Nigeria, high demand for safe drinking water cannot be overemphasized considering the inability of the government to provide adequate pipeborne water to the general public. Water is known to be the dwelling place for many bacterial species and other microorganisms which cause a variety of waterborne infections [1]. World Health Organization (WHO) estimated that 1.1 billion of the world's population does not have access to safe water. In addition to this, 80% of diseases and one-third of deaths in developing countries are due to consumption of contaminated water [2]. The associated health risks from the consumption of unsafe drinking water vary throughout the world depending on the chemical or microbiological contaminants present in the environment [3]. Many of the bacteria isolated in water distribution systems are opportunistic pathogens. The presence of high numbers of opportunistic pathogens in drinking water is of concern because these microorganisms can cause infection in certain segments of the population (newborn babies, the sick, and the elderly) [4]. According to the guideline set by the World Health Organisation, quality drinking water must not contain *Escherichia coli* or thermotolerant coliform bacteria, *Giardia,* eggs of worms, viruses, *Cryptosporidium* spp*, Legionella pneumophila, Entamoeba hystolitica* and other opportunistic pathogens such as *Clostridium* sp*ecies, Klebsiella* species and *Pseudomonas* [2]. The guideline further stated that the water should be tested against the presence of highly virulent pathogens such as *Salmonella typhi, Shigella dysenteriae* and *Vibrio cholerea* that are responsible for typhoid fever, bacillary dysentery and cholera diseases respectively. All the aforementioned bacterial species must not exist in water that is meant for drinking, hence, sources of water for packaged water are usually subjected to laboratory test by public analysts. It is expected that bacteria must not be found or detected in any 100 mL water sample. "Sachet water is not sterile" according to Linda [3]. Although, sachet water is assumed to be free from certain pathogens during treatment processes, presence of certain organisms are used to confirm the sterility of the water such as coliforms which act as indicator organisms used to assess the safety of water and thus give an idea of the degree of contamination associated with intake of such sachet water [4,5]. Antibiotics have revolutionized human medicine diversely, saving many lives because it has a major impact on the rate of survival of pathogens from infection. But with this great and remarkable benefit, it is sad that it is also the bedrock of many other diseases due to their resistance strains. Recently, major bacterial pathogens are becoming resistant to antibiotics, and these changing patterns caused a demand for new antibacterial agents. Antimicrobial resistance occurs when bacteria adjust or adapt in ways that permit them to stay alive in the presence of antibiotics designed to kill them. Bacteria evolve resistance to these drugs, typically by acquiring chromosomal mutations and multidrug resistant plasmids which has become a public health concern [6,7,8]. Antibiotics were formally defined to distinguish them as biochemicals produced by microorganism from the organic chemicals synthesized in the laboratory. But due to recent development, the distinction between both is no longer meaningful due to the fact that the biochemical structures of many naturally occurring antibiotics are now being synthesized by organic chemists and currently, many antibiotics used in medicine are in the chemically modified forms of the microbial biosynthetic forms [9].

Antibiotic resistance occurs when the sensitivity of an organism decreases against an antibiotic when compared to officially available breakpoints, usually measured as a decrease in "inhibition zone diameter". The increased use of antibiotics is often associated with increased resistance of bacteria to these chemicals, especially in the hospital setting [10]. A lot of transmissible diseases are waterborne. Many harmful microbial contaminants have been confirmed to be associated with potable water sources. Many people have resorted to patronizing sachet water with the belief that it is 'pure' - hence, fondly called 'pure water'. It is possible that this so called pure water is not pure after all; hence it may harbour harmful microorganisms as producers of such water may not pay adequate attention to microbiological quality. Identification of the major harmful microbial contaminants (*Escherichia coli, Salmonella, Shigella*, etc.) present in the sachet water is important in assessing its safety. Free from contamination with faecal matter is the most important parameter for determining water quality because human faecal matter is generally considered to be a greater risk to human health as it is more likely to contain enteric pathogens [11]. There is need to constantly assess the quality of water sources available to members of any community at intervals. This will help monitor and prevent the sudden outbreak of waterborne infections. It is also important to know the antibiotics susceptibility pattern of microorganism common in an environment in case of any outbreak. This research was borne as a result of the widespread use of sachet water in Nigeria especially in Akwa Ibom State, conflicting results on the safety conducted at different locations in the country and lack of data on safety of sachet water locally available. This research was aimed at determining the antibiotic resistant pattern of bacterial isolates obtained from sachet water by testing them against some of the commonly used antibiotics.

2. MATERIALS AND METHODS

2.1Study Area

Three major areas in Uyo metropolis, Akwa Ibom State where strategically selected for this study. The areas comprised of towns where sachet-packaged drinking water is sold by hawkers. They included: Abak road, Aka road and Oron road.

2.2 Sample Collections

A total of thirty (30) sachet water of six different brands was collected randomly from various parts of Uyo metropolis in Akwa Ibom state and taken to the laboratory (Department of Microbiology, Akwa Ibom State University) for analysis. The samples were coded as; BC, GO, FD, RS, ML, and CV to reflect the respective brands. They were collected and transported in clean ice-parked containers and stored at 4.0^{o}C for 30-60 minutes to maintain the properties of the samples before commencement of analysis. Hygienic and aseptic techniques were applied during sampling of the sachet water.

2.3 Determination of Bacterial Loads of the Water Samples

2.3.1 Preparation of the samples

Using aseptic method, six (6) different beakers were labelled according to the 6 different brands of waters. Five sachets were mixed from each brand to obtain 100 ml homogenous sample in the beaker.

2.3.2 Pour plating method

One milliliter of appropriate dilutions (10^{-1} to 10^{-3}) was aseptically pipetted into sterile, labelled petri dishes in duplicates. Appropriate medium (Nutrient agar, Eosin Methylene Blue, MacConkey agar, Salmonella-Shigella Agar) at 45ºC were poured aseptically into the inoculated petri dishes and swirled gently to mix. They were inversely incubated at 37ºC for 24-48hours. At the end of the incubation period, colonies were counted and the counts for each plate expressed as colony forming units per millilitre (cfu/mL) of the sample inoculated.

Nutrient agar (NA) was used to determine the total viable bacterial Count, Eosin Methylene Blue agar (EMB) to enumerate *Escherichia coli*, MacConkey agar (MAC) for coliform count and Salmonella-Shigella agar (SSA) for the determination of *Salmonella* and *Shigella* counts. Culture media were prepared according to the respective Manufacturers specification and sterilized in an autoclave at 121ºC at 15 psi for 15 minutes.

2.3.3 Purification of colonies

Using a fresh nutrient agar medium, 24 hours colonies were picked using a sterile wire loop and streaked on its surface and incubated for 24

hours at 37ºC to obtain pure colonies. After incubation, discrete growths were observed on the lines of streak. Distinct colony was picked aseptically and cultured on a fresh nutrient agar slant and incubated for 24hours at 37ºC and stored in a refrigerator at 4ºC. The routine laboratory method of Cruickshank et al. [12] was used to characterize different isolates. The isolates were identified using their macroscopic, cultural, physiological and biochemical characteristics.

2.4 Morphological Characterization (Gram's reaction)

Gram staining was carried out as described by Olutiola et al. [13]. Pure colonies of each bacterial isolate was observed for morphological features using Bergey's Manual of Determinative Bacteriology as a standard for comparison. Cell shape was determined under X100 objective of the light microscope after Gram staining procedure. Bacterial smear was prepared on the slide using an inoculation loop. This was done by introducing a drop of distilled water on grease-free labelled slide followed by the sample and then smeared, air dried and heat fixed. The slide was flooded with crystal violet staining reagent for about 60 seconds, then washed using a gentle indirect stream of tap water for about 2 seconds. The slide was flooded with a mordant (Lugol's iodine) for 15-30seconds. The slide was decolorized using 70% ethanol for 10 seconds and washed off. Lastly, the slide was flooded with 0.5% counter stain (safranin) for 30 seconds, and then washed using indirect stream of tap water and air dried. A drop of immersion oil was dropped on the stained sample and observed under the microscope.

2.5 Biochemical Characterization and Identification of Isolates

Pure cultures of bacterial isolates were subjected to various biochemical tests according to standard techniques described by Olutiola et al. [13] Biochemical tests carried out include; Catalase test, Coagulase test, Indole test, Oxidase test, Citrate test, Fermentation of glucose, lactose, sucrose, maltose and mannitol [14]. Bacterial isolates were identified according to Bergey's Manual of Determinative Bacteriology [15].

2.6 Antimicrobial Sensitivity Testing

Commercially available antibiotic impregnated 8mm sensitivity discs (Abtek Biological Ltd, UK) were used to determine the drug sensitivity profile of the isolates. Seventeen different antibiotic discs comprising of Tarivid (OFX), Nalidixic acid (NA), Peflacine (PEF), Gentamycin (CN), Augumentin (AU), Ciproflox (CPX), Septrin (SXT), Ceporek (CEP), Streptomycin (S), Ampicillin (PN) for Gram negative and Levoxin (Lev), Amoxicillin (Amx), Norfloxacin (NB), Chloramphenicol (CH), Erythromycin (E), Ampiclox (APX), Rifampin (RD), Streptomycin (S), Ciproflox (CPX), Gentamycin (CN) for Gram positive organisms . The antimicrobial sensitivity test of each isolate was carried out as described by the Kirby –Bauer disc diffusion method as recommended by the National Committee for Clinical Laboratory Standards [16].

Procedures: The turbidity of the bacterial suspensions was compared with 0.5 Macfarland's standard by inoculating the organism into 10ml peptone water and incubate. The standardized bacterial suspension was then inoculated on to Muller Hinton Agar and left to dry for 10 minutes, before placing the antimicrobial sensitivity discs. After incubation, the diameter of the zone of inhibition were measured and compared with zone diameter of interpretative chart [17,18] to determine the sensitivity of the isolates to antibiotics.

3. RESULTS

All the water samples collected and analyzed were National Agency for Food and Drug Administration and Control (NAFDAC) approved and had factory addresses on them (Table 1). They were all odourless, colourless and clear in appearance; had no batch number, also none had production and expiration dates meaning that the duration between production and consumption cannot be determined. Only FD contained little particles in it. All were the same net volume of 50 cl.

Table 2 shows the Total viable count (TVC) after 48 hours of water samples on different media. All the water samples were contaminated with bacteria. A higher value of TVC on Nutrient agar (NA) was $1.34X10^2$ cfu/ml from sample FD, Eosin Methylene Blue agar (EMB) plate was $3.10X10^1$ cfu/ml from sample ML, MacConkey agar (MAC) plate was $2.50x10^1$ cfu/ml from sample ML and on Salmonella Shigella agar (SSA) plate it was $0.5x10^1$ cfu/ml from sample FD. The highest number of organisms (on all the media) was $1.34X10^2$ cfu/ml in FD sachet water and the lowest was $2.5X10^1$ cfu/ml in CV sachet water.

Out of 29 bacterial isolates, seven (7) distinct isolates were obtained while others where replicates of the seven. *Klebsiella* sp. had the highest frequency showing seven (7) out of 29 representing 24.14%, followed by both *Staphylococcus* sp. *and Psuedomonas* sp. with the frequency of five (5) out of 29 isolates representing 17.24%. Other bacteria isolated included; *Escherichia* sp. with the frequency of four (4) out of 29 representing 13.79%, *Salmonella* sp. and *Citobacter sp*. with frequency of 3 out of 29 representing 10.34% and *Bacillus* sp. with the least frequency two (2) out 29 representing 6.90% as shown in Fig. 1.

Six brands of sachet water were analyzed and a total of seven bacterial isolates were identified from the sachet water samples. The isolates were initially differentiated on the basis of the cultural and morphological characteristics after which they were subjected to various biochemical tests. These tests revealed their probable identity as *Klebsiella* sp., *Escherichia* sp., *Staphylococcus* sp., *Salmonella* sp., *Pseudomonas* sp., *Citrobacter* sp., *Bacillus* sp.

Table 1. Physical examination of the sampled sachet water brands sold in uyo metropolis for compliance. Table pattern according to dada, 2009

Sample code	Nafdac	Production./ best fore date	Producers' name & address	Colour	Appear-ance	Odour	Floating particles	Batch no:	Net volume
BC	+	_	+	_	_	_	None	_	50CL
FD	+	_	+	_	_	_	Few	_	50CL
RS	+	_	+	_	_	_	None	_	50CL
CV	+	_	+	_	_	_	None	_	50CL
ML	+	_	+	_	_	_	None	_	50CL
GO	+	_	+	_	_	_	None	_	50CL

Key: +: displayed on sample sachet; -: not displayed on sample sachet

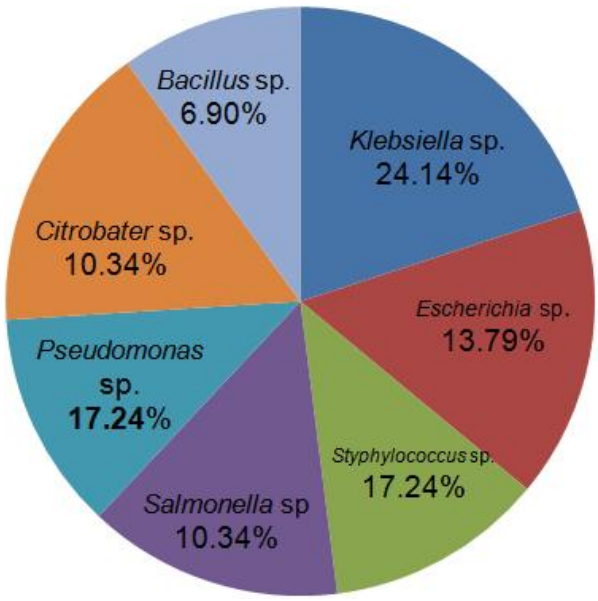

Fig. 1. Percentage frequency of bacteria isolates obtained from sachet water sold in Uyo metropolis

Table 2. Total viable count (TVC) after 48hours of culturing sachet water samples on different media

Sample/ Media	Total viable count (cfu/mL)	EMB	Total coliform count (cfu/mL)	SSA
BC	1.10×10^2	3.0×10^1	1.5×10^1	0
FD	1.34×10^2	2.9×10^1	0.9×10^1	0.5×10^1
RS	7.0×10^1	0.8×10^1	1.4×10^1	0
CV	2.5×10^1	3.1×10^1	2.0×10^1	0
ML	2.0×10^1	4.5×10^1	2.5×10^1	0.2×10^1
GO	1.18×10^2	1.8×10^1	1.2×10^1	0.1×10^1

key: NA: Nutrient Agar; EMB: Eosin Methylene blue agar; MAC: MacConkey agar; SSA: Salmonela Shigella Agar

Klebsiella sp. was most resistant to NA, CN, AU, CPX, S, PN, CEP (70%), followed by *Escherichia* sp and *Salmonella* sp. *Escherichia* sp was resistant to 6 (NA, CN, AU, SXT, S, PN and CEP) out of the 10 antibiotics tested against it. Same number of antibiotic resistance was recorded for *Salmonella* sp. (NA, CN, AU, S, PN and CEP). The least resistant gram negative isolate was *Citrobacter* sp. (NA, CPX, S, and PN) and *Pseudomonas* sp. All the Gram negative isolates were resistant to PN and NA. The Gram positive organisms were less resistant to all the antibiotics they were exposed to. *Bacillus* sp. was resistant to only ciproflox while *Staphylococcus* sp. was resistant to amoxicillin and Gentamycin Table 3.

4. DISCUSSION

This study was carried out to determine the bacteriological quality and the antibiotics susceptibility pattern of the bacterial isolates from sachet water sold in Uyo with the view of creating public health awareness concerning drinking such water. In Nigeria, sachet water is largely taken and they are obtained either from surface or underground sources, and are subjected to various treatment to make it fit for human consumption, but unfortunately, most of them still fall below the WHO standard from the physical and microbiological analysis [19]. From this analysis, one (1) out of six water samples had particles in it. Meanwhile, all the samples collected were odourless, colourless, and registered with NAFDAC. Bacterial occurrence was recorded in all the sachet-water samples and the TVC for some were higher than what is acceptable for drinking water (1.0×10^1 cfu/ml) [20].

The presence of pathogenic bacteria was recorded which is above the WHO standard for potable water [4]. High occurrence of *Klebsiella* sp. was recorded, followed by *Staphylococcus* sp. Others included *Pseudomonas* sp, *Escherichia* sp., *Salmonella* sp, *Citrobacter* sp. and the least frequent was *Bacillus* sp. Total Viable Count on EMB and MAC for coliform bacteria and the various values obtained for each water sample signified possible faecal contamination. This indicates that the sachet-water samples were contaminated especially with faecal materials, and are therefore not safe for drinking. Presence of coliforms (*Escherichia* sp. and *Klebsiella* sp. and Citrobacter sp.) maybe that some of the water were prepared from shallow and contaminated boreholes. Most of these bacteria are indigenous to aquatic environments [20]. The occurrence of *Salmonella* in the water samples could be as a result is also as a result of contaminated water and improper treatment; *Pseudomonas* sp. were also found in the water samples analyzed and are considered opportunistic pathogens and *Staphylococcus* sp. isolated from the water samples may have entered the water during packaging or handling since the organism is a normal flora of the human skin [21]. The ingestion of these bacteria with contaminated water constitutes public health risks to the immunocompromised members of the population, especially newborn babies, elderly and sick [22]. The presence of relatively heavy load of bacteria in water packaged for drinking purposes has been previously documented in literature [23,24,25,26]. The result of the antibiotics susceptibility testing showed various percentages of antibiotic resistance among the bacterial isolates from packaged water samples. *Escherichia* sp. was highly resistant to six (6) antibiotics and sensitive to only four antibiotics which were; Tarivia (OFX), Gentamycin (CN), Peflacine (PEF) and Ciproflox (CPX). *Klebsiella* sp. was resistant to seven (7) antibiotics and sensitive to Tarivia (OFX), Peflacine (PEF) and Septrin (SXT). *Bacillus sp.* was sensitive to all antibiotics tested and resistant to only Streptomycin (S). *Staphylococcus* sp. was also highly sensitive to all the antibiotics except Amoxicillin (AMX) and Gentamycin (CN). *Pseudomonas* sp. was also sensitive to most antibiotics except Nalidixic acid (NA), Augumentin (AU), Ampicillin (PN) and Ceporek (CEP). *Citrobacter sp.* was sensitive to the antibiotics and resistant to only four antibiotics, namely: Nalidixic acid (NA), Septrin (SXT), Streptomycin (S), Ampicillin (PN). *Salmonella sp.* was highly resistant to all the antibiotics except four; Tarivid (OFX), Peflacine(PEF), Ciproflox (CPX) and Septrin (SXT). Generally most of the isolates were resistant to Amoxil, Ceporex, Augmentin, Ampicillin, Nalidixic acid and Stretomycin. The resistance exhibited by *Pseudomonas aeruginosa and E. coli* to some of the antibiotics corroborates earlier report from South Eastern Nigeria [27]. The presence of the same type of enteric bacteria in almost all brands shows common source of contamination. It is documented that bacteria habour series of antibiotic resistant genes which can be transferred to others horizontally [28].

Therefore, from observation made from this study, a lot of sachet water producers and sellers

Table 3. Antibiotics susceptibility pattern of bacterial isolate from sachet water sold in uyo metropolis

		Gram Positive Isolates										Gram Negative Isolates										
S/N	**Isolate**	**AMX**	**S**	**NB**	**CPX**	**CH**	**E**	**LEV**	**CN**	**APX**	**RD**	**OFX**	**NA**	**PEF**	**CN**	**AU**	**CPX**	**SXT**	**S**	**PN**	**CEP**	**% Resistance**
1	*Escherichia* sp.											S	R	S	S	R	S	R	R	R	R	60
2	*Klebsiella* sp											S	R	S	R	R	R	S	R	R	R	70
3	*Bacillus* sp.	S	R	S	S	S	S	S	S	S	S											10
4	*S. aureus*	R	S	S	S	S	S	S	R	S	S											20
5	*Pseudomonas* sp.											S	R	S	S	R	S	S	S	R	R	40
6	*Citrobacter* sp.											S	R	S	S	S	S	R	R	R	S	40
7	*Salmonella* sp.											S	R	S	R	R	S	S	R	R	R	60

Key: Tarivid (OFX), Nalidixic acid (NA), Peflacine (PEF), Gentamycin (CN), Augumentin (AU), Ciproflox (CPX), Septrin (SXT), Ceporek (CEP), Streptomycin(S), Ampicillin(PN) for Gram negative and Levoxin (Lev), Amoxicillin (Amx), Norfloxacin (NB), Chloramphenicol (CH), Erythromycine (E), Ampiclox (APX), Rifampin (RD), Streptomycin (S), Ciproflox (CPX), Gentamycin (CN)

have emerged making it their major source of income. With this, appropriate health authorities should ensure that producers comply with the government regulations since some of these packaged water may have been produced under unhygienic conditions. Water can be seen as one of the most important, as well as one of the most abundant of those compounds and it is particularly, vital to living organisms [29]. Also, water is like the life wire of the body and as the basis of life; it is a critical part of human diet. Water constitutes about 90% by weight of the human body [30]. So, water should be treated and the necessary biochemical and microbiological test should be carried out to protect the general public from water-borne disease outbreak.

5. CONCLUSION

This study revealed that bacteriological quality of the sachet water brands sold failed to meet the standards for drinking water, even though the bacterial load did not exceed the allowable limits of microbial load. However, the bulk of sachet water brands were contaminated by coliform bacteria. It is therefore necessary for sachet water brands to be properly treated and handled to meet the WHO standard for drinking water. To minimise the problem of poor quality of sachet water, government agencies like the NAFDAC and the Environmental Protection Agency should ensure that packaged water manufacturers comply with good manufacturing practices. It is a serious threat to the people of the area if proper measurements are not taken by the concerned authorities. The water sources were contaminated with *Klebsiella* sp*., Escherichia* sp*., Staphylococcus* sp*., Salmonella* sp*., Pseudomonas* sp*., Citrobacter* sp*.,* and *Bacillus* sp. thus posing a very serious threats to the society. Antibiotic resistance is considered a major problem because many disease causing bacteria are becoming more resistant to the commonly used antibiotics. *Klebsiella* sp*., Escherichia* sp*., Citrobacter* sp. isolated from the samples, showed greater antibiotic resistances. The overuse and misuse of antibiotics can create the conditions for the development of antibiotic resistant bacteria.

6. RECOMMENDATION

There is need for NAFDAC to intensify efforts in the routine monitoring of activities in the packaged drinking water industries ensuring the safety of sachet drinking water through comprehensive regulatory programs at both the federal and state levels. Also, sample collection and testing of market samples will be a good way of detecting if the water is truly 'pure' as claimed by these producing companies. High emphasis should also be placed on enforcing compliance with Good Manufacturing Practice (GMP) with emphasis on management of raw water source to the consumer product point. Hence, routine monitoring of producers of sachet water should be enforced to ensure adherence to drinking water standards.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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