British Microbiology Research Journal
12(4): 1-9, 2016, Article no.BMRJ.16291
ISSN: 2231-0886, NLM ID: 101608140

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Microbial Evaluation and Antibiotic Susceptibility Profile of Isolates of Popular Sachet Water Brands Sold in Anambra State

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Authors' contributions

This work was carried out in collaboration between all authors. Authors OAL and COU designed the study, performed the statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed literature searches. Authors NIM, KUC and OEC managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2016/16291
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Complete Peer review History: http://sciencedomain.org/review-history/12880

Original Research Article

Received 22nd January 2015
Accepted 9th December 2015
Published 4th January 2016

ABSTRACT

Aims: To determine the microbiological quality of 5 brands of sachet water popularly consumed in Anambra state.
Study Design: To validate the level of water treatment, and determine the type of bacteria and fungi contaminants, level of contamination and presence of resistant pathogens in the sachet water brands.
Place and Duration of Study: Samples were collected based on the popularity and availability from different cities (Awka, Onitsha and Nnewi) in Anambra state. Analyses of the samples were

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conducted in the Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Awka, and National Food and Drug Administration and Control (NAFDAC), Nigeria. Both the sampling and analyses were done between April 2013 and May 2013.
Methodology: 5 sachets of each of the sachet water brands were purchased from strategic locations in Anambra State and they were analysed using Heterotrophic Plate Count (HPC), total and fecal coliform count (TCC & FCC) and antibiotic susceptibility testing.
Results: All the brands passed the HPC test. Brand 4 failed the TCC & FCC test, as the fecal coliform, *E. coli* was detected. *Aspergillus niger* was the most frequently encountered fungi with an occurrence rate of 100%. *Staphylococcus aureus* was the most frequently isolated bacteria with an occurrence rate of 60%. 53% of the isolated microorganisms are multi-drug resistant.
Conclusion: Sachet water sold in Anambra state is of commendable microbial quality as four of the five brands passed the TCC & FCC test. However, some organisms isolated are multi-drug resistant and they can transfer their resistance to potentially virulent microbes.

Keywords: Potable drinking water; microbiological quality; multi-drug resistant.

1. INTRODUCTION

Ensuring the availability of safe good quality drinking water is still a problem in Nigeria and other parts of the world. 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 environs. Waterborne diseases can be deadly especially in children.

Safe drinking water can be defined as having acceptable quality in terms of its physical, chemical, and microbiological parameters so that it can be safely used for drinking and cooking [1]. The absence of safe drinking water leads to a multitude of diseases (termed water borne diseases) including cholera, bacillary dysentery, hepatitis, polio, schistosomiasis, among others [1-3], with cholera causing about 50% of all diarrheal cases [1]. According to Federal Ministry of Health statistics, only about 30% of Nigerians have access to potable water [4]. As a result, the ever increasing populace has resorted to the sachet water popularly called “pure water” [5,6].

Water sources used for drinking purposes include: streams, ground water, and springs, among others. Of these, groundwater is the most common source of drinking water especially in the tropics [7].

Source water used in sachet water production has to be treated in order to make it potable for human consumption. The main objective of a water treatment system therefore, is to take water from the best available source and to subject it to processing to ensure that is safe for human consumption (potable) and aesthetically acceptable to consumers [8]. Some treatment processes that can be employed include filtration, chlorination, ozonation, UV disinfection, among others. In general, the United States Environmental Protection Agency (US EPA), recommends a multi-barrier treatment approach which includes filtration and disinfection, bearing in mind that no particular treatment process can handle all forms of contaminants [9].

Sachet water is not sterile. Its assumed safety comes from the fact that certain harmful pathogens have supposedly been removed by the treatment processes. Identification of the major harmful microbial contaminants (*Escherichia coli, Salmonella, Shigella*, etc.) present in the sachet water is important in assessing its safety. Since it is practically impossible to identify all pathogens in a sachet water sample, a selected number of micro-organisms have been selected to serve as indicators of water safety and they include the coliforms, streptococci from stool, among others [10]. These indicators are used to assess the safety of water and thus give an idea of the degree of contamination associated with intake of such sachet water.

Antibiotic resistance can be said to occur when an organism shows a decreased sensitivity, usually measured as a decrease in “inhibition zone diameter”, against an antibiotic when compared to officially available breakpoints. The mechanism of antibiotic resistance can either be genetic or biochemical. Bisht et al. [11], identified several factors that contribute to the occurrence of resistance such as; incorrect use of antibiotics, patient-related factors, hospital prescriptions practices, veterinary prescription practices, use of monotherapy, commercial promotion, over-the-counter sale of antibiotics,

under use of microbiological testing and globalization.

This research was borne as a result of the widespread use of sachet water in Nigeria, conflicting results on the safety conducted at different locations in the country and lack of data on safety of sachet water locally available in Anambra state. In Anambra state, seasonal increase in the number of children admitted in the hospital for food and water-borne infections do occur year-in-year-out, especially during the dry season. Therefore, assessing the safety of sachet water available in Anambra state by determining the efficiency of treatment approaches employed in its production, characterizing possible contaminants present and determining the antibiotic susceptibility profile of bacterial isolates, is important especially as it pertains to the vulnerable children population.

2. METHODOLOGY

2.1 Study Area

The study area is Anambra State located in the South-East region of Nigeria.

2.2 Preparation of Working Solutions

Equal volumes (30 ml) from 5 samples or sachets of each of the 5 sachet water brands were pooled together to make a combined water sample solution for each of the sachet water brands. The working solutions were sealed tightly and used for further studies.

2.3 Heterotrophic Plate Count

This was carried out using the single agar layer plate count technique [12]. 1 ml of each of the working solution was aseptically transferred using sterile syringes into sterile Petri dishes. 20 ml of molten agar cooled to 40℃ was poured aseptically into the same Petri dishes containing the samples, swirled and allowed to solidify. The Petri dishes were inverted and incubated. Peptone water agar (PWA), produced according to the protocol by was used for the bacterial heterotrophic plate count, Sabouraud dextrose agar (SDA) (LIFESAVE Biotech, USA) was used for the fungi heterotrophic plate count. The plates were incubated for 24 hrs at 37℃ for the inoculated PWA and 3 days at room temperature for inoculated SDA. After incubation the colonies were counted with a colony counter (0671M.JG.052, Medica Instrument MFG. CO., Mumbai).

2.4 Total and Fecal Coliform Count

This was carried out using the single agar layer plate count technique [12]. Here, 1 ml of each of the working solution of the various batches was aseptically transferred using sterile syringes into sterile Petri dishes. 20 ml of chromocult molten agar cooled to 40℃ was poured aseptically into the plates containing the samples swirled and allowed to solidify. This study was done in duplicate (1A and 1B etc). Petri dishes were inverted and incubated for 72 hr. The chromocult media is a differential media which gives a pink colour to coliforms except for *Escherichia coli* which appears as blue colonies.

2.5 Isolation of Bacteria and Fungi

The bacteria isolation was carried out using 3 different media namely: azide blood agar (azide from Lab. M. Ltd, UK), Levine EMB agar and nutrient agar (LIFESAVE Biotech, USA). It was done at two different time intervals employing two different techniques; the streak plate technique with the stock solutions of the samples and secondly, three weeks after the first test, using the pour-plate technique with a 10^{-4} dilution. This was to ensure that none of the organisms present were being inhibited by chlorine used in preservation of drinking sachet water or other preservation techniques. All inoculated plates were incubated for 24 hrs at 37℃.

The fungi isolation was performed by inoculating 1ml of each sample on Sabouraud dextrose agar using the streaking technique. The plates were incubated at room temperature for 5 days.

2.6 Identification of Fungi

The identification was done as described Watanabe [13] and Cheesbrough [14].

2.7 Identification of Bacteria

This was done using a sequential process of gram staining, spore staining and a set of biochemical tests including: catalase, coagulase, carbohydrate (lactose, glucose and mannitol (all from M&B, England)) fermentation, oxidase, and MR and VP tests [14,15]. Each characterized

microbe was transferred aseptically to nutrient agar slants. The agar slants were incubated at 37℃ for 24 hr after which they were stored in the refrigerator at a temperature of 4℃. All characterized isolates were standardized to McFarland's 0.5 turbidity standard prior to carrying out any microbiological assay.

The media (from section 2.3 to 2.7) with no mentioned manufacturer were prepared using their various component according to the protocol published in ASM microbe library [16].

2.8 Antibiotic Susceptibility Testing

The standardized nutrient broth cultures of the isolates were subsequently swabbed unto the surface of Mueller-Hinton agar in duplicate and the selected antibiotic discs (Ceftriaxone 30 µg, Gentamycin 10 µg, Erythromycin 30 µg, Levofloxacin 20 µg, Augumentin 30 µg, Ciprofloxacin 10 µg, Ampiclox 20 µg: Optundisc, Optun Laboratories Nigeria Ltd., Enugu, Nigeria) were aseptically placed on the surface of the agar. Pre-diffusion was allowed for 15 minutes, after which it was incubated for 24 hrs at 37℃.

3. RESULTS AND DISCUSSION

3.1 Heterotrophic Plate Count

Tables 1 and 2 show the average HPC of all water brands for both bacteria and fungi. All samples passed the HPC test based on the US EPA and UK standards as their HPC were below 500 cfu/ml and 100 cfu/ml respectively [17]. The samples also passed the WHO and NAFDAC standards with the limit of 100 cfu/ml [18,19]. This implies that the water treatment strategies employed at the sites of their production are efficient, with brand 5 being the most efficient and that of brand 1 being the least. Canadian drinking water guidelines has specified no maximum acceptable limit for HPC but rather advocated that an increase in the HPC values should be used to assess the treatment strategies [20]. This suggests that the heterotrophic plate counts should be done regularly in order to accurately assess the efficiency of the treatment strategies. There is no stated limit for HPC of fungi.

However, HPC alone cannot give an indication on the risk of the infection on the consumption of sachet water [20]. Other tests aimed at detection of certain indicator organisms are thus being used to confirm the safety of drinking water.

3.2 Total and Fecal Coliform Count

Table 3 shows the result of the total and fecal coliform count. Brands 1, 2, 3 and 5 passed the tests. Their values falls within the acceptable limits (0 cfu/ml for fecal coliform and 1 cfu/ml for total coliform) used in interpreting the test [17,19]. They are thus safe for drinking and will not cause any disease on consumption. However, brand 4 did not pass the test with an average of 5 cfu/ml count for *Escherichia coli*, an indication of fecal contamination of the drinking water either due to unsatisfactory treatment of source water or exogenous introduction during production. Enterotoxigenic *E. coli* is associated with the probably fatal diarrhoeal illness which is an important aspect of drinking water quality [21]. Fig. 1 shows the results of this experiment.

Table 1. Bacteria heterotrophic plate count

Sample	Average heterotrophic plate count (cfu/ml)
1	11
2	3
3	2
4	2
5	0
Negative control	0

Table 2. Fungi heterotrophic plate count

Sample	Average heterotrophic plate count (cfu/ml)
1	95
2	0
3	1
4	4
5	5
Negative control	0

Table 3. Total and fecal coliform count test

Samples	Total coliform count (CFU/ml)	Fecal coliform count (CFU/ml)
$\mathbf{1_A}$	0	0
$\mathbf{1_B}$	1	0
$\mathbf{2_A}$	0	0
$\mathbf{2_B}$	0	0
$\mathbf{3_A}$	0	0
$\mathbf{3_B}$	0	0
$\mathbf{4_A}$	3	3
$\mathbf{4_B}$	6	6
$\mathbf{5_A}$	0	0
$\mathbf{5_B}$	0	0

Fig. 1. Total and fecal coliform count tests, showing the blue and pink colonies

The blue colour signifies that coliforms are present in one sample from the second batch of sachet water, while pink colonies shows that *E. coli* is present in one of the samples of that same batch.

3.3 Isolation and Characterization of Fungi Isolates

Ten fungi isolates were obtained as shown in the Table 4. Three different fungi were identified, namely: *Aspergillus niger*, *Aspergillus fumigatus and Penicillium* spp. with frequencies of occurrence of 100%, 80% and 20% respectively.

Aspergilliosis is caused by *Aspergillus fumigatus* in 80% of all cases while *Aspergillus niger* appears as the causative agent in rare cases. However these organisms are not a health risk except in immune-compromised individuals [22]. Greater pro-active steps should therefore be used in treatment of sachet water. This study has joined a few of the studies that have recently reported the isolation of fungi from drinking water [23,24].

3.4 Isolation and Characterization of Bacteria Isolates

A total of fifteen isolates were obtained and where identified as six different species of microbe. These include: *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Bacillus cereus*, *Bacillus megaterium* and *Pseudomonas aeruginosa*. Their frequencies of occurrence are as shown in Table 5. The predominant bacteria found in water include; *Acinetobacter*, *Aeromonas*, *Enterobacter*, *Flavobacterium*, *Klebsiella*, *Pseudomonas* and *Bacillus* spp. etc [25]. All the identified isolates have been isolated previously [3,20]. In addition, all the isolates identified are normal heterotrophs found in man. They do not typically cause any gastro-intestinal infection, except under unique conditions like in vulnerable human population for example or in non-ingestion accidental or purposeful exposures to such water [26].

Staphylococcus aureus had the highest frequency of occurrence while *Pseudomonas aeruginosa & Staphylococcus epidermidis* had the least frequency of occurrence across the brands. *Staphylococcus aureus* may present a source of concern when sachet water is used for other purposes, apart from drinking, such as washing of open wounds. *Pseudomonas* is an opportunistic and emerging pathogen of foodborne and waterborne diseases. *Pseudomonas* has also been known to multiply abundantly in low nutrient water [25]; however, their acquisition from the environment, including water is responsible for a less significant number of healthcare-associated infections compared to

other forms of transmission like person-to-person contact for example. Furthermore, there has been a record of UTI cause by waterborne *Pseudomonas aeruginosa* in the community [25,26]. The occurrence of *Pseudomonas aeruginosa* in this study is low unlike a previous study [27]. *Bacillus cereus* has also been associated with "food poisoning" [14].

The TCC & FCC showed the presence of coliforms in batch 1 & 4, but these could not be isolated. Their quantity in the water brands is probably too infinitesimal to be isolated.

3.5 Antibiotic Susceptibility Testing of Bacteria Isolates

Table [6] shows the inhibition zone diameters of the different isolates while Table 7 shows the interpretation of the IZD of each bacterial isolate, using Clinical and Laboratories Standards [28]. Table 6 shows the wide-spread resistance of micro-organisms to conventionally used antimicrobial agents. It has been suggested that the environment (in this case water) could serve as a medium for transmitting resistance traits [29,30].

Table 4. Frequency of occurrence of fungi isolated from different sachet water samples

Samples / Fungi	1	2	3	4	5	Frequency rate
Aspergillus niger	+	+	+	+	+	100%
Penicillium spp.	+	+	-	+	+	80%
Aspergillus fumigatus	-	+	-	-	-	20%

KEY: + = Present; - = Absent

Table 5. Frequency of occurrence of bacteria isolated from different sachet water samples

Sample / Isolated organism	1	2	3	4	5	Frequency rate
Staphylococcus aureus	+	++	-	+	-	60%
Staphylococcus saprophyticus	++	-	-	+	-	40%
Staphylococcus epidermidis	-	++	-	-	-	20%
Bacillus cereus	+	-	-	+	-	40%
Bacillus megaterium	-	-	-	+	+	40%
Pseudomonas aeruginosa	-	+	-	-	-	20%

KEY: + = Present; - = Absent; ++ = Present and isolated again from the dilution, three weeks later

Table 6. Inhibition zone diameter of the different bacterial isolates to different antimicrobial agents

Isolates	CRO	CN	E	L	AG	S	CPX	APX
S. aureus	2	0	0	0	0	0	3	0
S. saprophyticus	18	5	12	15	5	0	15	0
S. aureus	13	6	1	0	0	0	20	0
B. cereus	7	0	0	0	0	0	5	0
B. cereus	13	8	0	0	0	0	2	0
S. saprophyticus	12	12	11	3	1	5	18	0
S. epidermidis	6	16	18	20	0	9	2	5
P. aeruginosa	12	3	0	0	0	0	20	0
B. megaterium	15	10	0	0	0	0	3	0
S. saprophyticus	9	5	16	10	0	10	18	0
S. aureus	20	14	20	20	8	18	18	16
B. megaterium	14	17	10	7	8	17	28	0
S. aureus	12	6	5	12	6	1	18	7
S. epidermidis	36	3	7	0	2	9	44	24
B. megaterium	22	18	18	10	11	10	38	8

Table 7. Susceptibility profile of each isolate to different antimicrobial agents

Isolates	CRO	CN	E	L	AUG	CPX	APX
S. aureus	R	R	R	R	R	R	R
S. saprophyticus	I	R	R	R	R	R	R
S. aureus	R	R	R	R	R	R	R
B. cereus	N/A	N/A	N/A	N/A	N/A	N/A	N/A
B. cereus	N/A	N/A	N/A	N/A	N/A	N/A	N/A
S. saprophyticus	R	R	R	R	R	I	R
S. epidermidis	R	S	I	S	R	R	R
P. aeruginosa	R	R	R	R	R	I	R
B. megaterium	N/A	N/A	N/A	N/A	N/A	N/A	N/A
S. saprophyticus	R	R	I	R	R	I	R
S. aureus	I	I	I	S	R	I	R
B. megaterium	N/A	N/A	N/A	N/A	N/A	N/A	N/A
S. aureus	R	R	R	R	R	I	R
S. epidermidis	S	R	R	R	R	S	R
B. megaterium	N/A	N/A	N/A	N/A	N/A	N/A	N/A

KEY: CRO - Ceftriaxone; CN - Gentamycin; E - Erythromycin; L - Levofloxacin; AUG - Augumentin; CPX – Ciprofloxacin; APX - Ampiclox (Ampicillin-Cloxacillin); S – sensitive; I – Intermediate sensitivity; R – resistant; N/A – Breakpoints not available for comparison

The level of resistance encountered ranged from as low as to two antibiotics to as high as to seven. 53% of the isolated microorganisms are multi-drug resistant. These organism acquired resistance to at least one agent in three or more antimicrobial categories [31]. This increased level(s) of resistance with most isolates therefore serves to support the claim that the issue of MDR strains of several micro-organisms is gradually becoming a global problem.

4. CONCLUSION

This study reveals the presence of some heterotrophic bacteria including opportunistic pathogens like *Pseudomonas aeruginosa* and *Bacillus* spp., and it indicates that there is risk of infection, especially to the vulnerable population; including children, on consumption of poorly regulated sachet water. There was also the presence of coliform bacteria in two out of the five brands analyzed. This is especially important in the vulnerable population. The water treatment processes appear to be efficient as their HPCs (both bacteria and fungi) were within limit, but the total and fecal coliform count reveals that this treatment was not efficient for one of the brands. This study also joins other studies in revealing the presence of opportunistic pathogenic moulds. The popular sachet water available in Anambra state is of fair microbial quality.

The issue of more than half of the isolates being multi-drug resistant underscores the environment, indeed water, as a medium through which bacteria resistance can spread to regular human pathogens. Regulatory agencies should therefore employ stricter measures in tackling the issue of improper; drug use, water treatment measures and disposal of antimicrobial agents including chemical disinfectants.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/12880