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MICROBIAL RISK ASSESSMENT OF SACHET WATER IN RUMUEPIRIKOM, PORT HARCOURT: FOCUS ON VIRULENCE ATTRIBUTES

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Abstract: Population and virulent attributes of bacteria in some commonly consumed sachet water supplies in Rumuepirikom area of Port Harcourt were investigated using Aerobic Plate Count and Most Probable Number (MPN) techniques. Fifty (50) samples of sachet water were analyzed, and comprised of 10 samples each of Bread of Life, Preson, Uzodhu, Judose and Obi Ronix sachet water. Results revealed detectable numbers of heterotrophic bacteria and coliform bacteria in the drinking water samples. However, viable cells of Salmonella, Shigella and Vibrio cholerae were not detected. Results also showed that the densities of the bacterial contaminants varied with the different brands of sachet water samples. Counts of total heterotrophic bacteria were reasonably high. The MPN results showed presence of coliforms in all brands of water with Uzodhu and Judose sachet waters having the highest counts. Five bacterial species:
Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis, Escherichia coli and Klebsiella pneumoniae were isolated. Among the isolates, Staphylococcus aureus was the most virulent, being able to elaborate lipase, hemolysin, coagulase and DNase on test media while Bacillus subtilis were the least virulent. On the other hand, the isolates demonstrated varying degree of responses to chemotherapeutic agents; Pseudomonas aeruginosa was the most resistant organism isolated from the sachet water samples. The quality assessment of the water samples showed that most of the sachet water supplies analyzed had high coliform and heterotrophic bacterial densities above WHO permissible limits for drinking water. Also virulent bacteria capable of causing disease were isolated. Thus, there is need for regular sanitary inspection of sachet water services so that only those companies which constantly produce water of acceptable bacteriological standards are permitted in the market. banks customers in a caring fashion, understand their needs and also have their best interest at heart.

Keywords: Population, Virulent, Attributes, Bacteria, Sachet Water

INTRODUCTION

Knowing the water quality is an essential safeguard for ensuring safe drinking and wholesome water. Primarily, water available for human usage are sourced from ground water and surface water which are exploited through various means like shallow wells, deep wells, hand pumps, tube wells or public water treatment works (Tariq and Ahmed, 1985). Sachet water is water obtained by means of technological process from the base sources namely underground or surface water supplies. It is water that is processed and packaged in small closed polythene locally and popularly referred to as “pure

water". Sachet water is a fast growing secondary source of drinking water among the urban and rural populations of Nigeria. Sachet water as a drinking water has gained general acceptance in Port Harcourt and among Nigeria populace due to a number of factors. These factors include lack or complete non-existence of public pipeborne water in Port Harcourt, simplified production process, low-capital intensiveness of production, low price, convenience and portability of the water. Somehow, the growth in population has placed limitation on the public water work services that are available to most parts of the city and its environs. Sachet water has been reported to be contaminated by virulent bacteria (Odeyemi. 2015). However, drinking water supplies are liable to be contaminated with sewage or other organic or inorganic mater and stands as a vehicle of water borne disease such as typhoid fever, cholera, paratyphoid and bacillary dysentery (Cruickshank *et al*., 1975). In safe guarding public water supplies, public health authorities rely on information obtained from the results of frequent bacteriological analysis (Itah *et al*., 1996). The determination of bacterial population and their virulent factor is important for consumer's protection (Maoyu and Zhang, 1989), specifically sachet water consumers. Thus, this research was aimed at determining the population and virulent attributes of bacteria in sachet water sold and consumed in Rumuepirikom area with a view to determining the bacterial resistance to antibiotics which is of public health importance for the treatment of emerging waterborne diseases.

MATERIALS AND METHODS

***Description of Study Area*:** Rumuepirikom area is located in Obio/Akpor Local Government Area of Rivers State. The area is a Subhub of Port Harcourt metropolis and contributes economically and commercially to the development of Port Harcourt. The demographic status of Port Harcourt makes it one of the largest cities in Nigeria. It is an empirical knowledge that some of the sachet waters sold and consumed in the Rumuepirikom area are also commonly sold and consumed in some other parts of Port Harcourt that share borders with the study area of this research.

Sample Collection: Fifty sachet water samples were collected randomly in batches at various distribution points in various parts of the study area from May to December, 2015. Ten (10) sachet water samples each were collected from five (5) brands of water namely: Preson, Bread of Life, Obi Ronix, Uzodhe and Judose water which were coded for the purpose of the study as SWSP, SWSB, SWSO, SWSU and SWSJ brands respectively. The samples were then transported to the laboratory and analyzed within two hours of collection.

Enumeration of Bacterial Population: The bacterial populations in the sachet water were enumerated using spread plate method as described by Harrigan and McCane (1976); Collins and Lyne (1976). Total heterotrophic, *Salmonella/Shigella* and *Vibrio* bacteria were enumerated by plating undiluted portions of the samples on freshly prepared Nutrient agar, Shigella/Salmonella agar, and TCBS agar plates respectively. Counts were then made on colonies that appeared after incubation at 37^{o}C for 24hrs. In addition, MPN method (Presumptive and Confirmatory tests) as described by Prescott *et al*. (1996) was used to estimate the coliform presence and there population size. Completed test was carried out to confirm suspected colonies to be coliform bacteria (Prescott *et al*., 1996).

Identification of Bacterial isolates: The isolates were identified using the identification schemes of Harrigan and McCance (1976). The following characterizations were carried out which include: Gram staining, coagulase, catalase, oxidase, indole production, citrate utilization, methyl red and Voges Proskauer and sugar fermentation.

Determination of Virulent Features of the Bacterial Isolates: Isolates were screened using Dnase, lipase, coagulase, lecithinase and heamolysin test media to determine their ability to cause

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disease on consumers. The isolates were streaked on the test media as described by Young and Wood (1977) and incubated at 37^{o}C for 24 hours; thereafter the results were recorded.

Antibiotic Assay of the Bacterial Isolates: The bacterial isolates were screened for their antibiotics sensitivity pattern using the agar diffusion technique described by Nwachukwu and Emeruem (2007). Sensitivity discs containing commonly used antibiotics were impregnated on freshly prepared nutrient agar media already seeded with the test bacteria and incubated overnight for 24hrs at 37^{o}C. Thereafter, the zones and sizes of inhibition were measured and recorded.

Statistical Analysis: The procedure used by Thrusfeild (2005) was used to process some data obtained and were analyzed using one way analysis of variance (ANOVA) with expressions noted as Mean±Standard Deviation. Statistical significance was set at $P<0.05$. Data obtained were also analyzed using descriptive statistics (bar and pie charts).

RESULTS

Bacteriological loads of the Sachet Water Samples

Results in Tables 1 and 2 showed the mean densities of the bacterial groups obtained from the sachet water samples analyzed. The samples contained detectable numbers of heterotrophic bacteria, total coliform and fecal coliform bacteria. However, viable cells of *Salmonella, Shigella* and *Vibrio cholerae* were not detected. The results also showed that the densities of the bacterial contaminants varied with the different brands of sachet water samples.

Table 1: Mean±SD Densities of Bacteria in the different Brands of Sachet Water

Brand of Water	Heterotrophic bacteria (CFU ML^{-1})	*Salmonella/Shigella* bacteria (CFU ML^{-1})	*Vibrio* bacteria (CFU ML^{-1})
Bread of life	6.8±1.20X10^{2}	ND	ND
Preson	7.8±1.94X10^{2}	ND	ND
Uzodhu	1.01±2.02X10^{2}	ND	ND
Judose	1.03±0.64X10^{3}	ND	ND
Obi Ronix	8.0±1.07X10^{2}	ND	ND

Key: ND = Not detected; CFU ML^{-1} = colony forming unit per milliliter

Heterotrophic bacteria were found in all brands of the water samples and their densities ranged from 6.8±1.20X10^{2}CFU ML^{-1} for Bread of life sachet water to 1.03±0.64X10^{3}CFU ML^{-1} for Judose sachet water. Statistical analysis revealed a significant difference ($P<0.05$) in bacterial loads among brands of the sachet water samples. Results presented in Table 2 showed the presumptive and confirmatory tests of the sachet water samples which revealed that all brands were contaminated with coliform bacteria. Mean data obtained from presumptive test showed that Judose water had the highest coliform numbers (40coliforms 100ml^{-1}), followed by Uzodhu water (35coliforms 100ml^{-1}), while Bread of Life sachet water had the least number of coliform bacteria (17coliforms 100ml^{-1}). However, the confirmatory test revealed that Judose sachet water was the most contaminated with 17coliforms 100ml^{-1} of water while Bread of Life was the least contaminated with 9 coliforms100ml^{-1} of water analyzed.

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Table 2: Most Probable Number (MPN) of Coliform bacteria Estimated in 100ml of Sachet Water Samples.

Brand of Sachet Water	Coliform Presumptive Test (100ml)	Coliform Confirmatory Test (100ml)
Uzodhu	35	13
Judose	40	17
Bread of Life	17	9
Obi Ronix	20	12
Preson	30	11

Characterization of bacterial Isolates

Table 3 shows the morphological and biochemical characterization of bacterial isolates. Five (5) isolates were identified in all brands of sachet water samples analyzed. Grams reaction classified the isolates in two distinct bacterial groups. Other features of the isolates are listed in Table 3.

Table 3: Morphological and Biochemical Characteristics of Bacteria Isolated from the different brands of Sachet Water

Coa	Mol	Cat	Suc	Lac	Oxi	Ure	Mal	Ind	Glu	Cit	MR	VP	GST	Description	Isolates
		+	+	+		+	+		+	+		+		Small short rods	*Klebsiella Pneumoniae*
	+	+		+			+		+	+	+		+	Rodshaped	*Bacillus subtilis*
	+	+			+				+	+				Rods	*Pseudomonas aeruginosa*
	+	+		+				+	+		+			Short slender rods	*Escherichia coli*
+		+	+	+		+	+		+	+	+	+	+	Grape-like clusters	*Staphylococcus aureus*

Key: + = Positive, - = Negative, GLU=glucose, SUC= sucrose, LAC= lactose, MAL=maltose, COA= coagulase, MOL = motility CAT= catalase, OXI= oxidase, IND= indole, URE= urease, MR=methyl red, VP=Vogues Proskauer and CIT= citrate, GST = Gram Stain

Prevalence of Bacterial Isolates in the Sachet Water Samples

Table 4 shows the distribution and frequency of occurrence of bacterial isolates, while their prevalence in sachet water is illustrated in Figure 1. Results showed that *Staphylococcus aureus* with 40% prevalence rate was the most frequently isolated bacterium while *Bacillus subtilis* with 6% prevalence rate was the least encountered bacterial contaminant. The results also revealed that their occurrences in the sachet water varied with the brands of water samples.

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Table 4: Distribution and Frequency of Occurrence of the Bacterial Isolates in the different brands of Water samples

Isolate	Obi Ronix (n = 10)	Bread of Life (n = 10)	Preson (n= 10)	Uzodhu (n = 10)	Judose (n= 10)	Frequency of Occurrence
Staphylococcus aureus	4	3	3	6	4	20
Klebsiella pneumoniae	1 1	- -	3	5		
Bacillus subtilis	1	- -	-	2	3	
Escherichia coli	1 -	2 1	3	7		
Pseudomonas aeruginosa	2 3	2 4	2	13		

Virulence Factors Exhibited by the Bacterial Isolates

Table 3 shows the virulent properties of the bacterial isolates. The virulent factors vary with the different bacterial isolates. From the results, heamolysin activity was produced by all isolates namely: *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae*, *Bacillus subtilis* and *Pseudomonas aeruginosa*. None of the isolates elaborated lecithinase while coagulase was produced by *Staphylococcus aureus* only. *Staphylococcus aureus* and *Pseudomonas aeruginosa* expressed lipase activity while DNase production was exhibited by *Pseudomonas aeruginosa*

Table 5: Virulent Properties of bacteria isolated from the brands of sachet water

Isolates	DNase	Lipase	Coagulase	Hemolysin	Lecithinase
Staphylococcus aureus	+	+	+	+	
Escherichia coli				+	
Klebsiella pneumonia				+	
Pseudomonas aeruginosa	+	+		+	
Bacillus subtilis				+	

Key: + = Positive, - =Negative and *Staphylococcus aureus* only. Ranking the virulence potential of the isolates showed that *Staphylococcus aureus* with 40% disease causing potential was the most virulent of the bacterial species isolated from the sachet water samples (Fig. 1), followed by *Pseudomonas aeruginosa* (26%), *Escherichia coli* (14%), *Klebsiella pneumoniae* (10%) and *Bacillus subtilis* (6%).

Chemotherapeutic Sensitivity Pattern of *Staphylococcus aureus* and *Bacillus subtilis* Isolated from the Water Samples

Fig. 2 shows that *Staphylococcus aureus* was sensitive to Amoxil, Streptomycin, Rifampicin, Erythromycin and Chloramphenicol with inhibition zone sizes of 20,18,17,18, and 26mm respectively. However, *Staphylococcus aureus* strain showed resistance to Levofoxacin, Ampiclox, Norfloxacin and Ciproflox. On the other hand, *Bacillus subtilis* was more sensitive to

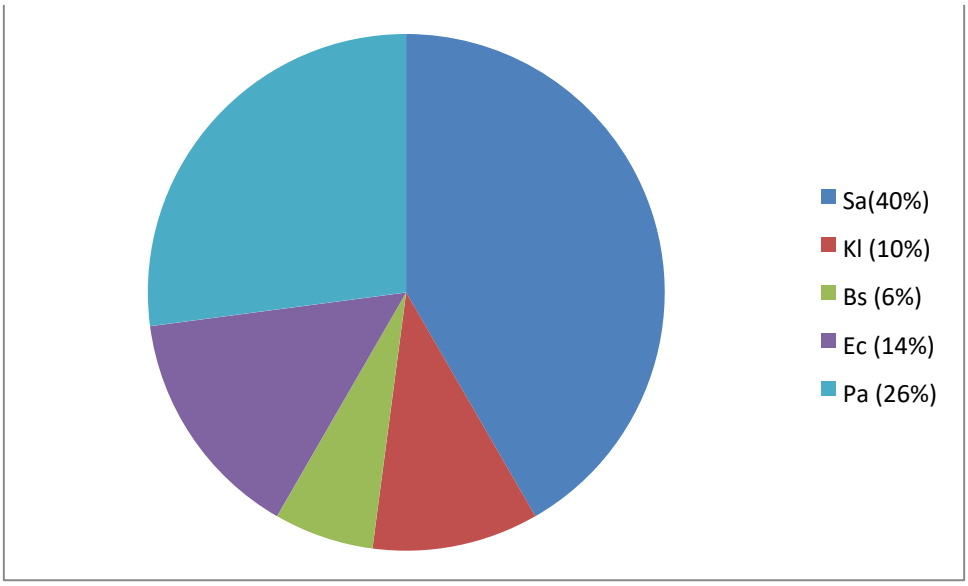

Fig. 1: Disease causing potential of bacteria isolated from sachet water samples
Key: *Sa =Staphylococcus aureus, Kl =Klebsiella pneumoiniae, Bs =Bacillus subtilis, Ec =Escherichia coli, Pa = Pseudomonas aeruginosa*

Norfloxacin (16mm); followed by Erythromycin (15mm) and Streptomycin (10mm) but resistant to Ciproflox, Amoxil, Rifampicin, Chloramphenicol, Ampiclox and Levofoxacin. The antibiogram of the Gram positive bacterial isolates showed that Chloramphenicol was the most potent drug against *Staphylococus aureus* while Norflaxacin was comparatively the best antibiotic against *Bacillus subtilis*.

Chemotherapeutic Sensitivity Pattern of *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* Isolated from the Water Samples

Results in Fig.3 showed the antibiotics susceptibility of Gram's negative bacteria; *Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa,* isolated from the sachet water samples. *Escherichia coli* were highly inhibited (20mm) by Gentamycin and Ampicillin. The fecal coliform was also sensitive to Ceporex (18mm) but resistant to Augmentin, Streptomycin, Nalidixic acid and Septrin. *Klebsiella pneumoniae* was sensitive to Reflacine (20mm), Gentamycine (18mm) and Streptomycin (19mm) but resistant to Tarivid and Septrin. The results also showed that *Pseudomonas aeruginosa* was resistant to all the antibiotic drugs assayed although a zone of inhibition of 6mm was recorded for Tarivid, Ampicilin and Streptomycin. The antibiogram results presented in Figure 3 revealed the high potency of Gentamycin and Ampicillin against *E. coli*, Reflacine against *Klebsiella pneumoniae* while none of the antibiotics was effective against *Pseudomonas aeruginosa*.

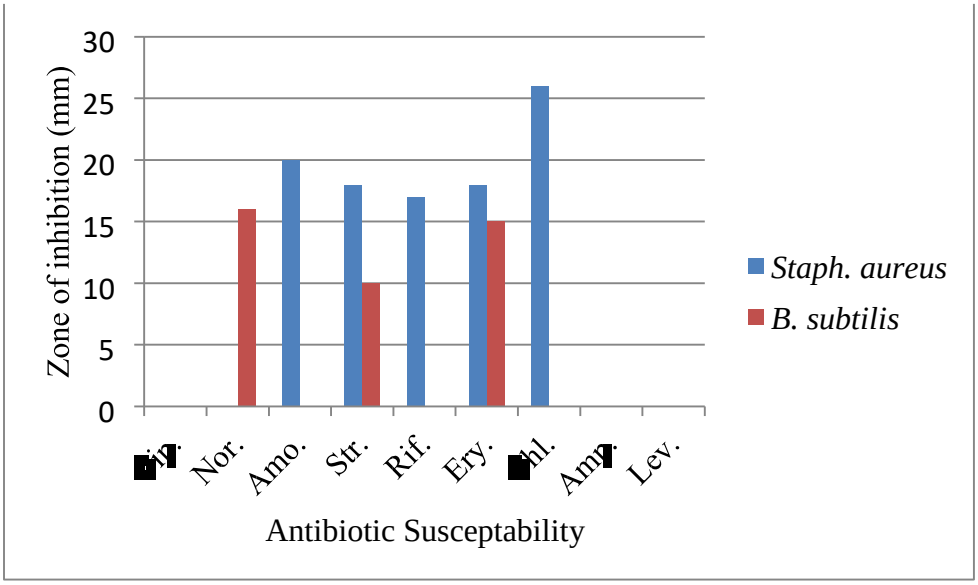

Fig. 2: Antibiogram of the Gram's Positive Bacteria Isolated from the Sachet Water Samples
Key: Cip = Ciproflox, Nor = Norfloxacin, Amo = Amoxil, Str = Streptomycin, Rif = Rifampicin, Ery = Erythromycin, Chl = Chloramphenicol, Amp = Ampiclox, Lev = Levofoxacin

DISCUSSION

The research findings revealed variation in the bacterial loads of the sachet water between the different brands marketed in the study area. The samples contained detectable numbers of heterotrophic and coliform bacteria. However, viable cells of *Salmonella, Shigella* and *Vibrio cholerae* were not detected. Basically, Judose and Uzodhu sachet water samples were the most contaminated. Ideally, the brands of sachet water are expected to be pure in quality at its source, but average bacterial numbers were high for heterotrophic and coliform bacteria, exceeded the acceptable limits recommended by WHO (2014). WHO (2014) regulatory limits for piped water stipulates that heterotrophic bacterial load should not exceed 100CFU ML^{-1} in drinking water, while coliform bacterial numbers should be less than two (2) in 100ml for total coliform bacteria and 0 in 100ml for faecal coliform bacteria. The reason for the high bacterial counts of the analyzed waters could be rather due to a few factors namely: the unhygienic conditions in which the sachet waters are packaged and poor chlorination process. The characterization of the bacterial isolates (Table 3) has shown that the sachet water samples were contaminated with coliforms and pathogenic bacteria. However, *Salmonella, Shigella* and *Vibrio cholerae* were not isolated from the water brands. Other isolates encountered are *Staphylococcus aureus, Pseudomonas aeruginosa, and Klebsiella pneumoniae*.

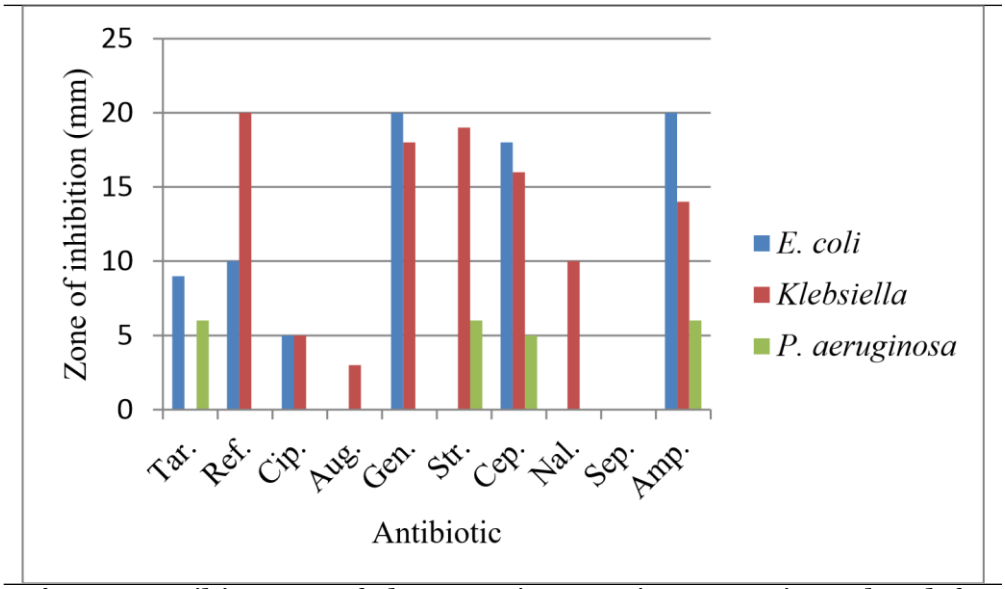

Fig. 3: Antibiogram of the Gram's Negative Bacteria Isolated from Sachet Water Samples

Key: Tar = Tarivid, Ref =Reflacine, Cip = Ciproflox, Aug = Augmentin, Gen =Gentamycin, Str = Streptomycin, Cep = Ceporex, Nal = Nalidixic acid, Sep = Septrin, Amp = Ampicillin.

The ability of pathogenic bacteria to cause disease in a susceptible host is determined by multiple virulence factors acting individually or together at different stages of infection. Virulence factors are often involved in direct interactions with the host tissues or in concealing the bacterial surface from the host's defense mechanisms (Wu *et al*., 2008). All isolates screened for pathogenicity were able to express at least one virulent factor (Table 5) as exemplified by *Staphylococcus aureus* (4), *Escherichia coli* (1), *Klebsiella pneumoniae* (1), *Pseudomonas aeruginosa* (3) and *Bacillus subtilis* (3), some of which have been implicated in infectious disease initiation in humans. Studies have shown that environmental strains of *Escherichia coli* do not readily produce significant virulence (Tharanga, 2007). The same author reported comparatively, strong virulent ability of some *Pseudomonas aeruginosa* strains. *Bacillus subtilis* which is one of the isolates that expressed the least virulent attribute in this study had previously been reported to have low virulence ability (Ihde and Armstrong, 1973).

Bacterial lecithinases are of special interest because of the possible role of these enzymes in pathogenicity (NME ICT, 2016). However, this study has shown that most bacteria contaminants of sachet water in Rumuepirikom area of Port Harcourt failed to produce lecithinase. *Bacillus subtilis*, an aerobic spore former and fermenter of mannitol unlike *Bacillus cereus*, *Clostridium perfringens* and *Pseudomonas fluorescens* lacked the ability to produce lecithinase (NME ICT, 2006). On the other hand, hemolysin production was common among the bacteria isolated in this study. Hemolysin has the capacity to lyse red blood cells (Passmore and Robson, 1973). The most virulent isolate, *Staphylococcus aureus* has been reported to have the capability of initiating a disease process in the case of direct or indirect human exposure to improperly treated drinking water. *Staphylococcus aureus* has virulent abilities that can enable it cause diseases such as food poisoning, toxic shock syndrome, boils and a lot more. Similar finding had earlier been reported by Nilsson and Dahlstrom (2005). *Klebsiella pneumoniae* and other infectious Enterobacteriaceae have also been implicated in

disease outbreaks and are thus, major pathogens of concern in contaminated drinking water (Nilsson and Dahlstrom, 2005).

The antibiotic susceptibility assay results have shown that the bacterial isolates exhibited variable response to antibiotic treatments. The Gram positive bacteria were resistant to Septrin only while the Gram negative bacteria were completely resistant to Ciproflox, Ampiclox and Levofloxacin. This study has shown that among the sachet water contaminants, *Pseudomonas aeruginosa,* a Gram negative bacterium was the most resistant to chemotherapeutic agents. This finding agreed with report by MDH (2010) that *Pseudomonas aeruginosa* had an intrinsically advanced antibiotic resistant property. MDH (2010) reported that *Pseudomonas* treatment is dually complicated by the bacterium resistance profile which may lead to treatment failure and expose patients to untoward adverse effects from advanced antibiotic drug regimens. It is also in consonance with reports from Chang *et al.* (2003). The wide use of antibiotics in the treatment of bacterial infections has led to the emergence and spread of resistant strains. Multidrug resistant strain of *Staphylococcus aureus* is a major cause of nosocomial infections. The marked antibiotic resistance pattern showed by some of the sachet water bacterial contaminants is attributable to worldwide dissemination of resistance genes that is on the rise recently (Chang *et al.,* 2003). In conclusion, this study has shown that consumption of sachet water in the studied area may be unsafe to the consumers. The loads of indicator bacteria (fecal coliform) were high though potent pathogens like *Salmonella* was not isolated. However, the infective dose in people whose local or general natural defense mechanisms are impaired would be significantly low. The people likely to be at risk would be the very old or the very young as well as patients undergoing immunosuppressive therapy. The findings from this study recommends the need for regular inspection of sachet water facilities so that only those companies which consistently produce water of acceptable bacteriological quality are allowed into the market community.

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