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Enumeration of microbial contaminants in sachet water: a public health challenge

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ABSTRACT

Accessibility and availability of fresh clean water is a key to sustainable development and essential element in health, food production and poverty reduction. In the present study, we have collected water sachet containing CM/L number and they were analysed for physical and bacteriological nature. The organisms isolated in this study were *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas vesicularis* and *Pseudomonas aeruginosae*. The harmful effects of these isolates were evidenced by antibiotic resistance, heavy metal tolerance and antibacterial activity. They were resistant to the antibiotics like amoxiclav, methicillin, chloramphenicol and streptomycin. They showed tolerance to the heavy metals at 5 mM conc. except for lead. For antibacterial activity, they were tested against human pathogens *Klebsiella pnemoniae*, *Proteus mirabilis, Micrococcus leuteus* and *Salmonella paratyphium*. But at the same time these organisms could be exploited for the industrial production of amylase, protease and cellulase.

Keywords: Sachet Water; Bacterial Contaminants; Pathogens; Industrially Useful Bacteria

1. INTRODUCTION

Bottled water is defined as water that is intended for human consumption and this is sealed in bottles or other container with no added ingredients except that it may contain safe and suitable fluorides. Its price is within the reach of tautology. Small scale entrepreneurs introduced small nylon sachets which are electrically heated and sealed at both ends to the market and is popularly called pure water. It finds patronage from members of low socio-economic class.

Besides, majority of the water sachets do not carry the National Food and Drug Administration Control (NAFD AC) approval number. This means that they are either not registered or the procedures have not completed the registration of their products with NAFDAC. It was therefore considered implication of sachet water. Accessibility and availability of fresh clean water is a key to sustainable development and essential element in health, food production and poverty reduction. 1.2 billion People around the world lack access to safe water and 2.5 billion are not provided with adequate sanitation (Third World water forum on water, 2003). In metropolis as a whole pipe born water is inadequate both in quality and quantity.

Consequently water born diseases such as cholera and typhoid often have their epidemic during the dry season. Typhoid remains a great socio-economic problem in developing countries. Proliferation of intestine is associated with high mortality with wound infection occurring in 50-75% of surviours [1]. Controlling wound sepsis or wound infection also affected mortality [2,3].

Since this health problem was largely traceable to unhygienic water supply, an alternative to the seemingly inadequate water supply was found in bottled water.

Drinking water including bottled water, may reasonably to expected to contain at least small amounts of some contaminants includes microbial pathogens, organic pollutants like heavy metals. The presence of contaminants does not necessarily indicated that water poses a health risk. Environmental Protection Agency (EPA) sets standards for approximately 90 contaminants in drinking water. EPA standards, along with each contaminant's likely source and health effects, are available at www.epa.gov/safewater/mcl.html.

Microbiological contamination of water has long been a concern to the public. There is a concern in increasing due to outbreak of coliform bacteria, and protozoans like Giardiasis, Cryptosporidiosis.

Coliforms are not a single type of bacteria but a group of bacteria that includes *Klebsiella*, *Proteus*, *E. coli*, and *Salmonella*. Coliform organisms are not necessarily

pathogens and are rarely found in bottled water, they serve as an indicator of insanitation or possible contamination.

Microbial potability of bottled and packaged drinking water hawked in Ilorin metropolis was done by selecting 81 samples containing 11 brands of drinking water packaged and hawked in cellophane bags, did not met drinking water standards. *Pseudomonas* was frequently recovered as a contaminant of packaged water [4]. Bottled mineral water consumption has significantly increased in Brazil. Public health determines the parasitological and microbiological status of some brands and found occurrence of Cryptosporidial oocysts and Guardia cyst in bottled mineral water [5]. An assessment of the health and social economic implantations of sachet water in Ibadam Nigeria [6] selected 78 samples from 20 brands of sachet water from hawkers/vendors. Bacteria obtained include: *Klebsiella* sp., *Streptococcus faecalis* and *Pseudomonas aeruginosae*. By sterile filtration of water, broad diversity of viable bacteria was isolated by using 0.2 µ filter for the removal of microorganisms and is commonly referred as 'sterile filtration'. 19 bacterial taxa were isolated by the acclimatization method from 0.2 micron filtered fresh water samples. *Cryptosporidium parvum* infection in Bergen and Norway was found during the large water borne Giardiasis outbreak [7].

The enforcement of the regulation guiding water quality before the National Agency for Food and Drug Administration Control (NAFDAC) to employ with the checking water qualities guideline values as recommended by World Health Organization (W.H.O) becomes urgent.

The water sachets are the products of middle class entrepreneurs and some small scale business ventures. The objective of the present study was to find out the quality of sachet water. We have collected seven different brands of water sachet containing CM/L number and the samples were subjected to physical and bacteriological analysis.

2. MATERIALS AND METHODS

2.1. Media Used

LB (Luria–Bertani) Agar, King's B medium, Nutrient Agar and EMB (Eosine Methylene Blue) Agar were used to screen the sachet water sample for bacterial contamination.

2.2. Collection of Samples

Seven sachet water samples supplied in and around Pallavaram were collected. All of them contained CM/L number along with ISI–14543, Ozonized and UV treated. Some of them were Reverse Osmosis processed.

3. PHYSICAL PARAMETERS

pH: pH was checked for all the water samples immediately after opened.

4. BACTERIOLOGICAL ANALYSIS ISOLATION AND IDENTIFICATION OF BACTERIA

4.1. Isolation of Bacteria

Seven different sachet water samples were taken up for the present study. The samples include Freeze, VSP, VPZ, Aqua fresh, Jai, Hi-tech and Sakthi. The water samples were serially diluted and spread on EMB medium, Nutrient agar, King's B medium and kept for 24 hours incubation at 37˚C. The isolated bacterial colonies were purified to homogeneity by quadrant streaking, store in LBA, NA and KBA slants periodically subcultured.

4.2. Identification of Organism

The bacteria isolated were identified based on the biochemical tests outlined in the Bergey's Manual of determinative bacteriology [8].

4.3. Antibiotic Resistance/Susceptibility Screening

The sensitivity/resistance of the isolates to various antibiotics such as Chloramphenicol, Ceffriaxone, Amoxiclav, methicillin, Nalidixic acid and Streptomycin was studied by inoculating a loopful of the overnight grown cultures on Nutrient Agar plates amended with 30µg/ml concentrations of the appropriate antibiotics and incubated at 37˚C. After 24 hours of incubation, the plates were observed for growth. Nutrient agar plates without antibiotics served as control. The minimum concentration at which no growth was taken as the MIC (Minimum Inhibitory Concentration).

4.4. Heavy Metal Tolerance Spectrum

The tolerance of the bacterial isolates to various heavy metals such as zinc (zinc sulphate), lead (lead acetate), copper (copper sulphate), chromium (potassium chromate) was studied by inoculating loopful of overnight grown cultures on Nutrient Agar plates amended with 1, 3 and 5mM concentrations of heavy metals and incubated at 37˚C. After 24 hours of incubation, the plates were observed for growth. Nutrient agar plates without heavy metal served as control. The minimum concentration at which there was no growth was taken as the MIC

value.

4.5. Screening for Extra Cellular Enzyme Production

Four industrially important extra cellular enzymes were selected and screened for primary extra cellular enzyme production.

4.6. Protease

Nutrient agar along with 1% Gelatin (substrate) and 1% casein (substrate) was taken in separate conical flasks and autoclaved. They were poured into respective petriplates (triplicates). After solidifying, isolates were streaked on them and kept for incubation at 37˚C. After 24 hours of incubation, the plates were stained with 15% $HgCl_2$ (indicator) and observed for zone of inhibition.

4.7. Amylase

Nutrient agar along with 1% soluble starch (substrate) was taken in a conical flask and then autoclaved. They were poured into respective petriplates (triplicates). After solidifying, isolates were streaked on them and kept for incubation at 37˚C. After 24 hours of incubation, the plates were stained with Gram's Iodine (indicator) and observed for zone of inhibition.

4.8. Cellulase

Nutrient agar along with 1% cellulose (substrate) was taken in a conical flask and then autoclaved. They were poured into respective petriplates (triplicates). After solidifying, isolates were streaked on them and kept for incubation at 37˚C. After 24 hours of incubation, the plates were stained with 0.3% of Congo red (indicator); plates were kept in orbital shaker (mild shaking) for 15 minutes. Congo red was discarded and then 1 N NaCl was added to the plate and kept in shaker for 10 minutes and observed for zone of inhibition.

4.9. Antibacterial Activity of Pure Bacterial Isolates

Four bacterial isolates (*Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas vesicularis* and *Pseudomonas aeruginosae*) from sachet water were selected for antibacterial activity against the four human pathogenic bacteria (*Micrococcus luteus*, *Salmonella paratyphium*, *Proteus mirabilis* and *Klebsiella pneumoniae*) obtained from CAS Botany, University of Madras. Isolated bacteria were grown in King's B Broth and kept for incubation at 37˚C in orbital shaking incubator for 48 hours. After 48 hours incubation, samples were transferred to sterile centrifuge tubes and centrifuged at 8000 rpm for 15 minutes at 4˚C. Then supernatant was collected and it is filtered using 0.45 µ membrane filter. Filtered supernatant was stored in refrigerator for further study.

Human pathogens as mentioned above were grown in Nutrient Broth and incubated for 24 hours in orbital shaking incubator at 37˚C. After 24 hours pathogens were stored in refrigerator for further study.

Nutrient agar plates were prepared and pathogens were swabbed on it and well were made on the plates with the help of borer. 100 µl of filtered supernatant of bacterial isolates was added into the wells and kept for incubation for 48 hours. After 48 hours, plates were observed for antibacterial activity.

4.10. Antibacterial Activity of Isolates with Organic Solvent

The supernatant of the bacterial isolates as mentioned above was taken. To that equal amount of Ethyl Acetate (EA), an universally proved polar organic solvent that could dissolve many compounds was added and kept in orbital shaker for one hour or more preferably overnight. Then transfer the organic layer and distribute it equally in to different conical flasks and cover the flask with cheese cloth to prevent contamination and after complete drying, add EA. To the residue presenting the flask, the supernatant was used for further study.

Human pathogens as mentioned above were grown in Nutrient Broth and incubated for 24 hours in orbital shaking incubator at 37˚C. After 24 hours, pathogens were stored in refrigerator for further study.

Nutrient agar plates were prepared and pathogens were swabbed on it and well were made on the plates with the help of borer. 100 µl of filtered EA supernatant was added into the wells and keep it for incubation for 48hours. After 48 hours, the plates were observed for antibacterial activity.

5. RESULTS AND DISCUSSION

The objective of the present study was to find out the quality of sachet water. For this, we randomly selected seven different brands of sachet water and they were collected from in and around pallavaram, Chennai. The samples were subjected to physical and bacteriological analysis. The water samples were assessed for coliform and other bacteria using Nutrient agar, KB agar and EMB agar. The results for this study support an earlier observation that the sachet water being produced is of questionable quality [9,10].

As useful as sachet water is to the society, the result of the analysis raised doubts as to its quality. The pH of water sachets has an upper range of 9.26 (**Table 1**), a value higher than the upper limit of pH 8.5, as recommended by W.H.O. Even though pH has no direct effect on health, its indirect action on physiological processes

Table 1. pH of the sachet water samples.

Name of the sachet	pH
Freeze	9.20
VSP	9.26
VPZ	8.30
Aqua fresh	8.45
Jai	9.10
Hi-tech	8.38
Sakthi	8.20

cannot be over emphasized [6] got the upper pH unit of 9.7 among the 78 samples from 20 brands of sachet water tested.

Bacteriological analyses results (**Table 2**) showed that all the seven different brands of sachet water produced growth after 24 hours of incubation. The organisms isolated in this study are *Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas vesicularis*, and *Pseudomonas aeruginosae*. [11] reported that pure water vending machine may not be so pure, after all, because investigations found bacteria like *E. coli* in the machine. Isolation of bacteria from pure water samples especially *Streptococcus facealis* indicates possible contamination from human excreta.

The procedure most often than not are those people who may not know and are very little about the quality of water sachets they produced. Some even imitate other good products. For the price, pure water sachets are affordable, to the middle class society and some of the lower class also. Thus this type of packaged water is now popularly and freely served at the common people parties and social functions.

Most of the small scale producers of pure water may not be able to afford the price or space for a bore hole in their premises, hence they still depend on the already condemned public water supply and water from doubtful environmental sources, for the sources of the water they use in packaging their products, some of them under very poor environmental conditions. It should be noted that the seven brands of sachet water selected for the present study carried the CM/L number and ISI certified but even they showed bacterial contamination. In the present study, *Pseudomonas* was isolated from all the samples and we are suggesting that the *Pseudomonas* also be included as an added indicator for determining their safety standard. Like us, Olayemi (1999) was frequently isolated *Pseudomonas* from packaged waters.

Table 2. Biochemical characterization of the isolates from the sachet water.

Biochemical test	Bacterial isolates			
	Proteus mirabilis	***Klebsiella pneumoniae***	***Pseudomonas vesicularis***	***Pseudomonas aeruginosa***
Colony morphology	Large, transparent, circular, raised, entire moist colony	Small, non-pigmented irregular lobate, raised moist colonies	Small, pigmented, circular, flat, entire dry colonies	Small, pigmented, circular, flat, entire dry colonies
Gram's staining	Gram negative rods	Gram negative rods	Gram negative rods	Gram negative rods
Motility	Motile	Non-motile	Active motile	Active motile
Catalase	+	+	+	+
Oxidase	–	–	–	+
Indole	–	–	–	+
Methyl red	+	–	–	–
VP test	–	+	–	–
Citrate test	+	+	–	+
Nitrate test	+	–	–	+
Starch hydrolysis	–	–	–	–
TSI	Alkaline slant, acid butt, H_2S positive, No gas production	Acid butt, alkaline slant, No gas and H_2S production	Alkaline butt, acid slant, No gas and H_2S production	Alkaline butt, acid slant, No gas and H_2S production

The isolates of the present study (*Proteus mirabilis*, *Klebsiella pneumoniae*, *Pseudomonas vesicularis* and *Pseudomonas aeruginosae*) were tested for their antibiotic resistance against ceffriaxone, nalidixic acid, amoxiclav, methicillin, chloramphenicol and streptomycin (**Table 3**). All of the isolates are sensitive to ceffriaxone and nalidixic acid and are resistant to amoxiclav, methicillin, chloramphenicol and streptomycin. That is, their presence in the water sachet pose a danger for those who are taking. The consuming public also must be informed of the consequences of consuming packaged water. Even though the water sachets are UV-treated and ozonized, it could be also recommended that produced packaged water should endeavor to disinfect their products with solar radiation, which is simple to construct and easy to maintain.

Proteus mirabilis, *Klebsiella pneumoniae*, *Pseudomonas vesicularis* and *Pseudomonas aeruginosae* were tested for their heavy metal tolerance. For this study, we selected only four heavy metals namely, copper (copper sulphate), chromium (potassium dichromate), zinc (zinc sulphate) and lead (lead acetate) at a concentration of 1 mM, 3 mM and 5 mM. All of the four isolates showed growth at 1 mM and 3 mM concentration. But at 5 mM concentration, the heavy metal except lead seems to be toxic to the isolates and no growth was observed. This result indicates that these organisms are resistant to heavy metals at 1 mM and 3 mM concentration. For lead, they are showing tolerance even at 5 mM concentration (**Table 4**).

We screened three of the industrially important extra cellular enzymes (protease, amylase and cellulase) production by our test organisms. For protease enzyme, two different substrates were used as nitrogen source *i.e.*, Gelatin and casein. All the test organisms showed protease production by plate assay method. For amylase production, starch served as a sole carbon source and the *Proteus mirabilis* and *Klebsiella pneumoniae* is the producer of the sole carbon source but this was not produced by any of the organisms tested. The isolates of sachet water shows some harmful (antibiotic resistance, heavy metal resistance) and useful properties. Useful property includes industrial enzyme production. So we can exploit these organisms for the mass production of industrial enzymes like protease and amylase (**Table 5**).

Among four bacteria *Klebsiella pneumoniae* and *Proteus mirabilis* were considered as human pathogens. To know its antibacterial property, we selected four human pathogens like *Klebsiella pneumoniae*, *Proteus mirabilis*, *Micrococcus luteus* and *Salmonella paratyphium*. *Proteus mirabilis* and *Klebsiella pneumoniae* showed antibacterial activity against *Micrococcus luteus* and *Salmonella paratyphium*. *Pseudomonas vesicularis* showed no antibacterial activity against *Proteus mirabilis*, *Micrococcus luteus* and *Salmonella paratyphium* but it formed zone of clearance against *Klebsiella pneumoniae*. *Pseudomonas aeruginosa* showed antibacterial activity against *Proteus mirabilis* and *Salmonella paratyphium* but no activity for *Klebsiella pneumoniae* and *Micrococcus luteus* (**Table 6**). The same result was observed for ethyl acetate fractions of bacterial isolates except *Proteus mirabilis* showed antibacterial activity against *Klebsiella pneumoniae* (**Table 7**).

Since *Proteus mirabilis* and *Klebsiella pneumoniae* itself was a human pathogen, it does not showed antibacterial activity against the same human pathogen brought from outside. But we could not exploit this to control *Micrococcus luteus* and *Salmonella paratyphium*. *Pseudomonas vesicularis* showed activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* showed activity against *Proteus mirabilis* and *Salmonella paratyphium*. We could also exploit these two isolates against

Table 3. Antibiotic resistance/sensitivity spectrum of isolates from sachet water.

Antibiotic	Concentration µg/ml	Bacterial isolates			
		Proteus mirabilis	***Klebsiella pneumoniae***	***Pseudomonas vesicularis***	***Pseudomonas aeruginosa***
Ceffriaxone	30	+	+	+	+
Chloramphenicol	30	–	–	–	–
Amoxiclav	30	–	–	–	–
Methicillin	30	–	–	–	–
Nalidixic acid	30	+	+	+	+
Streptomycin	30	–	–	–	–

+ ➔ sensitive

– ➔ resistant

Table 4. Heavy metal tolerance spectrum of isolated strains from sachet water.

Heavy metals	Concentration µg/ml	Bacterial isolates			
		Proteus mirabilis	*Klebsiella pneumoniae*	*Pseudomonas vesicularis*	*Pseudomonas aeruginosa*
Copper (copper sulphate)	1	+	+	+	+
	3	+	+	+	+
	5	+	+	–	–
Chromium (potassium chromate)	1	+	+	+	+
	3	+	+	+	+
	5	+	–	–	+
Zinc (Zinc sulphate)	1	+	+	+	+
	3	+	+	+	+
	5	+	–	–	+
Lead (Lead acetate)	1	+	+	+	+
	3	+	+	+	+
	5	+	+	+	+

+ ➔ sensitive
– ➔ resistant

Table 5. Screening for extra cellular enzyme production.

Name of the enzyme	Bacterial isolates			
	Proteus mirabilis	*Klebsiella pneumoniae*	*Pseudomonas vesicularis*	*Pseudomonas aeruginosa*
Protease (Gelatin)	+	+	+	+
Protease (Casein)	+	+	+	+
Amylase (Starch)	+	+	+	+
Cellulase (Cellulose)	–	–	–	–

+ ➔ presence of activity
– ➔ absence of activity

Table 6. Antibacterial activity of pure bacterial isolates.

Name of the isolates	Pathogens			
	Proteus mirabilis	*Klebsiella pneumoniae*	*Micrococcus luteus*	*Salmonella paratyphium*
Proteus mirabilis	–	–	+	+
Klebsiella pneumoniae	–	–	+	+
Pseudomonas vesicularis	–	–	+	+
Pseudomonas aeruginosa	+	+	+	+

+ ➔ Presence of activity
– ➔ Absence of activity

Table 7. Antibacterial activity of isolates with organic solvent (Ethyl Acetate).

Name of the isolates	Pathogens			
	Proteus mirabilis	***Klebsiella pneumoniae***	***Micrococcus luteus***	***Salmonella paratyphium***
Proteus mirabilis	–	–	+	+
Klebsiella pneumoniae	+	–	+	+
Pseudomonas vesicularis	–	–	+	+
Pseudomonas aeruginosa	–	+	+	+

+ ➔ Presence of activity

– ➔ Absence of activity

Klebsiella pneumoniae, *Proteus mirabilis* and *Salmonella paratyphium*. The same happened when the isolates when dissolved in Ethyl acetate, an organic solvent.

But the presence of pathogens in drinking water is serious health risks. Here the isolates like *Proteus mirabilis*, was a causative agent of urinary tract infection and *Klebsiella pneumoniae* was a causative of Pneumonia. The Environmental officers in the local Government employment owe it a duty to educate about the health risk in taking this kind of contaminant sachet water and to create awareness to the public when drinking water standards are violated.

6. CONCLUSIONS

People are increasingly concerned about the safety of their drinking water. As improvements in analytical methods allow detecting impurities at very low concentrations in water, water supplies once consider pure are found to have contaminants. The enforcement of the regulation guiding water quality before the national agency for Food and Drug Administration Control (NAFDAC) to comply with the drinking water qualities guidelines values as recommended by W.H.O. becomes urgent.

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