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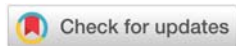
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Short Communication

A comparative bacteriological study of bottled drinking water In Nigeria

Abstract

Bacteriological examination of six brands of bottled drinking water samples produced by different bottled water producers in Nigeria was carried out to compare their bacterial quality. The bottled drinking water were bought from various sales outlets in Nigeria. The biochemical characteristics of the isolates and the sugar fermentation reactions of the isolates were studied. Results of the bacteriological examination showed varied degrees of bacterial load. Causes of contamination of the bottled drinking water and recommendations for improving their microbial quality were also highlighted.

secretion of hormones, enzymes and other biochemical body functions [1].

In view of the varieties of function of water and the magnitude of its requirement, studies are necessary to avert the danger of water shortage, water dehydration and disease outbreak. Bacteriological tests offer the most delicate test for detection of recent and therefore potentially dangerous water pollution [2].

Though a water supply may pass all laboratory tests, we have to bear in mind that hazard may arise from pollution of the water source through cross-connection, back siphonage, leaks in mains and service reservoirs [3].

Reports indicate that there is isolation of some pathogenic organisms like *Salmonella*, *Shigella* and *Escherichia coli* from drinking water. Most reported cases in Typhoid fever were not unconnected with drinking of sachet packaged water [4].

Organisms which are well characterized and mostly present can include strains of *Salmonella*, *Shigella*, *Leptospira*, Enteropathogenic *Escherichia coli*, *Francisella*, *Vibrio*, *Mycobacteria*, Human enteric viruses, Cysts of *Entamoeba histolytica*, *Giardia lamblia*, or other pathogenic protozoans and larvae of various pathogenic worms.

Water of good quality has a low total bacterial count fewer than 100 cfu per milliliters [5].

Salmonella strains have been frequently detected in drinking water sources and this have been correlated with outbreaks of Salmonellosis [1].

Since safe drinking water is essential for good health,

Introduction

Water is the most valuable of liquids in the world. The better our quality of life, the more water we consume, so, it is not surprising that the water being used by an entire society becomes polluted.

Nature is a great purifying system; water passes from the sky to the earth, from the earth to rivers to the sea, and from the sea, it returns to the sky. But this natural process is breaking down in a global scale.

Water is one of the most indispensable needs for the continued existence of all things including man.

Pollution of water sources has become a major source of epidemics in urban and rural communities and this is more evident where there is no proper supply of potable water.

If there is no adequate measure as to the provision of water, man may resort to obtaining water for different uses.

To stay healthy, it is therefore essential to ensure good and safe distribution of drinking water to the people.

Basically, water is as important as food in the nutrition of man and must be supplied regularly and in sufficient quality and quantity. Water is needed in the body for digestion, translocation, absorption, and excretion of metabolic wastes,

drinking water must be free of pathogens. This study is aimed at establishing the bacterial quality of six brands of bottled water produced and distributed in Nigeria by establishing the level of their bacterial load, the species distribution and potential public health significance [6].

Materials and Methods

Sample collection

Six bottled water were collected from different areas in Eastern Nigeria. They were bought from various sales outlets like open markets, supermarkets and street hawkers. The bottled water were bought from Enugu, Benin, Owerri, Port Harcourt, Aba and Onitsha areas of Eastern Nigeria after thorough inspection for possible tear or leak on the bottle so as to prevent bacterial contamination of the samples. These places are more than five kilometers from each other.

Bacterial examination

Coliform test: The most probable number (MPN) multiple tube fermentation technique divided into presumptive, confirmed and completed tests was used to enumerate coliforms [6,7].

Presumptive test: Sterile distilled water was used for serial dilution. This was achieved by distributing 9ml of sterile distilled water to make up 10 ml of 10^{-1} dilution. Using sterile pipette, 1ml, 0.1ml, 0.01ml water samples were inoculated into replicated batches of 3 tubes containing 10ml sterile lactose broth. The test tubes were incubated at 30°C for 24 hours. The tubes were then examined for gas formation. If no gas was formed in the inverted vials, these tubes were further incubated for another 24 hours. Presence and absence of gas formation was recorded.

Confirmed test: Using a sterile pipette, 10ml of sterile Brilliant Green Lactose Bile Broth was distributed with a set of tubes with inverted vials. The broth was sterilized and cooled. A metal loop of about 3mm in diameter was used to inoculate a loopful of medium from positive tubes of presumptive test into sterile Brilliant Green Lactose Bile Broth tubes. The inoculated tubes were incubated at 35°C for 48 hours. Presence and absence of gas formation was recorded.

Completed test: The melted Eosin Methylene Blue (EMB) agar was distributed into plates using the pour plate method and allowed to solidify. Using a sterile wire loop, a loopful of broth from positive tubes of the confirmed test was streaked on the EMB agar plates. The plates were incubated at 35°C for 24–48 hours. Gram stained preparation from those agar slant cultures corresponding to the secondary lactose broth tubes that showed gas formation was examined thoroughly and findings were recorded.

Standard plate count: Using standard plate count agar, plating was done with pour plate method and incubated at 35°C for 24 hours. Three plates each were used for a particular bottle water sample. The average number of colonies from the three

plates was taken and recorded. Water of good quality has a low total bacterial count, fewer than 100 cfu per milliliters [5,8].

Morphological identification: Gram staining

The reaction of each bacteria isolate to gram staining technique was carried out. The stain was checked, prior to use, for correct staining reactions using a smear containing known gram positive and gram negative organisms [9]

A thin smear of the bacteria colonies were made on grease-free slides using sterile wire loop and normal saline. These were subsequently air-dried and heat fixed by passing the slide through a flame. The fixed smear was covered with crystal violet stain for 60 seconds. After this, the stain was rapidly washed off with clean water. The water was tipped off and the smear was covered with Lugol's iodine for 30 seconds. The iodine was washed off with clean water. Acetone-alcohol was used to decolorize rapidly for few seconds and clean water was used to wash it off. The smear was then covered with neutral red stain for two minutes. The stain was washed off with clean water and the back of the slide wiped with cotton wool and placed in a draining rack for the smear to air-dry. The smear was examined microscopically, first with the 40x objective to check the staining and to see the distribution of the materials on the slide and then with the oil immersion objective 100x to report the bacteria and cells. The light microscope was used to observe the gram stain reaction and morphology of cells. Colour differentiation (pink and purple) was used to differentiate into gram negative and gram positive. Organisms that retain the primary crystal violet colour after decolourization and flushing with neutral red were gram positive while organisms that retain the neutral red colour are gram negative [7,10].

Biochemical test for identification of the bacterial isolates

Biochemical tests were carried out as confirmatory tests to identify the bacteria isolates and to differentiate the coliforms suspected into groups (i.e. IMVIC reactions: where I =Indole, M=Methyl red, VI=Voges proskauer reaction and C=Citrate) [11]. These tests were carried out on the bacterial isolates as follows:

Methyl red test: This was done to detect the production of sufficient acid during fermentation of glucose and maintenance of acid condition (pH 4.5 or below). This is shown by change in colour of the methyl red indicator solution. Exactly 5ml of buffered glucose broth was inoculated with each bacterial isolate and incubated at 35°C for 48 hours. Exactly 5 drops of methyl red reagent was added. Development of bright-red colour is indicative of positive results while yellow colour is indicative of negative results [8].

Voges proskauer test: Methyl red broth prepared during the methyl red test and inoculated with isolates were tested to know whether the organisms after producing acid from glucose as they did during the methyl red test are capable of converting the acid to acetyl methyl carbinol or 2, 3 butanediol which are neutral substances.

To 1ml of culture of each isolate, 0.5ml of 6% alcoholic solution of x-naphtol and 0.5 ml of 16% potassium hydroxide (KOH) solution was added. If a pink colouration appears after 5 minutes, it will be recorded as positive, but negative result can be left for up to one hour to check for slow reaction. The tubes were held in an almost horizontal position and vigorous shaking carried out intermittently.

Indole test: This test was performed to demonstrate the ability of the bacterial isolates to decompose the amino acid tryptophan to indole which accumulates in the medium. It was then tested by a colourmetric reaction with paradimethyl amino benzaldehyde. Each of the 5ml portions of the sterile tryptone broth was inoculated and incubated at 35°C for 24 hours. After the time lapse, about 0.3 to 0.4 ml of the test reagent (Kovac's reagent) was added. The Kovac's reagent consists of amyl or isoamyl alcohol 75ml, para-dimethyl-amino benzaldehyde 5g, and concentrated HCL 25 ml. A dark red colour in the amyl alcohol surface layer was taken to constitute a positive indole test, while the original colour of the reagent was taken as a negative test.

Citrate utilization test: This test was done to check for the ability of the organism to utilize citrate as the sole carbon energy source for growth and an ammonium salt as the sole source of Nitrogen. Each test tube of the sterilized citrate medium was inoculated a loopful of each of the bacterial isolates. The tubes were then incubated at 35°C for 72–96 hours. Changes in colour of the medium from blue to green indicates positive result. A sterilized and uninoculated citrate medium served as a control.

Other biochemical tests: Other biochemical tests like the motility, pigmentation, starch hydrolysis, Nitrate reduction, Gelatin hydrolysis, catalase, oxidase and Hydrogen sulphide tests were carried out using standard procedures [8,12].

Sugar fermentation tests

The sugar tested were glucose, lactose, arabinose, maltose, mannitol, raffinose, sucrose and xylose.

Exactly 1% of each sugar was prepared in peptone water using Andrade's incubator. Exactly 5ml of this solution was dispersed into test tubes. A Durham tube was inverted and inserted into each medium and sterilized at 115°C for 15 mins. Each of the test tubes were inoculated with the test organism by emulsifying the organism by the side of the tube and mixing with the solution. Incubation was carried out at 37°C for 24 hours. Acid production (fermentation) was shown by change in colour of the medium from pale to deep pink reddish, while gas production was shown by a displacement, showed no colour change [13,14].

Results

In the bacteriological analysis of the six brands of bottled drinking water, the standard plate count showed that the bottled water contains varied number of bacterial colonies in each plate and the result is shown below.

Heterotrophic plate count

Enugu bottled water (Ebw)	Dilution 10 ⁰
Plate	Number of Colonies
EBW ₁	83
EBW ₂	87
EBW ₃	80
Total	250

Average = 250/3 = 8.3 colonies. Standard Plate Count = 8.3 x 10⁻¹ cfu/ml

Benin bottled water (Bbw)	Dilution 10 ⁰
Plate	Number of Colonies
BBW ₁	87
BBW ₂	85
BBW ₃	89
Total	261

Average = 261/3 = 8.7 colonies. Standard Plate Count = 8.7 x 10⁻¹ cfu/ml

Owerri bottled water (Owbw)	Dilution 10 ⁰
Plate	Number of Colonies
OWBW ₁	89
OWBW ₂	75
OWBW ₃	68
Total ss	232

Average = 232/3 = 7.7 colonies. Standard plate count = 7.7 x 10⁻¹ cfu/ml

Port-Harcourt bottled water (Phbw)	Dilution 10 ⁰
Plate	Number of Colonies
PHBW ₁	78
PHBW ₂	89
PHBW ₃	93
Total	260

Average = 260/3 = 8.6 colonies. Standard plate count = 8.6 x 10⁻¹ cfu/ml

Aba bottled water (Abw)	Dilution 10 ⁰
Plate	Number of Colonies
ABW ₁	93
ABW ₂	84
ABW ₃	69
Total	246

Average = 246/3 = 8.2 colonies. Standard plate count = 8.2 x 10⁻¹ cfu/ml

Onitsha bottled water (Onbw)	Dilution 10 ⁰
Plate	Number of Colonies
ONBW ₁	94
ONBW ₂	83
ONBW ₃	87
Total	264

Average = 264/3 = 8.8 colonies. Standard plate count = 8.8 x 10⁻¹ cfu/ml

From these colonies, isolates were taken and purified by subculture and subsequently identified using biochemical tests which are collectively called IMVIC reactions [10,15].

The Enugu bottled water sample showed growth of *Bacillus* species and *Staphylococcus aureus*, the Benin bottled water sample was contaminated with *Staphylococcus aureus*, the Owerri bottled water sample showed growth of *Staphylococcus aureus*, and *Enterobacter species*, the Port-harcourt bottled water sample showed growth of *Staphylococcus aureus* and *Enterobacter species*, the Aba bottled water sample showed growth of *Staphylococcus aureus* and *Bacillus* species while the Onitsha bottled water sample was contaminated with *Staphylococcus* species and *Bacillus* species. The results of the biochemical characteristics and sugar fermentation reactions of the isolates are shown in tables 1,2 below.

Discussion

The bacterial analysis revealed the presence of bacteria in the six samples of bottled water. The presence of bacteria in the samples could be due to the integrity of the bottle used i.e; loose caps, cracks, breakages and apertures or openings, weak seals etc, which may be caused during packaging and transportation.

The presence of the organisms in “supposedly pure and treated” water should concern the producers and the consumers even if these organisms are not pathogenic [16].

Table 1: The biochemical characteristics of the isolates in bottled water.

Test	EBW	EBW	BBW	OWBW	OWBW	PHBW	PHBW	ABW	ABW	ONBW	ONBW
	Bacillus sp.	Staphylococcus aureus	Staphylococcus aureus	Staphylococcus aureus	Enterobacter species	Staphylococcus aureus	Enterobacter species	Staphylococcus aureus	Bacillus species	Bacillus species	Staphylococcus aureus
Motility at 22 °C	+	-	-	-	+	-	+	-	+	+	-
Gram reaction	+	+	+	+	+	+	+	+	+	+	+
Pigmentation	-	-	-	-	-	-	-	-	-	-	-
Citrate reduction	+	+	+		+ ²	+	+ ²	+	+	+	+
Starch reduction									+	+	
Nitrate reduction	+	+	+	+		+		+	+	+	+
Gelatin hydrolysis	+				d		d		+	+	
Catalase	+	+	+	+	+	+	+	+	+	+	+
Oxidase	-	-	-	-	-	-	-	-	-	-	-
Coagulase	-	+	+	+		+		+	-	-	+
Indole	-				-		-		-	-	
Hydrogen sulphide					-		-				
Voges proskauer	+	+	+	+	+	+	+	+	+	+	+
Methyl red					-		-				

KEY: + = Positive - = Negative

X2 = A minority of the strains give a negative result.

D = Slow reaction.

Table 2: Sugar fermentation reactions of the isolates in bottled water.

Test	EBW	EBW	BBW	OWBW	OWBW	PHBW	PHBW	ABW	ABW	ONBW	ONBW
	Bacillus species	Staphylococcus aureus	Staphylococcus aureus	Staphylococcus aureus	Enterobacter species	Staphylococcus aureus	Enterobacter species	Staphylococcus aureus	Bacillus species	Bacillus species	Staphylococcus aureus
Glucose	+	+	+	+	+	+	+	+	+	+	+
Lactose	-	+	+	+	+	+	+	+	-	-	+
Arabinose	-				+		+		-	-	
Maltose		+	+	+		+		+			+
Mannitol	+	+	+	+	+	+	+	+	+	+	+
Raffinose	-				+		+		-	-	
Sucrose	-	+	+	+	+	+	+	+	-	-	+
Xylose	-	+	+	+	+	+	+	+	-	-	+

KEY: + = Positive

- = Negative

It is evident that the quality of the water we drink affect our health, therefore to improve our health status, the producers of bottled water should pay more attention to the processing methods. This will go a long way in protecting public health, save time, energy and money likely to be lost through illness from consumption of contaminated water.

Great care should also be taken during the production processes, handling, packaging and distribution of bottled water to consumers.

Bacterial quality of all the products examined showed intolerable levels of bacterial load with Onitsha bottled water sample showing a higher bacterial load than the others i.e; 8.8×10^{-1} cfu/ml.

Effective treatment should be carried out on all the samples. The bottled water producers should change the seal of their bottle to be more tamper proof. They should use spot caps to seal their bottles. The spot caps should have a dust cover and constructed in such a way that one sucks the water instead of opening it to contamination by *Staphylococcus aureus* and other bacteria when one is about to drink from the bottle.

The present cap used by the producers is open to opportunistic invasion by *Staphylococcus* species and other microbes whenever they are opened for use.

The contamination by the *Bacillus* species and *Enterobacter* species can be solved by the improvement of hygiene in the factory, proper housekeeping, efficient cleaning, avoiding of overnight storage of water and proper disinfections.

The higher bacterial load in the bottled water from Benin, Port Harcourt and Onitsha may be due to the structural integrity of the bottle/container. The Benin, Port Harcourt and Onitsha bottled water is ordinary polyethylene. The bottled water producers should change their bottle /container to polyethylene tetrathylate for better structural integrity of their products to avoid cracks, breakages, openings and apertures on the containers which facilitates microbial contamination. There should be periodic checks and analysis on the methods and strategies used in the production and supply of their products.

Bacteriological tests must be carried out on the products before packaging and distribution to the sales outlets [17].

There should be proper education of the populace on the medical and economic importance of drinking impure water.

Competent personnel of good integrity should be employed in monitoring the production processes and supply of bottled water to the consumers.

The government should provide safe drinking water and sanitation to the people and there should be coordinated support in meeting the national and international drinking water standards by thorough and unbiased monitoring of water firms.

The presence of *Enterobacter* species which exists at higher temperature may be from its contamination of bottled water due to poor storage while the presence of *Bacillus* species may be from machinery processing and inadequate chemical usage.

The presence of *Staphylococcus* species is because it is highly commensal and ubiquitous.

The products should therefore be properly stored and bacteriological tests of the air quality and environment of the factory should also be carried out regularly.

At present, increased activities of the Nigerian National Agency for Food and Drug Administration and Control (NAFDAC) has helped to decrease the number of “mushroom” manufacturers of bottled water and helped to close down water firms with substandard water treatment and purification systems. The activities of NAFDAC has helped in decreasing disease outbreaks likely to result from consumption of impure water packages.

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