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Original Research Article

Bacteriological Examination of Bottled Drinking Water by MPN Method

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Abstract

In this study, the bacteriological quality of bottled water in Mandalay, Myanmar was detected by the use of MPN method. Nineteen brands of Bottled Drinking Water (BDW) samples were analysed for total coliform count as the primary presumption of coliform contamination. 7 water samples were contaminated in a range from 6 MPN/ 100ml to 16 MPN/100ml. The faecal coliform, the indicator bacterium *Escherichia coli* was detected in 5 out of 19 samples by confirmation on EMB showing metallic sheen and completed tests were re-confirmed by further biochemical reactions. Antibiotic sensitivity and plasmid DNA extraction from the *E.coli* isolates were examined. (40%) of 2 out of 5 strains contained plasmids with molecular weights of more than 23kb. The overall results showed that 63.15 % among the subjected bottled water in Mandalay were noted to be microbiologically clean and safe for drinking.
Keywords: Bacteriological examination**,** Bottle Drinking Water, MPN method, coliform, *Escherichia coli*.

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INTRODUCTION

Globally, the consumption of bottled water has increased every year by ten percent including the developing countries of Asia and South America with the fastest growth consumption [1]. Residents of larger communities are more likely to use the bottled water as the primary drinking water source [2]. People who live in large cities in Myanmar also depend on bottled water for drinking that may safer and more convenient than tap water. Gleick, Lalumandier and Anadu [1, 3, 4] have stated that bottled water has been used instead of tap water for its convenience, better taste, and perceived purity. In Myanmar, the bottled water considered as a food product is regulated by the Food and Drug Administration (FDA), Department of Health under the administration of Ministry of Health and Sports [5] for its quality, safety and protection. It was found that there has been a dramatic increase in bottled water production in Myanmar. Food microbiology laboratory, department of Food and Drug Administration (Yangon Branch) [6] has reported that there have been 320 bottled water industries in 2008 that has been increased to 657 in 2013. According to FDA (Myanmar) standard, it is not allowed to exceed 100 CFU per ml by means of standard plate count method and total coliform and *E. coli* must be free in 100 ml of water as regulated by World Health Organization drinking water guidelines [6]. Typically, there are three main parameters for drinking water quality standard such as physical, chemical and microbiological. Among them, microbial contamination is a major concern of water-related health burden [7]. The biggest health threat worldwide like water-borne diseases has been contributed between 70-80% of health problems in developing countries [8]. The presence of both non-coliform species like heterotrophic bacteria and total coliform bacteria may cause the special health risk for infants and young children and immunocompromised adults [9]. Department of Medical Research, Yangon, Myanmar have reported on the prevalence of acute diarrhoea in children under 5 years due to the high level of drinking-water contamination: (90%) of water samples were contaminated with thermotolerant (faecal) coliforms [10]. According to the recommendation by World Health Organisation (WHO), the microbial quality of drinking-water is measured using faecal indicator bacteria, preferably *Escherichia coli* that indicate the presence of faecal contamination rather than identifying pathogens directly [7]. Edberg [11] also reported that it is not necessary for analyzing drinking water for all pathogens but requiring an indicator of fecal pollution for Public health protection. It has been stated that *Escherichia coli* was chosen as the biological indicator for the safety of water treatment since 1890s [11]. The

presence of *E.coli* bacteria as indicator determines the risk of pathogenic contamination from fecal origin [12, 13]. The most commonly used test for indicator organism is to determine the presence of *Escherichia coli* which only indicates the possibility of fecal contamination as well as the occurrence of diseases, not the actual presence of fecal pathogens [14].

In this study, we used the Multiple Fermentation Tube or Most Probable Number (MPN) method according to [7] that has been focused for the analysis of drinking-water for many years with satisfactory results. The results for the presence of coliform bacteria are represented as a most probable number (MPN) index that would give the results shown by the test and not a count of the actual number of indicator bacteria present in the sample [15]. This study was conducted with the aim to find out the bacteriological contamination of bottled drinking water in accordance with the results of the acceptable limit [16]. The objectives of this study were to enumerate fecal indicator bacteria by the most probable number (MPN), to estimate the level of contamination in the water sample in the presence of indicator coliform *Escherichia coli*, to observe the antibiotic sensitivity pattern of *E. coli* isolates and to screen for plasmids from occurrence of *E. coli* isolates that may threaten to public health.

MATERIALS AND METHODS

Sample Collection

Nineteen different brands of bottled drinking water available in commercial market were collected randomly from Mandalay, Myanmar. The samples were bought as 1litre and 20litre plastic bottle packaging and then subjected for bacteriological analysis within 24hrs.

MPN Method

The procedure for testing bottled drinking water was done aseptically. MPN Method was conducted in three steps:

1) Presumptive test
2) Confirmed test
3) Completed test

1) Presumptive test

Presumptive test functions as the primary presumption for the presence of Gram negative coliform bacteria in the samples. In this test, MacConkey broth is commonly used for lactose fermentation for the presence of the indicator bromocresol purple. The inverted Durham's tube is used for the detection of gas formation by Gram negative coliform bacteria. The color changes of media into yellow and on collection of gas in Durham's tube can be assumed that coliform bacteria are present in these samples. 5 of 10ml of water samples were inoculated into each of 10ml of presumptive broth (double strength). 1 of 50ml water sample was added to a tube containing 50ml of presumptive broth (double strength). After 48 hour incubation at 37ºC, the number of positive tubes were recorded from each set and compare with standard chart to give presumptive coliform count per 100ml water sample.

2) Confirmed Test

In the confirmed test, positive samples from presumptive test were selected to determine the coliforms are of indicator bacteria of fecal origin *Escherichia coli*. Eosine Methylene Blue (EMB) media was used to differentiate *Escherichia coli* from Gram negative coliform bacteria by the production of greenish metallic sheen that confirms the presence of indicator bacteria *E. coli*. The production of color indication from colonies can be observed after 24hours incubation at 37ºC by streaking loopful sample from positive tubes.

3) Complete Test

The bacterial colonies on EMB media from confirmed test were inoculated in LB broth at 44.5^{o}C with Durham's tube and subculture the colony on Mac Conkey agar plate. Presence of faecal indicator *E.coli* is confirmed by the production of gas and color changes in media. For further complete confirmation, a satisfactory differentiation within the coliform group was done by indole, methyl red, Voges-Proskauer and sodium citrate (IMViC) tests which are commonly recommended for such differential determination according to Bergey's Manual of Systematic Bacteriology [17].

Antibiotic Susceptibility Test

The Kirby-Bauer test known as the disc diffusion test has been used for years as a standard by [18]. Antibiotics susceptibility test was performed by disc diffusion technique in which Mueller-Hinton agar that was recommended by WHO as a relatively simple medium. The commonly used antibiotics such as Chloramphenicol, Tetracycline, Nalixidic acid, Ciprofloxacin, Gentamycin and Streptomycin were subjected against the isolated *E coli*.

Plasmid Extraction

According to method of Bimboim & Doly [19], mini preparation of the plasmid DNA was done from *E.coli* isolates. The extracted plasmids were fractionated by 0.8% agarose gel electrophoresis at 70V for 1hr using TAE 1x buffer. Plasmid sizes were determined using standard DNA molecular weight marker λ DNA/ Hind III digest marker.

RESULTS AND DISCUSSIONS

MPN Method

Bacteriological examination of water for total coliform count was done by MPN method for 19 brands of bottled drinking water. The presumptive coliform count by MPN method was shown in Table-1. 7 brands had total coliform counts ranging 6 MPN/ 100 ml to 16 MPN/100 ml. 12 brands had no coliform contamination (<1 MPN / 100ml). It was found that 5 brands out of 19

were contaminated with *E.coli*. The presence of indicator organism *Escherichia coli* isolates for faecal contamination was confirmed by the production of greenish metallic sheen on EMB media as shown in Figure 1(a). The results for confirmed test of MPN method were presented in Table-2. The cultural examination for complete test was shown in Figure 1(b) and Table-3. Further complete identification of *E.coli* isolates was done by biochemical reactions as shown in Figure-2 and described in Table-4.

Antibiotic Sensitivity Test

The data for antibiotic sensitivity test showed that all the *E.coil* isolates are sensitive to Chloramphenicol, Tetracycline, Nalixidic acid and low Ciprofloxacin resistant strains were found (Isolate R and A3). These isolates were resistant to Gentamycin (Isolate G, S2, and A3) and the two resistant for Streptomycin (Isolate G, S2) and (Isolate E) intermediate results for Streptomycin. Antibiotic Sensitivity Pattern of the Isolated *Escherichia coli* was represented in Table-5.

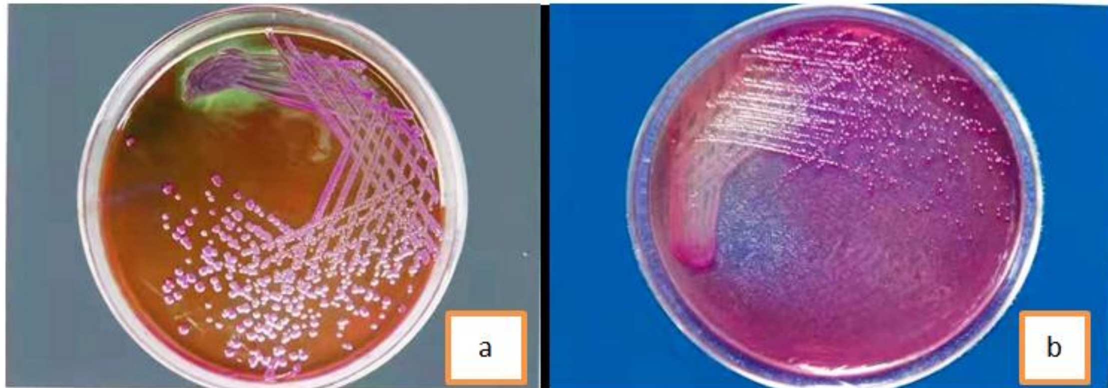

Fig-1: Pure culture of *Escherichia coli* isolate by the production of greenish metallic sheen (a) on EMB media (b) pink colonies on Mac Conkey's media

Fig-2: Biochemical Characterization by iMViC tests for *Escherichia coli* isolate

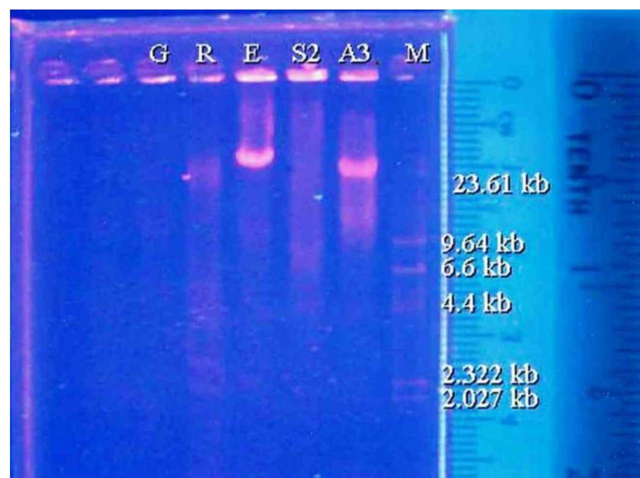

Fig-3: Agarose Gel Electrophoresis of Extracted Plasmids

M = λ DNA/ Hind III digest marker , Lane 3,4,5,6,7= Isolate G,R,E,S2,A3

Table-1: Total Coliform Count by MPN Method

Sample Identification		Number of tubes giving positive reaction		MPN Index per 100ml	95 % confidence limits	
Sample Code	Sample Type	1*50ml	5*10ml		Lower	Upper
Y	1 L	0	0	<1	_	_
E1	1 L	0	0	<1	_	_
A1	1 L	0	0	<1	_	_
P	1 L	0	0	<1	_	_
O1	1 L	0	0	<1	_	_
A2	1 L	0	0	<1	_	_
D	1 L	0	0	<1	_	_
S1	1 L	0	0	<1	_	_
W	1 L	0	0	<1	_	_
L	1 L	0	0	<1	_	_
K	1 L	0	0	<1	_	_
M	1 L	0	0	<1	_	_
N	1 L	1	2	6	1	15
G	1 L	1	2	6	1	15
R	20 L	1	3	9	2	21
E	20 L	1	3	9	2	21
O2	20 L	1	3	9	2	21
S2	20 L	1	4	16	4	40
A3	20 L	1	4	16	4	40

*All the experiments have done for triplicates and the results were reproducible.
One representative data was represented.

Table-2: Results for the Comfirmed Test

Sample Code	Growth on EMB	Production of Greenish Metallic Sheen	Result
G	+	+	Non-potable
R	+	+	Non-potable
E	+	+	Non-potable
S2	+	+	Non-potable
A3	+	+	Non-potable

Table-3: Complete tests for *E.coli* isolates

Isolate Tests	G	R	E	S2	A3
LB broth (+/-)	+	+	+	+	+
Microscopic Morphology	Short,G (-)ve straight rods 2-4 μm by 0.4 μ m	Similar	similar	similar	Similar
Motility	+	+	+	+	+
Colonial morphology: On EMB agar	circular raised and smooth colony about 2mm to 3 mm greenish metallic sheen on the surface of colonies or dark centre colony	similar	similar	similar	Similar
On MacConkey's agar	Pink colored colonies	Similar	similar	similar	Similar

Table-4: Biochemical Characterization of *E.coli* isolates: (IMViC) tests

IsolateTest	G	R	E	S2	A3
Indole Test	+	+	+	+	+
MR Test	+	+	+	+	+
VP Test	-	-	-	-	-
Citrate Test	-	-	-	-	-

Table-5: Antibiotic Sensitivity Test of the *Escherichia coli* Isolates

No.	Antibiotics	Inhibition Zone Diameter				
		G	R	E	S2	A3
1.	Chloramphenicol	29 mm (S)	28 mm (S)	23 mm (S)	25 mm (S)	27 mm (S)
2.	Tetracycline	21 mm (S)	21 mm (S)	21 mm (S)	23 mm (S)	23 mm (S)
3.	Nalixidic acid	24 mm (S)	28 mm (S)	21 mm (S)	24 mm (S)	28 mm (S)
4.	Ciprofloxacin	22 mm (S)	9 mm (R)	25 mm (S)	11 mm (R)	10 mm (R)
5.	Gentamycin	10 mm (R)	15 mm (S)	15 mm (S)	11 mm (R)	10 mm (R)
6.	Streptomycin	9 mm (R)	22 mm (S)	14 mm (I)	8 mm (R)	22 mm (S)

Note: S = Sensitive, R = Resistant, I = Intermediate
*All the experiments have done for triplicates and the results were reproducible. One representative data was represented.

Plasmid Extraction

In the agarose gel fractionation of the extracted plasmids, the extracted plasmid DNA was visualised using the ultraviolet light illuminator and sizes of plasmid were estimated using standard DNA molecular weight markers at λ DNA/ Hind III marker. 2 bands

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were found from 2 isolates out of 5 isolates of *E.coli* were shown in Figure-3. It was found that all plasmids are more than 23 kb in size (i.e. E, and A3).

DISCUSSIONS

The methods of drinking water survey are designed to show the potential presence of pathogens by screening for faecal indicator organisms which themselves are not necessarily pathogens [14]. The indicator bacterium, *Bacterium coli* (*Echerichia coli*) [11] is still used as the major target bacterium. The screening tests for the possible presence of indicator species were established by fermentation of lactose with the production of acid and gas at 37°C. However, lactose negative species of *Salmonella* and *Shigella* that can cause waterborne outbreaks of diseases are not detected by the standard screening methods. The numbers of bottled drinking water brands are now commercially available in Myanmar. With the results from bacteriological analysis, 7 of the brands tested were contaminated with coliform bacteria. The standard of water for drinking permits absolutely no contamination of water (0 coliform/100ml) [7]. But some of the brands (36.84 %) representing 7 brands were not suitable for drinking. Other forms of bacteria, such as cocci were also found in some of the brands. Further identification test had been done and that revealed the presence of *Escherichia coli* in 5 of these brands. As *E.coli* is an indicator of fecal contamination, the safety level of water for drinking is very low and the quality assurance of these brands is to be questioned. However, 12 brands (63.16% of total) represent with no contamination, neither coliforms nor any other bacteria were found in them. In these cases, they can be said to be microbiologically clean. Whether they are pathogenic or not, it is not a good practice to contain some kind of bacteria in potable water, especially if it is a bottled drinking water. One notable fact is that the large 20L bottles were found to be more contaminated with coliform and *E. coli* relative to 1L bottles.

The present study of the bacteriological analysis of bottle water by MPN method observed that 7 out of 19 samples (36.8%) were not reached the standard of 0 Most Probable Number (MPN) per 100 ml of water as recommended by WHO drinking water guidelines. It was noted that the bottle water samples in Yangon (32.6%) among 30 out of 92 samples were less contaminated with coliform than those of Mandalay. The cause of bacterial contamination for bottled drinking water may be due to the poor processing such as treatment system, filling and sealing, storage and distribution [6]. In 2010, Htoo [20] from University of Public Health, Yangon have reported on hygenic practices of workers in bottled drinking water factories in northern District, Yangon that recommended to train the workers in industries for working experiences and to educate for the knowledge on hygienic practices. The similar problem like bacterial contamination in drinking water that has also been examined by MPN method was also found in other foreign countries [21-24].

According to the antibiotic sensitivity test, the results indicated that the drug resistant strains are existence in bottled drinking water brands. In the agarose gel fractionation of the extracted plasmids, 2 bands of more than 23 kb in size are found from 2 isolates (i.e. E, A3). Out of 5 isolates of *E.coli*, Isolate E represented intermediate results for Streptomycin and A3 were resistant to Gentamycin respectively.

Genetic analysis of *E.coli* isolates by plasmid extraction showed that some large size plasmids were of more than 23 kilobasepairs occurred in some isolates (i.e. E, A3). The resistance genes encoding for antibiotic defence mechanisms are located on the bacterial chromosome or on extra chromosomal plasmids. Plasmids serve a central role, as the vehicles for antibiotic resistance gene and their subsequent dissemination [25]. The high bacterial count in the water sample in the presence of antibiotic-resistant bacteria makes the risk more serious that highlights certain critical problems associated with water safety [26]. It can be assumed that the the *E. coli* isolates which carrying antibiotic resistance traits may be serious for health problem dealing with water bore diseases.

CONCLUSIONS

19 brands of bottled-purified drinking water were bought from local markets in Mandalay and analyzed for coliform contamination. 7 brands had coliform contamination ranging from 6 MPN/ 100ml to 16 MPN/100ml. Other 12 brands had no coliform contamination (<1 MPN/100ml) and no *E.coli* contamination. These 12 brands are suitable for drinking. Among 19 of brands, 5 brands are concluded to be highly contaminated as they contained *E.coil*: 5 strains of *E.coil* have been isolated from them. Hence only 63.15% of the 19 brands studied are microbiologically clean and regarded as suitable for drinking. Therefore, this study may provide the information about bacteriological quality of bottled drinking water in Mandalay by means of MPN method.

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