Culture - independent Pathogenic Bacterial Communities in Bottled Mineral Water

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Abstract **-** Bottled mineral water (BMW) is an alternative to mains water and consider it to be better and safer. Access to safe BMW from the bacteria involving potential health hazard is essential to health. Cultivation-independent technique PCR-based single-strand conformation polymorphism (SSCP) for genetic profiling of PCR-amplified 16S rRNA genes was performed using Com primer set targeting the 16S rRNA genes for detection of pathogenic bacteria in bottled mineral water from the final product of six factories for BMW in Wadi El-natron region- Egypt.

These factories use often ozone technology to treat large quantities of water because of its effectiveness in purifying and conditioning water. A total of 27 single products were isolated from the profiles by PCR re-amplification and cloning. Sequence analysis of 27 SSCP bands revealed that the 16S rRNA sequences were clustered into seven operational taxonomic units (OTUs) and the compositions of the communities of the six samples were all common.

The results showed that most communities from phyla Alphaproteobacteria and certainly in the Sphingomonas sp. Culture-independent approaches produced complementary information, thus generating a more accurate view for the bacterial community in the BMW, particularly in the disinfection step, as it constitutes the final barrier before BMW distribution to the consumer

Keywords **-** Bottled mineral water, Pathogenic bacteria, Culture- independent, PCR-SSCP, 16S rRNA genes.

I. INTRODUCTION

Bottled mineral water (BMW) is often recommended for patients with immune-system deficiencies as well as marketed as ideal for infant nutrition and reconstitution of foods (Warburton, 1993). Mineral water is an oligotrophic environment; their content of viable bacterial cell is as low as 10cfu ml-1 (Ferreira et al., 1994). These lows, count of native organisms are of little concern to the healthy consumer. Outbreaks of infectious bacteria via the use of contaminated tap drinking water still pose a serious health threat worldwide, despite that drinking water is one of the most closely monitored and strictly regulated resources. Both bottled water and tap water may contain the same microorganisms, which originated from the same sources (Papapetropoulou et al., 1997; Ahmed et al., 2013).

The European Union Council Directive on the quality of water intended for human consumption restricts the presence of E. coli, Clostridium perfringens, Pseudomonas aeruginosa, enterococci and coliforms and recommends a total heterotrophic colony count only in the case of water offered for sale in bottles or containers (The Council of the European Union 1998; Ahmed et al., 2013). By using conventional cultivation techniques, less than 1% of the bacterial population can be cultivated from oligotrophic systems such as the drinking water distribution system and the related microbial population (Kalmbach et al., 1997; Falcone-Dias et al., 2015). The real significance of of pathogenic bacteria in the drinking water is poorly understood (Guzman et al., 2010).

Sphingomonas spp. are widely distributed in nature and are resistant to many disinfecting and toxic chemicals (Laskin and White, 1999), and has been isolated from drinking water and drinking water distribution systems. Some Sphingomonas strains are well known for metabolizing complex organic pollutants, but some are opportunistic human pathogens as Sphingomonas paucimobilis and S.

parapaucimobilis (Anon, 2000, Guzman et al., 2010, Gesumaria et al., 2011). A developed protocol was used, which allows the application of single-strand-conformation polymorphism (SSCP) (Orita et al., 1989; Hayashi, 1991; Gasser et al., 2007) for the culture-independent assessment of microbial-community diversity (Schwieger and Tebbe, 1998; Gasser et al., 2007; Jean and Georges , 2008;

Keskes et al., 2012). The SSCP method has the potential to be more easily applied (Lee et al., 1996) and the SSCP produced a number of sharp bands and differentiated the bacterial community structures (Tomoyuki et al., 2006). SSCP was optimized to analyze only one of the complementary single strands (Schwieger and Tebbe, 1998; Meng-zhi et al., 2008), by preferentially degrading with lambda exonuclease the one strand generated with a phosphorylated primer.

This development aims to avoid heteroduplex formations, or overlapping of forward-reverse strands from different amplicons during separation, allowing the separation of mixtures of fragments of identical size but different in sequence. The application of this modified technique was focused on studies of taxonomic shifts in microbial communities by targeting 16S rRNA genes (Peters et al., 2000; Schmalenberger et al., 2001; Schwieger and Tebbe, 2000, 2003). However, a potential application to assess diversity of functional genes was foreseen (Stach and Burns, 2002).

The aim of this study was to identify the common uncultured bacterial community via SSCP in the BMW from the end product of six factories for mineral water located in Wadi El-natroon region, which is one of the largest industrial regions for mineral water in Egypt.

II. GEOMETRY OF THE MODEL AND MATERIAL PROPERTIES

A. DNA Extraction from the six water samples

Six water samples equal volumes each 2 liters were collected from the final product of BMW from six factories in Wadi El-natroon region, The filtration was implemented under sterile conditions with a filtering device actuated by a motor driven pump, the filter sandwich with the bacteria was cut into small pieces with a sterile scalpel and transferred to The MULTIMIX 2 Tissue Matrix Tub, add 978 µl Sodium Phosphate Buffer and 122 µl MT Buffer from fast DNA SPIN Kit for Soil Bio 101 and follow the DNA extraction according to the protocol for DNA extraction with the Fastprep DNA kit for soil (Bio 101). DNA was visualized on 1% agarose gels. Yield of genomic DNA was determined spectrophotometrically by measuring the absorbance at 260 nm.

Purity was determined by calculating the ratio of absorbance at 260 nm to absorbance at 280 nm (a pure DNA has an A260/A280 The DNA extracts from water samples containing approximately 200 ng ml-1 DNA were 50- or 100-fold diluted in Tris-HCl buffer (10 mM, pH 8.0) and used as template DNA in PCR.

B. SSCP for six BMW samples collected from the final product of six factories in Wadi El-natroon region

The primers Com1 (5`CAGCAGCCGCGGTAATAC3`) targeting the position (519-536) and Com2-Ph (5`CCGTCAATTCCTTTGAGTTT3`) targeting the position (907-926) were chosen for the amplification of bacterial 16S rRNA genes (Schwieger and Tebbe 1998). Single-stranded DNA (ssDNA) from PCR products was obtained as previously described (Schwieger and Tebbe, 1998). Briefly, PCR as performed with one of the primers being 5` phosphorylated, Each amplification was carried out using 2 ng DNA template in a final volume of 50 µl, starting with an initial denaturation for 15 min at 95°C.

A total of 30 cycles (30s at 95°C, 30s at 55°C, and 1 min at 72°C was followed by a final elongation for 10 min at 72°C. Amplification was achieved using Hot Star Taq DNA polymerase (Fermentas). PCR products were eluted from agarose gels (see Figure. 1), and the phosphorylated strands were digested by lambda exonuclease (NEB). The remaining single-strands were purified with GeneJET PCR Purification Kit (Fermentas), dried by vacuum centrifugation, resuspended in 6 µl of loading buffer (95% formamide, 0.25% bromophenol blue and 0.25% xylene cyanol), and denatured for 5 minutes at 94 °C, followed by instant cooling on water ice bath for 3 minutes.

The separation conditions were standardized in a DCode System for PCR-SSCP, optimized running parameters were 120V (10 mA) for 18 h at a constant temperature of 26°C on 20 cm x 20 cm x 0,75 mm 0.6X MDE gels in 0.7X TBE (Sambrook et al., 1989) as a running buffer. Optimal results were obtained when ssDNA obtained from 100 - 400 ng dsDNA was loaded onto the gels and a slightly enhanced resolution was achieved when the amplified single-strands of the reverse primer were subjected to PCR-SSCP analysis. For nucleic acid detection, gels were silver stained as reported previously (Bassam et al., 1991).

Single-strand electrophoretic mobilities corresponding to different conformations were excised from dried gels, and DNA extracted by the “Crush and Soak” method (Sambrook et al., 1989) PCR reamplification of the excised and eluted single-strands was made with the same primers used to generate the original dsDNA fragment.

C. Data Deposition

The sequences reported in this paper have been deposited in the GenBank database (27 sequences for the independent culture bacteria accession numbers (JF793682- JF793708).

D. RESULTS

Com Primer set were used to amplify the eubacterial 16S rRNA gene sequences (see Figure 1) including the variable regions yielded complex SSCP patterns, SSCP community profiling showed highly diverse and distinct microbial communities for the BMW water samples, BMW sample 5 displayed the largest numbers of bands, while BMW sample 6 displayed less numbers of bands (see Figure 2 A, B). By PCR, the opposite strands were regenerated and the products were reamplified. SSCP gel electrophoresis was used to evaluate the purities and identities of the reamplified 27 products, as shown for products obtained from PCR targeting the hypervariable samples 16S rRNA genes only 27 were appeared (see Figure 3). In most cases, reamplification products corresponded to the expected positions in the community patterns and no additional products were observed. These products were then directly used for cloning and DNA sequencing.

To identify the predominant products by DNA sequencing, a total of 27 different DNA single strands ("bands") were excised, 27 sequences were in good appearance. By comparing the 27 sequences accession numbers from JF793682- JF793708 with the related taxa, revealed 7different operational taxonomic units OTUs of bacteria, and that the most compositions of the communities of the six BMW samples were all common. The phylogenetic tree (see Figure 4) constructed from the partial sequences of the 16S rRNA amplicons from the 6 BMW samples showed a predominance of Alphaproteobacteria, especially family Sphingomonas sp. (Table 1).

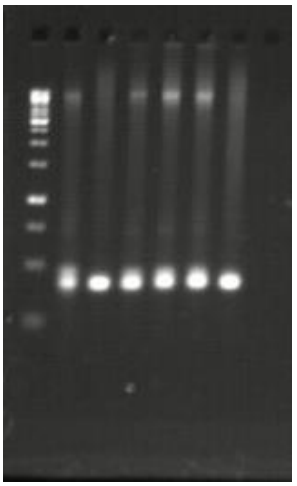

Fig .1. PCR with com primers targeting the 16SrRNA genes for the four DNA extracted samples from the six water samples collected from the final product of BMW from six factories in Wadi El-natroon region (lanes 1, 2, 3, 4,5,6) , C is the controle without DNA and M is 1Kb DNA Ladder GeneRuler™, Fermentas.

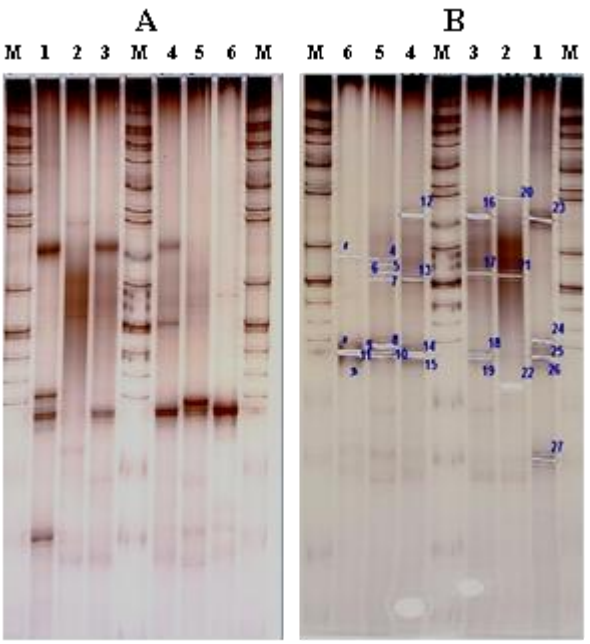

Fig .2. (A) SSCP on a polyacrylamide gel for the Six BMW samples before cutting the bands, (B) after cutting the bands.

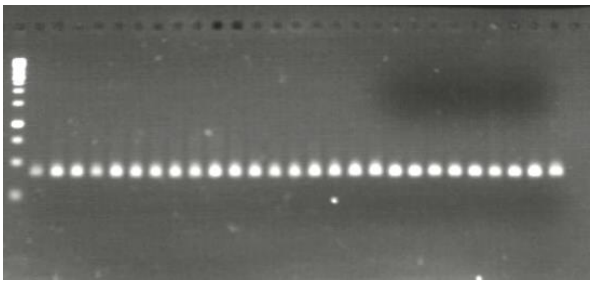

Fig .3. PCR reamplification of the excised and eluted single-strands with the same com primers to generate the original dsDNA fragment lans 1-27 are the product of the reamplification, C is the control without any template, M is 1Kb DNA Ladder GeneRuler™, Fermentas.

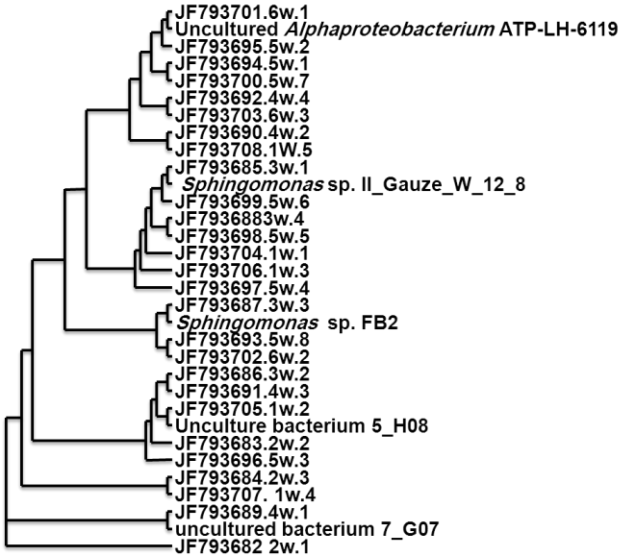

Fig .4. Phylogenetic tree of partial 16S rRNA gene sequences for the SSCP bands from the six BMW samples and its high similarity.

Table 1. Phylogenetic assignment of sequences of prominent bands in SSCP gel profiles of the six BMW samples communities., showed the sample number and the band number i.e 6W1 means BMW sample number 6 and 1 is the band number in this sample.

Band Number	OUT#	Accession Number	bp	Most closest related sequence	Similarity (%)	Accession Number
6w.1	1	JF793701	348	uncultured alpha proteobacterium ATB-LH-6119	98.7	FJ535117
6w.2	3	JF793702	353	uncultured bacterium; BF0001B016	89.6	AM696990
6w.3	1	JF793703	345	uncultured alpha proteobacterium ATB-LH-6119	97.9	FJ535117
5w.1	1	JF793694	344	uncultured bacterium; BF0001B016	94.7	AM696990
5w.2	1	JF793695	351	uncultured bacterium; 13_D04	96.2	FN421620
5w.3	4	JF793696	350	uncultured bacterium; BF0002B019	100	AM697069
5w.4	2	JF793697	346	*Sphingomonas* sp. II_Gauze_W_12_8	97.2	FJ267571
5w.5	2	JF793698	358	uncultured bacterium; BF0002B019	97.2	AM697069
5w.6	2	JF793699	353	uncultured alpha proteobacterium; ATB-LH-6119	95.6	FJ535117
5w.7	1	JF793700	344	uncultured alpha proteobacterium ATB-LH-6119	98.7	FJ535117
4w.1	6	JF793689	355	uncultured bacterium BF0002B019	97.4	AM697069
4w.2	1	JF793690	391	uncultured bacterium; BF0001B024	96.4	AM696998
4w.3	4	JF793691	343	uncultured bacterium; 5_H08	92.8	FN421996
4w.4	1	JF793692	345	uncultured alpha ATB-LH-6119	98.7	FJ535117
4w.5	3	JF793693	344	uncultured bacterium; 4_H07	94.6	FN421778
3w.1	2	JF793685	347	uncultured bacterium; 2_E05	91.1	FN421872
3w.2	4	JF793686	352	uncultured bacterium; 13_F05	94.6	FN421638
3w.3	3	JF793687	359	*Sphingomonas* sp. FB2	95.1	AM933496
3w.4	2	JF793688	355	uncultured bacterium BF0002B019	96.2	AM697069
2w.1	7	JF793682	359	*Sphingomonas* sp Ens34	96.2	DQ339629
2w.2	4	JF793683	362	uncultured bacterium BF0002B019	96.4	AM697069
2w.3	5	JF793684	387	uncultured bacterium7_G07	95.1	FN421898
1w.1	2	JF793704	353	uncultured bacterium; 4_H03	95.7	FN421776
1w.2	4	JF793705	345	uncultured alpha proteobacterium ATB-LH-6119	94.6	FJ535117
1w.3	2	JF793706	392	uncultured bacterium1_C11	94.4	FN421586
1w.4	5	JF793707	396	unculturedbacterium1_D12	96.7	FN421594
1w.5	1	JF793708	389	uncultured bacterium; BF0002B019	92.6	AM697069

III. DISCUSSION

The cultivation independent methods, based on amplification of environmental DNA followed by acrylamide gel electrophoresis, separate sequence specific DNA fragments of the same length, have the potential for accurate comparison of environmental samples in a short period of time. In this study, we have shown that SSCP analysis of 16S rRNA genes amplified from directly extracted DNA from the six BMW samples from the different end product manufactories can be used to visualize all the community structures included the pathogenic species in the BMW, and also this indicate the high potential of this technique to monitor microbial communities and their variation qualitatively and quantitatively (Peters et al., 2000).

Culture-independent techniques were used for the detection of pathogenic bacteria in BMW at potentially critical control points in the end products of BMW six factories in Wadi El-natroon region – Egypt. PCR-SSCP in the BMW from the end product samples. BMW sample number 5 displayed the largest bands than the other bands maybe possibly due to different reasons, because the factory was near from urban but the other factories were a little bit far, and may be also due to the handling of the workers.

As more gene sequences become available, PCR-SSCP-mediated monitoring of different subgroups or microorganisms, due to optimized primer design, will become even more attractive, almost all of these OTUs from the indirect culture (Table 2) in this study are belonging to the phyla Alphaproteobacteria (Falcone-Dias et al. 2015) and certainly related to Sphingomonas species, which are widely distributed in nature. Sphingomonas have been recovered from sea water (Cavicchioli et al. 1999; Gesumaria et al., 2011), sea ice (Bowman et al. 1997), river water (Tabata et al. 1999), waste water (Neef et al. 1999), polluted ground water (Männistö et al. 1999), mineral water (Ferreira et al. 1996; Vachee et al. 1997), sterile water' used in hospitals (Oie et al. 1998), drinking water (Gauthier et al. 1999).

The widespread distribution of sphingomonas can be explained by their ability to survive and grow at low temperature, low nutrient concentration and in toxic chemical environments (Laskin and White, 1999). Because sphingomonas are relatively slow growing (The Council of the European Union 1998). Therefore, the presence of sphingomonas in BMW and also in the drinking water environment may be much more common than has been reported so far and deserves further study not only by culture bacteria but also by culture- independent bacterial molecular techniques. Two species of Sphingomonas, S. paucimobilis and S. parapaucimobilis, have been classifed to Hazard Group 2 in the European Community regulations (Anon, 2000), all species of Sphingomonas contain glycosphingolipids in their cell envelope. Glycosphingolipids (Kawahara et al., 1999).

Glycosphingolipids of sphingomonas have been shown to induce tumour necrosis factor and other monokine production in human mononuclear cells (Krziwon et al. 1995), stimulate phagosytosis and phagosome lysosome fusion (Miyazaki et al. 1995), activate the human complement system (Wiese et al. 1996) and inhibit protein kinase C and possibly function as endogenous modulators of cell function and as second messengers (Hannun and Bell, 1989). These factors may partly explain the pathogenic features of hospital sphingomonas infections.

V. CONCLUSION

This study provides a better method for understanding the uncultured bacterial community in the BMW and providing new information that might be used for improving BMW quality and safety. The above findings underline the fact that BMW sold in Egypt and tested as free from culture pathogenic still have uncultured potential bacterial pathogens. Therefore, in a country such as Egypt , where the quality of tap water is uncertain, it is recommended that there should be stringent regulation of bottled water quality whilst boiled tap water could be a safe alternative.

It is important that consumers are aware that the perception that bottled water is always safer than tap water can be misleading as the former can also contain the same microorganisms commonly found in tap water. This study gives an attention that BMW is not completely safe either for BMW producer to try to solve BMW problems or for the user to be careful during use BMW for infant nutrition and reconstitution of foods.

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