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BIOLOGICAL METHODS OF WATER TREATMENT

Monitoring the Quality of Mineral Bottled Water Concerning to Potential Pathogenic Bacteria and Nitrate Levels[1]

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Abstract—The diversity of cultured pathogenic bacteria in the bottled mineral water (BMW) was investigated using selective media. The pure isolates from these selective media, which showed hemolytic activity on the blood agar media and antibiotic resistance, were identified by 16S rRNA gene technique. The seven obtained strains were belonged to the genus *Pseudomonas*, *Bacillus*, *Acinetobacter*, *Stenotrophomonas*, and *Exiguobacterium*, and were mostly closed to the pathogenic strains. The increasing of ozone concentration from air-fed ozone generators eliminate the growth of bacteria included the pathogenic bacteria, but in other side it increases the amount of nitrates and nitrites in the final product of the BMW. These findings revealed that the BMW either has potential pathogenic bacteria or high levels of nitrates and all these products may effect on the health of the end user.

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Keywords: bottled mineral water, pathogenic bacteria, 16S rRNA gene sequence, ozone and nitrates.

INTRODUCTION

Bottled mineral water (BMW) as an oligotrophic environment should have viable bacterial cell content as low as 10 cfu ml^{-1} [1–2]. These lows, count of native organisms are of little concern to the healthy consumer. Bacterial communities of BMW and tap water originated from the same sources may contain the same bacterial communities [3]. Waterborne pathogens may infect 350 million people within those people 10–20 million succumbing to severe cases [4]. This phenomenon is far from being restricted to developing countries but also threatens developed countries. From 1991 to 1999 in the USA 430,000 cases were infected by 126 waterborne infectious diseases outbreaks [5]. BMW represent one of the largest sectors by volume in the Egyptian soft drinks market [6]. The treatments for producing bottled water processed by sedimentation, sand filtration, carbon filtration, green sand filtration, microfiltration, ultraviolet disinfection and ozone disinfection. Bacteria enter the potable water as a result of its attachment to carbon fines in carbon filter [7]. Inducing of BMW contamination may be from the bacteria and impurities which clog the filter or from potential pathogen bacteria remain in the underground mineral water or during the bottling process itself [8]. Because there is no effective sterilization process of commercially mineral waters for removal of all microorganisms, where the pathogen may be having a way to cause infection for end user. Several bacterial species contaminated the bottled drinking water as: *E. coli, Salmonella typhimurium, Pseudomonas aeruginosa, Campylobacter jejuni and Aeromonas hydrophila* [9]. In Germany hospital the intensive care units infected with *P. aeruginosa* as a result of contamination BMW used for the preparation of orally administered medications and oral fluid replacement [10]. Also *Salmonella sp*. was isolated from BMW from the markets by randomly selection and in the local BMW factory [11]. In 1974, there was an outbreak of cholera by *Vibrio cholera* associated with bottled mineral waters in Portugal, 2467 bacteriological confirmed cases, 48 were dying, while 82 patients had a history of drinking bottled mineral water from one particular source other than bottled water, this means that bottled water was not the only cause of the outbreak. *Vibrio cholera* was found in the water source after 36 cases had visited the spa served by the same water source as the bottled water [12, 13].

Gram-positive cocci isolated from bottled mineral waters as *Staphylococcus micrococci*, *S. yours*, *S. epidermidis*, *S. hominis* and *S. wameri* were identified [9]. The autochthonous flora of bottled water is a complex ecosystem with great heterogeneity, they are generally psychrophilic and oligocarbotrophic and they multiply rapidly in the bottled water as (*Acinetobacter*, *Moraxella*, *Aeromonas*, *Xanthomonas* etc.). The autochthonous flora has the potential for the causing diseases are not clear. *Aeromonas* is sometimes associated with wound infec-

[1] The text was submitted by the authors in English.

tions and suspected to be a causative agent of diarrhea. Several *Pseudomonas sp*. can cause disease in humans. *P. cepacia* is increasingly identified as a cause of serious chest infections in children with cystic fibrosis. *Acinetobacter sp*. can be a problem on intensive care units. *Mycobacterium sp*. may be a cause of pulmonary disease. *Moraxella* can cause infections of the eye and upper respiratory tract. Spreads of waterborne infectious diseases via the use of BMW even in low count still pose a serious health threat worldwide.

BMW consumers are spread all over Egypt, and any waterborne infectious diseases will be difficult to control. In this study, not only potential bacterial pathogens were screened but also chemical and physical conditions were examined to understand the environmental condition for these pathogens, as well as the effect of ozone treatment on BMW and nitrogen oxide formation.

EXPERIMENTAL

Water Samples

Fifty samples were collected from treatment processing units of BMW and also from the final product (BMW) from 8 factories for BMW in Wadi El Natroon region, where most of the Egyptian BMW manufactories are in Wadi El Natroon region. Samples were collected aseptically from several treatments processing units as: sand filter, carbon filter, green sand filter, 1 micro filter 0.45 micro filter, storage tanks, ozone disinfection towers and final product. The collected water samples were filtered from bacteria using a thin filter 0.45 μm (Sartorius stadium Biotech). The retained bacteria on the filter were grown on selective media: mannitol salt agar, Endo agar base, Difco^{tm} Pseudomonas isolation agar and Clostridium agar [14], each trapped bacterium on the filter was grown into a colony.

Monitoring Physical and Chemical Conditions of the Water Samples

Physical conditions as pH, temperature and turbidity were measured, while the chemical conditions as total hardness, calcium, magnesium, bicarbonate, potassium, chloride, sulphate, total dissolved salts (TDS), ammonia, nitrates, nitrite, silica, manganese and iron were detected, all the chemical analysis were determined by the Procedures recommended in the standard methods for the examination of water and wastewater [15], nitrate levels were detected by DR 2800 Spectrophotometer (HACH) with use Catalogue number: DOC022.53.00725 and its chemical reagent.

Growth on Blood Nutrient Agar Media

The pure colonies on the filter membrane grown on selective media: mannitol salt agar, endo agar base, Difco^{tm} Pseudomonas isolation agar and Clostridium agar were purified on nutrient agar media, and cultured on blood agar plates and incubated at 37°C for 24 h. The observation of clear zones around the bacterial colonies showed β-haemolysis, whereas green zones around the colonies suggested α-haemolysis and no haemolysis was called γ-haemolysis.

Antibiotic Susceptibility Tests for Potentially Pathogenic Heterotrophic Bacteria

The antibiotic susceptibility of the bacterial isolates were tested using the disk diffusion method. Ten types of antibiotic disks were placed on the inoculated plates with sterile forceps antibiotics were purchased from bioanalyse; these antibiotics were: ceftriaxone (30 μg), ampicillin (10 μg), cefprozil (30 μg), ofloxacin (5 μg), amikacin (30 μg), ciprofloxacin (5 μg), amoxicillin/clavulanic acid (20 μg/10 μg), chloraphenicol (30 μg), cloxacillin (1 μg) and rifamycin (30 μg).

Phylogenetic Analysis

Genomic DNA of the pure isolates were extracted using DNA purification GeneJET™ Genomic DNA Purification kit (Thermo Scientific), using gram-positive bacteria genomic DNA purification protocol. Three primers were used in the amplification of 16S rRNA. These include: Bact27f (5'- AGAGTTTGATC (A/C)-TGGCTCAG-3'), Bact1492r(5'-TACGG(C/T)TACCTTGTTACGACTT-3'), and Bact1098r (5'-AAGGGTTGCGCTCGTTGCG-3') [16]. Theoretically, amplification with Bact27f -1492r should yield 1505bp and amplification with Bact27f -1098r should yield 1108bp from the 16S rRNA. PCR amplification was performed in a total volume of 50 μl in model T Personal thermocycler (Biometra). Each PCR mixture contained 25 ng of template DNA, 0.6 μM of each primer, 1.75 mM $MgCl_2$, 200 μM of dNTPs, 1.25 U of Taq

polymerase in Buffer A (M1865, Promega Chemicals, Madison, WI). PCR program started with 94°C for 3 min, followed by 30 cycles (94°C for 1 min, 53°C for 1 min, and 72°C for 2 min) and a final extension at 72°C for 8 min. PCR products were checked for expected size on 1.5% agarose gels. The PCR product was purified by Gene JET Gel Extraction kit (#K0691) (Thermo Scientific), sequenced, the nucleotide sequences were analyzed (http://www.ncbi.nlm.nih.gov/blast/), and aligned using MEGA software version 3.1 [17]. TreeView version 1.6.6 was used for the construction of phylogenetic tree.

Data Deposition

The 16S rRNA sequences for the seven possibly pathogenic strains reported in this study have been deposited in the GenBankwith accession numbers (JQ396174- JQ396180).

RESULTS AND DISCUSSION

Chemical and Physical Analysis of the Water Samples

To perform a risk analysis for pathogens in BMW, it is necessary, to better understand the composition of water from, calcium, magnesium, bicarbonate alkalinity, potassium, chloride, sulphate, ammonia, nitrates, nitrites, silica, iron, manganese, and the total dissolved solids (Table 1) which serve either a structural or functional role in the nutritional requirements of the bacteria. BMW cannot be subjected to any type of disinfection that modifies or eliminates their biological components, and they always contain bacteria that are primarily a natural component of these water. Inorganic ions as phosphorus, calcium, and Iron serve as main cellular inorganic cation, cofactor for certain enzymes, phosphorus plays an important role in nucleotides formation, phospholipids, LPS, teichoic acids in gram positive bacteria.

Calcium salts function as a component of endospores. Iron salts serve as components of cytochromes and certain nonheme iron-proteins. The approval process for a BMW requires always evidence of the stability in physical and chemical characteristics. In the BMW the nutrients and metabolites present in low concentration can be dramatically modified through bottling, under the influence of increasing temperature and oxygenation, adsorbed, and concentrated onto the surface and, thus can be more available to the bacteria [18].

Table 1. The chemical analysis and ozone during the treatments for producing bottled mineral water

Chemical analysis, mg/L	Well	Sand filter	Carbon filter	Green sand	Tank 30 m^3	Ozone tower	Product
Total hardness	42	43	42	42	42	40	40
Calcium	8.8	8.8	8.8	8.8	8.8	8.8	8.8
Magnesium	4.3	4.4					
Bicarbonate alkalinity	251.32	251.32	251.32	251.32	251.32	251.32	251.32
Potassium	3.8	3.8	3.8	3.8	3.8	3.8	3.8
Chloride	44	43	42	42	43	42	42
Sulphate	28	28	28	28	28	28	28
TDS	348	345	344	345	345	345	345
Ammonia	0.001	0.001	0.001	0.001	0.001	0.001	0.001
Nitrate	0.6	0.2	0.2	0.02	0.2	0.2	0.4
Nitrite	0.023	0.022	0.021	0.02	0.023	0.023	0.6
Silica	18	18	18	18	18	18	18
Iron	0.04	0.04	0.04	–	–	–	–
Manganese	0.023			–	–	–	–
Ozone, ppm	0.023	0.023	0.023	–	–	–	0.4

Chemical analysis results showed no significant difference in inorganic chemical components after each stage of water treatment and all the analysis were in the standard rang of the drinking water quality according to WHO but there are slightly differences in iron, manganese, nitrite and nitrate (see Table 1).

The nitrite and nitrate differences may be as a result of the activity of sand filter anthracite, which remove nitrite and nitrate partially, but the differences in iron and manganese content may be resulted from the activity of green sand filter. Ozone technology is effective to treat huge amount of water, it can keep water sanitized throughout a facility. In the tested bottled water samples, after ozone application maintain 0.1 to 0.4 ppm residual ozone (see Table 1). International Bottled Water Association (IBWA) recommends that ozone be applied in the 1.0 to 2.0 mg/L range for a period of four to 10 min contact time to safely ensure disinfection. Presence of ozone can be suitable for rinsing and cleaning bottles and disinfecting production equipment, and reducing the bacterial growth in unchlorinated water found within the distribution system. Another benefit is that ozone will not lead to the formation of harmful trihalomethanes (THM), forming with the addition of chlorine to raw water containing humic materials. Despite these advantages for ozone treatment, there are also disadvantage, it will initiate facultative anaerobic bacteria as *P. aeruginosa*, as it is well adapted to proliferate in conditions of partial or total oxygen depletion. This organism can achieve anaerobic growth with nitrate as a terminal electron acceptor, and, in its absence, it is also able to ferment arginine by substrate-level phosphorylation [19–20]. Ozone generators usually use air as an oxygen source for ozone production so that nitrogen and nitrous oxides are carried into the water. In the presence of ozone and oxygen atoms, NO is oxidized to NO_2 and NO_3 [21].

The formation of nitrous oxide and dinitrogenpentoxide from air-fed ozone generators was investigated in [22]. The results showed that as increasing the concentration of ozone in disinfection processes before the bottling process, increasing the level of nitrate concentration (Fig. 1). The physical conditions pH, turbidity and temperature were detected regularly through this study to show the properties of sites that colonized by bacterial community, the results indicated that the turbidity may be increased or decreased according to the load of impurities and effectiveness of treatment step. The pH was slightly alkaline about 8 which indicate that the obtained microorganism were slightly alkaliphilic and the temperature was about 20°C.

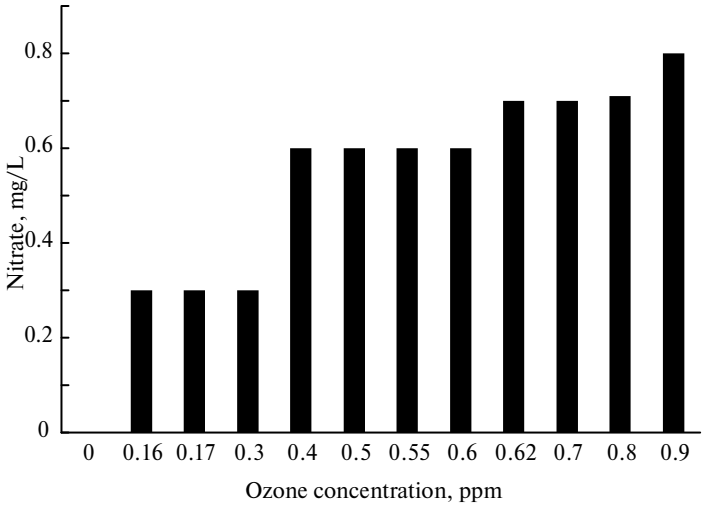

Fig. 1. The relation of increasing of ozone concentration for BMW disinfection and the concentration of nitrate.

Isolation of Pathogenic Culture Bacteria from BMW

Microbiological analysis of BMW at source has always revealed that the presence of some bacteria that are capable of growth and can form colonies on appropriate culture media. After bottling, the number of viable counts increases rapidly, attaining 104–105 cfu/mL within 3–7 days [23], and then the bacterial colonies remain constant about 104 cfu/mL.

These heterotrophic bacteria are also psychrotrophic, because they can grow at temperatures as low as 5°C as in winter in all the BMW process but in final product no bacterial growth was found in all the media (Table 2) as a reason of using ozone for disinfection and also may be the temperature is not suitable to grow the persistent bacteria, and this indicted by presence of bacterial growth in the final product in the summer where the temperature about 35°C, the decreasing or elimination of bacterial growth may be due to the absence of growth factor requirements such as vitamins, amino acids or nucleotides and are, therefore, prototrophic, in contrast to auxotrophic bacteria, which require many of these growth factors. The rapid multiplication of heterotrophic bacteria in flasks containing natural mineral water has been documented by many investigators, as described in our review [23]. However, a possible explanation of growth is a debatable point.

It is important to reduce public health risks, especially with regard to putative bacterial pathogens growing in water [24]. By isolation and identification of bacteria in the BMW that causes unacceptable health problems.

Community structure is generally considered to be related to the types of organisms present in an environment and to their relative proportions. For natural mineral waters, all the data have been obtained, thus far, by culture methods.

Table 2. The bacterial growth on the selective media (Nutrient agar media, Endo agar base, Difco^{tm} Pseudomonas agar) during the four seasons

Sample source		Well	Sand	Carbon	Green sand	Tank 30 m^3	Product
seasons	media		filter				
Spring	Nutrient agar media	+	+	+	+	+	+
	Endo agar base	+	+	+	+	+	–
	Difco^{tm} Pseudomonas agar	+	+	+	+	+	+
	Streptococcus selective	+	+	+	+	+	–
	Manitol agar	+	+	+	+	+	+
	Clostridum salt agar	+	+	+	+	+	+
Summer	Nutrient agar media	+	+	+	+	+	+
	Endo agar base	+	+	+	+	+	+
	Difco^{tm} Pseudomonas agar	+	+	+	+	+	+
	Streptococcus selective	+	+	+	+	+	+
	Manitol agar	+	+	+	+	+	+
	Clostridum salt agar	+	+	+	+	+	+
Winter	Nutrient agar media	+	+	+	+	+	–
	Endo agar base	+	+	+	+	+	–
	Difco^{tm} Pseudomonas agar	+	+	+	+	+	–
	Streptococcus selective	+	+	+	+	+	–
	Manitol agar	+	+	+	+	+	–
	Clostridum salt agar	+	+	+	+	+	–

Bacteria belonging to the alpha, beta and gamma subclasses of the *Proteobacteria* and members of the genera *Cytophaga–Flavobacterium–Bacteroides* are the most common bacteria isolated from bottled mineral water. The retained bacteria on the filter were cultured on selective media: mannitol salt agar, Endo agar base, Difco^{tm} Pseudomonas isolation agar and Clostridium agar. The results were monitored for 2 years to understand the reason for contamination and history for detection the pathogen through the treatment processing units and transferring possibility to the end product during the seasons (spring, summer and winter). The total bacterial counts were decrease in the end product than the well, but the bacteria still present as a result of the resistance of some bacteria especially which grow in Difco^{tm} Pseudomonas isolation agar and then persist till final product. The contaminated well will lead to the filter contamination and the to the end product, if the well disinfected, the total bacterial count will decrease, and sometimes the growth on selective media were negative this mean disinfection of the well and cleaning the filters continuously lead to almost bacterial free product. The bottling processes also were source of pollution especially in the manual production line [8]. Virulent and avirulent strains were morphologically distinct in that the virulent strains have a polysaccharide capsule surrounding the bacterium and form smooth, shiny-surfaced colonies when grown on agar plates [25]. Smooth, shiny-surfaced colonies which grow on selective media were purified for antibiotics disk diffusion test and blood agar test**.** From screening of 50 water samples for the cultural bacteria, 240 isolates were purified and applied to blood agar hemolysis test, 6 isolates have the capability for complete blood hemolysis (M1, M3, M10, M15, M18, and M125) and one isolates M123 showed incomplete blood agar hemolysis, the seven isolates, which showed complete and incomplete blood agar hemolysis have been applied to antibiotics disk diffusion test, 4 isolates (M1, M18, M3, and M15) showed antibiotic resistance for more than 3 antibiotic disc types and have the capability for blood hemolysis and two isolates (M10, M125) showed less antibiotic resistant but have the capability to complete blood hemolysis. One isolates (M123) showed antibiotic resistant against cloxacillin and with incomplete blood hemolysis test. Most of the 250 isolates showed antibacterial

resistant against cloxacillin. Antibacterial resistance is usually quantified as the minimum concentration required asserting a definite effect (e.g. growth inhibition) on a population of cells. Susceptible organisms can become insensitive by mutation or by incorporation of the genetic information which encodes the resistance [26]. Foundation and spread of antibiotic-resistant bacteria (ARB) are a threat to public health in worldwide, where the aquatic ecosystems were recognized as a reservoir for ARB and antibiotic resistance genes (ARG) [25–32]. The presence of trace levels of antibiotics and ARB in source water and finished drinking water may also greatly affect public health and is an emerging issue for the general public and the drinking water industry [33, 34]. Virulence is an important factor for pathogenicity of microorganisms and is defined as the capacity of the organism to invade tissues, multiply and produce toxic effects [35]. Virulence characteristics such as β-haemolytic activity were evidence of the pathogenic potential as study on *Aeromonas hydrophila* [36].

Characterization of Potential Pathogenic Bacteria by 16S rRNA

The isolates (M1, M3, M10, M15, M18, M123 and M125) which showed activity against antibiotic resistance or blood hemolysis or both were characterized with nearly complete 16S rRNA gene sequence of these isolate (corresponding to position 15-1494 according to the *E. coli* numbering system) were determined. Analysis using the RDPII database revealed that the isolate M1 belongs to *Acinetobacter*, isolate M3, M123 belong to *Bacillus*, isolate M10 belongs to *Stenotrophomonas*, M15 and M18 belong to *Pseudomonas*, M125 belongs to *Exiguobacterium* and the characterized strains in this study closely related to the pathogenic genus. The phylogenetic relationships between the characterized strains (*Acinetobacter sp.* HM01, *Stenotrophomonas sp.* HM02, *Bacillus sp.* HM03, *Bacillus sp.* HM07, *Exiguobacterium sp.* HM04, *Pseudomonas sp.* HM05, and *Pseudomonas sp.* HM06 as well as type strains of the related taxa were determined by a neighbor-joining distance analysis of their 16S rRNA gene sequences (Fig. 2), the phylogenetic analysis revealed that the isolated strains *P. sp.* HM05, and *P. sp.* HM06 showed 99% similarity with *P. aeruginosa* and *P. mendocina,* respectively. *P. aeruginosa* is pathogenic only when introduced into areas devoid of normal defences to cause systemic disease. These processes are promoted by the pili, enzymes, and toxins. Lipopolysaccharide plays a direct role in causing fever, shock, oliguria, leukocytosis and leukopenia, disseminated intravascular coagulation, and adult respiratory distress syndrome, while *P. mendocina,* was first isolated from soil and water samples collected in the province of Mendoza, Argentina [37]. *P. mendocina* has been reported as causative agent for endocarditis [38]. *Bacillus sp.* HM03 and *Bacillus sp.* HM07 showed high similarity 100 and 97% with *B. subtilis* strain CRB115 and *B. pumilus* strain X22 respectively, where *B. subtilis* is only known to cause disease in severely immunocompromised patients, and can conversely be used as a probiotic in healthy individuals [39–41]. *B. subtilis* spores can survive the extreme heat during cooking, the molecular assays revealed that most of *B. sp.* have a positive amylase activity and high heat resistance [42], and this may be reasons that this strain persist in BMW after all these treatments for producing BMW, while *B. pumilus* may cause human infection, where three cases of cutaneous infection caused by *B. pumilus* that occurred in 3 shepherds, 2 of whom were members of the same family [43]. *B. pumilus* made food poisoning and large numbers of *B. pumilus* were isolated from food poisoning cases [44]. *Stenotrophomonas sp.* HM02 showed 99% similarity with *S. maltophilia* strain ZFJ-4, *S.maltophilia* commonly present in a wide variety of aquatic as potable water, soil, and rhizosphere environments. *S. maltophilia* is more resistant to disinfectants used with potable water than other waterborne bacteria [45], and may cause hospital and community-acquired infections as: pneumonia, bacteremia, dermatitis and soft tissue sepsis [46–49]. *S. maltophilia* is an increasingly recognized nosocomial pathogen, particularly for immunocompromised patients [50].

Exiguobacterium sp. HM04 showed 99% similarity with *E. acetylicum* strain LT5, where *E. acetylicum* belongs to coryneform bacteria, the centers for disease control has reported a number of *E. strains* from various clinical sources (e.g. Skin, wounds, cerebro-spinal fluid) [51]. A case of catheter-related bacteraemia caused by *E. acetylicum* is reported in an elderly patient. *Acinetobacter sp.* HM01 showed 99% similarity with *Acinetobacter sp.* 407, members of the genus *Acinetobacter* are ubiquitous in soil, water, food and sewage, *Acinetobacter* have been isolated from various types of opportunistic infections, including sepsis, septicaemia, pneumonia, endocarditis, meningitis, and UTI [52]. *A. baumannii,* are implicated in a wide spectrum of diseases as: bacteriaemia, secondary meningitis, urinary tract infection and have role as agents of nosocomial pneumonia in intensive care units [53].

The quality of bottled waters in Egypt were studied and revealed that more than half (54.8%) of biological parameters were violated the Egyptian standards and a percentage of 28.6% of all bottled water samples were contaminated with coliform [6]. Characterization and test the potential pathogenic bacteria from BMW will eliminate the spreading of waterborne infectious diseases and then increase the water quality.

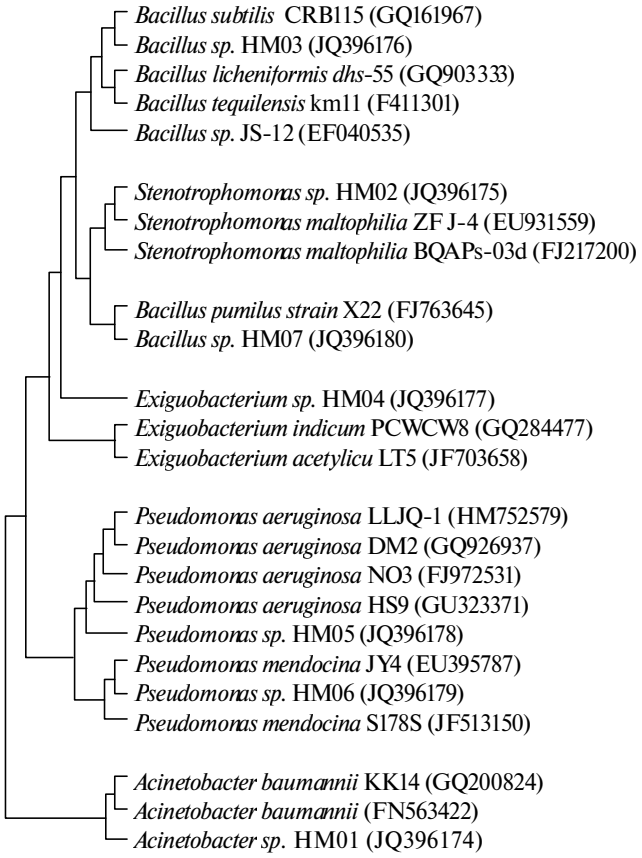

Fig. 2. Phylogenetic relationship based on the comparison of the 16S rRNA gene sequences of the identified members of BMW with related pathogenic species with validly published names. The sequences were aligned using ClustalW implemented in MEGA software version 3, the scale bar represents 0.01 substitutions per site and the GenBank accession numbers of sequences are in parentheses.

CONCLUSIONS

This study provides a better understanding of the microbial, chemical, and physical condition of BMW by detecting and identifying bacterial pathogens, the chemical and physical conditions were examined to understand the environmental condition for these pathogens, as well as the effect of ozone treatment on BMW and nitrogen oxide formation, this study providing new information that might be used for improving BMW quality and safety. The observations reported here indicate that the bacterial growth of BMW is apparently influenced by the temperature. Hence the increasing of bacterial growth in summer than the other seasons is worthwhile questions to address in future studies are whether there are methods to eliminate the microbial growth in summer within this relatively extensive BMW utility and whether there are detectable quantitative changes in the various pathogens within the water samples in real time (upon collection) over the year. As most BMW factories are using ozone treatment on BMW as an effective method for bacterial disinfection, where ozone generators usually use air as an oxygen source for ozone production so that nitrogen and nitrous oxides are carried into the water, In the presence of ozone and oxygen atoms, NO is oxidized to NO_2^- and NO_3^- [20], It is possible that methemoglobinemia associated with nitrate plus bacterial contamination of drinking water. Elevated nitrate concentrations in drinking water (greater than 50 ppm) impact on cancer incidence and mortality in Valencia, Spain [54–55]; also, nitrite compounds can produce hypotension in humans as a result of direct action on smooth muscle [56]. 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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