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Microbial Contaminants in Drinking Water Collected from Different Regions of Kuwait

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Abstract**—**Water plays a major role in maintaining life on earth, but it can also serve as a matrix for pathogenic organisms, posing substantial health threats to humans. Although, outbreaks of diseases attributable to drinking water may not be common in industrialized countries, they still occur and can lead to serious acute, chronic, or sometimes fatal health consequences. The analysis of drinking water samples from different regions of Kuwait was performed in this study for bacterial and viral contaminations. Drinking tap water samples were collected from 15 different locations of the six Kuwait governorates. All samples were analyzed by confocal microscopy for the presence of bacteria. The samples were cultured *in vitro* to detect cultivable organisms. DNA was isolated from the cultured organisms and the identity of the bacteria was determined by sequencing the bacterial 16S rRNA genes, followed by BLAST analysis in the database of NCBI, USA. RNA was extracted from water samples and analyzed by real-time PCR for the detection of viruses with potential health risks, i.e. Astrovirus, Enterovirus, Norovirus, Rotavirus, and Hepatitis A. Confocal microscopy showed the presence of bacteria in some water samples. The 16S rRNA gene sequencing of culture grown organisms, followed by BLAST analysis, identified the presence of several non-pathogenic bacterial species. However, one sample had *Acinetobacter baumannii,* which often causes opportunistic infections in immunocompromised people, but none of the studied viruses could be detected in the drinking water samples analyzed. The results indicate that drinking water samples analyzed from various locations in Kuwait are relatively safe for drinking and do not contain many harmful pathogens.

Keywords**—**Drinking water, 16S rRNA, microbial diversity, viruses, Kuwait.

I. Introduction

WATER has a primary contribution in sustaining life on earth, but when contaminated, it can also be a source of pathogens responsible for morbidities and mortalities in humans [1], [2]. Normally, infectious disease outbreaks due to contaminated drinking water do not occur in industrially developed countries of the world, but infections have been reported in many countries leading to clinical symptoms and deaths [1]. To aggravate this effect, sources of potable water are either becoming scarce or being polluted due to human interventions. This comes as a result of population growth and urbanization which are ultimately causing a global water crisis with a potential threat for lives and sustainable development [2]. Hence, adequate evaluation of microbial water quality is necessary to detect signs of disease outbreaks. Standard methods of analysis rely on culturing indicator microbes such as *Escherichia coli* and fecal enterococci present in water samples [3]. However, disadvantages associated with these methods include lengthy time of analysis as well as under estimation of total bacteria in water as a result of overlooking un-culturable microorganisms [3], [4]. This emphasizes the importance of adopting innovative and novel microbial risk-assessment methods such as real time PCR and DNA sequencing [5], [6]. A good example is 16S rRNA gene-based fingerprinting which can provide an overview on the bacterial community independent of cultivation, which in turn overcomes the restriction of detecting only the few bacteria growing under the cultivation conditions [5], [7], [8].

The different sources of water in Kuwait suggest variation in water quality [9]; this in turn highlights the need for a thorough assessment of contaminants hypothesized to be present in potable water in Kuwait. Previous studies assessing water quality in Kuwait had focused on a single contaminant, looked at contaminants using old methods, and/or neglected to study some contaminants such as viruses [10]-[12]. Hence, the present study aimed to overcome these shortcomings via the employment of advanced analytical and molecular techniques to identify microbial diversity and health risks in Kuwaiti drinking water samples.

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II. Materials and Methods

A. Sample Collection

Drinking tap water samples of 1 L each were obtained from 15 places of public use located in various governorates of Kuwait, i.e. Ahmadi, Asma, Farwaniya, Hawally, Mubarak Al-Kabeer and Jahra. All samples were collected in sterile pyrogen-free bottles containing sodium thiosulfate (20 mg/L) to inactivate residual chlorine and ozone. Samples were transported on ice and were either analyzed directly or stored at 4 °C and processed within 24 h of collection. Commercial nuclease-free water was used as the negative control.

B. Analysis of Samples for Bacterial Contamination by Confocal Microscopy

The tape water samples (n = 15), nuclease-free water (negative control) and nuclease-free water seeded with bacteria (positive control) were used for analysis by confocal microscopy (LSM 700, Carl Zeiss Microscopy GmbH, Munich, Germany). All samples were treated with 2% formaldehyde to fix bacteria and stained with Sybr Gold dye (ThermoFisher Scientific, Waltham, MA, USA) at 1: 10,000 concentrations for 15 minutes at room temperature in the dark. Five-milliliter aliquots of the fixed and stained water samples were filtered on 0.2-µm pore size Anodisc filters (Whatman-GE Health Care, Buckinghamshire, UK) and mounted on

microscopic glass slides. Slides were analyzed directly with a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Inc., Oberkochen, Germany) using a Zeiss 63x oil immersion objective lens. Multichannel images of SYBR gold fluorescence were acquired using the 488-nm laser line of a 25-mW argon laser with a 560 long-pass emission filter.

C. Sample Processing and Bacterial Culture

One liter aliquots of the collected tape water samples were centrifuged at 10,000 x g for 30 minutes at 4 °C. From the hundred milliliter pellet, one milliliter was cultured on a nutrient agar plate and incubated at 37 °C for three days. Single colonies were then picked using a sterile inoculation loop and cultured in nutrient broth for an additional 48 hours at 37 °C. The cultures were then centrifuged and the pellets were washed with sterile ice cold PBS and re-centrifuged.

D. DNA Extraction and Quantification

DNA was extracted from the pallets of bacterial cultures or from filters of filtered water samples using the DNA Mini Kit from Qiagen (Hilden, Germany) according to the manufacturer's instructions. DNA concentrations were determined using Epoch microplate spectrophotometer from Biotek (Winooski, VT, USA) and DNA quality was assessed using 2100 Bioanalyzer from Agilent Technologies (Santa Clara, CA, USA).

E. Amplification of 16S rRNA Genes from Environmental Samples

50 nanograms of high quality bacterial DNA was amplified using primers which were designed to prepare amplicons of the fourth hypervariable (V4) domain of microbial 16S ribosomal RNA (rRNA) genes (Table I).

TABLE I
PRIMER SEQUENCES FOR AMPLIFYING THE 16S RRNA GENE

Primer	Sequence
341 Forward Primer	5'-CCTACGGGGGGCAGCAG- 3'
1046 Reverse Primer	5'-CGACAGCCATGCACCACCT-3'

The amplicons were purified using Qiagen PCR purification kit (Hilden, Germany) according to the manufacturer's instructions. Sequencing PCR products were prepared and purified using the BigDye® Terminator V3.1 Cycle Sequencing kit and BigDye® XTerminator Purification Kit, respectively (Applied Biosystems, Foster City, CA, USA). The purified sequencing products were then analyzed on an ABI PRISM-3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

F. Analysis of 16S rRNA Sequence Data

The 16S rRNA sequence data were cleaned and analyzed using the Basic Logical Alignment Search Tool from The National Center for Biotechnology Information (NCBI-BLAST), USA where the resulting 16S gene bacterial sequences were queried against the 16S ribosomal RNA sequences database using nucleotide blast. Database sequences resembling the queried sequence were then generated and analyzed along with the sequence results using MEGA5.2 software. Multiple sequence alignment was performed using ClustalW. Subsequently, a phylogenetic tree was constructed from the aligned sequences to calculate the distance.

G. Identification of Viruses in Drinking Water

Viral RNA was extracted from 100 milliliter of centrifuged water samples. The nucleic acid extractions and amplifications were carried out according to the manufacturer's instructions using kits for the detection of Hepatitis A virus and Enterovirus, or the multiplex kit for the detection of Astrovirus, Norovirus, and Rotavirus (Sacace Biotechnologies, Como, Italy). Amplifications were performed using 7500 Fast Instrument from Applied Biosystems (Foster City, CA, USA). The threshold cycle (Ct) values for internal control (IC), Astrovirus, Norovirus, Rotavirus, Hepatitis A, and Enterovirus were exported and analyzed using Excel and plotted against the different controls and samples.

III. Results and Discussion

Waterborne diseases are a worldwide problem that is responsible for death of more than 2.2 million people pear year and a larger number of human beings suffer from waterborne diseases every day, including diarrhea and other gastrointestinal diseases as well as systematic illnesses [14], [15]. Among the people dying due to waterborne diseases, about 1.4 million are children [14]. It has been suggested that waterborne diseases result into an economic loss of about US$ 1 billion in the United States of America annually [16], and the global loss amounts to about US$ 12 billion per year [17]. Waterborne infections are the result of ingestion, airborne or contact with contaminated water by different types of infectious agents including pathogenic bacteria and viruses [18]. The global problem gets aggravated because it is estimated that 0.8 billion people do not have facilities to get access to a source of purified and pathogen-free water [14]. In the present research, experiments were performed to determine the presence of pathogenic bacteria and viruses in drinking water samples obtained from different governorates of Kuwait.

In this study, culture, fluorescence microscopy and various molecular methods were implemented to complement each other and to provide an idea about the biodiversity of microbial life in drinking water samples in Kuwait. The analysis of nuclease-free water samples by confocal microscopy did not show the presence of any bacteria, whereas micrographs from both tap and contaminated water samples showed an increasing number of bacteria (data not shown).

Filtered water samples yielded low quality DNA and minute concentrations that deemed them unsuitable for 16S rRNA gene sequence analysis directly from the water samples. This in turn led to culturing water samples on nutrient agar. Colonies from samples showing growth were further cultured in nutrient broth and the DNA were extracted, amplified and sequenced as given in the materials and methods. The PCR amplification products of the 16S rRNA gene are shown in

Fig. 1. Some samples showed weak amplification or did not yield the amplification product of the expected size (lanes 11, 14 and 17) (Fig. 1). These results suggested that most probably that the growths on the nutrient agar might have been due to overwhelmingly fungal contaminations.

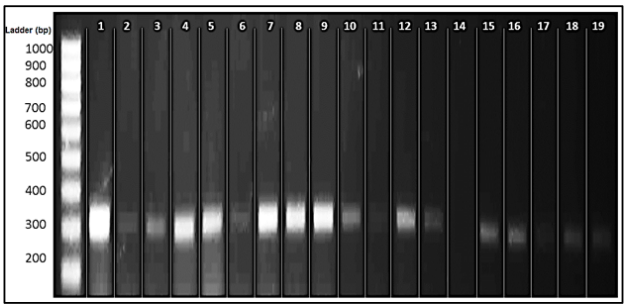

Fig. 1 Agarose gel electrophoresis of the PCR products amplified with the primers for 16S rRNA gene and DNA isolated from the microorganisms grown from the water samples. The majority of samples contained the amplified product in different degrees of abundance

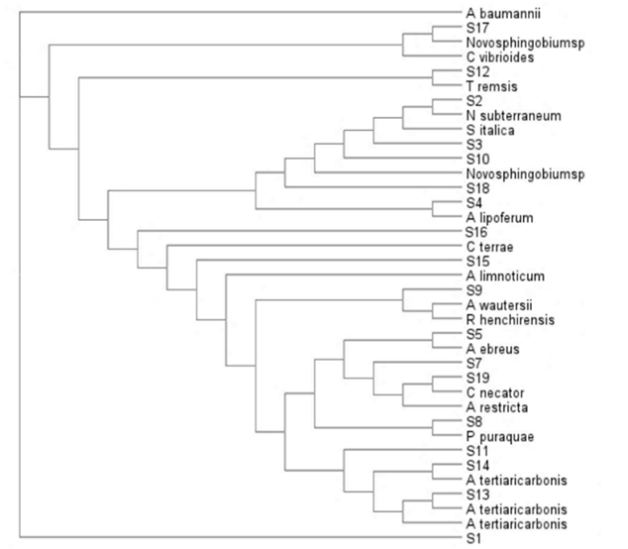

Fig. 2 Phylogenetic tree based on the 16S rRNA gene sequences representing the distance between the sample sequences and the bacterial species closest to the sequence based on the BLAST results

The phylogenetic tree of analyzed microbial samples and their NCBI-BLAST analysis results are depicted in Fig. 2. It indicates the evolutionary relationships of the 16S rRNA gene among the different species identified. This comes as a result of molecular phylogenetic analysis where the sequences generated were aligned and a tree was constructed using the neighbor-joining method [19]. In this method, genetic distance is used as a clustering method, where the distance is often defined as the fraction of mismatches at aligned positions, with gaps either ignored or counted as mismatches [13]. Although, some of the listed bacteria such as *Pelomonas puraquae* and *Acidovorax wautersii* were clinical isolates, these species and the rest of the bacterial species identified are either novel species and/or identified as environmental bacteria with little known health effects in relation to being hazardous.

Of the bacteria identified in Fig. 2, the only bacterium with known health effects, as a pathogenic organism, is *Acinetobacter baumannii* which has been previously isolated from environmental samples [20], and was found to cause opportunistic infections in immuno-compromised individuals [21]. *Acinetobacter baumannii,* a Gram-negative cocco-bacillus, is a ubiquitous organism and has been found as a part of the normal flora in various parts of the human body, e.g. normal skin, throat and rectum, etc. [22]. It can colonize patients in intensive care units and also can contaminate inanimate hospital surfaces and devices [23]. Although usually a colonizer, *Acinetobacter baumannii* can be the cause of severe and lethal infections, in both community and hospital settings, in immunocompromised individuals, predominantly causing aspiration pneumonia and catheter-associated bacteremia [24]. It can also cause infections of the urinary tract, bloodstream, skin, and soft tissues. *Acinetobacter baumannii* is most pathogenic organism in the genus *Acinetobacter* and is responsible for approximately 80% of *Acinetobacter* infections reported in the world [25]. This organism forms strong biofilm as a part of its virulence and pathogenesis mechanisms, and elimination of the identified source of infection requires several intervention strategies [26]. It has also emerged as a cause of nosocomial outbreaks and is characterized by increasing multidrug-resistance throughout the globe [27]. In addition, *Acinetobacter baumannii* has become a highly resistant organism to most clinically available antibiotics (due to emergence of multidrug resistant, extensive drug resistant and pandrug resistance strains) and hence it is threatening the existence of humanity at the global level [28]. It has evolved a large number of drug resistant resistance mechanisms that include β-lactamases, aminoglycoside-modifying enzymes, efflux pumps, permeability defects, and modifications of target sites [29].

Among the viruses, Astrovirus, Norovirus, Rotavirus, Hepatitis A, and Enterovirus have been associated with water contamination and health risks [30]-[33], and therefore their presence in drinking water was assessed in this study. Apart from the positive controls of the respective viral families assessed, no viral RNA was detected in the drinking water samples analyzed (data not shown). These results suggest that the studied pathogenic viruses may not be a health concern in the drinking water of Kuwait.

The molecular techniques used in this study for bacterial and viral detections and identifications were 16S rRNA gene sequencing, virus-specific PCRs and a multiplex PCR, respectively. These methodologies have limitations in detecting and identifying microorganisms as contaminant in drinking water samples. In the case of 16S rRNA gene sequence analysis for bacterial identification, the genome database is limited, and hence the analysis can miss the organisms due to sequences being unassigned in the data base [34]. In case of viruses, the species-specific PCRs and

multiplex PCRs are limited in detecting the number of viral species [35]. In contrast, the new and emerging technologies like whole genome sequencing and whole metagenome sequencing techniques are versatile and capable of detection and characterization of all possible pathogens in any given specimen [36]. Therefore, in future, such techniques should be used in the detection and characterization of bacterial and viral pathogens in drinking water samples.

IV. Conclusion

Within the perimeter of the tests used in this study, the preliminary results indicate that the drinking water samples obtained from various governorates of Kuwait and analyzed in this study are relatively safe and do not contain many harmful and pathogenic organisms. However, further studies are needed to confirm the results with a larger number of drinking water samples, a large variety of pathogens and more sensitive detection methods. Examples include collecting a larger amount of water per sampling point and optimizing the direct DNA extraction to eliminate the need to culture the bacteria and the restrictions associated with culturing microorganisms. Nevertheless, this study presents an insight on the microbial biodiversity of Kuwaiti drinking water which suggests that pathogen contamination in the drinking water samples of Kuwait is limited.

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