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Pseudomonas spp. - The most Popular Water Contaminants of Fresh Water Storage Tanks in Al-Ahsa Providence, KSA

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Pseudomonas species **were isolated from drinking water tanks at Al-Hofuf city from Al-hasa- providence ofthe eastern region of the Kingdom of Saudi Arabia. Water from the drinking water tanks was analyzed to determine the prevailing microorganisms. Fifteen (15) water samples were screened on SS agar medium; only four (4) water samples were highly infected with members of family*entrobactereaceae*. Identification of the bacterial isolates was done using API-20E kit provided from biomeurexin addition to sequencing 16srRNA. The evolutionary history wasinferred using the Neighbor-Joining Method based on the16s rRNA. Identification of bacteria isolated on SSagar plates indicated *Pseudomonas* species as the principal bacterial species involved; three bacterial species belongs to genus*Pseudomonas*; these are; *Pseudomonas mendocina*, *Pseudomonas alcaligenes*, *Pseudomonas* sp. and one species of*Delftiatsuruhatensis*were found to be the major contaminants of drinking water tanks at studied area and provide a possible health risk.**

Key words: Kingdom of Saudi Arabia(KSA), Eastern providence, Water contamination, *Pseudomonas* spp.

Many different microbes have demonstrated the ability to survive in water distribution system, with some possessing the ability to grow and/or produce biofilms. Some of these organisms may beprimary pathogens (i.e., those that cause disease in healthyindividuals), while others may beopportunistic pathogens (i.e., those that cause disease in individuals with underlying conditions that may facilitate infection). Microbes can enter water distribution systems through a wide range of avenues, including treatment processes or through deficiencies of the water distribution system infrastructure. Microbial presence in the distribution system can result in colonization of the distribution system infrastructure. Once biofilm development begins, subsequent material, organisms and contamination. introduced to the distribution system can become entrained in the biofilm. The biofilm can protect microbes from disinfection and allow microbes injured by environmental stress and disinfectants to recover and grow. In addition, biofilms may increase pipe corrosion, adversely affect pipehydraulics and reduce the utility of total coliforms as indicator organisms. Microbial growth inbiofilms may result in deterioration of water quality, generation of bad tastes and odors, and proliferation of macro invertebrates. The primary intestinal bacterial waterborne pathogens include *Shigella*, *Salmonella*, *Yersiniaenterocolitica*, *Campylobacter jejuni*, and *Escherichia coli* O157. The potential for them to attachto biofilms exists, and limited growth in some circumstances cannot be ruled out. One primarypathogen that may be waterborne, *Helicobacter pylori*, was found to survive at least 192 hours onstainless steel

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coupons (inserts used to monitor biofilm buildup) in a chemostat (17). Park, et al., (21) have also noted the presence of *H. pylori* in biofilms of drinking watermains. In another study, two non-pathogenic *E. coli* strains injected into a pilot distribution systemwith a biofilm (20°C) grew slightly in the biofilm before eventually dying out (11). Thebuilding plumbing system may act either as a direct conduit for pathogens sloughed off fromdistribution system biofilms or as an amplifier of these pathogens. A waterborne disease outbreakcaused by *E. coli* O157 persisted for weeks after the suspected source – contaminated water metersand main breaks–were replaced or repaired (26). Although a biofilm was notimplicated, the potential exists for biofilms to prolong the survival of some microbes. In anotherstudy, *Salmonella typhimurium*was able to grow for a short time at 24ºC in non-sterile tap water(5).Under the Safe Drinking Water Act (SDWA), EPA sets legal limits on the levels of certain contaminants in drinking water. The legal limits reflect both the level that protects human health and the level that water systems can achieve using the best available technology. Besides prescribing these legal limits, EPA rules set water-testing schedules and methods that water systems must follow. The rules also list acceptable techniques for treating contaminated water. SDWA gives individual states the opportunity to set and enforce their own drinking water standards if the standards are at least as strong as EPA's national standards. Most states and territories directly oversee the water systems within their borders(9).Ground water considered an important source of drinking in the Kingdom of Saudi Arabia. In general, the chemical and biological character of ground water is acceptable for most uses. The quality of ground water in some parts of the country, particularly shallow ground water, is changing as a result of human activities(1). Ground water is less susceptible to bacterial pollution than surface water because the soil and rocks through which ground water flows screen out most of the bacteria. Bacteria, however, occasionally find their way into ground water, sometimes in dangerously high concentrations. But freedom from bacterial pollution alone does not mean that the water is fit to drink. Many unseen dissolved mineral and organic constituents are present in ground water in various concentrations. Most are harmless or even beneficial; though occurring infrequently, others are harmful, and a few may be highly toxic. Water is a solvent and dissolves minerals from the rocks with which it comes in contact. Ground water may contain dissolved minerals and gases that give it the tangy taste enjoyed by many people. Without these minerals and gases, the water would taste flat. The most common dissolved mineral substances are sodium, calcium, magnesium, potassium, chloride, bicarbonate, and sulfate. In water chemistry, these substances are called common constituents. Water typically is not considered desirable for drinking if the quantity of dissolved minerals exceeds 1,000 mg/L (milligrams per liter). Water with a few thousand mg/L of dissolved minerals is classed as slightly saline, but it is sometimes used in areas where less-mineralized water is not available. Water from some wells and springs contains very large concentrations of dissolved minerals and cannot be tolerated by humans and other animals or plants. Many parts of the Nation are underlain at depth by highly saline ground water that has only very limited uses. Dissolved mineral constituents can be hazardous to animals or plants in large concentrations; for example, too much sodium in the water may be harmful to people who have heart trouble. Boron is a mineral that is good for plants in small amounts, but is toxic to some plants in only slightly larger concentrations(8; 9). Water that contains a lot of calcium and magnesium is said to be hard. The hardness of water is expressed in terms of the amount of calcium carbonate-the principal constituent of limestone-or equivalent minerals that would be formed if the water were evaporated. Water is considered soft if it contains 0 to 60 mg/L of hardness, moderately hard from 61 to 120 mg/L, hard between 121 and 180 mg/L, and very hard if more than 180 mg/L. Very hard water is not desirable for many domestic uses; it will leave a scaly deposit on the inside of pipes, boilers, and tanks. Hard water can be softened at a fairly reasonable cost, but it is not always desirable to remove all the minerals that make water hard. Extremely soft water is likely to corrode metals, although it is preferred for laundering, dishwashing, and bathing.

The microbial guidelines seek to ensure that drinking water is free of microorganisms that can cause disease. The provision of such a supply

is of paramount importance to the health of a community(30). The most common and widespread health risk associated with drinking water is contamination, either directly or indirectly, by human or animal excreta and the microorganisms contained in human and animal waste. If the contamination is recent, and those contributing to the contamination include carriers of communicable enteric diseases (diseases of the gut), some of the microorganisms that cause these diseases may be present in the water. Drinking such contaminated water or using it in food preparation may cause new cases of infection. Those at greatest risk of infection are infants and young children, people whose immune system is suppressed, the sick, and the elderly. Pathogenic (disease-causing) organisms of concern include bacteria, viruses and protozoa; the diseases they cause vary in severity from mild gastroenteritis to severe and sometimes fatal diarrhoea, dysentery, hepatitis, cholera or typhoid fever. The classic waterborne diseases are caused by organisms originating in the gut of humans or other animals. However, many organisms of environmental origin that are not normally associated with the gastrointestinal system are found in water, and some of these organisms may, under certain circumstances, cause disease in humans. Such organisms include the protozoan *Naegleriafowleri,*a number of bacteria, including *Pseudomonas, Klebsiella and Legionella* spp, and some species of environmental mycobacteria.

Inthe current study we are going to investigate the drinking water quality of Al-Ahsaprefecture in the eastern region of the Kingdom of Saudi Arabia and analyze the possible microbial pathogenic microbes inhabiting the water tanks in houses, hospitals, schools, governmental buildings that affect the peoples health. Also trying to setguidelines to prevent the possible contamination in the future.

MATERIALS AND METHODS

Water sampling:

Water samples were collected in 500 ml sterile bottles. Water samples were collected from residential sites (Houses) as well as sites of initial treatment of waterat Al Hofuf city, governorate of Al-Ahsa; kingdom of Saudi Arabia; with the purpose to study the quality of drinking water. Water samples were collected from water tanks over fifteen sites;eightsamplescollected from the water tanks of regular houses; four sampled were collected from water tanks that feeds farms; and three samples were collected from water distribution stations feeds the rest of Al-Ahsa governorate these are; Ain Al- Houwairate,; Ain Al-hara and Ain Al -Swedera. The material of the tanks cement or ceramics was considered.

Preparation of samples / standard solution and calibration curve:

15 samples were collected from different locations representing drinking water tanks from houses, water tanks from farms and water tanks from water treatment stations (represented waste water treatments use for irrigations). 1 liter of each sample was stored at 4 R"C for experimental work. All samples were filtered through whatman filter paper no 1. Then the pH and conductivity of water samples were measured directlyby putting about 50 ml of the water sample in a clean 100 ml beaker and using pH meter directly. pH was measured accurately using a pH meter. The pH meter was calibrated before pHmeasurements. For calibration standard buffers of pH 4.00, 7.00 and 10.00 were used. The electrical conductivity is determined by filling a conductivity meter cell with the water sample, the pH and the EC is determined by the method of (29). Four different concentration standards were prepared with rang of linearity (average to each element) with a volume of 50 ml. the emission was measured for potassium sodium and calcium standards using flame photometer (Jenway, model PFP7, England) the emission was plotted against the concentrations in excel to get the calibration curve. Samples were diluted in different manner (See their specific tables). While for Cu, Zn, Fe, Co, Al and Mg, the absorbance was measured using atomic absorption (spectrometer: iCE 3000 C113300264 v1.30 from thermo). Their calibration curves were obtained by plotting standards absorbance against the concentrations in excel, for analysis of these elements samples were pr-concentrated 10 times by tacking 100 ml of sample and genteelly evaporated on hotplate to reduce volume to 10 ml. 1 ml of concentrated nitric acid was added to each sample for digestion process. However for analysis of Mg all samples were diluted 100 times except No 8 and 9 which

were diluted five times. The concentration of the elements in each sample was calculated using the equation from its calibration curve.

Screening of water samples on Microbial Culturing medium:

Microbial Culturing kit refill package (Catalog #166-5021EDU) and *Salmonella, Shigella* Agar medium (SS agar medium DS205D)(provided from mastgrp. lot#302935) were prepared according tothe instructions provided by the manufacturing company and used for bacterial cultivation and isolation. The agar plates streaked with heavy inoculumof the water samples and bacterial culture were examined after 18-24 hours of aerobic incubation at 35Cº. Pathogenic *Pseudomonas* spp isolates were primarily identified by their transparent colonies with brownish color on SS agar medium; changing the color of the growth medium from pink to yellow. The growth of bacterial isolates were fair to good based on the number of colonies appeared on the agar plate.

Phenotypic characterization of the strains using API20 (Biomerieux, France):

Phenotypic characteristics of the strains were investigated using the using API20E kitprovided from(Biomerieux, France) according to the manufacturer's instructions.

Phylogeny of the isolated bacterial species based on the 16s rRNA sequences:

The evolutionary history was inferred using the Neighbor-Joining method (25). The optimal tree with the sum of branch length = 0.38956404 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (12). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (28) and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1378 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (27).

RESULTS AND DISCUSSION

While taste is subjective, there is a difference in purity between drinking waterchoices.According to asurvey, around 1.1 people in the world have no access to safe drinking water supplies and 2.6 billion people lack adequate sanitation(31). The chemical and chemical parameters of the water samples used in this study were measured using standard analytical techniques as described in the materials and methods. Sodium(Na), Potassium(K)and Magnesium (Mg)are measured using Flame Atomic Spectroscopy(FAS), whereas, Calcium is measured using Flame Photometry (FP).The chemistry of the drinking water from the residential houses in Al-Hofuf city can be summarized in following order:

Na (min.191ppm-max. 566ppm)>Ca (min.78.5ppm-max325.5ppm)>Mg(min.41.3ppm-max. 140.6ppm)>K(min.21.8ppm- max. 46.2ppm)>Zn(min.<1.0ppm- max. 132.3ppm) based on the data we obtainedin the current study**(**data not shown). Three portable water samples were used as referencesthrough out; Cloud; Hana, and Nestle water. Theresult obtained from the water analysis did not meet qualifications outlined by the EPA (Environmental protection Agency or GCC (Gulf Cooperation Councilfor drinking water).The concentrations of thecationswere higher than the maximum concentration level permitted by the EPA, WHO and GCC (8, 19, 31). this resultagreed with

Table 1. Physical parameters

Sample ID	Sample ID	pH	Conductivity (mS/cm)
01	1	7.75	1.087
02	2	7.69	1.50
03	3	7.80	1.289
04	4	7.89	1.067
05	5	7.53	1.52
06	6	7.69	1.543
07	7	7.79	1.168
08	8	8.03	0.128
09	9	8.11	0.162
10	10	7.87	1.316
11	11	7.88	1.971
12	12	7.77	1.732
13	13	7.92	2.695
14	14	7.95	2.037
15	15	7.95	1.324

similar study done on the chemistry of under groundwater of Al-Ahsa in by Al-Zarah (2, 3); he found that the cations of the underground water of Al-Ahsa province can be outlined in the following order Na>Ca>Mg (3)Meanwhile; the concentrations of Cupper (Cu), Cobalt (Co),Iron(Fe), and Aluminum (Al) were minor. Concerning cupper concentration the absorbance of all samples were less than the absorbance of the lower standard, so after pre-concentration ten times the concentration of cupper in all samples were less than 10 ppb, also iron in all samples were less than 50 ppb; cobalt were less than 5 ppb; aluminum were less than 1 ppm (data not shown). In2014;Al-Zarahmentioned that regular evaluation of the water hardness of the tap water showing that there is considerable increase in the concentration of Ca and Mg compared to the portable water. The investigation performed on Al-Hofuf drinking water quality was totally different it has been reported that 85.4% of the water supply network and 79.1% of the tap water were exceeded the limits of hardness of water according to the WHO limits (2, 3) That was true for the past 3-4 years; we have been inforced to dilute the water samples 100 time in order to detect those cationsin tap waters ; however; recently during 2013-2014 there is a remarked improvement in the quality of tap water and the concentration ofCa and Mg along with other cations went dawn (personal consultation with faculty members of department of chemistry at KFU). In this investigation we have observed thatsample number 8 and 9 mentioned in (**Table 1**) showed lower reading for all the tested cations due to desalting of water through specific water filtering system connected to the water tanks. The pHand the electrical conductivity of water samples were in the normal rang (**Table 1**); This is consistent with the normal salinity range of the studied samples.

Table 2. Results of API20 for different bacterial strains isolated

Sample ID	Test performed	Strain 4	Strain 5	Strain 9	Strain 11	Strain 14
1	ONPG	-	-	-	-	-
2	arginine	+	+	+	+	-
3	lysine	-	-	-	-	-
4	ornithine	-	-	-	-	-
5	citrate	-	-	-	+	-
6	Na thiosulfate	-	-	-	-	-
7	urea	+	+	+	-	-
8	tryptophan	+	-	+	+	+
9	Indole production	-	-	-	+	-
10	Na pyruvate	-	-	-	-	-
11	charcoal gelatin	-	-	-	-	-
12	glucose	-	+	-	+	+
13	mannitol	+	+	+	+	+
14	inositol	+	+	+	+	+
15	sorbitol	+	+	+	+	+
16	rhamnose	+	+	+	-	+
17	sucrose	-	+	-	-	-
18	melibiose	-	+	-	-	-
19	amygdalin	-	+	-	-	-
20	arabinose	-	+	-	-	-

Phenotypic characteristics of the strains were investigated using the using API20 (Biomerieux, France) according to the manufacturer's instructions

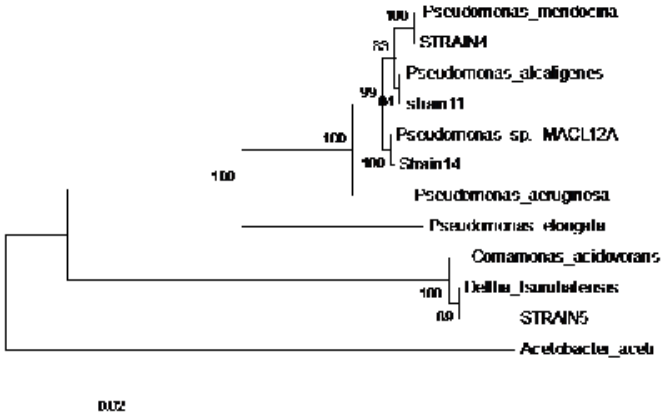
Table 3. Results of 16s rDNA sequences blasting against NCBI database

Strain identity	Related species	Identity %	Coverage %
4	*Pseudomonas mendocina*	100	99
5	*Delftiatsuruhatensis*	98	99
11	*Pseudomonas alcaligenes*	99	99
14	*Pseudomonas* sp.	99	99

Microbial Analysis:

The potential for increased risks of infection is an important concern with drinking water tanks. We performed microbial analysis on water samples taken from different sources of drinking water to search for the presence of members of the family *entrobacteriaceae* or any other genera that have phylogenetic relatedness with enteric bacteria.The samples were analyzed for the standard hygiene indicators such as coliforms, *Escherichia coli*, *Enterococci*, *Pseudomonas aeruginosa* and *Legionella pneumophila* which can form biofilm on warm water pipe surface (4). Most of the water samples used in this survey withdrawn from drinking water tanks from houses and underground water supplies. Most of the water tanks manufactured form cement and ceramic that developed a clear microbial biofilm on their walls; the biofilm layer was clearly observed by naked eyes. Fifteen(15) bacterial species were isolated; out of which four (4) species

were remarkably abundant. Thosebacterial isolates were further identified using API-20E kit provided from biomeurexin addition to sequencing 16srRNA. The evolutionary history was inferred using the Neighbor-Joining Method based on the16srRNA. The resulting data stated that there were three bacterial species belongs to genus *Pseudomonas*; these are; *Pseudomonas mendocina*, *Pseudomonasal caligenes*, *Pseudomonas* sp. and one species of *Delftiatsuruhatens is* found to be major contaminants ofdrinking water tanks at studied sites(Fig.1; Table 2&3). The presence of *Pseudomonas species* as the major hygiene contaminants under this conditions is a strong evidence for the water contamination with members ofthe family *Pseudomonaceae*that has phylogenetic relatedness with the family *entericbacteriaceae*; their members are famous human and plant pathogens[13,18]. *Pseudomonas*is a genus of Gram-negative aerobic gamma-proteobacteria containing 191 validly described species [11, 15]. The members of the genus demonstrate a great deal of metabolic diversity, and consequently are able to colonize a wide range of niches[10, 11]. The predominance of *Pseudomonas* species overthe other bacterial species attributed to their ability to survive and adapt to nutrient-rich or poor conditions resulting in their colonization and survival in wide range of internal and external environments[13,22]. *Pseudomonas aeruginosa* for example is able to adapt to different environments partly because of the high percentage of transcriptional regulators that allow the cells to adapt rapidly to changing environmental conditions[16].Although *DelftiaTsuruhatensis* is not amajor contaminant of drinking water since it is known by its ability to degrade organic waste[6,14, 16, 24] therefore, its appearance in the drinking water samples indicates the presence of some forms of organic wastecontamination that can support bacterial growth and metabolism[7]. In fact, we find this result is consistent with the source of water from which *Delftiatsuruhatensis* isolated where we observed very clear and heavy biofilm layer of the internal walls of water tanks of residential houses that is manufactured from cement and ceramic and was 22 year old. The age of these containers open for the possibility of a biological or a chemical degradation of the internal surface of the tank material[14,23]. In addition, there were two representative of coliform bacteria belonging to the genus *Esherichia coli* were detected from drinking water tanks of residential houses; the colony counts was minor(2-3 colony/1ml) in fifteen drinking water samples surveyed; although of the low magnitude it is still represent a public health concerns. The limited number of coliform species



The evolutionary history was inferred using the Neighbor-Joining method[1]. The optimal tree with the sum of branch length = 0.38956404 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches[2]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method[3] and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1378 positions in the final dataset. Evolutionary analyses were conducted in MEGA5[4].

Fig. 1. Evolutionary relationships of taxa

(onlydetected in two sites out of the fifteen sites involved in this survey) indicates that this contamination is highly due to the contamination of the water tanksbut not to the entire water pipes. No more information is available about the quality or the quantity ofmicrobial contamination of the drinking water in Al-Ahsaprovince, recent work has been done on the drinking water quality on Al-Ahsa province, KSA, stated that microbial contamination was more prevalent than the chemical contamination; 58% of the studied water samples were contaminated with bacteria, particularly the fecal coliform bacteria were represented by 8%, while the percentage of *E.coli* found in the samples were about 1%[1, 2, 3]. Microbial contamination of drinking water has long been recognized as a source of acute gastrointestinal illness(AGII)[21]. To protect against this, multi-tiered approaches involving combinations of source water protection, filtration, disinfection and residual disinfection is a must.

CONCLUSION

Generally, all the water samples used in this study exceeds the limits of water hardness permissible limits according to EPA (9), WHO (World Health Organization, 31), or GCC (Gulf CooperationCouncil, 19) drinking water standards. However, recently there is a considerable improvementin the water quality compared to the past years, more work is needed to meet the quantification and qualification recommended values. Moreover, The presented investigation showed that many water tanks of buildings and work organizations at theeastern part of Kingdom of Saudi Arabia (Al-hasa providence; city of Al-hofuf) are contaminated with *Pseudomonas spp*. This is especially true for houses and farms. Our study also focused the light on the of age of water tanks and the body structure of the water tank(means build form cement or ceramic material or both) may be a potential source for *Pseudomonas spp*. and *DelftiaTsuruhatensis*contamination . We recommend that water samples should be examined periodically; the contamination should be quantifiedand the systems should be assessed for the reasons of contamination. After words; measures for removal of the contamination should be undertaken (disinfection, flushing).

Ongoing work

Currently, in a parallel study we are investigating the fungal contamination of thestudied areas and expanding to cover the drinking water pipes from the initial point of treatment till reaches the residential areas. Also, we concerning the arsenic contamination and arsenic respiring bacteria in the underground water in the eastern coast of the kingdom of Saudi Arabia.

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