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Bacteriological Drinking Water Potability at Al-Madinah Al-Mounwwarah in Relation to Plasmid-Linked Multidrug-Resistance

Idriss Mouneer Al-Turk, Atef M. Diab*

Faculty of Applied Medical Sciences, Taibah University, Al-Madinah Al- Mounwwarah, KSA

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Abstract: The principle drinking water sources at the Kingdom of Saudi Arabia (KSA), including Al-Madinah Al-Mounwwarah are the desalinated and underground well water. The present study focused on evaluating this drinking water sources portability from the bacteriological point of view. Searching for any possible correlation between bacteria present in the studied drinking water and those which have been isolated from the final effluent waste water of hospitals, is a crucial target. Water samples were collected from underground well water, underground reservoirs and taps at the nearest neighbourhood to previously-studied five hospitals namely; King Fahd, Ohod, Al-Mouassat, Women and Maternity and Saudi-German. Water samples from two additional sites namely; Al-Eskan and Al-Bsateen, away from these hospitals were investigated as control remote areas. Drinking bottled water (6 trade marks) processed at Al-Madinah Al-Mounwwarah namely; Doraq, Nada, Quba, Sarat, Taibah and Watanya, were also studied. Qualitative and quantitative bacteriological full analyses for water samples collected from the 27 sites, including enumeration of total viable (TVB), total coliform (TC) and faecal coliform (FC) bacteria in addition to the identification of the purified isolates to the specific level using API 20E strips and its software program, have been accomplished. Antibiotic-resistance profiles, expressed in MIC μg/ml, of the most important Gram– ve rods against 9 antibiotics namely; bacitracin, chloramephincol, erythromycin, impenim, penicillin G, rifampicin, streptomycin, tetracycline and vancomycin, were also drown up. Screaning for plasmids in the antibiotic-resistant isolates was fully studied. Results showed high and fluctuated TVB, TC and FC bacterial counts ranged between 100 : 1000, 90 : 800 and 1 : 3 cfu/100 ml, respectively, in the studied underground well water, indicating its unacceptable quality for drinking. Underground reservoirs and tap water samples were free from TC and FC, giving acceptable drinking water according to both the local and international standards, although having low to moderate TVB counts (1 : 15 cfu/ 100 ml). No direct correlation between the bacterial counts and the neiborhood hospitals, compared with counts detected in water samples from the two remote control areas. The study clearly-assured the bacteriologically-high grade quality of all the six investigated bottled water, showing no counts for TC and/or FC, so safe to drink. The low TVB counts (3 : 24 cfu/ 100 ml) seemed, numerically-potable according to standards, but health threatens are alarming because of the ability of some of these commensals to accept extrachromosomal elements changing their characteristics and impose pathogenic behaviours. Identification revealed that 74% were Gram –ve, 56% of it are rods, while 18% are cocci, only 26% were Gram +ve rods, 19% are spore-formers and 7% are non spore-formers, while no Gram +ve cocci were detected. Gram –ve rods belonged to 8 strains, 7 species and 7 genera namely; *Escherichia coli* 1, *Citrobacter frundii, Providencia stuartii, Proteus vulgaris, Cryseomonas leteula* , *Stenotrophomonas maltophili* (which have been heavily-isolated from almost all the studied bottled water but non of the other sources), *Aeromonas hydrophila* (group 1&2). The most distributed species were *Aeromonas hydrophila* (group 1), *Cryseomonas leteula* and *Stenotrophomonas maltophilia*. All of the 8 studied strains resist from 3 : 9 of the antibiotics at MIC(s) from 50 :100 μg/ml. Molecular studies resulted in the detection of plasmids in 5 out of the 8 strains. Miniprep, alkaline lysis and 1% agarose gel protocols, along with electrophoresis against Hind III partially-digested Lambda phage, showed only one plasmid in each, all of the same size; 23130 bp. It is very important here to record that only 3 isolates namely; *Escherichia coli* 1, *Provedencia stuartii* and *Stenotrophomonas maltophilia* , out of 5 isolates of these were identical; from the points of antibiotic-resistance profiles, MIC(s) and plasmid content, with 3 of those isolated from hospitals waste water in the previous study.

Keywords: Ptability, Plasmid DNA, Antibiotic-resistant bacteria, Escaping of pathogenic bacteria from hospitals wastewater to drinking water.

*Corresponding: E-mail: nogodbutgod@hotmail.com, Tel: 0502439981, Fax: 8475790

Introduction

AL-Madinah AL-Munwwarah (now 1 million capita, 1.5 millions in 2040) is a fast growing community. The infrastructure mainly, factories, hospitals, universities, giant superstores are in a daily settlement process, the logarithmic increase in population density, in addition to the huge number of visitors to the mosque of Mohammed the Great Prophet of Islam (peace be on him), comprises a unique culture and environment. The many and continuously reported strange health complains and cases of illness represent a major community and governmental challenge. Drinking water plays an important role as a possible source of some of these infections. Groundwater comprises a fair share of AL-Madinah drinking water, besides the desalinated sea water as the principle source of drinking water. The amount , as well as the quality of water, as it is depleting and deteriorating, is the problem of human priority. Bacteria occupies a first place terror causing many disorders, diseases and epidemics. The combination and interaction between bacteria and their antibiotic residuals, resulting in antibiotic-resistant pathogenic and/or opportunistic bacteria, comprises a threat since the early 1960s.

The world is heading for a public health crisis. Even old infectious diseases such as tuberculosis, cholera and diphtheria are coming back worldwide. One third of the 52 million deaths from all causes in the world in1995 were due to infectious diseases; over half of these are young children. *Aeromonas* species, for example, specially motile ones, have long been recognized as primary pathogens (Khashe *et al* 1996) and increasingly known as aetiological agent of disease syndromes such as gastroenteritis (Altwegg *et al* 1991), otitis Vizmanos *et al* 1994), vaginitis and kidney infections including pyelonephritis (Rusin *et al* 1997a,b). *Aeromonas* species are also noted to be important pathogens of pediatrics and immuno-compromised patients causing diarrhoea or dysentery leading to dryness and even more to death in many cases (Essers *et al* 2000 and Ogunc *et al* 2000). The problem was duly recorded in scientific literature and then ignored for 15 years (Hignite and Azarnoff 1977). Hospitals and the health care industry are the major sources of these problems, especially antibiotics and chemotherapy chemicals (Steger 1997). When a human or an animal is given a drug, anywhere from 50% to 90% of it is excreted unchanged. The remainder is excreted in the form of metabolites; chemicals produced as byproducts of the body's interaction with the drug. Drugs are also designed to have particular characteristics. For example, 30% of the drugs manufactured between 1992 and 1995 are lipophilic, meaning that they tend to dissolve in fat but not in water. This gives them the ability to pass through cell membranes and act inside cells. Unfortunately, this means that, once they are excreted into the environment, they enter food chains and concentrate as they move upward into larger predators. Comparing the amounts of the many times more of these antibiotics drained from hospitals than from other sources (Brown 2004).

In view of the increasing interest in the possible role played by hospital and municipal wastewater systems in the selection of antibiotic-resistant bacteria, biofilms were investigated using *Enterobacteriaceae*, and heterotrophic bacteria as indicator organisms (Schwartz *et al* 2000, Schwartz *et al* 2003). Many drugs are also designed to be persistent, so that they can retain their chemical structure long enough to do their therapeutic work. Unfortunately, after they are excreted, such drugs also tend to persist in the environment.

The transferable extra-chromosomal DNA elements such as plasmids, are exchangeable amongst bacterial genera and even families; vertical distribution, and/or bacterial species of the same genus; horizontal distribution, drew attention to concentrate and focus on the identity of these elements and the mechanisms by which they transfer, accepted and expressed within their hosts. Diab *et al* 1989 investigated the plasmid-linked resistance patterns of 13 gram negative ampicillin-resistant isolates. For six of them, plasmids were detected and found responsible for resistance to β-lactam containing antibiotics, ampicillin in particular with relatively high $MIC_{(S)}$. Plasmid-linked resistance, especially for pathogenic bacterial isolates, are still of critical importance (Diab *et al* 2001, Gilbert 2003, Lancashire *et al* 2005, Somwang *et al* 2005, Diab *et al* 2006 and 2008 a, b). Hospital effluent with its high content of multi-drug resistance-plasmids, could pose a grave problem for the community(Chitnis *et al* 2004 and Diab *et al* 2008 b). Several studies showed that antibiotic resistance characteristics can be transferred to sensitive recipient organisms in the environment and DNA coding for antibiotic resistance may be conjugally transferred between similar microorganisms (Ramteke *et a*l 1990 and Harnett *et al.*, 1998, Merz *et al* 2004).

All in all, the massive uncontrolled use of antimicrobial agents, antibiotics in particular, was and still one of major threatens that man enrolled in his "Agenda 21" (UNIDO 1994). The infection, pathogenicity, treatment and antibiotic-resistance cycle have been extensively-studied. The tendency of bacteria to accept and the power to express genetic information encoded in extrachromosomal elements such as plasmids are tremendous, nevertheless to the taxonomical and phylogentic aspects (Brooun *et al* 2000, Randall and Woodward 2001, Kwon *et al* 2003, Nishino and Yamaguchi 2004, Buriánková *et al* 2004, Koutsolioutsou *et al* 2005, Perreten *et al* 2005 and Wiuff *et al* 2005).

The major changes in the use of antibiotics necessitates a national strategic programme for the rational use of antimicrobial agents and surveillance of resistance like that followed in Swedish Strategic programme "STRAMA"(Molstad and Otto 1999).

The Kingdom of Saudi Arabia (KSA), has its own national drinking water standards, guidelines and actions. The "Presidency of Meteorology and Environment" has implemented an over all regulations for the environmental protection and development, at the name "General Environmental Regulations and Rules for Implementation in the Kingdom of Saudi Arabia"(2001). Environmental protection standards came in appendix-1, and in the B-4- section, the biological pollutants were guided with the total coliform counts at 70 (MPN)/ 100ml (average for 30 day period), for the receiving water guidelines. For drinking water, bottled and un-bottled, the Saudi Arabian Standards Orgnization, in its issues No. 409/1984 and No. 409/2000, described in details the standards and action plans.

The present study aimed at investigation of the bacteriological quality of drinking water resources at Al-Madinah Al-Mounwwarah including bottled water produced locally, tap water, stored water in underground reservoirs and raw (untreated) well water, around the previously-studied hospitals neighborhood. Tracing any bacterial contaminants that may be escaped from hospitals wastewater and reached drinking water sources through infiltration, seepages and leakages. Matching of the identical traced bacterial isolates species; if there is any, from the quantitative and qualitative distribution patterns, antibiotic resistance profiles and plasmid ecology points of views.

This is a part of a research project sponsored by the Deanship of Scientific Research, Taibah University.

Materials and Methods

Sampling

Sampling localities: In this complementary study, sampling localities were designed to be from the nearest points to the previously-studied hospitals. The final wastewater of five hospitals were extensively-studied looking for plasmid-linked antibiotic resistant bacterial strains. So localities for drinking water sources , including raw well water (W), underground reservoirs (R) and tap water (T), were named after the hospitals they geographically-represent. Then 15 sampling localities namely; K.Fahd W., K. Fahd R., K. Fahd T., Al-Mouassat W., Al-Mouassat R., Al-Mouassat T., Ohod W., Ohod R., Ohod T., Saudi German W., Saudi German R., Saudi German T., Women and Maternity W., Women and Maternity R., and Women and Maternity T. In addition two remote areas from the previously-studied hospitals were sampled as control localities. Bsateen and Eskan were chosen and samples from wells, reservoirs and taps were investigated. Locally-bottled drinking water and are mostly popular in use in the market were also studied. Doraq, Nada, Quba, Sarat,, Taiba, and Wataniya were the trade names of the investigated bottled-water staff. Then a total of 27 sampled drinking water localities were studied to cover the needs of the research objectives.

Sampling protocol: The previously-described localities of drinking water types were sampled three successive times on monthly basis. Quantitative results are represented as the arithmetic mean of experimental readings. Samples (1500 ml) were aseptically-collected in sterile pyrogen-free glass containers, and bottled water made available for laboratory examination adapting the recommended procedures from APHA (1992), (Standard Methods of Examination of Water and Wastewater). The guidelines of concern from (WHO 2007) was also recognized.

Samples processing: Counts of bacteria were investigated in all the water samples described using the standard membrane filtration method. On plate count, MacConkey and Endo agar culture media, poured in 50 mm UV-sterilized plastic Petri dishes, the harvested bacterial catchs from 100 ml water

samples on 0.45 μm, 49 mm in diameter Millipore filters, were placed face up on solid agar media, bubbles avoided, and directly incubated at 35˚ C for 24 hours (plate count and MacConkey), while Endo plates were incubated at 44.5˚ C for 48 hours. Counts were recorded before isolates were picked up, streaked, described, purified, sub-cultured on nutrient agar, stained for microscopic investigations and preserved for further identification and plasmid studies.

Antibiotic assay: Serial dilutions; 10, 30, 50, 70 and 100 μg/ml, were prepared from 9 antibiotics namely; bacitracin, chloramephincol, erythromycin, impenim, penicillin G, rifampicin, streptomycin, tetracycline and vancomycin, comprising the most wide spread and commonly prescribed by physicians and as representatives of different antibiotic families. Sterile saline solution, 5 ml aliquots (0.85 % NaCl) were inoculated with 200 μl of bacterial suspensions adjusted to a density of approx. 10^7 to 10^8 using basic 0.5 McFarland standard solution, (NCCLS 1990).

Identification of bacterial isolates: Bacterial identification was based on colony morphology ion nutrient agar culture medium (Oxoid), bacterioscopy of Gram-stained bacterial streaks, motility, standard oxidation, oxidase production, glucose fermentation, urease activity, H2S production (from sulfer-containing aminoacids), indole from tryptophan, use of citrate and decarboxylation of lysine, arginine and ornithine. Bacterial inocula from purified 24 hours freshly-cultured single colonies were). adjusted to a growth density of approx. 10^7 to 10^8 using basic 0.5 McFarland standard solution , (NCCLS 1990). API 20E strips (Murray *et al* 1999 Api Web) computer program (from BioMerieux, Inc.) and API protocol adopted from the science advisory board, helped in identifying the bacterial isolates (153) up to a 95% confidence ID results.

Detection and Extraction of Plasmid DNA: The miniprep. Alkaline lysis method described in even all the Molecular Cell Biology Lab. Manuals, for the detection, extraction and isolation of plasmid DNA was adopted. LB broth plus appropriate antibiotics in culture tubes with individual bacterial colonies were shaked at 37 ^oC overnight. Cell pellets are resuspended in 100 μL alkaline extraction with solution I composed of 50 mM glucose, 25 mM tris (pH 8.0) and 10 mM EDTA, combined with fresh 20% SDS, solution II by combining 1 mL 2 M NaOH, 0.5 mL 20% SDS, and 8.5 mL H_2O. 150 μL of ice-cold solution III [3 M KOAc were added, brought to pH 5.5 by adding glacial acetic acid] to each tube. A white precipitate of denatured proteins and cell debris is formed. 200μL phase separation mixture of phenol chloroform (1:1) was added to each Tube. Closed microfuge were inverted tubes several times to mix the phases well. The samples were centrifuged for1 minute in a microfuge. Using a pipettor or a Pasteur pipet, the upper (aqueous) phase was transferred to new microfuge tubes. Precipitate DNA using 300μl cold isopropanol to each tube. Fresh TE [50 mM Tris (pH 8.0); 20 mM Nacl and 5 mM EDTA] plus digestion of RNA using RNase by adding 20 μL (5 mg/mL) RNase in TE buffer was prepared. 5 μL of 3 M NaOAc and 125μL cold ethanol to DNA were added. Samples can be stored at –20 ^oC indefinitely. The pellets were resuspended in 40 μL 1 mM Tris and 0.1 mM EDTA, pH 7.5. Sterile non-pyrogenic water for intravenous injection was the quality of water used in all preparations through the study, (Pharmaceutical Solutions Industry Ltd, KSA In Cooperation with Fresenius Kabi, Germany .

Agarose Gel Electrophoresis: Agarose gel electrophoresis was carried out using the tris-borate EDTA buffer (TBE). Gels were prepared by adding (0.7% - 1%) agarose and 0.5 μg/ml ethidium bromide (5-7 μL ethidium bromide from stock solution of 10 mg/mL) to the TBE buffer as described by Hammad and Dora (1993). Electrophoretic runs proceeded at 70-90 V for 2-3 hours. The previously mentioned conditions were acclimatized according to specifity of each group of bacteria in the run.

Results

Results hereafter will introduce three major parts of the study. First is the bacteriological study including quantitative and qualitative analyses, as well as the distribution patterns of the identified bacterial species in the different drinking water sources. Second is the bioassay investigating antibiotic-resistance profile of the identified bacterial isolates. Third is the molecular genetics including plasmid detection, isolation and plasmid size determination.

Bacteriological study

1) Quantitative results of bacteriological analysis

A) King Fahd Hospital neighbourhood: The mean bacterial counts enumerated as (TVB) on plate count agar reached 82 cfu/100 ml, 16 cfu/100 ml and 12 cfu/100 ml for raw well water, underground reservoir and tap water, respectively. The mean bacterial counts of (TC) showed reading of 74 cfu/100 ml, for only raw well water, while underground reservoir and tap water samples were TC-free. All samples were FC-free, (Figure 1).

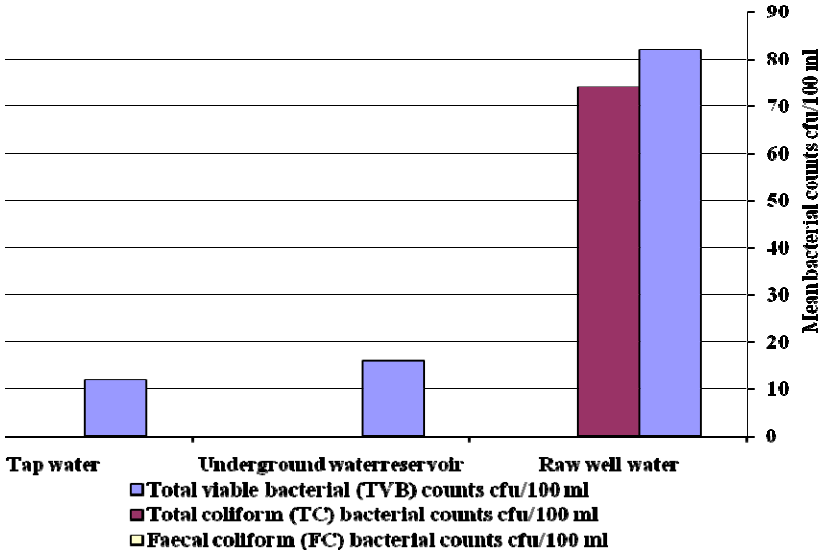


Figure 1. Showing the total viable bacterial (TVB), total coliform (TC) and faecal coliform (FC) mean counts cfu/100ml in drinking water samples collected from the hospital neighborhood weel water, underground reservoir and tap water of King Fahd hospital.

B) Al-Mouassat Hospital neighbourhood: The mean bacterial counts enumerated as (TVB) on plate count agar reached 997 cfu/100 ml, 15 cfu/100 ml and 13 cfu/100 ml for raw well water, underground reservoir and tap water, respectively. The mean bacterial counts of (TC) showed reading of 877 cfu/100 ml, for only raw well water, while underground reservoir and tap water samples were TC-free. All samples were FC-free, (Figure 2).

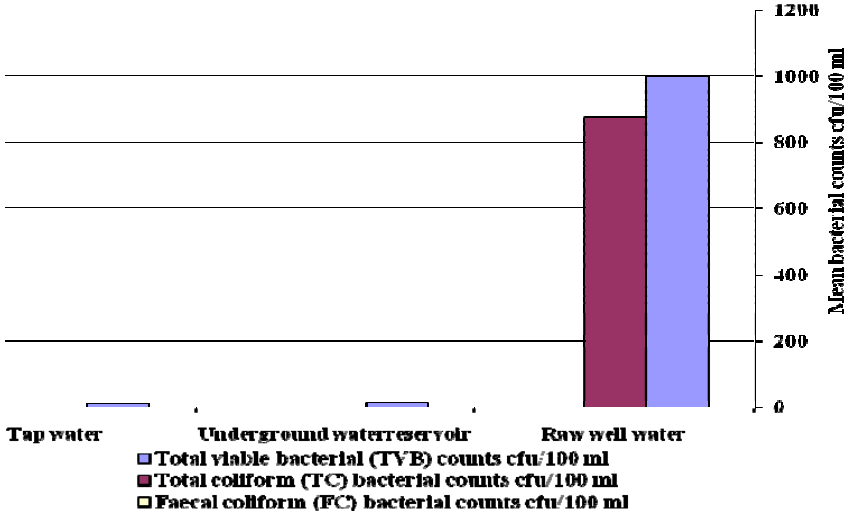


Figure 2. Showing the total viable bacterial (TVB), total coliform (TC) and faecal coliform (FC) mean counts cfu/100ml in drinking water samples collected from the hospital neighborhood weel water, underground reservoir and tap water of Al-Moussat hospital.

C) Ohod Hospital neighbourhood: The mean bacterial counts enumerated as (TVB) on plate count agar reached 263 cfu/100 ml, 8 cfu/100 ml and 6 cfu/100 ml for raw well water, underground reservoir and tap water, respectively. The mean bacterial counts of (TC) showed reading of 141 cfu/100 ml, for only raw well water, while underground reservoir and tap water samples were TC-free. All samples were FC-free except raw well water exhibited 3 cfu/100 ml, (Figure 3).

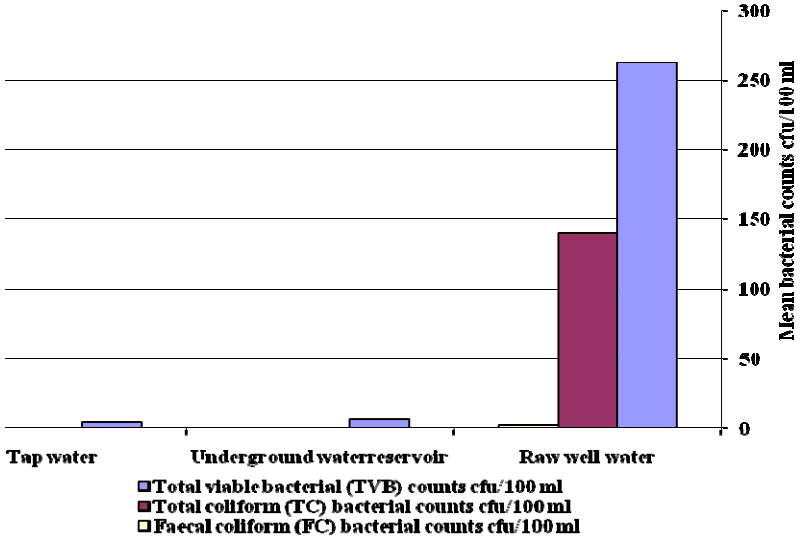


Figure 3. Showing the total viable bacterial (TVB), total coliform (TC) and faecal coliform (FC) mean counts cfu/100ml in drinking water samples collected from the hospital neighborhood weel water, underground reservoir and tap water of Ohod hospital.

D) Saudi-German Hospital neighbourhood: The mean bacterial counts enumerated as (TVB) on plate count agar reached 241 cfu/100 ml, 4 cfu/100 ml and 1 cfu/100 ml for raw well water, underground reservoir and tap water, respectively. The mean bacterial counts of (TC) showed reading of 127 cfu/100 ml, for only raw well water, while underground reservoir and tap water samples were TC-free. All samples were FC-free, (Figure 4).

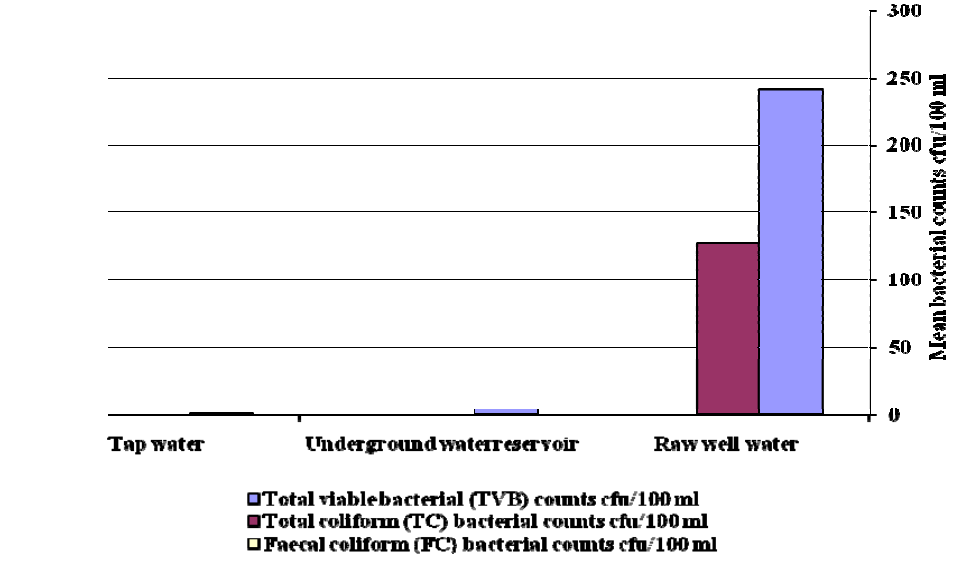


Figure 4. Showing the total viable bacterial (TVB), total coliform (TC) and faecal coliform (FC) mean counts cfu/100ml in drinking water samples collected from the hospital neighborhood weel water, underground reservoir and tap water of Saudi-German hospital.

E) Women and Maternity Hospital neighbourhood: The mean bacterial counts enumerated as (TVB) on plate count agar reached 891 cfu/100 ml, 35 cfu/100 ml and 29 cfu/100 ml for raw well water,

underground reservoir and tap water, respectively. The mean bacterial counts of (TC) showed reading of 794 cfu/100 ml, for only raw well water, while underground reservoir and tap water samples were TC-free. All samples were FC-free except raw well water exhibited 5 cfu/100 ml, (Figure 5).

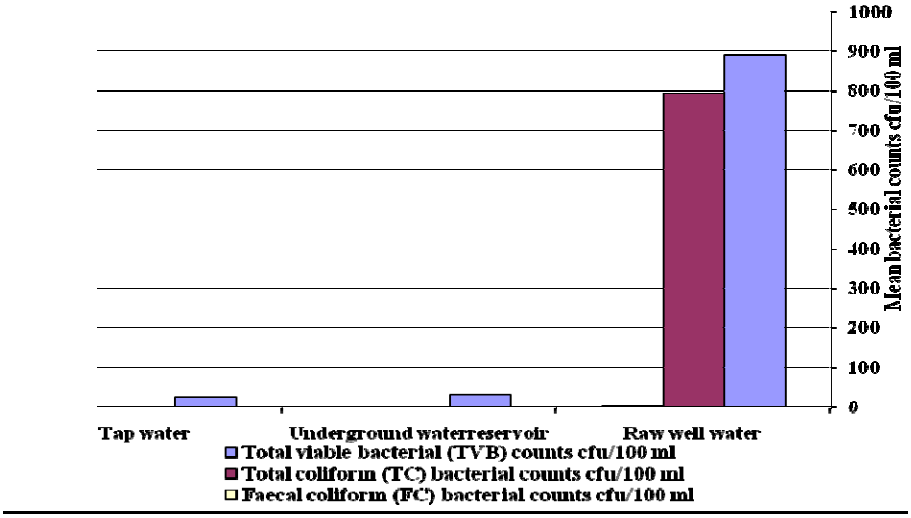


Figure 5. Showing the total viable bacterial (TVB), total coliform (TC) and faecal coliform (FC) mean counts cfu/100ml in drinking water samples collected from the hospital neighborhood weel water, underground reservoir and tap water of Women and Maternity hospital.

F) Bsateen region – Remote Control Area 1: The mean bacterial counts enumerated as (TVB) on plate count agar reached 453 cfu/100 ml, 8 cfu/100 ml and 00 cfu/100 ml for raw well water, underground reservoir and tap water, respectively. The mean bacterial counts of (TC) showed reading of 396 cfu/100 ml, for only raw well water, while underground reservoir and tap water samples were TC-free. All samples were FC-free, (Figures 6 and 7).

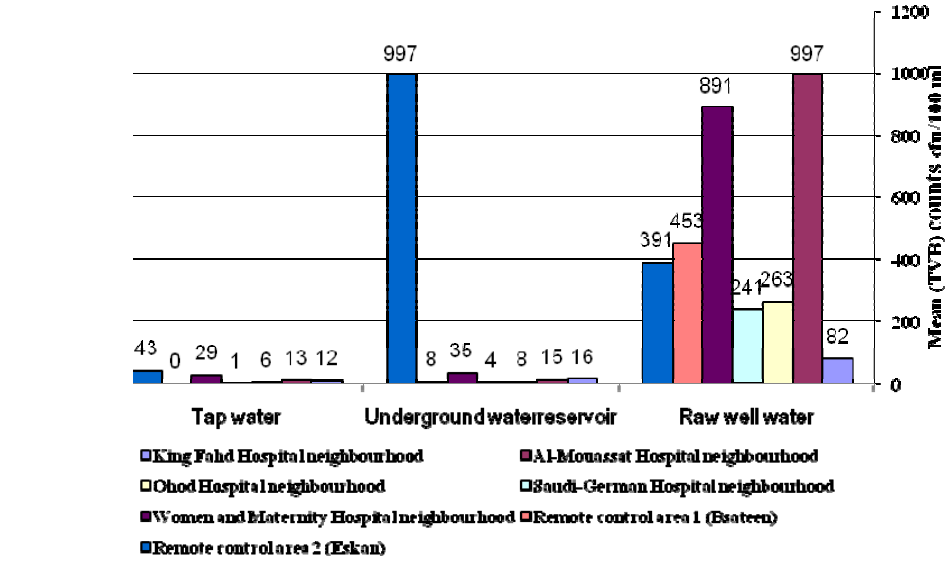


Figure 6. Comparing the total viable bacterial (TVB) mean counts cfu/100ml in raw well water, underground reservoir and tap water samples of the studied hospital-neighborhood regions and tow remote control areas.

G) Eskan region – Remote Control Area 2: The mean bacterial counts enumerated as (TVB) on plate count agar reached 391 cfu/100 ml, 997 cfu/100 ml and 43 cfu/100 ml for raw well water, underground reservoir and tap water, respectively. The mean bacterial counts of (TC) showed reading of 327 cfu/100 ml and 442 cfu/100 ml for raw well water and underground reservoir, respectively, while tap water samples were TC-free. All samples were FC-free except raw well water exhibited 9

cfu/100 ml (most probably *E. coli* with remarkable metallic green sheen) on Endo agar plates after 48 incubation hours, (Figures 6 and 7).

H) Locally-processed Bottled Water: The mean bacterial counts enumerated as (TVB) on plate count agar reached 2, 4, 11, 4, 24 and 2 cfu/100 ml for the 6 studied bottled water staff respectively named Doraq, Quba, Nada, Sarat, Taiba and Wataniya, (Fig.8). All samples were absolutely free from both TC and FC bacteria.

Qualitative results of bacteriological analysis

The macro, as well as micro-morphological characteristics of the enumerated bacterial colonies were recorded and matched as a basic tool of grouping and differentiating between the isolates to be picked up for further identification. Gram reaction for 57 isolates, showed the superiority of G –ve bacteria (74%) over the G +ve ones (26%), Fig. (9). Gram –ve rods represented 56%, while the G-ve cocci represented only 18%, Fig. (10). The G +ve isolates were all rods, with 19% spore formers and 7% non spore formers, out of the total isolated and identified species, (Fig.10).

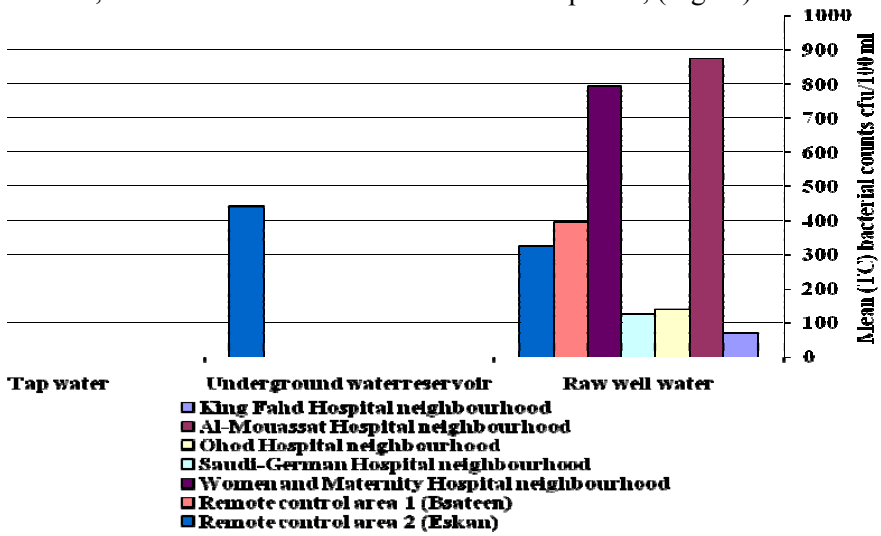


Figure 7. Comparing the total coliform (TC) bacterial mean counts cfu/100 ml in raw well Water, underground reservoir and tap water samples of the studied hospital-neighbrhood regions and the control areas.

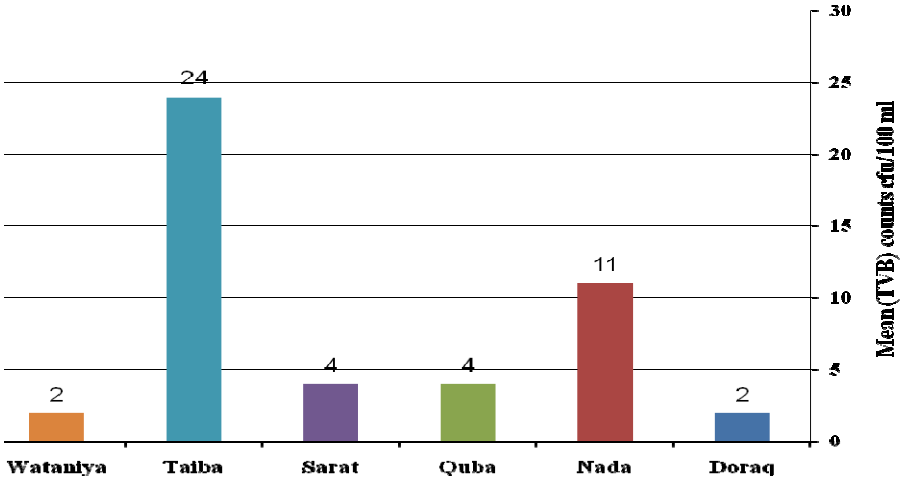


Figure 8. Recording the mean counts of total viable bacteria (TVB) cfu/100ml, detected in the 6 investigated locally-processed drinking bottled water in Al-Madinah Al-Mounawwarah

The identification of G-ve rods using API 20 E proved the presence of seven species, belonging to seven genera and three families, (Fig.11). Family: *Enterobacteriaceae* was the most presented by four genera, each by one species namely; *Escherichia coli* 1, *Citrobacter freundii*, *Providencia*

stuartii and *Proteus vulgaris*. Family: *Pseudomonadaceae* came in the second position with two genera, each has one representative species namely; *Chryseomonas luteola* and *Stenotrophomonas maltophilia*. Finally came family: *Vibrionaceae* with only one genus and one species *Aeromonas hydrophila* (group 1 and 2).

The spread capacity of each of the 8 isolates, considering group 1 and group 2 of *Aeromonas hydrophila* as two different isolates, in the different sampled sites showed that *Aeromonas hydrophila* group 2 was the most spreading species (5 sites), *Citrobacter freundii*, *Proteus vulgaris*, *Chryseomonas luteola* and *Stenotrophomonas maltophilia* (4 sites each) and *Escherichia coli*, *Providencia stuartii* and *Aeromonas hydrophila* group 1 (2 sites each), (Fig.12).

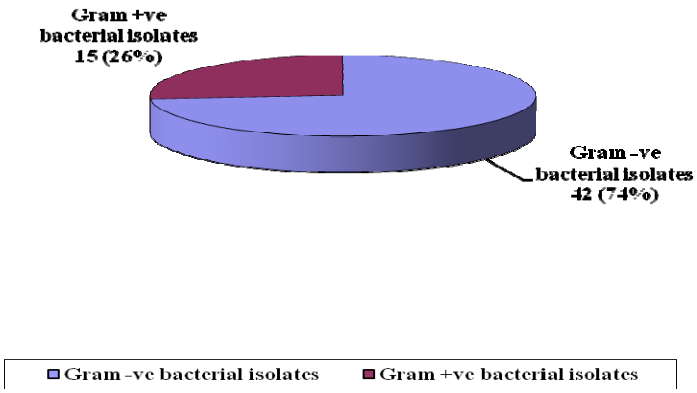


Figure 9. Showing the distribution % of Gram-ve to Gram +ve bacteria isolated from different drinking water sources at Al-Madinah Al-Mounawwarah.

The antibiotic assay for the eight identified G –ve species, against nine antibiotics showed that four species; *Escherichia coli* 1, *Chryseomonas luteola*, *Aeromonas hydrophila* group 1 and group 2, resisted the all nine antibiotics, *Stenotrophomonas maltophilia* resisted 8 antibiotics, *Citrobacter freundii* and *Providencia stuartii* resisted 6 antibiotics and *Proteus vulgaris* resisted only 3 antibiotics, (Fig.13). The minimum inhibitory concentrations (MIC s) µg/ml of the eight identified species to the nine selected antibiotics were as shown in (Table 1). *Chryseomonas luteola* exhibited the highest MIC readings being more than 100 µg/ml for all the nine studied antibiotics. The most, relatively, sensitive isolate was the *Proteus vulgaris* as it resisted only three out of the nine antibiotics to MIC (s) between 70 and 100 ug/ml, (Table 1).

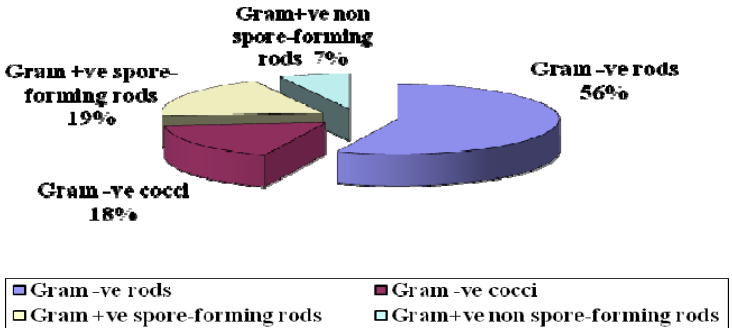


Figure 10. Showing the distribution % of Gram-ve rods to cocci and Gram +ve spore to non-spore forming rods isolated from different drinking water sources at Al-Madinah Al-Mounawwarah

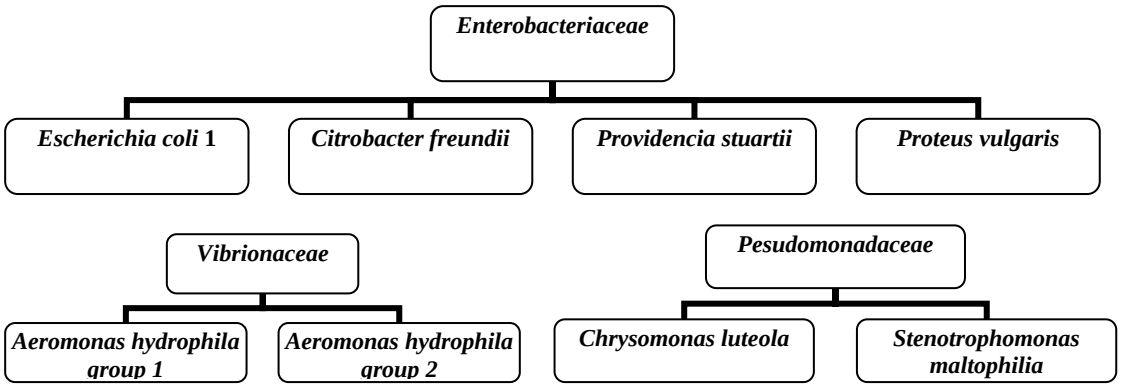


Figure 11. Diagrammatic presentation of the (Gram –ve rods) families, that the bacterial genera and species isolated from drinking water sources at Al-Madinah Al-Mounwwarah, are affiliated.

Table 1. Showing the results of the minimum inhibitory concentration (MIC) µg/ml of eight identified bacterial species to the nine studied antibiotics, where Bacit = bacitracin, Chlora = chloramephenicol, Eryth = erythromycin, Imp = impenim, Pini. G = penicillin G, Rifam = rifampicin, Strepto = streptomycin, Tetra = tetracycline, Vanco = vancomycin, +++ MIC more than 100 µg/ml, ++ MIC up to 70 µg/ml, + MIC up to 50 µg/ml and - = sensitive.

Isolate	Bacit	Chlora	Eryth	Imp	Pini.G	Rifam	Strepto	Tetra	Vanco
Escherichia coli 1	+++	+	+++	+++	+++	+++	+	++	+++
Citrobacter freundii	+++	+	+	+++	+++	+++	+++	+++	+++
Providencia stuartii	+++	-	-	++	+	-	++	++	-
Proteus vulgaris	+++	-	-	-	-	-	++	++	-
Cryseomonas luteola	+++	+++	+++	+++	+++	+++	+++	+++	+++
Stenotrophomonas maltophilia	+++	+	+	+++	+	+	-	+	+++
Aeromonas hydrophila (group 1)	+	+	+++	+++	+++	+	+	+	+++
Aeromonas hydrophila (group 2)	+	-	-	+	-	-	-	+	-

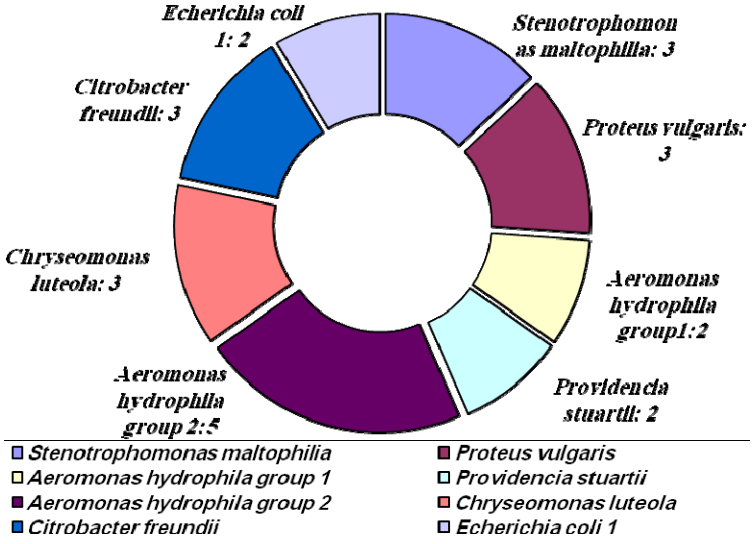


Figure 12. Showing the spread capacity of each of the identified bacterial species in relation to each other in the drinking water sources at Al-Madinah Al-Mounawwarah

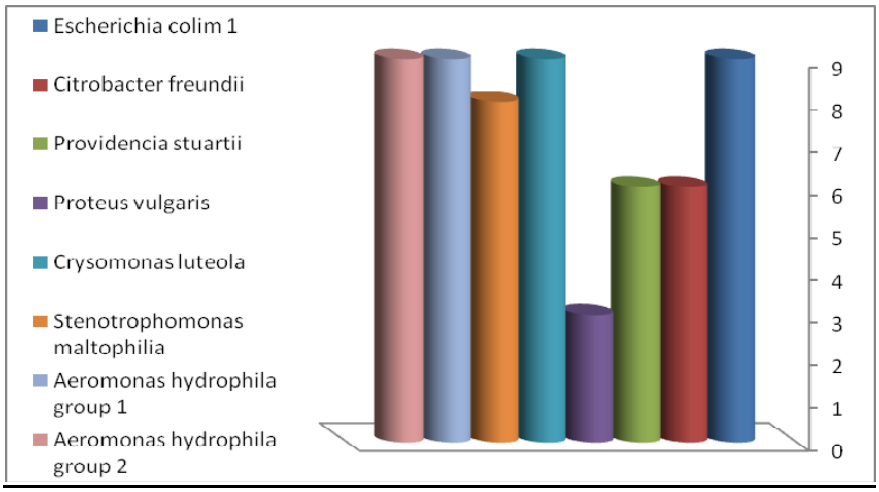
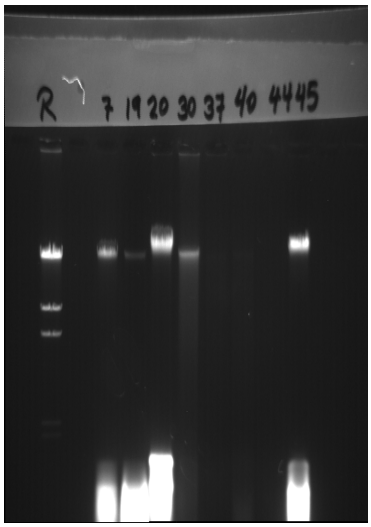


Figure 13. Showing the number of antibiotics that each of the 8 bacterial strains isolated from drinking water at Al-Madinah Al-Mounawwarah, resists out of 9 studied antibiotics.

Molecular study: Investigating the presence of plasmid in the eight strains isolated from drinking water sources under study at Al-Madinah Al-Mounwwarah namely; *Escherichia coli* 1, *Citrobacter freundii*, *Providencia stuartii*, *Proteus vulgaris*, *Chryseomonas luteola*, *Stenotrophomonas maltophilia*, *Aeromonas hydrophila* (group 1) and *Aeromonas hydrophila* (group 2), using miniprep. and alkaline lysis protocols resulted in detecting plasmids in five of them namely; *Stenotrophomonas maltophilia*, *Proteus vulgaris*, *Aeromonas hydrophila* (group 1), *Providencia stuartii* and *Escherichia coli* 1, Fig. (14). The detected plasmid was sized against the standard fragments of digested Lambda phage with Hind III, and found 23,130 bp.



Fragment Size/bp	R	7	19	20	30	37	40	44	45
23,130►	–	–	–	–	–				–
9,416►	–								
6,557►	–								
4,361►	–								
2,322►	–								
2,027►	–								

Figure 14. The only plasmid type detected in this study visualized in a group of five out of eight representative isolates. Full lane analysis is as follows:

1. R is the λ phage, HindIII-digested with its well known 6 fragments
2. Isolate No. 7, *Stenotrophomonas maltophilia* with 1 plasmid, 23,130 bp.
3. Isolate No. 19, *Proteus vulgaris* with 1 plasmid, 23,130 bp.
4. Isolate No. 20, *Aeromonas hydrophila* (group 1) with 1 plasmid, 23,130 bp.
5. Isolate No. 30, *Providencia stuartii* with 1 plasmid, 23,130 bp.
6. Isolate No. 37, *Aeromonas hydrophila* (group 2) with no plasmids.
7. Isolate No. 40, *Chryseomonas luteola* with no plasmids.
8. Isolate No. 44, *Citrobacter freundii* with no plasmids.
9. Isolate No. 45, *Escherichia coli* 1 with 1 plasmid, 23,130 bp.

Discussion

Bacterial counts of drinking water samples from different sources will be individually discussed according to the source of isolation and in accordance with the order in which results have been presented. The bacterial counts (TVB, TC and FC cfu/100 ml) detected in raw or untreated drinking well water, underground reservoirs and taps at and around the previously-studied hospitals neibourhood, will discussed first, followed by bottled drinking water, those only processed at Al-Madinah Al-Mounwwarah.

For untreated well water total viable counts ranged between 82 cfu/ 100 ml at King Fahd hospital neibourhood and 1000 cfu/ 100 ml at Al-Mouassat neibourhood, proved unacceptable drinking water qulity. The relatively high total coliform counts ranged between 73 cfu/ 100 ml at King Fahd hospital neibourhood and 870 cfu/ 100 ml at Al-Mouassat neibourhood, confirmed the unpotability conditions of these water sources. In addition, the recorded faecal coliform counts, although in low numbers, at the two wells around Ohod hospital and Women and Maternity hospital neibourhoods, categorize them as health risk and source of disease. They sure accept these faecal pollutants, mainly *E. coli* from a continuous human source. They sould be closed and prohibited until disinfected and re-examined according to the time schedule and guidelines for drinking well water programs of WHO 2002a&b, 2007 and the NCCLS (1990) quality assurance. Water contaminated with feces should be disinfected with liquid bleach, thoroughly flushed to remove bleach residue and retested. Due to potential for re-growth of bacteria in distribution lines, these should also be disinfected and cleared prior to retesting. The original source of contamination should be determined so changes can be made to prevent future contamination from backflow, flooding, poor construction or other causes. The higher bacterial counts; TVB/ TC/ FC, detected in the well waters of the two control areas remote from the previously-studied hospitals, made it difficult to nominate the hospitals as direct and sole source of bacterial pollution for neibourhood groundwater, as cited by Mayhall (1996). The absence of geographic pattern for the pollution indicators distribution may be, then, real. Of nearly 12,000 samples analysed for Total Coliform bacteria, 15% had concentrations greater than the drinking water guideline of 10 CFU per 100 ml water, 80% of samples had between 1 and 10 CFU/100 ml and 5% of samples had less than or equal to 1 CFU/100 ml (Somwang *et al* 2005). The study found no geographic pattern of occurrence of Total Coliform organisms above the guideline. But it still possible they had a role in it even if partially. This finding is in line with that the very close area surrounding the well has much more influence than far one in constituting the microbial population, bacterial in particular (Ong *et al* 1999). The use of re-cycled wastewater in irrigation of farms close to these wells, with highprosity and rapid infiltration of water deep in land, may give a good excuse for the high bacterial counts and the presence of coliforms from human origin in these well water, this concept has been also discussed by Blumenthal *et al* (2001).

Recent study (Somwang *et al* 2005) without assurance, indicated the hospital final effluent, even treated, as the first suspect in polluting the near aquifer of ground water. The need of a long term monitoring program studying the underground bacteriological water quality in relation to environmental circumstances, like that New Mexico Environment Department, Ground Water Quality Bureau (2003) has established, is a priority. Many statistical observations worldwide, pointing hospitals as a real threat of pollution to underground water and health risks (Replidyne, 2006).

Fortunetally, most of the well water sources at Al-Madinah used for irrigation and swimming pools, but rarely used for drinking. This minimizes the probability of health risks expected from such polluted water.

Except for the King Fahd hospital neibourhood, all the studied underground water reservoirs were found bacteriologically accepted. In most cases these underground reservoirs lack monitoring and routin disinfection. The careless behavior of the owners and the lack of governmental surveillance program may explain such results. On site programs developed by different researchers (Franceys *et al* 1992 and Mara 1996), gave good recovery results. It is important here to record the absolute absence of total and faecal coliforms from all the studied water samples collected from houses taps; neibourhood of the previously-sutdied 5 hospitals and 2 remote control areas.

Counts of bacterial pollution indicators (total and faecal coliforms cfu/100ml), and in reference to both the local (KSA) and international standards, clearly-indicated purity at the high grade potability bacteriological conditions of all the studied bottled water (6 local commercial trade marks) being T/Fcoliform-free. The relatively high counts of total viable bacteria in two (Nada 11 cfu/100ml

and Taibah 24 cfu/100ml) of the investigated locally-produced drinking bottled water types make them suspicious, as recommended by other investigators and authorities who believe , and our results agreed with them, that the crowded TVB counts emphasize risk originated from their over growth that may mask coliforms from being culturable on plates, may be because of their poor capabilities of gram –ve bacteria in general and coliforms in particular of competition for nutrients (Colwell and Huq 1994, Islam *et al* 2001). and on the other hand, the powerful extracellular enzymatic system of gram +ve and other commensal bacteria.

Regarding the bacteriological quality of of the studied drinking water sources, based upon the identification results of the strains isolated, the high percentage of Gram –ve bacteria (74%) in relation to that of the Gram +ve ones (26%) may indicate the fresh and continious source of these bacteria introduced to water, (WHO, 2007). The short-term living mode of Gram –ve bacteria in water and the lack of certain essential nutrients may explain this conclusion (Engelbrecht 2005, Schwartz, 2000;Umbreit, 1991). The study focused, then on the (56%) of detected Gram –ve rods as the mostsuspected group in health problems that directly-linked to the water pollution indicators. In addition it represented the highest percentage of existence compared with other groups; Gram –ve cocci (18%), Gram +ve spore-forming rods (19%) and Gram +ve non spore-forming rods (7%).

The identification of four spcies of *Escherichia, Citrobacter, Providencia and Proteus,* family: Enterobacteriaceae, with *Escherichia coli* with them confirms this group as a powerfull indicator for pollution measurements, (Engelbrecht 2005). While the presence of two species of *Aeromonas,* family: Vibrionaceae, suspect their source as healthy-hazarous and pathogenic, (WHO 2002b). The importance of having two spceis of *Chryseomons* and *Stenotrophomonas*, family Pseudomonadaceae, reflects the possibility of water contamination with residuals of pharmaceutical drugs, antibiotics in particular, because of their well known wide range of multidrugresistance patterns (Janet, 2005; Diab, 2008). The first three species those distributed in drinkingwater sources were; *Aeromonas hydrophila* group 2, *Stenotrophomonas maltophila* and *Escherichia coli*, in a desnding order. Such a distribution profile encourage the point of having hospitals, so far, as a prosecuted source of the problem. Srenthing this idea the antibiotic resistance (β- lactamase production is, for sure, the main cause of such resistance (Bradford *et al,* 2001; Lepper *et al,* 2002, Koch, 2003) profiles of the studied isolates which is positively with that these isolates are well-trained on tolerating and resisting the investigated nine antibiotics through being in contact with them at the same ecosystem for enough period. Final effluent of hospital wastewater supposed to be the the theater of such phenomenon (Houndt and Ochman, 2001; Diab, 2008).

Special intrest, in this study, was paid to *Stenotrophomonas* which is a Gram –ve, non-lactose fermentor, pigment-producing rods that resist cephalosporines, penicillines, sulphonamides, steroids, aminoglycosides and quinolones (e.g.: ofloxacin), with known efflux system. *Stenotrophomonas* is a genus belonging to family *Pseudomonadaceae*. It has many species, *maltophilia* is one which is the most frequently-isolated from both clinical and environmental samples. Its significant potential as opportunistic pathogen being the second most frequently isolated non-lactose fermentor from a variety of nosocomial infections, causing 70% of otitis externa (swimmer's ear) and dermatitis especially those associated with contaminated whirlpool baths, as well as a source of biocontrol and bioremediation activities are well known (Romanenko *et al* 2008). It also has a wide range of multi-drug-resistance including different generations of cephalosporines (β-lactam containing antibiotics). The hospital outbreaks related to antiseptics and disinfectants resistance has been reported for *Stenotrophomonas maltophilia*, (David 2008). Fatty acids analyses of clinical strains proved a relation between growth temperature (30–37˚C) and antibiotic susceptibility profiles (Hejnar *et al* 2003). Marine isolates exhibited differences in pigmentation, NaCl tolerance and substrate utilization pattern. Some strains displayed haemolytic and remarkable inhibitory activity against a number of fungal cultures and Gram +ve bacteria, but very weak or non against *Candida albicans*. The ability of some strains, DSM 50170, for example, to produce glucosylglycerol and accumulate trehalose as osmolytes, may explain the ability of such strain to tolerate salinity and grow at concentration of 3-5% (Roder *et al* 2005). *Stenotrophomonas maltophilia* has emerged linearly with the increased use of carbapenem (Sanyal and Mokaddas 1999). *Stenotrophomonas maltophilia* is a real cause of concern because of the reported increasing frequency of isolation from cystic fibrosis patients (Graff and Burns, 2002). They suggested that if PCR test with sensitivity and specificity, according to CLSI/NCCLS M100-S15 (Janet, 2005) approaching 100% has been developed, the reported

prevalence might increase dramatically. It has been cultured from the perirectal ulcers with intrinsic resistance to carbapenems and aminoglycosides (Micozzi *et al*, 2000).

Although the detected plasmid in five isolated strains in the present study namely; *Escherichia coli*, *Providencia stuartii*, *Proteus vulgaris*, *Aeromonas hydrophila* group 1 and *Stenotrophomonas maltophila*, was at the same size 23130 bp they showed different antibiotic profiles. This primiraly indicate they are not the same from the point of gene constitution. One remarkable finding is the presence of to strains of *Aeromonas hydrophila*, 1 and 2, and only strain 1 got plasmid, while 2 was plasmid-free. The plasmid existence in strain 1 sure participate in the antibiotic profile resistingall the nine sutdiedantibiotics at MIC(s) 50:100 µg/ml, while strain 2 resist only three and at not more than 50 µg/ml. Baucheron *et al* (2004) concluded similar findings for other members of Vibrionaceae. The result of having the same plasmid size, could be the same plasmid or not, inspite of the differences in taxonomic ranks at the generic as well as specific levels may indicate the presence of certaine inducing agent that inforced bacteria to keep, transfere and transform this plasmid, which encodesin turn for tolerating that agent. The gene ecology concept at the horizontal level may notbe clearly-recognized, but at the vertical level it is.

Obviously, the higher percentage of bacteria bearing plasmids, as well as the frequency of each plasmid, in hospitals wastewater than those in drinking water may be explained by the relative purity of drinking water being almost free from pharmaceutical residues and many of the essential growth nutrients. That is why the distribution of plasmids amongst bacterial population of drinking water is much less than those of the final wastewater of hospitals. Pharmaceutical residues, in particular, comprise the most effective inducers those enhance bacteria to keep, transcript, transfere and transform the genetic elements enabled them of expressing resistance capabilities they encode. Final wastewater of hospitals is ideal environment for this gene ecology exchange, while drinking water is not. Once the bacteria, in general, accepted plasmids under certain conditions, they should not easily give up with them even when environmental conditions are positively-changed. The plasmid contents of *Escherichia coli* strains and thermotolerant coliforms (TTC) and were isolated from sewage from a treatment plant before and after peracetic acid (PAA) disinfection . The plasmid profiles of 120 *E . coli* strains were analyzed . Although PAA disinfection effectively reduced the number of TTC and *E . coli* strains, the percentage of *E . coli* strains containing plasmids was not statistically different among water samples . The sizes of the plasmids found ranged from < 3 kb to > 56 kb, but plasmids of between 3 and 5 kb were encountered most frequently, (Arturo-Schaan *et al,* 1996).

Conclusions

1- Bacteriologically, bottled, underground reservoir and tap water (all are from desalinated water source) are safe to drink, while underground well water is not.

2- No direct correlation could be obtained between the bacterial counts in the studied water samples and their site of sampling whether neer or far from the hospitals.

3- The identification studies, antibiotic-resistance profiles and plasmid patterns for bacterial isolates from the present drinking water study and the previous hospital wastewater study, primarily, suggest a link and give a clew for the hospitals to be the source that, partially, involved in contaminating drinking water sources with bacteria at Al-Madinah Al-Mounwwarah.

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