MANSOURA JOURNAL OF BIOLOGY
Official Journal of Faculty of Science, Mansoura University, Egypt
E-mail: scimag@mans.edu.eg ISSN: 2974-492X

Bacterial Status of Some Drinking Waters from Dakahlyia and Alexandria Governorates of Egypt

Hayder G. Wajer[1,3], H. ElSheshtawy[2] and Yehia A. Osman[1]

[1]*Botany Department, Faculty of Science, Mansoura University, 35516, Egypt*

[2]*Agriculture Genetic Engineering Research Institute, ARC, Giza, Egypt*

[3]*Univ of Baghdad, Iraq*

Received: 30/ 9 /2019
Accepted: 14/10 /2019

Abstract: Availability of sanitized quality water is one of the basic rights of humans worldwide. The international standards require the drinking waters to be odorless, tasteless, and colorless besides being free of pathogenic microbes and toxic heavy metals. Using traditional microbiological techniques twelve bacterial colonies were isolated and identified from eight drinking water samples. Molecular biology tools such as random amplified polymorphic deoxyribonucleic acid fingerprinting, protein banding patterns, sequencing the gene encodes for a conserved region of the 16S rRNA, and Matrix-Assisted Lasar Desorption Ionization were used for identification of the multidrug-resistant bacterial isolates from drinking waters. All bacterial isolates belong to three genera named E. coli, Staphylococcus epidermis, and Bacillus halo... The accuracy of molecular techniques to the speciation of the bacterial isolates was beyond doubts. Moreover, Matrix-Assisted Lasar Desorption Ionization proved to be faster, highly accurate and cheaper to perform than other molecular biology techniques; considering the availability of the instrument and the existence of the bacterial spectra in its library.

keywords: Drinking water, Bacteria, MALDI, Molecular biology

1.Introduction

There should no doubts or problems with the safety of the direct use of municipal drinking waters. This water should be absolutely free of any human infectious pathogens such as bacteria and viruses. The waterborne pathogens can cause cholera, intestinal infections, typhoid fever, hepatitis, dysentery, hepatitis….etc. However, continuous monitoring of municipal drinking water is done by health official worldwide as precautionary routine to ensure public safety against waterborne microbial diseases. Diseases such as diarrhea have amounted to 2.2 million annual deaths among children and population at large in the developing countries. UNICEF reported in 2010 about 5,000 children were dying every day due to diarrheal diseases (11, 25, 26). The main cause of these diseases is known to be fecal bacteria such as *E. coli* and Enterococcus. All this constituted huge challenges to public health officials especially it affected more than half of the world population in the developing countries. Therefore, Egypt has developed a number of reviews of drinking water to quell any microbial threat to the health of its people. In the same time, it can take the necessary measures to prevent the entry of any microbial pathogen into the water distribution system after treatment process.

The worries have increased from the presence of more dangerous microbes such as Salmonella and campylobacter in drinking waters. Also, the rise of multidrug-resistant bacteria (MDR) and the presence or spread in the drinking water will not only complicate their health hazards but bigger challenges and threat to the whole population. Therefore, detection and then elimination of these harmful microbes from drinking water is a priority to the scientific community and the health community as well (15, 19, 23, 24). Standard tests of water sanitary require the presence of zero number of total coliforms per 100 ml

tested andless than 15 colony forming units (CFU) per 100 ml of Salmonella (21, 25, 26). Other dangerous bacteria that should be completely absent from drinking water include *Campylobacter Shigella, Yersinia or Vibrio cholera, Legionella pneumophila* (1, 22).

Every day we read about new microbial pathogenic agent isolated from drinking water bodies and that number has totaled more than a 1000 species of viruses, bacteria, fungi, protozoa, and parasitic worms (15, 22) which necessitates quantitative risk assessments to these waterborne pathogens. There are many factors which affect the choice of the quantitative methods including the isolation, sensitivity, and rapidity of the identification process. The available numerous methods for rapid detection and quantification of waterborne pathogenic bacteria in drinking water can be classified into classical bacteriological techniques, immunology-based, nucleic acid-based, and biosensor-based methods. The highly sophisticated modern molecular biology techniques have also been adopted for water microbiological analysis and evaluation. These included digital droplet PCR, DNA microarray, Next-generation sequencing (pyrosequencing, Illumina technology, and genomics), real-time PCR, multiplex PCR, and fluorescence in situ hybridization. However, the very cost-effective test, the basic total coliform techniques, is as informative as all other technologically advances and costly tests.

The increase of human leisure activities, the globalization of travel and trade, and different changes in drinking water treatment technology have affected the trust of the public in the municipal waters and converted them to bottled waters. This is another burden on drinking water industry that requires absolute sanitary standards and microbiological monitoring. Therefore, this study has taken the responsibility to test the degree of sanitary of the municipal drinking water in Mansoura city and some selected sites in Dakalyia and Alexandria Governorates. Our results demonstrated that the water treatment process in Dakahlyia does follow the standards and no serious problem is associated with the water samples tested.

Materials and Methods

Sample collection

Eight drinking water samples were collected from Dakahlyia and Alexandria Governorates.

Seven samples from Dakahlyia (Meet Khamis Water Treatment Station, Weesh, Neketah, Khaeriah, Belqas, El-Sinbilawain, laboratory tap) and one sample from ElMontazah dristrict, Alexandria (Fig. 1).

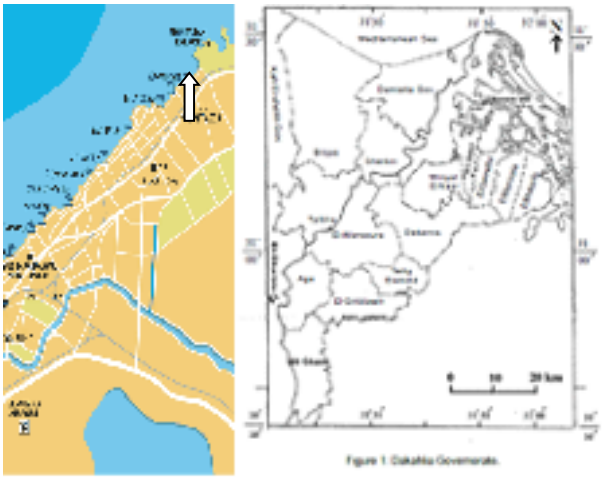

Fig. 1. A map showing locations of water samples; the white arrow points to the location of Alexandria Sample.

Bacterial growth media and isolation

Water samples were streaked onto Luria Bertani (LB) agar plates (contains g/L: Tyrptone 10g, yeast extract 5g, sodium chloride 10g and agar 15g), nutrient medium (Peptone 5g, yeast extract 3g, sodium chloride 5g, and agar 15g), 36.6g of TBX medium, MacConkey agar (49.53 g of ready-made mix was dissolved in 1L disitilled water, and Mueller Hinton agar (2.0g beef extract, 17.5g casein hydrolysate, 1.5g starch, and 17.0g agar). All bacterial isolates were grown in solid/liquid media were incubated at 37^0C for overnight before any further manipulations. All media constituents were dissolved in one liter distilled water and autoclaved at 121^oC for 20 minutes (10). The selected purified colonies were stored at -20°C in 20% glycerol stocks for longer preservation time.

Antibiotic sensitivity test

All 12 bacterial isolates were tested for their sensitivity to seven commercially available antibiotic discs by Kirby-Bauer disc diffusion method [4, 7]. Each disc type was saturated with a fixed concentration of the antibiotic and

used as recommended by the manufacturer (Table 1). The zones of inhibitions were recorded and analyzed according to the Clinical Laboratory Standard Institute (7). The antibiotic discs used and their concentrations were Ampicillin (10ug), Amoxicillin (25ug), Amoxycillin- Clavulanic acid (20-10ug), Aztreonam (30ug), Cefoxitin (30ug), Cefsoludin 30, Cephalexin (30ug), Chlorampheicl 30, Erythromycine (15ug), Imipenem (10ug), Kanamycin (30ug), Meropenem (10ug), Methicillin (10ug), Nalidixic Acid (30ug), Oxacillin (5ug), Penicillin (10ug), Streptomycin (10ug), Trimethoprim- sulfamethoxazol (1.25-23.75ug), and vancomycin (30ug).

Protein banding patterns

The total cellular proteins of all bacterial isolates from drinking water samples were analyzed by polyacrylamide gels electrophoresis in denaturing conditions (17). The gel consisted of a stacking layer (upper) and a separating layer (lower). Both layers consisted of the same components with varying concentrations; the lower layer is 12% and the upper layer is 4%. Tetramethylethylenediamine (TEMED) and ammonium persulphate (APS) were the initiator and gelling agents of the bis-polyacrylamide matrix. The loaded proteins were standardized by extrapolating their concentrations from a standard curve constructed using Bovine serum albumin (BSA).

Identification of Bacteria

The purified bacterial colonies were morphologically, biochemically and molecularly characterized according to the standard bacteriological techniques (25).

DNA Fingerprinting

The three multidrug-resistant isolates were further differentiated from each other by the DNA fingerprinting as described in the literature [1, 5, 9, 15, 19]. Four randomly constructed and chosen primers manufactured by OPERON were used. These were OPU-16 5′-CTGCGCTGGA-3′), (OPT-16 5′-GGTGAACGCT-3′), (OPK-02 5′-GTCTCCGCAA-3′) and (OPI-17 5′-GGTGGTGATG-3′. Each of the four primers should result in a different PCR product profile depending on the bacterial DNA templates which are called RAPD-PCR fingerprint. Each reaction final volume was 25ul (20 ng of bacterial template DNA, 20 pmol of primer, 1U of Taq DNA polymerase, and 1x buffered master mixture of dNTPs and covered by a tiny drop of mineral oil) heated at 94 ^{o}C for 5 minutes to denature the template DNA. This followed by 35 amplification cycles in each cycle the template DNA was denatured at 94°C for 1 min, the primer was annealed at 46°C for 45seonds and the polymerization was extended for 3 min at 72°C. At the end of the 45 cycles, a final extension step at 72°C for 7 min was performed. The yield of the PCR was analyzed by agarose gel electrophoresis, visualized by staining with ethidium bromide and photographed for analysis.

Plasmid DNA purification

Although the alkaline lysis technique is the gold-standard for preparation of plasmid DNA from bacteria, a lot of commercial kits are now available that simplified such procedures. One of these kits is manufactured by iNtRON Biotechnology (South Korea) called DNA-spin Plasmid DNA Purification kit. The kit contains resuspension buffer, lysis buffer, neutralization buffer, washing buffer A, washing buffer B, elution buffer, spin column and collection tube, RNase A (Lyophilized powder), and lysis viewer**.**

Mass spectrometry microbial identification system

A mass spectrometry (VITEK® MS) located at Zagazig University hospital was used for quick microbial identification. This instrument uses a laser to ionize matrices and the time ions take to reach a detector (MALDI-TOF MS) technology. A loopful of each designated bacteria loaded on the specific slide which inserted into a high-vacuum chamber. Within the closed chamber the sample was ionized by exposing to a precise laser burst to release a cloud of proteins accelerated by an electric charge. This cloud was passed through the ring electrode and the time of flight of each was recorded generating characteristic spectra for each bacterial isolate (1).

The 16S rRNA gene amplification and sequencing

One of the highly accurate molecular tests depends on the sequencing of conserved regions of the gene encodes for 16SrRNA. This gene was amplified using two universal oligonucleotide primers: forward -(5′-CAGGCCTAACACATGCAAGTC-3′) and reverse -(5′-CGGCGGAGTGTACAAGGC-3′). The DNA template used in the PCR was prepared from the different bacterial strains by Quick-gDNA miniprep kit-from Zymo Research, USA. MyTaq™ Red Mix from Bioline Company contains all the necessary reagents and stabilizer with no interfering materials with the PCR was used in the gene amplification. The annealing temperature for the primer pair was 60ºC depending on the melting temperature values. The amplified DNA fragments were extracted and purified from agarose gels by the DNA clean and concentrator kit-25 from Zymo Research, USA, as recommended by the supplier. The highly purified DNA was sequenced by an automated sequencer. The use of this kit removes not only the free dNTPs but also the DNA polymerases, as well.

RESULTS

Coliform bacteria in drinking water samples

Twelve morphologically distinctive bacteria isolated from all the collected water samples were tested for the presence of the pollution indicator *E. coli* and other coliforms (Fig. 1).

One bacterial colony was isolated from samples collected from Kharieh (#1), Belqas (#10), Weesh (#12) and Meet Khames Water Purification Station (#5). Two from Tap waters from Alexandria (6, 7), our Bacteriology Laboratory (8, 9), Neketah (2, 3) and Senbellaween (4, 11) samples and three isolated from each other water samples used for this study. After 24h of growth, only isolate number three produced acid and gas, isolates one, two, four, five, six, seven, eight, nine, ten, eleven and twelve produced gas and a slight change in the indicator color; into yellow. Isolates two, five, six, seven, and eight appeared red as the control (un-inoculated) tubes, while isolates one, four, nine, ten, eleven, and twelve are pink in color; i.e neither red (as control) nor yellow as the completely positive isolate three

Fig. 2. The coliform test for the twelve isolated bacteria from the drinking water samples collected from different locations in Dakahlyia and Alexandria Governorates, Egypt.

Antibiogram of bacteria from drinking water

The antibiotic sensitivity of the twelve bacterial isolates varied from highly sensitive, intermediate sensitive to resistant (Fig.2 and Table 1). Isolates one (Khariah) and two (Neketah) showed complete resistance to the seven antibiotics tested. These were followed by isolate seven that showed resistance to six antibiotics and an intermediate sensitivity to vancomycin only. Isolate three resisted ampicillin, penicillin G, gentamycin, vancomycin, and trimethoprim-suflanoaxonal and showed intermediate sensitivity to erythromycin and kanamycin. Isolate four resisted only three of the seven antibiotics tested; these were ampicillin, penicillin, and trimethoprim-suflanoaxonal. Each of isolates five and 10 showed resistance to only two antibiotics; these were erythromycin and penicillin and ampicillin and SXT, respectively. The rest of the isolates showed sensitivity to some antibiotics and intermediated sensitivity to others. Strains 8 and 9 showed the highest

sensitivity to the SXT with 30mm diameter of the inhibition zone.

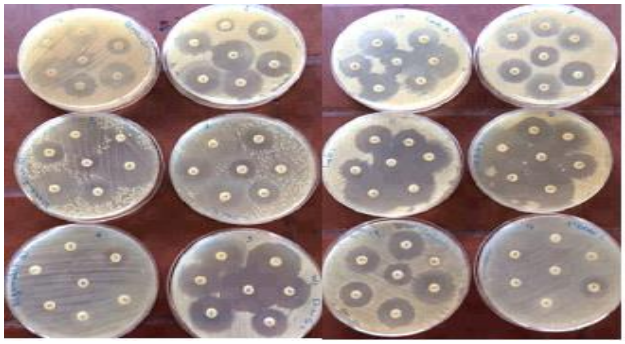

Fig. 3. The antibiogram of the twelve bacterial isolates from drinking water sample

Table 1. The sensitivity of twelve bacterial isolates (to seven antibiotics) from the drinking waters scored according to the CLSI standards (R, I, and S)

Isolate No.	AntibioticsZone of inhibition (mm)						
	Ampicillin	Erythromycine	Pencicillin G	Kanamycine	trimethoprim-suflanoaxonal	Vancomycine	Gentamycin
1	R	R	R	R	R	R	R
2	R	R	R	R	R	R	R
3	R	I	R	I	R	R	R
4	R	I	R	I	R	I	I
5	I	R	R	I	I	I	I
6	R	I	R	I	I	I	I
7	R	R	R	R	R	I	R
8	I	S	I	I	S	I	R
9	I	I	I	I	S	I	S
10	R	I	I	I	R	I	I
11	S	S	S	I	S	R	I
12	I	S	S	S	S	I	I

R. resistance (≤16mm) I: Intermediate, (from 16 to 21mm) S: sensitive (>21mm

The protein contents of the bacterial isolates from drinking waters

The protein banding patterns of the twelve bacterial isolates can be grouped into four major bacterial types. The isolates one, three, six, seven and 11 are closely similar while isolates number two, four and 12 showed unique protein banding patterns. The closely related bacteria isolated from four geographically different localities are isolated one from khiarieh village north of Mansoura city, isolate three from Nekeetah village south of Mansoura city, isolates six and seven from Alexandria, and isolate 11 from Senbellaween city east of Mansoura city. isolate two from Nekeetah, isolate four from Senbellaween and isolate 12 from Weesh (Fig. 2)

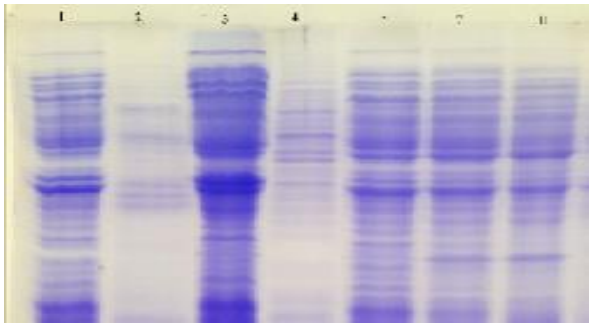

Fig. 4. The protein banding patterns of seven bacterial isolates from drinking water samples

16, OPK-02 and OPI-17 havd PCR products with organism three and primer OPT-16 did not.

DNA Fingerprinting of three MDR bacteria from drinking waters

From the antibiogram, three bacterial isolates showed resistances to all antibiotic tested. These three isolates were fingerprinted using four oligonucleotides primers OPU-16, OPT-16, OPK-02, and OPI-17 (Fig. 5). The four primers are numbered from 1 to 4 with one isolate, 5 to 8 with the second isolate and from 9 to 12 with the third isolate. Primers OPU-16 and OPK-02 have no PCR products with organism one, primer OPT-16 produced two distinct DNA bands (300 and 600bp), and primer OPI-17 produced a 900bp band and a faint 600bp band. Only primer OPT-16 produced three DNA fragments with organism two, while primers OPU-16 (no.5), OPK-02 and OPI-17 did not produce any PCR products. Primers OPU-

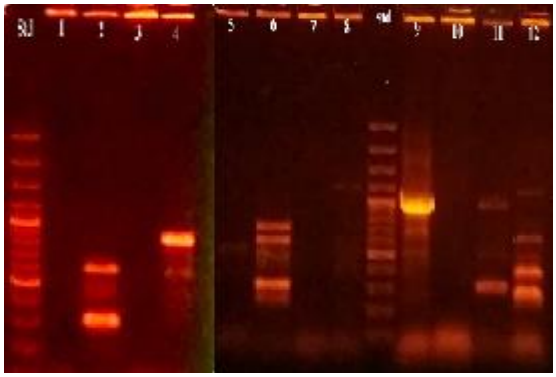

Fig. 5. DNA fingerprinting of the three MDR bacterial isolates from drinking water with OPU-16, OPT-16, OPK-02 and OPI-17 oligonucleotides random primers.

Automated identification of bacterial isolates using MALDI-TOF-MS

A special mass spectrophotometry technique called matrix-assisted laser desorption ionization (MADLI) technique was used to automatically identied the bacterial isolates obtained in this study. In this technique, the bacteria was mixed with a matrix which ionizes upon exposure to specific laser burst producing a cloud of proteins carrying specific electric charge that travels to a detector at the end of the process for identification (mass/charge, or m/z). The detector generates spectra for the different ions. The spectra are represented as mass over charge (m/z) and the intensity is the height of the peak. Comparing the acquired spectrum for each bacteria against the built-in library, the produced spectrum was used to name the bacteria under examination (Fig…6 A, B, and C). The spectra are definitely different from each other

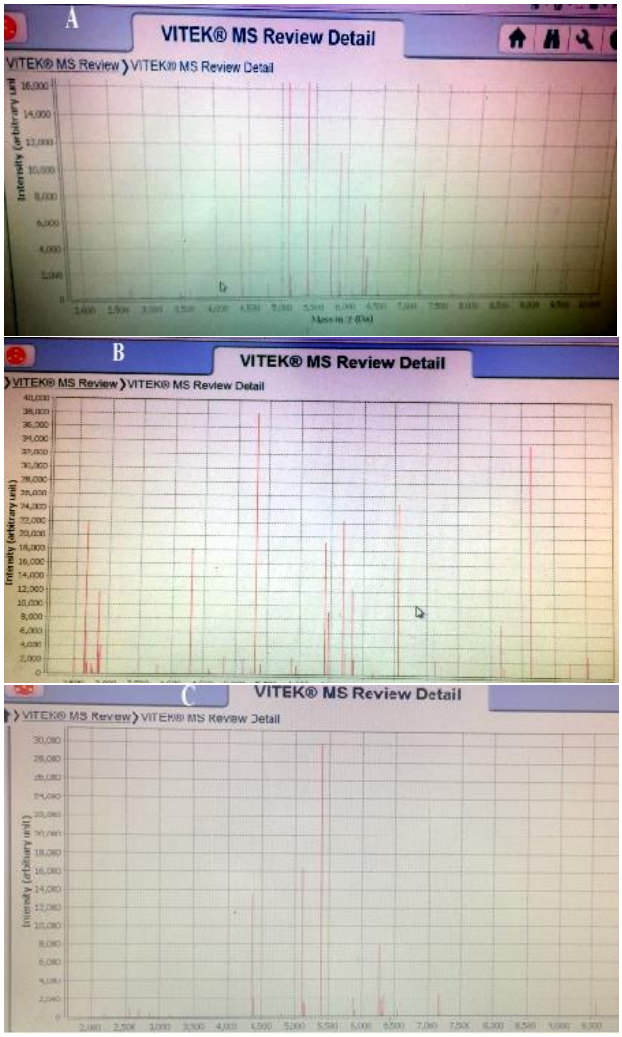

Fig.6. The MALDI spectra for the three MDR bacterial isolates from drinking waters. Panel A: isolate 1, panel B: isolate 2 and panel C: isolate 3.

Discussion

Without water and oxygen, human life is impossible on earth. The irony of the availability of clean water for drinking is dramatized in the fact that poor people have less access to mobile phones than safe drinking water. This scarcity of high-quality potable water to about 2.6 billion of human was highlighted by the different WHO reports. In specialized reports by the WHO and the UNICEF, the annual death reached 3.4 million among children due to water-related diseases. Annually, the waterborne diseases lead to the death of over 25 million from amongst them children (10, 15). Inadequate drinking water contributed to five million diarrhea deaths and inadequate sanitation lead to about three million deaths according to a retrospective data analysis collected from 145 countries (20). Water distribution pipeline systems are known to age over time, due to rusting or breakage and may contain pathogenic bacteria. Therefore, the availability of clean drinking water dictates regular examination to ensure safety and complete sanitization. This is to avoid any contamination due to Leaks from sewage pipes running side by side with the water distribution network. Since it is well documented, broken and/or rusty pipelines help in mixing sewage with drinking water and hence spread of potential pathogens to drinking water.

From the bacteriological or microbiological point of view presence of coliforms in drinking water is the gold-standard indicator of fecal contamination and hence unsuitability for human consumption. Three major bacterial species were isolated and identified in this study: *E. coli*, *Staphylococcus epidermis* and *Bacillus* species Luckily the total coliform counts (only one E. coli) in our samples did not exceed the permissible range and total absence of pathogenic bacteria such as *Salmonella*, *Shigella*, and *Vibrio cholera* from the collected samples during this study. These satisfactory results conform to the microbiological standards set by the WHO for the drinking water. The international standards demand the absence of any fecal pollution indicator and any pathogenic bacteria from natural water, soil, or

any other environmental source. However, the classical water examination does not include the detection of pathogenic bacteria and looks only for indicator bacteria such as *E. coli.* The presence of *E. coli* is a serious indicator of either human and/or animal fecal contamination or even both. The major concern is the association of any of six highly pathogenic microbes such as Campylobacter Jejuni, Hepatitis A, Giardia Lamblia, Salmonella, Legionella Pneumophila, Cryptosporidium with E. coli (14). However, some English researchers found another four biofilm-producing bacteria in Sheffield tap drinking. These biofilm-producing bacteria were not harmful themselves but they were helpful for the thriving of harmful bacteria. Moreover, they suggested the use of sophisticated DNA-based methods for water analysis instead of culturing bacterial techniques for pathogenic bacterial identification and hence a quicker response to clean and sanitize drinking water. (17). Many organizations like the Environmental Protection Agency (EPA;) in the USA did not agree with the British argument because it is not practical and expensive to test drinking water for all potential bacterial pathogens. The EPA even stated that the presence of coliform in drinking water does mean it is not unsafe for drinking, but rather it is a sign of risk that pathogens are present. However, the EPA requires zero total coliforms for every 100 milliliters of drinking water.

Regular drinking water checking for potential bacterial pathogens is highly important to take the correct measures to protect human from being infected. From the microbiological point of view all bottled water on the roadside of Dehli, 28% of tap water in Patiala, Punjab, India, 23 out of 24 samples from Ethiopia, 87.5% of bottled waters in Nepal were unsuitable for human drinking. These authors were able to isolate highly virulent human pathogenic bacteria from the water samples examined such as Enterobacter, Klebsiella, Salmonella and Shigella, Vibrio, rotavirus, all are predominantly fecal in origin (6, 15,16, 18, 23)

In conclusion, public drinking water systems are safe for the majority of people to use. However, special group of people such as the immunologically compromised group is at high risk if they drink inadequately disinfected and sanitized water. This last category of drinking water can be identified from its color, odor, and bad taste. Despite the assurance of the current drinking water the majority of people now depends on bottled regardless of their financial status and you find them everywhere around at homes, bus or railway stations, airports, restaurants, and hospitals….etc. Recently, a published report on the low quality of waters on major Airlines served to their passengers (DietDetective.com and the Hunter College New York City Food Policy Center at the City University of New York, USA). This and similar reports increased the distrust in public drinking water and hence created great demand for bottled waters which needed more scrutiny and attention to this life-dependent resource system.

The molecular fingerprinting widely used in place of the phenotypic characterization of isolated bacteria just to avoid the confusion and inconclusive results associated with it. The molecular methods included 16S rRNA sequencing, protein banding patterns, MALDI spectral analysis, and RAPD-PCR typing. These powerful methods not only identified the bacterial isolates at the species level and were able to make a sharp distinction between closely related species to the same genus. (2, 3, 8, 9, 10, 12, 13). Generally, the molecular methods need specific experiences and specially equipped laboratory which may not available all the time. However, these molecular techniques not only accurately identify bacterial content of the drinking water but were able to show the least amount of differences among them even it is a single nucleotide polymorphism in a single copy of the DNA from any bacterium under examination.

References

1. Ahmad, F., Babalola, O. O., and Tak, H. I (2012). Potential of MALDI-TOF mass spectrometry as a rapid detection technique in plant pathology**:** identification of plant-associated microorganisms. Anal. Bioanal. Chem. **404(4)**:1247-55.

2. Anukam KC, Agbakoba NR (2017) A comparative study of the oralmicrobiome

compositions of healthy postmenopausal, premenopausal, and prepubertal Nigerian females, using 16s rrna metagenomics methods. Niger *J Clin Pract* **20(10**):1250

3. Blanton JM, Kirega AT, Yang L, Chen T, Leonetti C, Tanner A, WadeW, Paster BJ, Dewhirst FE, Izard J (2012) The human oralmicrobiome: a status report. In: AADR Meeting, Tampa
4. Bauer, A. W., W. M. M. Kirby, J. C. Sherris, and M. Turck. (1966). Antibiotic susceptibility testing by a standardized single disk method. Am. *J. Clin. Pathol*. **36**:493-496.
5. Chiang, Y.-C., Lai, C.-H., Lin, C.-W., Chang, C.-Y., and Tsen, H.-Y. (2014). Improvement of strain discrimination by combination of superantigen profiles, PFGE, and RAPD for Staphylococcus aureus isolates from clinical samples and food-poisoning cases. Foodborne pathogens and disease, **11**, 468-477.
6. Chauhan A, Goyal P, Varma A, Jinda T. (2015). Microbiological evaluation of drinking water sold by roadside vendors of Delhi, India. Appl Water Sci DOI 10.1007/s13201-015-0315-x
7. Clinical Laboratory Standards Institute. (2006). Performance Standards for Antimicrobial disk Susceptibility tests; Approved standard— 9th ed. CLSI document M2-A9. 26:1. Clinical Laboratory Standards Institute, Wayne, PA, USA
8. Costello EK, Lauber CL, Hamady M, Fierer N, Gordon JI, Knight R (2009). Bacterial community variation in human body habitats across space and time. Science **326**(5960):1694–1697
9. Cullinan MP, Westerman B, Hamlet SM, Palmer JE, Faddy MJ, Lang NP, Seymour GJ (2001). A longitudinal study of interleukin-1 gene polymorphisms and periodontal disease in a general adult population. *J Clin Periodontol* **28**:1137–1144
10. Deshmukh, R.A., Joshi, K., Bhand S., Roy, U. (2016). Recent developments in detection and enumeration of waterborne bacteria: a retrospective minireview. Microbiology Open, **5(6**):901-922.
11. Dewhirst, F. E., Chen. T., Izard, J., Paster, B. J., Tanner, A. C., Yu, W. H., Lakshmanan, A., and Wade, W. G. (2010). The human microbiome. *J. Bacteriol*. **192(19):**5002-5017.
12. Dzwairo B, Hoko Z, Love D, Ghuza E. (2006)Assessment of the impacts of pit latrines on ground water quality in rural areas: A case study from Marondera district, Zimbabwe. Phys Chem Earth, **31**, , 779-788.
13. Eren, A. M., Borisy, G. G., Huse, S. M., Welch, M. J. L. (2014). Oligotyping analysis of the human oral microbiome. Proc Natl Acad Sci USA **111(28):**E2875–E2884
14. Gao, L., T. Xu, G. Huang, S. Jiang, Y. Gu2, F. Chen. (2018). Oral microbiomes: more and more importance in oral cavity and whole body. Protein Cell 2018, **9(5):**488–500 https://doi.org/10.1007/s13238-018-0548-1).
15. Bewley AF, Farwell DG (2017) Oral leukoplakia and oral cavity squamous cell carcinoma. Clin Dermatol **35(5):**461–467
16. Girones, R., et al., (2010). Molecular detection of pathogens in water–the pros and cons of molecular techniques. Water research. **44(15):**4325-4339.
17. João P. S. Cabral, (2010). Water Microbiology. Bacterial Pathogens and Water. Int. J. Environ. Res. Public Health 2010, 7, 3657-3703; doi:10.3390/ijerph7103657
18. Malathy, B. R., S. K. Sajeev, S. Thampy, K. Guruvayurappan, S. P. Ajitha. 2017. Bacteriological Analysis of Drinking Water by MPN Method from Chennai, India. *J. Environ. Sci*, *Toxicol. F. Technol.* (IOSR-JESTFT) **11** (7 Ver. II):57-64.
19. Pant ND, Poudyal N, Bhattacharya SK. (2016) Bacteriological quality of bottled drinking water versus municipal tap water in Dharan municipality, Nepal. *J Health Popul Nutr* **7**, , 17.
20. Payment, P., M. Waite, and A. Dufour, (2003). Introducing parameters for the assessment of drinking water quality. Assessing microbial safety of drinking water, **4:** 47-77.

21. Ramalingam, B., Sekar, R., Boxall, J.B. and Biggs, C. (2013). Aggregation and biofilm formation of bacteria isolated from domestic drinking water. Water Science and Technology: Water Supply, **13 (4).** 1016 - 1023. ISSN 1606-9749).
22. Singh AK, Gupta VK, Sharma B, Singla B, Kaur P, Walia G (2015). What are we drinking? Assessment of water quality in an urban city of Punjab, India. *J Family Med Prim Care.* **4,** , 514–518.
23. Tanchou, V., (2014). Review of methods for the rapid identification of pathogens in water samples. Thematic group: chemical and biological risks to the water sector. Report EUR, 26881.
24. United Nation Children's Fund (UNICEF) (2014) World Water Day 2025: 4,000 children die each day from a lack of safe water, http://www.unicef.org/wash/index_25637.html
25. Ustün AP, Bartram J, Clasen T, Colford JM Jr, Cumming O, Curtis V, Bonjour S, Dangour AD, De France J, Fewtrell L, Freeman MC, Gordon B, Hunter PR, Johnston RB, Mathers C, Mäusezahl D, Medlicott K, Neira M, Stocks M, Wolf J, Cairncross S. (2014). Burden of disease from inadequate water, sanitation and hygiene in low-and middle-incomesettings: a retrospective analysis of data from 145 countries. Trop Med Int Health. **19**:894-905
26. Willcox, W. R., S. P. Lapage, B. Holmes. (1980). A review of numerical methods in bacterial identification. Antonie van Leeuwenhoek, **46(3)**: 233–299
27. World Health Organization (WHO) (2010) Water Sanitation and Health. http://www.who.int/water_sanitation_health/diseases/en/.
28. World Health Organization (WHO) (2014) Water Quality and Health. Drinking water chlorination – A review of disinfection practices and issues. http://www.waterandhealth.org/drinkingwater/wp.html.
29. Zeyinudin A , Kebede B , Deribew A , Ali S , and Zemen E. (2011) Bacteriological analysis of drinking water. African *Journal of Microbiology* Research **5,** , 2638-2641.