



Faculty of Resource Science and Technology

**Microbial Diversity and Antimicrobial Resistance of
Bacterial Isolates in Zamzam Water**

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**Bachelor of Science with Honours
(Resource Biotechnology)
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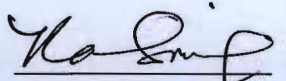


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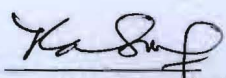
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Microbial Diversity and Antimicrobial Resistance of Bacterial Isolates in Zamzam Water

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A thesis submitted in partial fulfillment of requirement for degree of Bachelor of Science
with Honours (Resource Biotechnology)

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µm	
°C	
%	
P	
TBE	
TE	
TPC	
TYB	
V	
X	

LIST OF ABBREVIATIONS

APW	Alkaline Peptone Water
CFU	Colony Forming Unit
CN	Gentamicin
DA	Clindamycin
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
ddH ₂ O	Double distilled water
E	Erythromycin
FC	Fecal coliforms
LB	Luria Broth
MgCl ₂	Magnesium Chloride
MH	Mueller-Hinton
mL	milliliter
PCR	Polymerase Chain Reaction
Rpm	Revolution per minute
Spp.	Species (Plural)
U	Unit
UV	Ultraviolet
μL	Microliter
μm	Micrometer
°C	Degree Celsius
%	Percent
P	Penicillin G
TBE	Tris-borate-EDTA
TE	Tetracycline
TPC	Total plate count
TVB	Total viable bacteria
V	Voltage
X	Times

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ABSTRACT

Water resource can be contrived by human activities and spread waterborne disease thus raising concern of water quality standard among the consumers. In Mecca, Zamzam water is one of the natural drinking water accessible from a well. Objectives of this project are determination of microbial isolates in the Zamzam water and the association between biotic profile and the source of Zamzam water. A total of 8 samples of Zamzam water were collected (January 2014 until March 2015) including bottled mineral water and were analyzed using standard method following World Health Organization(WHO) and United State Environmental Protection Agency (US-EPA) and specific detection using PCR. Samples were tested for total viable count and coliform count. *Pseudomonas aeruginosa* from Zamzam water showed resistance towards multiple antibiotics (Penicillin G, Clindamycin, Tetracycline, Erythromycin, and Gentamycin) in disk diffusion method. Zamzam water shows high bacterial contamination with existence of *E. coli*, *Proteus mirabilis*, *Proteus vulgaris*, and *Pseudomonas aeruginosa* up to 4.5×10^2 CFU/ml. In conclusion, there was risk in waterborne disease from Zamzam water and association between source of Zamzam water and antimicrobial profile.

Keywords: Zamzam water, pathogenic, PCR, antimicrobial profile

ABSTRAK

Sumber air boleh dicemari oleh aktiviti manusia dan menjadi punca penyakit seterusnya menimbulkan kerisauan mengenai penyelarasan kualiti air dalam kalangan pengguna. Di Mekah, air Zamzam merupakan salah satu air minuman semulajadi daripada telaga. Tujuan kajian ini adalah untuk mengenalpasti hubungan bakteria dalam air Zamzam daripada sumber berbeza dengan sensitiviti antibiotik di mana 8 sampel air Zamzam telah diambil (Januari 2014 sehingga Mac 2015), diperbanding dengan air minuman biasa dan dikaji melalui teknik analisis air minuman selaras piawaian World Health Organization (WHO dan United State Environmental Protection Agency (US-EPA) serta pengesanan menggunakan PCR. Pengiraan bakteria berdaya (TVB) dan pengiraan koliform meliputi kajian ini. Kaedah resapan disk membuktikan *Pseudomonas aeruginosa* dalam air Zamzam merintangi antibiotik (Penicillin G, Clindamycin, Tetracycline, Erythromycin, dan Gentamycin). Majoriti bakteria yang dikenalpasti ialah *E. coli*, *Proteus mirabilis*, *Proteus vulgaris*, dan *Pseudomonas aeruginosa* kadar kiraan mencapai 4.5×10^2 CFU/ml. Kesimpulannya, air Zamzam mempunyai risiko penyebaran penyakit dan wujud kaitan antara sumber Air Zamzam dengan profil antimikrobial.

Kata kunci: Air Zamzam, patogen, PCR, profil antimikrobial

1.0 INTRODUCTION

The quality of water is of vital importance to public health especially when being consumed as source of drinking water. Located within the holiest place for Muslim, mosque in Mecca known as Masjid al-Haram, the Zamzam well produces continuous underground water to be consumed by millions of life. Muslim pilgrims drink it and bring back the bottle filled with Zamzam water to their home. Many Muslims believed that this water has magical effect in healing the disease. So the Zamzam water is used either medicinally or religiously. BBC reported in May 2011 that the Zamzam water is poisonous because of its high level in arsenic content (Shomar, 2012).

The Ministry of Agriculture and Water Resource (1971) from Saudi Arabia sent samples of Aabe Zamzam to be investigated by the European laboratories to test the portability of the water. From water sample tested by them, the Zamzam water has special physical that makes it advantageous drinking water and the differences between the main city water is it has slightly higher content of calcium and magnesium salt but more significantly, the water contain fluorides that have effective germicidal action. Besides it doesn't go mouldy nor does it change color, taste or smell (Koshak, 1983). Mashat (2010) confirmed that there isn't any sign of biological growth in the Zamzam water well. The Zamzam water has never been chemically treated except by sand filtration and microfiltration. Consequently, it may have diversity microbial that are potentially pathogenic.

Bacteriological studies in the Zamzam water are very minimal thus this research will involve bacteriological water analysis for Zamzam water to estimate the bacterial present and possible faecal contamination. Bacteria in water are not present individually but as clumps or in association with particulate matter. Pathogen bacteria are the causes of many waterborne diseases and its ability to cause disease is called pathogenicity. Bacterial indicator organism such as total coliforms, faecal coliforms and *E. coli* are used to detect

the existence of pathogenic organisms. Cowan (2012) stated that if there are presence of microorganism in a sample means that pathogen might also be there.

One of the world major problems for human is the microbial resistance to antibiotic and it is generally accepted that the major factor for the increase on the antibiotic resistance is an extensive use of antibiotics (Lukasova & Sustackova, 2003; Mukherjee et al., 2005). In order to find the association between isolates found in the Zamzam water and its antimicrobial resistance, Kirby-Bauer antibiotic testing or known as agar disk diffusion antibiotic sensitivity testing will be applied (Raphael et al., 1983; Atlas, 1997).

Bacterium isolates from sample were grown on agar plates in the presence of disk containing relevant antibiotics. Zone of inhibition (clear zone) will be measured including the diameter of the disc. By referring to the interpretation table, the sizes of zone inhibition were classified into susceptible (S), intermediate (I), and resistant (R) categories (Hudzicki, 2010). Zamzam water is chosen because this water might present a source of potential risk to health of consumer. Thus this study aids in contribution to the health department in all over the country as Zamzam water is consumes internationally and consumer for its safe water quality level and data collection can be used as baseline data in the future research.

The specific objectives of this project are to:

1. Determine the type of the bacterial species present in the Zamzam water.
2. Examine the antimicrobial resistance profile of indicator bacterial from the Zamzam water.
3. Determine the association between antimicrobial profile and the source of Zamzam water.

2.0 LITERATURE REVIEW

2.1 The Zamzam Water

The sample that were used in this study is Zamzam water. Samples were collected via vendors and acquaintances. The well is about 30.5 meters deep and ranging from 1.08 to 2.66 meter in diameter as displayed in Figure 2.1. The chemical analysis of Zamzam water contains some inorganic elements such as sodium (Na), Calcium (Ca), Magnesium (Mg), Potassium (K), bicarbonate (HCO_3), Chloride (Cl), Fluoride (F), Nitrate (NO_3^-), Sulfate (SO_4) and totally dissolved salts (Al-Zuhair, 2005). The water is alkaline (average pH is 8) with an average concentration of $15 \mu\text{g L}^{-1}$ (Shormar, 2012). Alkaline in nature, the Zamzam water can neutralize excess hydrochloric acid. Iodide, sulphate and nitrate contents are also much higher in the Zamzam water. The average concentrations of As and NO_3 showed values three times higher than the WHO standards ($27 \mu\text{g L}^{-1}$ and 150 mg L^{-1} , respectively). The averages of Ca and K were 95 and 50 mg L^{-1} , respectively.

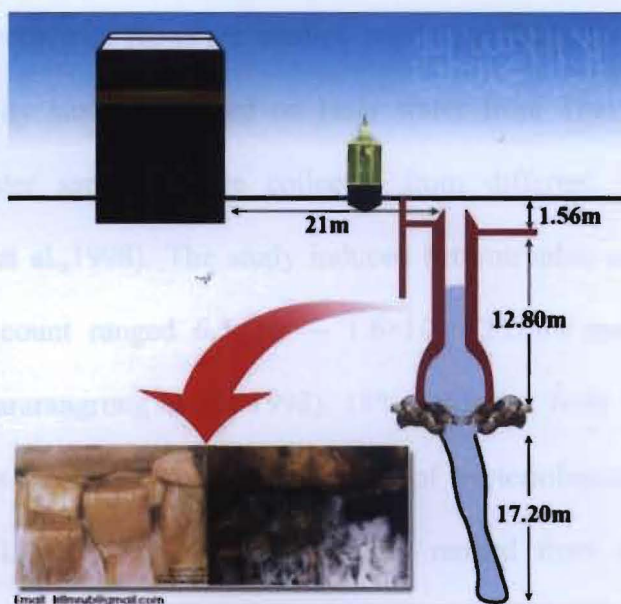


Figure 2.1: Illustration of Zamzam well based on its actual measurement (Jafri, 2012)

2.2 Drinking Water Quality Standard

Guidelines and legislation such as European Union Council Directive and World Health Organization (WHO) state that drinking water should contain pathogenic microorganism only in such low number that the risk for acquiring waterborne infections is below accepted limit (Szewzyk, 2000). WHO Guidelines for Drinking Water Quality 1984 has sets 5000 CFU/100 ml as the acceptable value of total coliform parameter and 300 TCU for color parameter in raw water. Malaysia has its own drinking quality standard which accepts none of total coliform and faecal *streptococci* detected in membrane filtration of 100 ml sample. Absent of *E. coli* in 100 ml indicates that the water is safe for drinking. The free residual chlorine should be within 0.2 and 5.0 mg/l. most of the parameters in Malaysia referred to WHO guidelines (MOH, 2000).

2.3 Microbiological Analysis in Drinking Water

Several studies to detect of *E. coli* in holy water in temples and churches in Asia and Europe have been reported. However studies regarding Zamzam water are quite rare. Bacteriological quality has been tested on Holy water from Thai temples in Songkhla where 76 holy water samples were collected from different 76 Buddhist temples (Phattharangrong et al., 1998). The study induced heterotrophic aerobic bacterial count and total coliform count ranged $6.5 \times 10^1 - 1.6 \times 10^5$ CFU/ml and 1600 MPN/100 ml respectively (Phattharangrong et al., 1998). 18% coliforms from 9 samples and 8% *E. coli* from 4 samples were examined in the study of bacteriological quality from water vendors in Tripoli-Libya. The coliforms counted ranged from 0.0 to 1.6×10^3 MPN (Nashnouch et al., 2009). In Izmir City, Turkey, a study of drinking water from wells has been conducted to determine the microbiological quality. 100 samples were collected and of them, 85% met the specifications set by Turkish Food Regulation for coliforms count.

Wells with motor pumps contained 33% coliforms, 47% aerobic count and 7% of *E. coli* (Sahika & Karapinar, 1991).

2.4 Pathogenic Indicator Microorganism

Pathogenic indicator microorganisms are very useful in detecting the existence of disease causing organism in water analysis. Houston (1900) reported that faecal streptococci were present in polluted water and absent in unpolluted water. Coliform bacteria most commonly used bacterial indicator of sanitary quality of foods and water. Coliform defined as rod shaped Gram-negative non-spore forming bacteria that can ferment lactose with the production of acid and gas incubated between 35°C to 37°C (APHA, 1995). Coliform are not normally causing serious illness and they are easy to culture and their presence is used to indicate that other pathogenic organism of fecal origin may be present. The typical genera of coliform bacteria include *Citrobacter*, *Enterobacter*, *Hafnia*, *Klebsiella*, fecal coliform and *Escherichia*. Total coliforms and *E. coli* counts are used worldwide as indicators for faecal contamination of drinking and recreational bathing water (Rompre et al., 2002).

2.4.1 *Citrobacter*

Citrobacter is Gram negative genus bacteria that can be found in soil, water wastewater and everywhere. One of the species, *C. freundii* strains is resistant to ampicillin and first generation of cephalosporin. This is due to its inducible ampC genes. Isolates from *Citrobacter* encodes resistance to other antibiotic as well as a result of plasmid-encoded resistance genes. *Citrobacter* is the most commonly isolated total coliform (Karla et al., 1980).

2.4.2 *Enterobacter*

Enterobacteriaceae is an anaerobic genus of common Gram negative. Several strains of these bacteria are pathogenic and may cause opportunistic infection. It does not belong to the fecal coliforms (or thermo tolerant coliforms) group of bacteria, unlike *Escherichia coli*, because it is incapable of growth at 44.5°C in the presence of bile salts. It is oxidase-negative, indole-negative, and urease variable (Cabral, 2010). In the previous survey of bacteriological quality in drinking water conducted in rural area of northern Rajasthan, India, *Enterobacter* species were identified together with another 9 bacterial species. The occurrences of these bacteria in drinking water are of primary importance because these constitute a major part of coliform organisms in open water as they generally live together in water than other intestinal pathogen which can be easily detected (Suthar et al., 2009). According to Klein and Casida (1967), if such bacteria are not detectable in 100 ml, the water can be said as potable water. Continuous consumption of such contaminated water may cause a serious health risk in local residents of that area especially in children.

2.4.3 *Hafnia*

Hafnia is the genus of *Enterobacteriaceae* family whose only species is the Gram negative, facultatively anaerobic, rod shaped bacterium *Hafnia alvei*. *H. alvei* is a commensal of the human gastrointestinal tract and not normally pathogenic, but may cause disease in immunocompromised patients. It is often resistant to multiple antibiotics, including the aminopenicillins. The Latin name is Copenhagen.

2.4.4 *Klebsiella*

It is named after the German microbiologist Edwin Klebs (1834–1913). They have no specific growth requirements and grow well on standard laboratory media, but grow best between 35 and 37°C and at pH 7.2. The *Klebsiella* organisms can lead to a wide range of

disease states, notably pneumonia, urinary tract infections, septicemia, meningitis, diarrhea, and soft tissue infections (Sieper et al., 2011). They can be found in water, soil, plants, insects, animals and humans (Bagley, 1985).

2.4.5 Fecal coliforms

Coliform bacteria generally originate in the intestines of warm-blooded animals. The term "thermo tolerant coliform" is more correct and is gaining acceptance over "faecal coliform" (WHO, 1996). The presence of fecal coliform in aquatic environments may indicate that the water has been contaminated with the fecal material of humans or other animals. Large quantities of fecal coliform bacteria in water are not harmful according to some authorities, but may indicate a higher risk of pathogens being present in the water (Fresno County Department of Public Health, 2009).

2.4.6 *Escherichia coli*

The genus *Escherichia* is commonly found in the lower intestine of warm-blooded organisms (endotherms) (Singleton, 1999). However, *E. coli* can be distinguished from the coliform group because most strain of *E. coli* is harmless but some can cause serious illness in humans. Infection symptoms and signs include bloody diarrhea, stomach cramps, vomiting and sometimes fever. Pneumonia may result from the *E. coli* and other respiratory illnesses and urinary tract infection (Todar, 2007).

2.5 Antimicrobial Resistance

To begin, the definition of "antibiotic," as first proposed by Selman Waksman, the discoverer of streptomycin and a pioneer in screening of soils for the presence of biological has been seriously over interpreted; it is simply a description of a use, a laboratory effect, or an activity of a chemical compound. At the risk of attack from purist

colleagues, the generic term “antibiotic” is used here to denote any class of organic molecule that inhibits or kills microbes by specific interactions with bacterial targets, without any consideration of the source of the particular compound or class.

Several studies showed that antibiotic resistance characteristic can be transferred to sensitive recipient organism in the environment and DNA coding for antibiotic resistance may be conjugally transferred between microorganisms (Ramteke et al., 1990; Harnett et al., 1998; Merz et al., 2004). The infection, pathogenicity and antibiotic resistance cycle have been studied extensively. According to Denise et al. (1995), the presence on antibiotic resistant bacteria in rural groundwater supplies used as drinking water source may have important public health implication. These bacteria are able to spread their genes into water indigenous microbes, which also contain resistance genes (Fernando et al., 2008). Antibiotic resistant bacteria in drinking water was analyzed by from seven communities for multiply antibiotic resistant (MAR) bacteria and the MAR bacterial isolates was screened against five antibiotic by replica plating. Overall, 33.9% of 2,653 standard plate count bacteria from treated drinking waters were MAR (Armstrong et al., 1981). Armstrong also stated that the Isolate identification revealed that MAR gram-positive cocci (*Staphylococcus*) and MAR gram-negative, nonfermentative rods (*Pseudomonas*, *Alcaligenes*, *Moraxella*-like group M, and *Acinetobacter*) were more common in drinking waters than in untreated source waters.

3.0 MATERIALS AND METHOD

3.1 Materials

(Materials are shown in Appendix 1)

3.2 Methodology

3.2.1 Sample Collection

A total of 9 samples were collected within 15 months which includes Umrah and Hajj seasons. Hajj season begins on early October every year whereas Umrah can be practiced in any months. Some samples were collected from family member, friends and acquaintances that went to Mecca, Saudi Arabia to perform prayers. Some other were collected from the vendor for examples, sample V was ordered directly from Saudi Arabia National Water Company, sample A was bought from vendor in Bangladesh, and sample K and M was collected from vendor located in Kuching and Kota Samarahan respectively. One bottle of drinking water from F&N Company was bought as control variable to be compared with data of isolates from the other samples of Zamzam water. The date of sampling was tabulated in the Table 3.1.

Table 3.1: Sample collections data

Date of collection	Season (Source)	Details
January 2014	Hajj (Direct source)	Zamzam water. Labeled as S.
	Hajj(Direct source)	Zamzam water. Labeled as F.
April 2014	Umrah (Vendor)	Zamzam water. Labeled as A.
September 2014	Umrah (Vendor)	Zamzam water. Labeled as V.
December 2014	Hajj (Direct source)	Zamzam water. Labeled as H.
January 2015	Umrah (Vendor)	Zamzam water. Labeled as K.
February 2015	Umrah (Direct Source)	Zamzam water. Labeled as I.

March 2015	Umrah (Vendor)	Zamzam water. Labeled as M.
	- (Vendor)	Drinking water. Labeled as WD. Act as control.

All the samples were transferred into sterilized 50mL centrifuge tube as shown in the Figure 3.1 with two replicates each as shown in the Figure 3.1 with corresponding labeled and stored in ice at 1⁰C-4⁰C. The first four samples collected were samples F, A, V, and S while sample H, K, M, I and WD were collected during the seconds sampling. All of the samples are brought to the lab for immediate process.



Figure 3.1: Zamzam water samples in 50mL centrifuge tube

3.2.2 Sample Processing

Physical test were performed to test its physical appearances and indicate properties detectable by the senses such as their color, odor, temperature, pH and turbidity (Sawant & Deshmukh, 2012). pH value and the temperature of each samples were evaluated by using pH and temperature meter (pH Scan30, Bante, Rome) as in Figure 3.2. The pH meter were rinsed with distilled H₂O before and after used in the samples to prevent cross

contamination. Water temperature, transparency and turbidity, odor of water, color of water, and pH data is to ensure the sample can be used for an accurate assessment of the condition of the source. The analysis results were recorded in the table.



Figure 3.2: Evaluation of pH value and temperature using pH meter

3.2.3 Enrichment Process

Enrichment broth were prepared and sterilized by autoclaving at 121°C for 15 minutes before mixed with the samples. 10mL from each sample were introduced into 90mL of nutrient broth (MERCK, New Jersey) in the conical flask for enrichment stage. The mixture was incubated in the 37°C incubator for 6 hours.

3.2.4 Serial Dilution

Alkaline Peptone Water (OXOID, England) was used as diluent for the enriched samples. 27 universal bottles filled with 9mL of APW each were autoclaved at 121°C for 15 minutes to ensure sterilization. After 6 hours of incubation, 1mL from each samples were added into the first sterilized APW respectively. Enriched samples were diluted until dilution 10^{-3} and Figure 3.3 shows the dilution process.

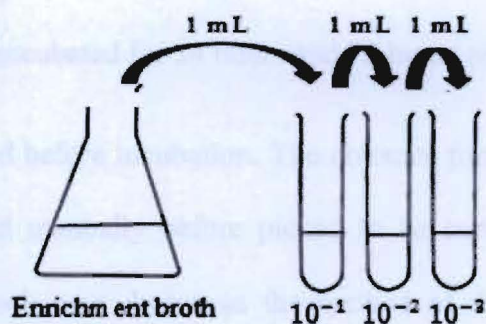


Figure 3.3: Enriched samples added into universal bottle filled with 9mL APW

3.2.5 Spread Plating and Bacterial Count

The next stage is spreading the dilution 10^{-1} , 10^{-2} , and 10^{-3} onto the Total Plate Count Agar (PCA) (OXOID, England). The composition of plate count agar are 0.5% peptone, 0.25% yeast extract, 0.1% glucose, 1.5% agar, and the pH was adjusted to neutral 25°C (Atlas, 2004). 100 μL of each dilution were spread plated onto PCA by using a glass stick spreader sterilized by 70% ethanol and flamed with duplicate set. Then, they were incubated in the 37°C incubator for 36 hours. The colonies formed were counted and documented in the table.

Since PCA allowed few numbers of bacteria grow on it, a few colonies was picked and enriched with LB broth (OXOID, England) before inoculated onto slant nutrient agar (PRONADISA, Madrid) for the next stage, which is biochemical test. The next step in screening the total viable bacteria count isolates for bacteria which commonly inhabit portable water consisted of testing their ability to grow on Reasoner's 2A agar (R2A) (OXOID, England). Another 100 μL of each serial dilution were spread onto the R2A agar. Later, they were incubated in the 37°C incubator for 72 hours. Other than that, 100 μL of serial dilution 10^{-1} until 10^{-3} were also plated on selective agar Eosine Methylene Blue agar