



Faculty of Resource Science and Technology

Occurrence of *Pseudomonas aeruginosa* in Drinking Water from Water Vending Machine (WVM)

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Occurrence of *Pseudomonas aeruginosa* in Drinking Water from Water
Vending Machine (WVM)

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



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ABSTRACT

The rise in popularity of water vending machine (WVM) due to urban modernization has given a rise to concerns regarding the quality and safety of the drinking water. Several data have shown the potential risk WVM could harbor. However, there is a lack of such data on its risk in Sarawak. Some studies have found that there is a potential risk of WVM being contaminated with the opportunistic pathogen *Pseudomonas aeruginosa*. In this research, the level of *P. aeruginosa* prevalence in WVM is studied. Over the course of 2 months from January 2021 to February 2021, sampling was done once a month from WVM located in Kota Samarahan and Kuching. A total of one hundred drinking water samples (n=100) were taken from Kota Samarahan (n=50) and Kuching (n=50). The physical condition, surrounding location, date of maintenance and whether the WVM had a license were observed. The drinking water were analyzed for pH, total dissolved solids (TDS) and temperature. Enumeration and isolation of *P. aeruginosa* was done using single dilution method for most probable number (MPN). Identification was done with polymerase chain reaction (PCR) targeting the *ETA* gene, Antibiotic susceptibility test (AST) done according to the M45 guideline. Biofilm forming capacity of *P. aeruginosa* was studied in vitro. The results concluded that 26% of WVM from Kota Samarahan and 36% from Kuching were positive for the presence of *ETA* positive *P. aeruginosa* and it was observed that many of the WVM were not in acceptable condition including the absence of dispensing area cover, low hygiene condition, unsanitary or unsuitable locations and a lack or absence of maintenance done to the WVM. The AST conducted showed that *P. aeruginosa* was most susceptible to Meropenem (64%) and it was most resistant to ciprofloxacin (28%). The biofilm forming capacities were mostly observed to be moderate

strength with 5 moderate strength and only 1 non-adhering based on *P. aeruginosa* biofilm-forming capacity in vitro. It is recommended that a stricter monitoring of WVM is needed to ensure the safety of the public.

Keywords: *Pseudomonas aeruginosa*, water vending machine (WVM), most probable number (MPN), ETA gene, biofilm

Kejadian Pseudomonas aeruginosa dalam Air Minuman Daripada Mesin Jual Air (MJA)

ABSTRAK

Pemodenan bandar membawa kepada kenaikan populariti water vending machine (WVM). Ini meningkatkan soalan tentang kualiti dan keselamatan air minuman MJA. Terdapat beberapa data mengenai potensi risiko daripada MJA. Bagaimanapun, terdapat kekurangan data mengenai hal ini di Sarawak. Terdapat beberapa penyelidikan yang menunjukkan MJA ada potensi untuk dicemari oleh bakteria Pseudomonas aeruginosa. Penyelidikan ini dijalankan untuk mengetahui tahap kelaziman P. aeruginosa dalam MJA. Sampel diambil dalam tempoh 2 bulan, dari Januari 2021 hingga Februari 2021 sekali sebulan. Sebanyak 50 sample minuman air (n=25) daripada Kota Samarahan (n=25) dan Kuching (n=25). Keadaan fizikal, keadaan kawasan sekeliling, tarikh servis dan kehadiran lesen MJA telah diperhatikan. Analisis pH, jumlah pepejal terlarut, dan suhu air minuman telah dilakukan. Enumerasi dan isolasi P. aeruginosa telah dilakukan menguna teknik dilusi tunggal bilangan paling berkemungkinan. P. aeruginosa telah diidentifikasi menggunakan teknik tindakbalas rantai polimerase menyasar gen ETA. Ujian ketahanan terhadap antibiotik dijalankan menggunakan garis panduan M45. Kebolehan P. aeruginosa untuk membentuk biofilem dikaji secara vitro. Hasil daripada penyelidikan ini menunjukkan 26% MJA daripada Kota Samarahan dan 36% daripada Kuching adalah positif kepada kehadiran ETA positif P. aeruginosa dan banyak MJA dijumpai dalam keadaan yang tidak menyenangkan seperti ketiadaan penutup bahagian pendispensan air, tahap kebersihan rendah, kawasan sekitar yg tidak bersih ataupun tidak sesuai dan kekurangan atau ketiadaan penyelenggaraan dilakukan terhadap MJA. Ketahanan terhadap antibiotik menunjukan tahap kelemahan paling tertinggi kepada Meropenem (64%) dan tahap

ketahanan paling tinggi kepada ciprofloxacin (28%). Kapasiti P. aeruginosa untuk membentuk biofilem adalah sederhana dengan 5 menunjukkan kadar ketahanan sederhana dan hanya 1 menunjukkan ketiadaan ketahanan berdasarkan kapasiti P. aeruginosa untuk membentuk biofilem dalam vitro. Adalah disyorkan utuk meningkatkan pemantauan yang lebih tegas bagi memastikan keselamatan orang awam.

Kata kunci: Pseudomonas aeruginosa, water vending machine (WVM), bilangan paling berkemungkinan, gen ETA, biofilem

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LIST OF ABBREVIATIONS

AGE	Agarose gel electrophoresis
AST	Antibiotic susceptibility test
ATTC	American type culture collection
CF	Cystic fibrosis
CFU	Colony forming unit
ddH ₂ O	Deionized distilled water
DNA	Deoxyribonucleic acid
EOW	Electrochemically oxidized water
ERW	Electrochemically reduced water
ETA	Exotoxin A
GAC	Granular activated carbon
HAP	Hospital-acquired pneumonia
HCAP	Healthcare-associated pneumonia
HPC	Heterotrophic plate count
LB	Luria-Bertani
LPS	Lipopolysaccharide
MHA	Mueller Hinton agar
MIC	Minimum Inhibitory Concentration
MPN	Most probable number

MOH	Malaysian Ministry of Health
OD _c	Cutoff value
OMV	Outer membrane vesicle
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
ppm	Part per million
QMRA	Quantitative microbial risk assessment
RO	Reverse osmosis
ROC	Reverse osmosis concentrate
SFA	Singapore Food Agency
TDS	Total dissolved solids
T3SS	Type III secretion system
T6SS	Type VI secretion system
TOC	Total organic carbon
TSB	Tryptic soy broth
TSS	Total suspended solids
UV	Ultraviolet
VAP	Ventilator-associated pneumonia
WHO	World Health Organization
WVM	Water Vending Machine

CHAPTER 1

INTRODUCTION

1.1 Study Background

In this current age of modernisation and public infrastructure a need for accessibility to the basic human necessities such as water is a must. This has caused the increase in popularity of machines such as water vending machine (WVM). Water vending machine (WVM) is an automated device which dispenses out clean filtered drinking water when the appropriate amount of money is inserted. Such machines provided a convenient access to potable drinking water at a cheap price for as low as 10 cents per litre. They were usually located in convenient places such as grocery stores, markets and housing areas. These machines provided an alternative to boiling tap water which takes up time and have an unfavourable taste due to the presence of toxins from leaking pipes and corrosion (Fauzi et al., 2020). According to Doria (2006), consumers often opt for alternative sources to drinking water other than tap due to 2 main reason, taste and health concerns as well as other factors. Hence, with factors which included convenience, safety and price, consumers would usually opt for water from WVM. However, filtered water from WVM could also became unsafe to consume due to risk factor such as improper maintenance, usage and low hygiene. This would introduce unwanted pathogens and bacteria into the filtration system, compromising the cleanliness and the safety level of the water dispensed.

There had been a few cases reported on the contamination of WVM in Malaysia. On 19 August 2014, The Star Online reported that almost all the drinking water sample taken from

WVM around the Klang Valley area were contaminated with harmful bacteria and that many of the samples did not have any traces of free chlorine or chloride which is needed for disinfection. (Aruna & Cameons, 2014). Another case on 2 September 2015 where 630 WVM and 786 water sample were tested in which 22 machines were confiscated where 4 had low hygiene levels and 10 were positive for presence of bacteria (Bernama, 2015). This was the result of poor maintenance and hygiene of the WVM. One particular case involved the detection of the bacterium *Pseudomonas aeruginosa* in a Malaysian brand of bottled drinking water done by the Singapore Food Agency (SFA) on 29 June 2019 (Bernama, 2019).

Pseudomonas aeruginosa is a Gram-negative aerobic bacterium with a rod shape. It is a non-spore forming bacterium and an oxidase-positive and a lactose non-fermenter. It is also ubiquitous meaning it could be found thriving anywhere in the natural environment such as soil, water, vegetation and even the human body. Part of what make *P. aeruginosa* significant in the scientific research community was the fact that it is an opportunistic pathogen that had caused a wide variety of infections especially amongst the immunocompromised individual such as cystic fibrosis (CF) patients and patients with severe wounds or burns. It took very low maintenance for *P. aeruginosa* to had flourished successfully in its environment. It survived in hospital setting and colonised the medical devices and also the water system. This gave it a higher chance for the bacteria to come into contact with immunocompromised individuals. Its multi-drug resistance was what made it hard to be treated in patients and to be eradicated from the water system. An array of virulence factor such as toxins and surface molecules also facilitated in its persistence and adaptability. There was also the fact that this bacterium could form biofilms providing an added layer of protection against antibiotics and the immune system.

Biofilms would form inside the water system and would be difficult to remove once established because chemical treatment or physical removal is needed.

1.2 Problem Statement

As the availability and usage of WVM increased due to factors such as convenience and low prices, there was an increased concern of the cleanliness and the quality of the drinking water. This was due to the fact that several cases had reported that these machines were prone to bacterial contamination due to the lack of regular and proper maintenance and improper usage by the consumers. Because of the poor maintenance provided to these machines there was a concern for potential contamination of the opportunistic pathogen *P. aeruginosa*.

Under normal circumstances, *P. aeruginosa* would not cause severe harm or infections in healthy individuals. Most cases of *P. aeruginosa* infections were reported in hospital settings involving immunocompromised individuals but there had been cases linked to contaminated drinking water. Although rare and these cases were usually linked to already vulnerable individuals due to pre-existing medical conditions. It is important that precautionary measures are taken.

Despite the risks that *P. aeruginosa* possesses due to its opportunistic nature, there is a lack of data on its occurrence in drinking water specifically from WVM. There were some researches pertaining to this issue conducted within Malaysia. However, there was a lack of it being done in Sarawak. Extensive research on the level of contamination of *P. aeruginosa* in drinking water from WVM was needed. These knowledges were needed to build effective strategies to prevent the presence of *P. aeruginosa* and ensured the safety of drinking water available to the public. Based on previous studies done on this topic, it was hypothesised that

WVM that adhere to the rules and regulations and strict hygiene practices, will exhibit lower level of *P. aeruginosa* contamination compared to those that does not adhere to the standard regulations and proper hygiene practices.

1.3 Objectives

This research aimed to fill in the knowledge gap about the extent of the contamination of *P. aeruginosa*. This was achieved by using the following objectives:

- i. Evaluation of the drinking water samples from water vending machine (WVM) around Kota Samarahan and Kuching using physical and chemical parameters
- ii. Profiling of *P. aeruginosa* from water vending machine (WVM)
- iii. Investigation of the resistance profile of *P. aeruginosa* through antibiotic susceptibility test from water vending machine (WVM)
- iv. Analysation of the biofilm formation of *P. aeruginosa* from water vending machine (WVM)

CHAPTER 2

LITERATURE REVIEW

2.1 Water Vending Machine (WVM)

Water vending machine (WVM) is defined as a self-service device that automatically dispenses a set amount of drinking water when appropriate amount of money is inserted and machine vended water means any water that does not come in a sealed container or bottle dispensed by a WVM (Malaysia & International Law Book Services, 2019). These WVM are equipped with a filtration system with different water treatment option such as reverse osmosis, ionization or distillation. The water vended from WVM must go through filtration, treatment and disinfection. Lifestyle changes and modern infrastructure has resulted in the rise in popularity of WVM which provides an accessible and convenient way to obtain clean drinking water. It is usually located at convenient locations such as convenience store, markets, shop lots and a near housing areas. Compared to buying bottled water, WVM are very cost efficient with a set price as low as 10 cents per litre. It is also considered environmentally safe, reducing the use of single-use plastics. Consuming water dispensed by WVM is also regarded as safe as oppose to tap water. Tap water is often associated with a displeasing nickel-like taste.

According to Bennet (2012), tap water can contribute to rash, skin burns, tooth enamel erosion and often contains toxic substances such as lead, barium, arsenic and many other harmful substances in reference to the New York Times investigation. In tap water, there are numerous microorganisms and toxins that have a potential to contribute to allergy and disease symptoms. As a result, the consumers choose to get other alternatives for better quality of

drinking water such as bottled water that is commercially sold in the shop, filtered water, water dispenser or the most common choice is WVM. However, the rise in popularity of WVM also arises some concerns. As according to Praveena et al. (2018), drinking water from WVM are not guarantee free from pathogen and harmful bacteria, as there are risks and factors that can contribute to contamination which could affect the standard and safety of the drinking water depending on the maintenance of the machine and the filtration system. Improper maintenance and handling by consumers can lead to cross contamination of the water dispensed.

2.2 Filtration System of Water Vending Machine (WVM)

Water vending machine (WVM) are equipped with a multi-staged filtration and purification system to ensure a clean and safe quality of drinking water. Depending on the make and model of each machine, the type of filtration system can vary. Common type of filtration method used in WVM is sediment filtration, activated carbon filtration, reverse osmosis (RO) filtration, alkali water filtration, and ultraviolet (UV) disinfection. Each filtration method has its advantages and disadvantages, so most WVM have a combination of filtration method to reach a certain quality level of drinking water dispensed. Figures 2.1 shows the general purification process of WVM.

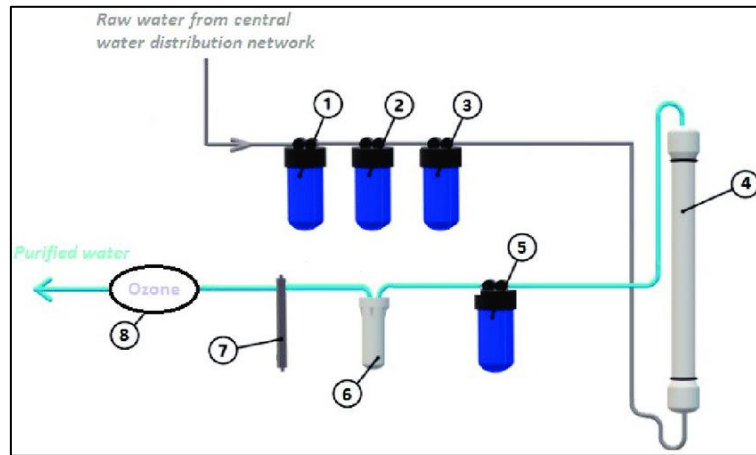


Figure 2.1: General filtration system found in WVM. 1: 5- μ g polypropylene sediment filtration cartridge; 2: granular activated carbon cartridge; 3: 1- μ g polypropylene sediment filtration cartridge; 4: reverse osmosis membrane stage; 5: granular activated carbon cartridge; 6: calcite-mineralization cartridge; 7: ultraviolet lamp; 8: ozonation (Dèdelè et al., 2022)

2.2.1 Sediment Filtration

Sediment filtration or also known as mechanical filtration is the first initial stage for water filtration is usually sediment filtration to remove large particles present in the water. This method involves physically filtering water by passing it through a filter medium to remove large particles such as sand, silt, clay, loose scale and organic matter (Saha et al., 2023). An example of the type of filters used for this method is mesh filter and ceramic filter. Based on the effectiveness of each filter in term of the particle size it is capable of removing, they are given a micron rating which indicates the size of particles in micrometre the filter is able to remove (Dvorak & Skipton, 2008). The micron rating is usually described as nominal or absolute. Absolute filters can filter approximately 99% of particles larger than its pore size while, nominal filter is can only filter out 90% of particles larger than its pore size (Wu et al., 2021).

2.2.2 Activated Carbon Filtration

Activated carbon filtration is commonly used in water filtration process. It can be used to filter out chlorine, some chemicals, organic compounds and certain heavy metals (Dvorak & Skipton, 2013). Activated carbons are carbons that have been processed to increase its surface area and porosity (Togibasa et al., 2023). Activation can be done through 2 different methods, which are steam activation and chemical activation. During steam activation method, the carbon is first heated in the absence of oxygen, steam is then introduced to activate the carbon (Sharma & Bhattacharya, 2016). Chemical activation involves heating the carbon source at low temperature with the presence of an activating agent (Gaňan et al., 2004). The steam activation results in an activated carbon with fine pore structure ideal for adsorbing liquid phase and vapour phase compound (Li et al., 2021). On the other hand, chemical activation results in a greater pore structure making it more suitable for absorbing larger molecules (Nandi et al., 2023). Different material can be used as the carbon source such as bituminous or anthracite coal, bone char, coconut shell and peat. There are 2 types of activated carbon filters, granular activated carbon (GAC) filter and block carbon filter. Granular activated carbon comprises of loose carbon granules, while block carbon filter have been compressed. The GAC filter is used as a polishing filter to remove low concentration compounds usually present in water such as algal toxin, pesticides, industrial micropollutants as well as improving the taste and odour by removing chlorine and hydrogen sulphide (Gabr et al., 2009).

2.2.3 Reverse Osmosis (RO)

Reverse osmosis (RO) is the process of separating solutes in a solution from the solvent by forcing the solvent through a semi permeable membrane to a lower solute concentration by

applying external pressure (Ahuchaogu et al., 2018). The pressure required must be more than the natural osmosis pressure to ensure the water travel from the concentrated solution to the diluted solution. The semi-permeable membrane only allows water to pass through, filtering out the dissolved salts, mineral and other contaminants. Table 2.1 shows the type of contaminants RO is able to remove. The water filtered using RO is free from biological contaminants and have low total suspended solids (TSS) and total dissolved solids (TDS) (Lamma et al., 2015). Reverse osmosis is a very efficient method in removing contaminant and producing water with high purity (Kumar & Dixit, 2021). However, there are also a few disadvantages to this method. Reverse osmosis process generates a lot of waste, called reverse osmosis concentrate (ROC) which carries all the contaminants filtered out and it requires proper disposal in order to avoid severe environmental impact (Vaishnav et al., 2023). Other than removing harmful minerals such as lead, RO also removes the beneficial mineral such as iron.

Table 2.1: Contaminants removed by RO (Dvorak & Skipton, 2014).

Ions and metals	Arsenic, Antimony, Aluminium, Barium, Beryllium, Cadmium, Calcium, Chloride, Chromium, Copper, Fluoride, Iron, Lead, Magnesium, Manganese, Mercury, Nitrate, Potassium, Radium, Selenium, Silver, Sodium, Sulphate, Thallium, Zinc
Particles	Asbestos, Protozoa cyst, Cryptosporidium
Pesticides	Endrin, Heptachlor, Lindane, Pentachlorophenol
Radionuclides	Radium, Uranium

2.2.4 Alkaline Water Filtration

Alkaline water filtration or also known as alkaline water ionizers rises the water's pH value through electrolysis. Water would pass through an electrolysis chamber with a positive and a negative electrode separated by a semi-permeable membrane (LeBaron et al., 2022). Figure 2.2 shows the basic of how alkaline water is prepared. The negative electrode will attract the positively charge alkaline ions such as hydrogen ions, producing electrochemically reduced water (ERW) and the positive electrode will attract the negatively charge acidic ions, producing electrochemically oxidized water (EOW) (Shirahata et al., 2012). This will create a separation between the alkaline ion and the acidic ions. The neutral water will react with both the alkaline and the acidic ions and is discharge into separate stream. The water with the alkaline mineral is discharged as alkaline ionized water. Alkaline ionized water has a negative oxidation reduction potential due to the presence of dissolved hydrogen ions and an alkaline pH due to the increased hydroxide ions (LeBaron et al., 2022). The presence of hydrogen ions and a negative oxygen reduction potential gives alkaline ionized water an anti-oxidative property (Vadthya et al., 2019). According to Tanaka et al. (2018), there are several beneficial effects of drinking alkaline ionized water on health such as improving gastrointestinal symptoms. However, there are several reports on its assumed effectiveness and antioxidative effects of the dissolved hydrogen on various diseases. According to LeBaron (2022), some of the health benefit claims are validated by the literature and others are not.

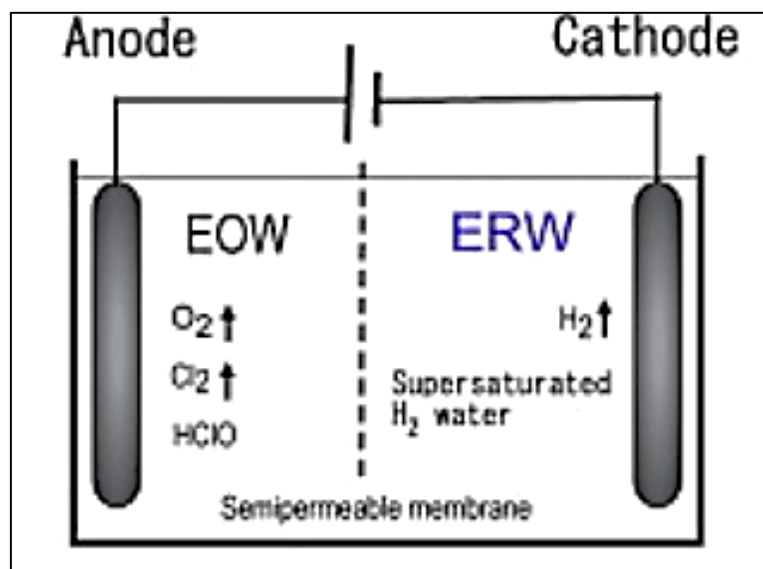


Figure 2.2: Basic principle of how alkaline water is produced (Shirahata et al., 2012)

2.2.5 Ultraviolet (UV) Disinfection

Ultraviolet (UV) water disinfection method eliminates water borne pathogens such as bacteria, virus and protozoa by using UV light, disrupting the deoxyribonucleic acid (DNA) rendering it inactive. UV disinfection of drinking water utilises UV-C light because of its shorter wavelength and higher energy level compared to UV-A and UV-B light (Kim et al., 2023). UV irradiation disrupts the DNA directly or act on pyrimidines producing pyrimidine dimers, causing the microbial DNA to lose its physical and chemical properties (Paidalwar & Khedikar, 2016). This prevents DNA replication and also its metabolic activities. After UV disinfection, microorganisms in the water are inactive and pose no threats to health. Ultraviolet disinfection offers many advantages in terms that it uses no chemicals, it does not produce any bi-products and it does not alter the taste, pH or other properties of the drinking water (Adegbola et al., 2012). Similar to other filtration or disinfection method, Ultraviolet disinfection also has its

negative side. Ultraviolet disinfection only provides a limited effectiveness as there is no residual effect on the water after UV disinfection (Bolton & Cotton, 2008).

2.3 Rules and Regulations of Water Vending Machine (WVM)

There are several rules and regulations implemented by the Malaysian government under the Food Act 1983 (Act 281) and Regulations (Malaysia & International Law Book Services, 2019). Any vendor who owns an WVM is required to have a licence to operate it for the purpose of business under Regulation 360C. Owners is required to follow certain requirements before installing an WVM. As stated in this act, owners must display their name and registered address on the machine and the WVM must be placed at a suitable location to prevent contamination. The area surrounding the machine must be accessible to cleaning to prevent any potential of harbouring pest. The WVM itself must also abide to certain conditions for it to be considered as operatable. The machine must be made in a way that is easy to clean and conduct maintenance of its exterior and interior components. All components which are in contact with water should be made with approved material which is resistant to corrosives and is non-absorbent and is able to withstand repeated cleaning and sanitising treatment. The dispensing spout must be recessed or shielded from corrosion. The WVM should be equipped to handle any drips, spillages and overflow of water and it must also be able to prevent any water backflow. It must be equipped with an approved disinfection system and a monitoring mechanism to halt all operations in case of a failure in the disinfection system. The doors of the dispensing compartment of the WVM must be self-closing and be able to close tightly to protect the dispensing spout from

contamination. Failure to comply with these regulations will result in a fine or conviction to the owner or operator.

2.4 Risk Factor of Water Vending Machine (WVM)

Water vending machine provides a convenience in terms of readily available drinking water source, safety and is cost effective. However, there are certain risk that needs to be addressed when drinking water from WVM such as contamination risk from microorganisms, maintenance practice, hygiene practice, regulation, and public health aspect. Although WVM are equipped with several filtration system, it is still possible for microorganisms to contaminate the drinking water (Tan et al., 2016).

Microbiological contamination in WVM could first be introduced through the water source. The water source of WVM must be an approved public supply of water under the Malaysian Food Act 1983 (Act 281) and Regulations (Malaysia & International Law Book Services, 2019). The water source must be in compliance with the water standard. Failure to meet the standard could increase the chance of contamination of the water supply. Water vending machine differ based on model and manufacturer with different types of filtration system. The lack of a standardised regulation regarding the make of WVM can lead to varying quality of drinking water dispensed. The filters used in WVM have a potential to cause contamination as well. Although carbon filtration is effective in filtering out contaminants, there is a chance for heterotrophic plate count (HPC) bacteria to colonize the carbon filter in a short period of time (Taylor et al., 1979). In WVM, HPC bacteria can rapidly grow given there is inadequate cleaning of the carbon filter, RO membrane or the UV lamp (Chaidez et al., 1999).

Water vending machine also lack temperature control of the water stored in the machine. This could promote bacterial growth in the drinking water.

Maintenance also plays a crucial role in the quality of the water. Each WVM requires regular maintenance to keep the machine functioning properly. Some of the maintenance done on WVM include annual replacement of UV lamps, frequent change of carbon filter, and yearly replacement of reverse osmosis membrane (Muhammad et al., 2020). Maintenance is required to regularly clean the machine and to change the water filters. Irregular maintenance can lead to build up of contaminants (Tabotabo-Picardal et al., 2018). Lack of regular and improper maintenance can also lead to biofilm formation inside the filtration system and on the nozzle (Ansahrullah & Shafie, 2021). Biofilms is a complex community of microorganism. Biofilm can occur on both internal and external components of the WVM. Biofilm can protect the microorganism from disinfection and would require chemical or physical removal. The duration of biofilm attachments depends on the type microorganism present, environmental factor and the surface of attachments. Environment provided by WVM can lead to formation of biofilm relatively fast.

Contamination can also occur through improper hygiene practices. Improper use by consumers can lead to cross contamination. Cross contamination can occur through the physical contact from consumer's hand or the exposure of fomites from containers use by consumers (Ang & Tham, 2020). Since WVM requires the consumer to use their own container to store the drinking water, this can lead to cross contamination at the nozzle. Under regulation, WVM is required to be equipped with a self-closing cover at the nozzle area. The absence of a cover to the dispensing area can lead to contamination from the surrounding such as dirt, wild animals or others (Ansahrullah & Shafie, 2021). The surface of the tubing, spigots, nozzles and drains

can support biofilm growth as it is not often maintained or sanitized when maintenance is done (Hile et al., 2020). The location hygiene of the WVM could also be a risk factor to the contamination of WVM. Improper installation site of the WVM such as nearby garbage and high dust generating area can lead to the abundance of bacteria on the nozzle and body of WVM (Precha et al., 2022). The water dispensed by WVM must meet certain standard to be considered as safe for consumption. There are 3 main aspects on water safety which are, physical property, chemical property and microbial property. Under bacteriological property, drinking water must not be over the maximum acceptable value.

2.5 Reported Cases of Water Vending Machine (WVM) Contamination

Due to the rise in popularity of WVM, concerns have risen on the safety of the drinking water dispensed. Several research have been conducted showing that drinking water dispensed by WVM are not necessarily safe. There have been cases in Malaysia about the contamination of WVM. Two cases involving WVM contamination were reported in the news. Water vending machine around the Klang Valley area was found to be contaminated with harmful bacteria and had no traces of free chlorine on 19 August 2014 (Aruna & Cameons, 2014).

Another research was done at Parit Raja, Batu Pahat, Johor in 2015 to investigate the quality of drinking water dispensed by WVM in terms of pH, TDS, turbidity, mineral content, total organic carbon (TOC), total colony forming unit (CFU) and total coliform. The TDS value and heavy metal were found to conform with the Food Act 1983 and the Malaysian drinking water quality. Coliform was not detected in any machine and the pH was only a bit above the

standard. However, the turbidity was 45 to 95 times higher than the standard set by the Malaysian Food Act 1983 which is 0.1 NTU (Hashim & Yusop, 2016)

A study was done in Seri Kembangan, Selangor in 2018 on the public health risk in regard to the microbial quality of drinking water from WVM. The study utilises epidemiological and Quantitative Microbial Risk Assessment (QMRA) approaches to estimate the burden of disease from *Escherichia coli*. It was found that the drinking water taken from the sampling area exceeded the Malaysian Drinking Water Quality and the World Health Organisation (WHO) drinking water quality guidelines with the highest recorded level of *E. coli* was 46-68 CFU/100 mL. The environmental condition of each WVM were also noted where most are located near dustbin and drains (Praveena et al., 2018).

Another study was done in Johor Bahru, Johor in 2020. The study aims to check for the presence of microorganisms cultured from the water and nozzle. Several samples were collected from 8 areas around Johor Bahru and several bacteria was identified. A total of 20 bacterial isolates were amplified with 16sRNA determined that the bacteria isolated were from the *Pseudomonas*, *Bacillus* and *Stenotrophomonas* genera. Physical and chemical parameters were also tested. Two samples were slightly above the standard level of pH and conductivity (Muhammad et al., 2020).

A study in Gombak, Selangor was done in 2021 to assess the physical, chemical and microbiological qualities of drinking water from WVM. This study also assessed the environmental condition. A total of 20 WVM with 10 registered and 10 were not registered were tested and the results were compared between the 2 categories. Based on the findings of this study, 1 registered WVM was found to be over the pH and total coliform limit. 5 of the non-registered machines was not in compliance with the Malaysian Ministry of Health (MOH) water

quality standard. Out of the 5, 4 was over the pH limit and 1 was found violate the total coliform standard. Registered WVM were found to have better conditions and cleanliness compared to non-registered WVM. The study concluded that non-registered WVM were more likely to be contaminated (Ansahrullah & Shafie, 2021).

2.6 *Pseudomonas aeruginosa*

2.6.1 Key Characteristic

Pseudomonas aeruginosa is a Gram-negative rod-shaped bacterium. It is asporogenous meaning it does not produce spores. *P. aeruginosa* is an aerobic bacterium but can be a facultative anaerobe depending on the availability of nitrate acting as an alternate electron receptor (Planet, 2023). It is ubiquitous, commonly found in soil, water, plants, animals and human. It is a non-fermenter as it is not able to ferment lactose and other carbohydrates but is able to derive energy through oxidation by oxidising glucose and xylose (Planet, 2023). It uses a single polar flagellum, with some strains having 2 or 3 flagella for motility (Iglewski, 1996). *P. aeruginosa* are able to produce pigments including pyocyanin, blue green pigment, pyoverdine, yellow green fluorescent pigment and pyorubin, red-brown pigment (Wu et al., 2015). It needs little nutritional requirements to flourish and can adapt to conditions that are usually not tolerable by other organisms. It commonly grows at a temperature between 25 °C to 37 °C but what differs it from other species is its ability to grow at 42 °C (Wu et al., 2015).

2.6.2 Virulence Factor

Pseudomonas aeruginosa can survive and colonise several environments due to a variety of virulence factor. One of the factors that contributes to its pathogenicity is the presence of lipopolysaccharide (LPS) on the outer membrane. Lipopolysaccharide is a large molecule made of lipid and polysaccharides composed of O-antigen and are able to poison the host cell using the endotoxicity of lipid A present in the LPS. The O-antigen are said to prevent phagocytosis and decrease the diffusion rate of antibiotic in chronic lung infections (Iglewski, 1996). This will result in the colonisation of *P. aeruginosa*. This enables tissue damage, attachment and also recognition by host receptor (Qin et al., 2022). When the host cell recognises the LPS, it can trigger an immune response to the bacteria, activating the hosts' immune cells. Although, activating the immune response is crucial for the host defence system, an excessive immune response will lead to inflammation and could harm the host cell, causing tissue damage (Qin et al., 2022).

Next, *P. aeruginosa* also have outer membrane vesicles (OMV) that contributes to its virulence. Outer membrane vesicles are bacterial components released into its surrounding to aid the bacterium in molecule transport, cell-to-cell communication, biofilm formation and interaction with host. It delivers a wide variety of bacterial protein and nucleic acids such as lipoprotein and toxins to neighbouring cells and host cell without the need of direct contact. Outer membrane vesicles can lead to acquisition of resistant genes, regulation of biofilm, and host immune escape (Qin et al., 2022). *Pseudomonas* quinolone signal (PQS) are transported through OMV which triggers quorum sensing and regulate *P. aeruginosa* group behaviour, production of virulence factor and formation of biofilm (Huszczyński et al., 2020).

Thirdly, *P. aeruginosa* contains 6 secretion system that aids it in colonization. Type III secretion system (T3SS) and Type VI secretion system (T6SS) plays the main role in *P. aeruginosa* virulence. T3SS delivers protein directly from the bacterium to the host cell. This type of secretion system regulates the injection of toxin proteins called effectors which are ExoU, ExoT, ExoY and ExoS (Diggle & Whiteley, 2020). This leads to rapid host cell death and preventing *P. aeruginosa* phagocytosis. T6SS aids in *P. aeruginosa* competition with competing pathogens. This secretion system delivers an enzyme called AmpDh3, a paralogous zinc protease to competing organisms leading to their death (Qin et al., 2022). Thus, promoting the colonization of *P. aeruginosa*.

P. aeruginosa also produces toxins that help it cause infections and contribute to its virulence. There are several pigments produced by *P. aeruginosa*, among it is the blue green pigment pyocyanin. Pyocyanin is toxic to host cell, causing damage to the tissue, inflammation and stopping cellular function plus it stops the growth of competing organisms (Iglewski, 1996). Exotoxin A (ETA), produced by 90% of all strain of *P. aeruginosa* inhibits protein production of host cell through ADP ribosylation activity on elongation factor which eventually causes cell death (Lee et al., 2005). Production of cytotoxin ExoU, ExoT, ExoY and ExoS also adds to the virulence of *P. aeruginosa*. ExoU shows high toxicity where it disrupts the membrane of eukaryotic cells (Lee et al., 2005).

The ability of *P. aeruginosa* to form biofilms that acts as a defensive measure can promote to its survival and colonisation. A biofilm is formed when free-floating planktonic cells combine together into a complex bacterial community, connected by an extracellular substance that enhance adhesion to surfaces (Olsen, 2015). Production of a type of exopolysaccharides called alginate in biofilm helps resist stressor such as bacteriophages and host immune system (Diggle

& Whiteley, 2020). Biofilms can also increase the bacterium's drug resistance. Biofilm allows easier gene transfer such as antibiotic resistance gene between the bacterium in a biofilm matrix. Bacteria can form an antibiotic tolerance in the biofilm state as the aggregated bacteria in an extracellular matrix is able to produce a structured environment resulting in an antibiotic tolerant subpopulation (Ciofu & Tolker-Nielsen, 2019). This ability to tolerate antibiotic treatment and host immune system is the reason of the widespread infection. A biofilm matrix also allows minimal contact by the antibiotics with the bacterium due to its thick layer of alginate. This furthers the difficulty in eradicating the infection. Metabolic activities are reduced in a biofilm due to the fact that nutrients are not readily available to the bacteria in the inner area of a biofilm. The low metabolic rate effects the synthesis of antibiotic-target protein (Ciofu & Tolker-Nielsen, 2019). The biofilm also aids in the adhesion of the colony to surfaces leading to faster colonisation.

Due to the increase use of antibiotic treatment, some strains of *P. aeruginosa* has acquired a resistance to antibiotics. Acquired antibiotic resistance results in higher prevalence of *P. aeruginosa* infection. Antibiotic resistance of *P. aeruginosa* is acquired through mutation resulting from the bacterium adapting to antibiotics in its environment. Several mechanism can be the factor in the bacterium resistance such as alteration of the antibiotic target protein and horizontal transfer of genetic material (CloECKaert et al., 2017). Strains of antibiotic-resistant *P. aeruginosa* are harder to eradicate due to it being less susceptible to antibiotic treatment. Antibiotic-resistant strains require a new treatment to be introduced which will require time and effort to determine the right treatment. Delay in administering treatment would make it harder to remove the biofilm. According to Stewart (2015), biofilms become less susceptible with age.

Resistant strain of *P. aeruginosa* could cause a more severe infection, leading to the colonisation of the bacteria causing tissue damage and inflammation.

2.6.3 Clinical Impact of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa have a huge clinical impact both in healthcare and public setting. It is an opportunistic pathogen where it rarely infects healthy individual but can easily infect those with weeken immune system and those with an underlying medical condition. *Pseudomonas aeruginosa* can cause an array of infections such as cystic fibrosis (CF), healthcare associated infections, respiratory infection, burn and wound infection, eye infection, bloodstream infection and urinary tract infection. Table 2.1 summarises the types of infections caused by *P. aeruginosa*.

Table 2.2: Description of infections caused by *P. aeruginosa* (Al-Wrafiy et al., 2017)

Infection	Route of Infection and Characteristics
Cystic fibrosis (CF)	Chronic, in individuals with genetic disease
Pneumonia: healthcare-associated pneumonia (HCAP), hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP)	Acute and nosocomial infection, the poor health status of the patients, the high carriage rate of often multidrug-resistant strains in hospital wards, and prior use of broad-spectrum antibiotics
Chronic lung infection	In an immunocompromised patient, cystic fibrosis patients, individuals receiving chemotherapy

Table 2.2 continued

Urinary tract infections	Acute and nosocomial infections, urinary catheterization
Skin and soft tissue infections	Acute and nosocomial infections, burn, wounds and surgical site infections
Diabetic foot infections	Community-acquired infections
Burn infection	Acute and nosocomial infection
Bacteraemia and septicaemia	Acute, in immunocompromised patients
Central nervous system infections	Meningitis and brain abscess by contiguous spread, bacteraemia or direct infection as in trauma and surgery
Otitis externa	Community-acquired infection, in immunocompromised patient such as those with diabetes mellitus and swimmer's ear
Corneal infections	Acute ulcerative keratitis, community-acquired infection usually associated with users of extended-wear soft contact lenses

CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling

Randomized sampling was done with a total of fifty (n=50) samples from Kota Samarahan and fifty (n=50) samples from Kuching, totalling up to one hundred (n=100) samples were collected per one time of sample collection from water vending machine (WVM). The sampling was at random dates. Sampling was done according to Hashim and Yusop (2016), which started with collection of drinking water samples from the WVM using a sterilized sampling bottle that was preserved in nitric acid solution with ratio 1:1 and rinsed with mili-Q ultra-pure water for 2 days. The samples were placed in the icebox with temperature around 4-6 °C. The samples collected was tested within 24 hours in the laboratory from the day of collection to avoid any changes to occur on the samples. The physical condition of the WVM were noted in terms of physical condition of WVM, condition of surrounding area of WVM, presence of maintenance done, and presence of a license on the WVM body. Physical condition of the WVM was assessed in terms of overall machine cleanliness and presence of cover to the dispensing area. The condition of the surrounding of the WVM were assessed in terms of where it was located and overall area cleanliness. The maintenance of the WVM were assessed with the presence of a maintenance sticker with a recent maintenance date.

3.2 Water Quality Analysis Using Physical and Chemical Parameters

The pH of the water samples was measured by using a calibrated pH meter. Meanwhile, the total dissolved solid meter was used to measure the TDS. The measurements were done in triplicates. The readings were done immediately on site with the drinking water collected inside the sampling bottle.

3.3 Enumeration and Identification of *Pseudomonas aeruginosa* Using Most Probable Number (MPN) Method

The microbiological analysis of the water samples includes isolation, identification and enumeration of the microorganisms following the methods described by da Silva et al. (2018). Most probable number (MPN) method is used to enumerate *Pseudomonas aeruginosa* utilising the single dilution test.

First is the presumptive test, the sample was vigorously shaken 25 times to homogenise the sample. A total of 10 mL of the samples was inoculated into 10 tubes containing double-strength asparagine broth. The samples were then incubated 37 °C for 24 to 48 hours. After the incubation period, the tubes were examined under long wave ultraviolet (UV) light in a dark room. Tubes that showed a green fluorescent pigment were considered as presumptive positive. Negative tubes were incubated again for another 24 to 48 hours.

Secondly, the confirmed test was done. From the positive presumptive tubes, 0.1 mL of the culture was inoculated into tubes containing 10 mL of acetamide broth and was incubated 37 °C for 24 to 36 hours. Any purple colouration observed after incubation was noted and considered as positive confirmed for *P. aeruginosa*. A third completed test was done to confirm

the observations from the confirmed test. Culture from the second step was inoculated into cetrimide agar and incubated at 37 °C for 18 to 24 hours. MPN calculation was done by taking note of the number of positive tubes and determine the MPN/mL based on the data retrieved from MPN table. Figure 3.1 shows a summarised view of the single dilution MPN test.

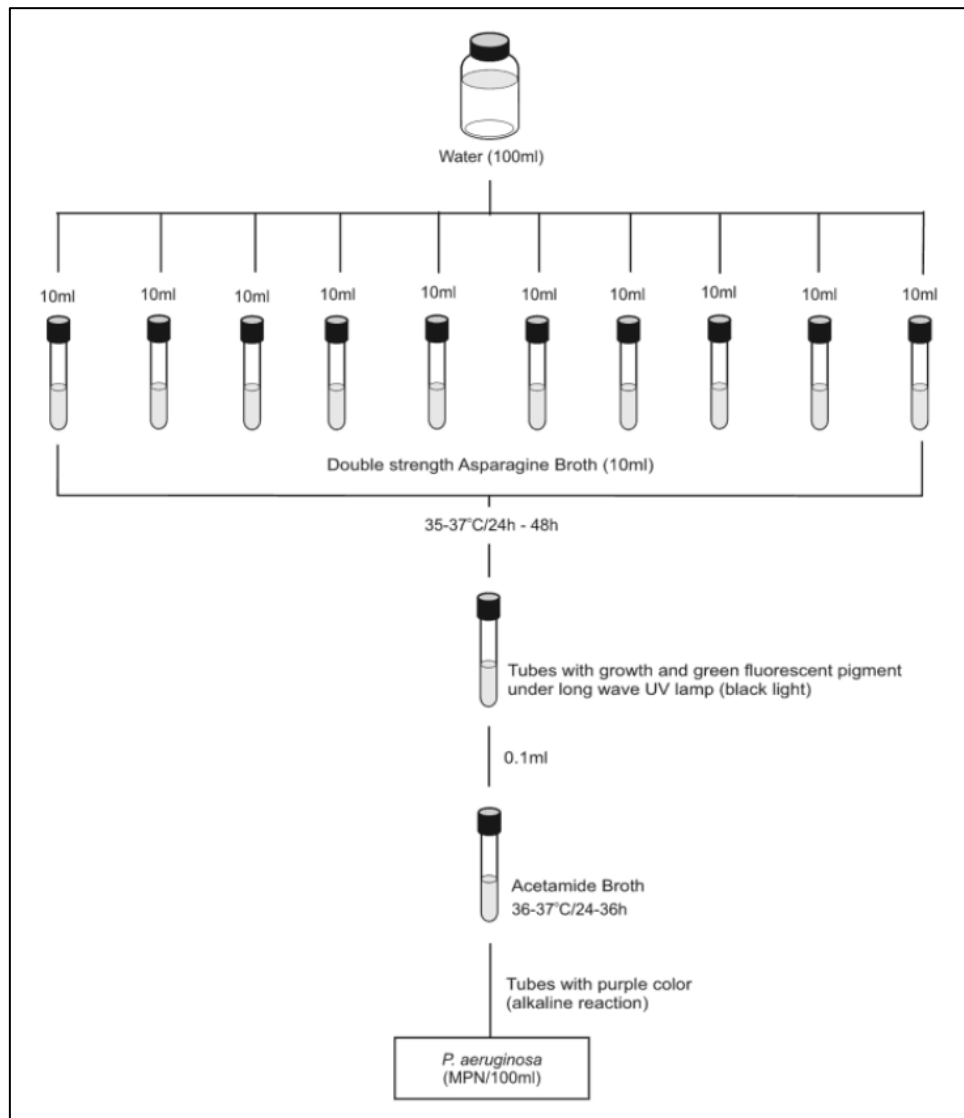


Figure 3.1: An overview of a single dilution MPN test (da Silva et al., 2018)

3.4 DNA Extraction

Deoxyribonucleic acid (DNA) extraction method was adjusted from Peng et al., (2013). Single colony was inoculated in 5 mL of Luria-Bertani (LB) broth and incubated at 37 °C for 18 to 24 hours. A total of 500 µL of the culture broth was transferred to a 1.5 mL centrifuge tube and was centrifuged at 10,000 rpm for 5 minutes. The resulting supernatant was discarded and the pellet was resuspended in 100 µL of deionized distilled water (ddH₂O). The mixture was then boiled for 10 minutes and was proceeded to be cooled in ice for 5 minutes. Next, each tube was centrifuged for 10 minutes at 10,000 rpm. The resulting supernatant is the DNA product to be used in polymerase chain reaction (PCR).

3.5 Polymerase Chain Reaction (PCR)

PCR method was adapted based on the method by Al-Ahmadi and Roodsari (2016). A total of 25 µL reaction mixture including 8 µL of exTEN PCR 2X Master Mix (Base Asia), 0.5 µL of each reverse and forward primer, 11 µL of nuclease-free water and 5 µL of DNA extract. One set of primer was used for PCR which targeted the exotoxin A (ETA) gene in *P. aeruginosa*. The forward primer used was ETA1 5' GACAACGCCCTCAGCATCACCAGC 3' and reverse primer ETA2 5' CGCTGGCCCATTCGCTCCAGCGCT 3' will amplify the ETA gene with an expected amplicon size of 396 bp sequence. DNA amplification was done using a thermal cycler (Eppendorf Mastercycler Gradient) with the protocol by Momtaz et al. (2011) is shown in Table 3.1.

Table 3.1: PCR conditions for ETA primer

PCR conditions	Temperature (°C)	Time (s)	Number of cycles
Initial denaturation	95	180	35 cycles
Denaturation	94	60	
Primer annealing	68	90	
Primer extension	72	60	
Final extension	72	600	

The PCR amplification result was viewed with agarose gel electrophoresis (AGE) using 1.5% agarose gel. A total of 5 µL of the PCR product was loaded into the agarose gel and was subjected to 120V and 208 mA for 45 minutes. Next, the gel is stained with 0.1% ethidium bromide (EtBr) for 1 hour. The bands that formed was observed by using a UV transilluminator.

3.6 Antibiotic Susceptibility Testing (AST)

Antibiotic susceptibility test (AST) was done to determine *P. aeruginosa* sensitivity towards various antibiotics. Disc diffusion method was done according to Bauer et al. (1966). Pure cultures of *P. aeruginosa* were isolated and lawned by using sterile cotton swabs on Mueller Hinton agar (MHA). The plates were then left to dry for 5 minutes. Disc containing the antibiotics was then placed on top of the agar by using sterile tweezers with decent space in between each disc. The plates were then incubated at 37 °C for 18 to 24 hours.

Table 3.2: Antibiotic used for the antibiotic susceptibility test (AST) for *P. aeruginosa*

Antibiotic	Abbreviation	Content (µg)
Meropenem	MEM10	10
Ciprofloxacin	CIP5	5
Ceftazidime	CAZ30	30

The lawn of inhibition was measured according to its diameter and was interpreted as Resistant (R), Intermediate (I) or Sensitive (S) based on the guidelines by Clinical and Laboratory Standards Institute (CLSI), M45 (Hindler et al., 2015). Interpretation of the result following CLSI guidelines for the antibiotic listed in Table 3. is shown in Table 3.3.

Table 3.3: Zone diameter and MIC breakpoint for *P. aeruginosa* (Hindler et al., 2015)

Antibiotic	Interpretive Categories and Zone Diameter Breakpoint, nearest whole mm			Interpretive Categories and Minimum Inhibitory Concentration (MIC) Breakpoints µg/ml		
	S	I	R	S	I	R
Meropenem	≥19	16-18 [^]	≤15	≤2	4 [^]	≥8
Ciprofloxacin	≥25	16-24 [^]	≤18	≤0.5	1 [^]	≥2
Cefrazidime	≥18	15-17 [^]	≤14	≤8	16 [^]	≥32

3.7 *Pseudomonas aeruginosa* Biofilm formation in Vitro

A total of 6 random ETA positive of *P. aeruginosa* and a positive control using *P. aeruginosa* ATCC 15442 were studied for its biofilm forming capabilities *in vitro* using polystyrene flat-bottomed 96-well microtiter plates as described according to the method described in Elexson et al. (2014). The wells of the microtiter plates were filled with 100 μ L of tryptic soy broth (TSB). A negative control was done using only fresh media. The microtiter plates were pipetted with 100 μ L each inoculum. The plates were incubated sealed with parafilm at 37 °C. After 24 hours of incubation, the medium and any cells that were not attached were discarded with thorough washing with sterile phosphate-buffered saline (PBS). The plates were dried by gently blotting with paper towels and was inverted to remove any residual medium. The washed wells were then stained with 110 μ L of 0.4% aqueous crystal violet solution for 45 minutes. Then the wells were washed twice with sterile distilled water. A total of 200 μ L of 95% ethanol was used to de-stain the wells and 100 μ L of the de-staining solution was then transferred to a new well. The concentration of the crystal violet stain contained in the de-staining solution was measured in terms of absorbance using microplate reader (VERSAMAX, Sunnyvale, CA, USA) at optical density (OD) 590 nm.

The degree of strength by *P. aeruginosa* to produce biofilm was calculated as cutoff value (OD_c). The average OD of the negative control and its standard deviation (SD) was calculated. The cutoff was calculated using the formula in Table 3.4. The OD of each isolate was calculated as the difference between the average optical density of the isolates ($OD_{isolates}$) and the cutoff value using the formula in Table 3.4. The results were then rated from non-adherence, weakest, moderate to strongest based on the biofilm strength based on the $OD_{isolates}$ compared to the cutoff value as shown in Table 3.5.

Table 3.4: Formula for OD_c and OD_{isolates} calculation

$$\text{OD}_c = \text{Average OD of negative control} + (3 \times \text{standard deviation (SD) of negative control})$$
$$\text{OD}_{\text{isolates}} = \text{Average OD}_{\text{isolates}} - \text{OD}_c$$

Table 3.5: *P. aeruginosa* biofilm-forming capacities

Result of calculation	Biofilm capacities
$\text{OD} \leq \text{OD}_c$	No biofilm
$\text{OD}_c < \text{OD} \leq 2\text{OD}_c$	Weak
$2\text{OD}_c < \text{OD} \leq 4\text{OD}_c$	Moderate
$4\text{OD}_c < \text{OD}$	Strong

CHAPTER 4

RESULTS

4.1 Water Vending Machine (WVM) Physical Condition

The water vending machine (WVM) physical condition was assessed according to overall machine condition including presence of cover to the dispensing area and overall cleanliness. The environment was assessed according to the condition of the location the WVM was situated in such as nearby drains or garbage bins. The machine was also checked for the presence of a maintenance sticker with a recent maintenance date. The number of machines displaying licences was also noted. All parameter were assessed based on the regulations set for WVM from the Food Hygiene Regulation 2009 Part V. The results were tabulated in Table 4.1.

Based on Table 4.1, the physical condition of the WVM in Samarahan and Kuching showed a majority of the WVM in good physical and hygiene level with 64% and 76% respectively. Both locations had the same amount of WVM without any cover to the dispensing area with 16%. Only 20% and 8% of WVM in Kota Samarahan and Kuching respectively had low hygiene level with some having litter in the dispensing area and some covered in stickers.

Based on the condition of the surrounding area of the WVM, WVM around Kuching area was better with 44% compared to Kota Samarahan with 32%. However, a higher portion of WVM in both cities had unsuitable surrounding condition with 68% in Kota Samarahan and 56% in Kuching. WVM in Kuching was observed to be better maintained with regular maintenance done with 76% while only 44% of the WVM in Kota Samarahan were regularly maintained. Majority of the WVM in Kota Samarahan and Kuching either did not have a license or was not displaying it on the WVM body with 80% and 76% respectively.

Table 4.1: Condition of the WVM around Kota Samarahan and Kuching area

Sample	Physical Condition of WVM			Condition of Surrounding area of WVM			Maintenance		Licensed	
	Acceptable	Not acceptable		Acceptable	Not acceptable		Yes	No	Yes	No
		No dispensing area cover	Unhygienic condition		Near drain	Near garbage				
Kota Samarahan (n=50)	32	8	10	16	32	2	22	28	10	40
Kuching (n=50)	38	8	4	22	28	0	38	12	12	38

4.2 Physical and Chemical Parameter

Table 4.2 shows the mean value of pH, total dissolved solids (TDS) AND temperature of drinking water from Kota Samarahan (n=50) and Kuching (n=50) area.

Table 4.2: Mean of physical and chemical parameter of water from WVM

Sample	pH		TDS (ppm)		Temperature (°C)	
	Samarahan	Kuching	Samarahan	Kuching	Samarahan	Kuching
1	7.34	8.03	53	46	26.3	28.3
2	7.49	7.85	68	53	26.6	30.4
3	7.67	8.3	73	47	26.8	27.7
4	7.76	9.29	69	96	26.6	28.2
5	7.58	9.18	67	77	27.1	28.5
6	7.49	9.18	66	54	28.9	28.3
7	7.67	7.94	69	57	31.2	28.0
8	7.67	7.67	69	53	28.6	27.7
9	7.49	8.21	61	56	27.4	28.0
10	7.67	7.85	58	56	26.3	29.0
11	7.85	9.18	55	56	24.7	28.2
12	7.85	7.85	41	53	25.5	30.0
13	7.67	8.84	55	55	27.7	29.2

Table 4.2 continued

14	7.58	7.85	47	52	25.5	29.2
15	7.67	7.94	58	51	24.8	29.7
16	7.67	7.58	54	47	25.5	28.0
17	7.76	7.42	60	47	26.3	27.7
18	8.21	7.98	53	61	27.7	28.2
19	7.94	7.66	62	51	27.1	26.6
20	7.85	7.5	59	55	26.3	30.2
21	8.12	7.42	47	53	30.2	27.9
22	7.85	7.34	64	61	30.0	31.5
23	7.94	7.5	51	55	27.7	28.3
24	8.03	7.5	52	55	26.9	28.3
25	7.85	7.42	58	52	27.5	30.6
26	8.03	7.5	58	46	29.7	28.0
27	8.03	7.34	57	46	27.8	29.2
28	8.12	7.74	66	45	29.0	28.6
29	7.94	7.66	62	58	26.3	28.7
30	7.76	7.9	63	50	25.5	26.9
31	7.4	8.22	60	58	27.7	29.7
32	7.85	7.66	55	52	28.4	27.7
33	7.94	7.34	68	53	30.2	28.6

Table 4.2 continued

34	7.58	7.9	54	59	27.4	27.3
35	7.76	7.42	54	53	28.6	27.8
36	7.67	7.58	52	57	24.0	27.4
37	7.76	7.34	52	56	23.5	27.2
38	7.76	7.9	55	59	23.6	27.3
39	7.31	7.34	55	57	23.5	27.5
40	7.49	7.26	55	56	24	27.2
41	7.49	8.03	58	50	25.5	31.5
42	7.58	7.49	63	33	25.5	26.0
43	7.76	7.58	61	53	26.0	29.2
44	7.67	7.57	65	48	26.0	28.3
45	7.67	7.22	65	58	26.3	29.3
46	7.58	7.4	57	48	26.6	29.5
47	-	7.49	-	49	-	29.5
48	7.34	7.31	54	49	26.3	29.5
49	7.42	7.67	60	49	25.8	29.7
50	7.42	7.22	58	58	24.8	32.4

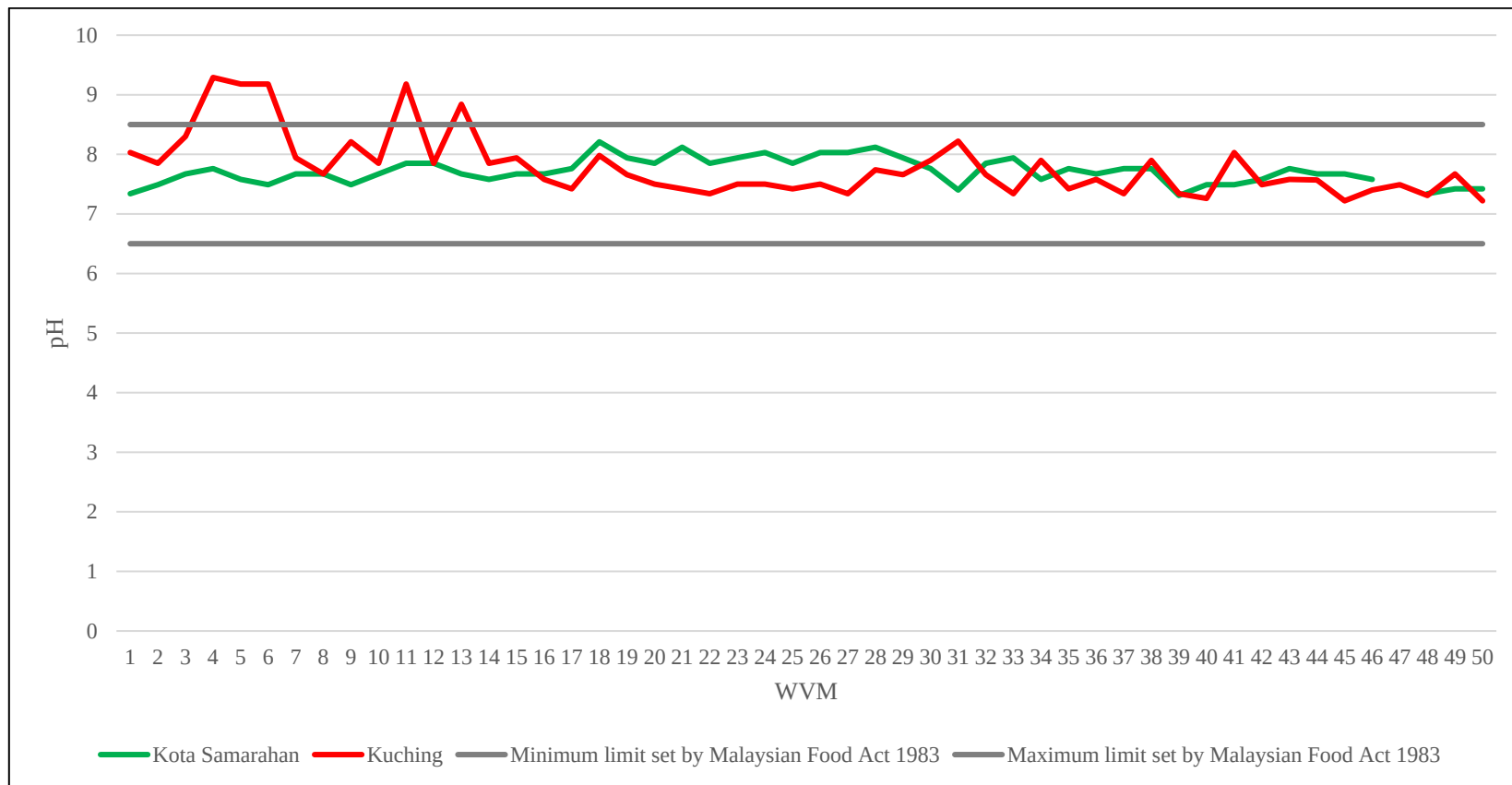


Figure 4.1: Mean pH value of water from WVM from Kota Samarahan and Kuching compared to the allowed range set by the Malaysian Food Act 1983

Figure 4.1 shows the mean value of pH of water taken from WVM in Kota Samarahan and Kuching in comparison to the allowed pH range set by the Malaysian Food Act 1983 (Act 281) and Regulations under Subregulations 360B(3) and 360C(3) which is between 6.5 to 8.5. Based on the figure, almost all of the drinking water are in the allowed range. Only 5 of the drinking water dispensed by WVM from Kuching was over the maximum limit by 9.29% from WVM4, 8% from WVM5, WVM6 and WVM11, and 4% from WVM13. All of the WVM from Kota Samarahan complied with the Malaysian Food Act 1983 in terms of pH. Based on the data in Figure 4.1, it can be seen that the pH value in Kota Samarahan is more constant than when compared to the pH value in Kuching which is shown to fluctuate more. The drinking water in Kuching recorded both the lowest and the highest reading with 7.22 and 9.29 respectively, showing the biggest variability among the 2 cities.

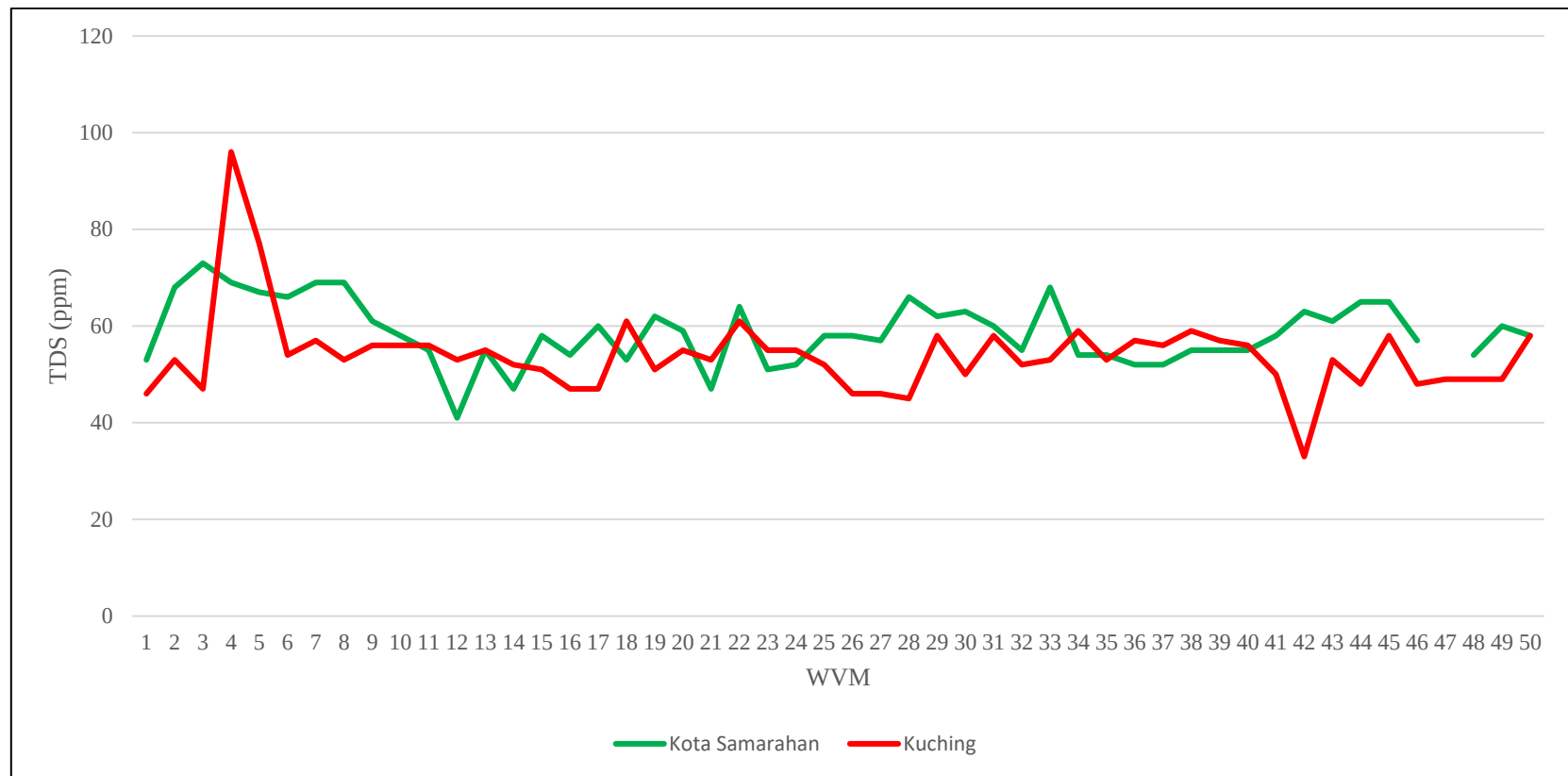


Figure 4.2: Mean TDS (ppm) value of drinking water from WVM from Kota Samarahan and Kuching

Figure 4.2 shows the mean value of total dissolved solids (TDS) of drinking water collected from WVM around Kota Samarahan and Kuching area. According to Malaysian Ministry of Health (MOH) national standard for drinking quality standards, the allowed level of TDS in drinking water is 1000 mg/L which is equal to 1000 ppm (Ministry of Health Malaysia, 2004). TDS refers to the total inorganic and organic matter dissolved in water. The results based on Figure 4.2 shows that the drinking water from WVM shows that the TDS present is very minimal with all WVM having TDS below 100 ppm. The highest and lowest recorded TDS level was from Kuching from WVM5 with is 96 ppm and the lowest was from WVM 42 with 33 ppm. The drinking water from WVM from Kota Samarahan ranged between 41 to 73 ppm.

As for the result of the drinking water from Kota Samarahan and Kuching is shown in Figure 4.3. The temperature of drinking water in Kota Samarahan ranged from 23.5 °C to 31.2 °C, while the temperature in Kuching ranged from 26.0 °C to 32.4 °C. The lowest recorded drinking water temperature was from Kota Samarahan at 23.5 °C, while the highest recorded was from Kuching at 32.4 °C According to Wu et al. (2015), *Pseudomonas aeruginosa* had a optimal growth temperature ranging from 25 °C to 37°C. Based on the temperature of drinking water results, majority of the WVM fall within this temperature range except 5 WVM from Kota Samarahan that fall under 25°C.

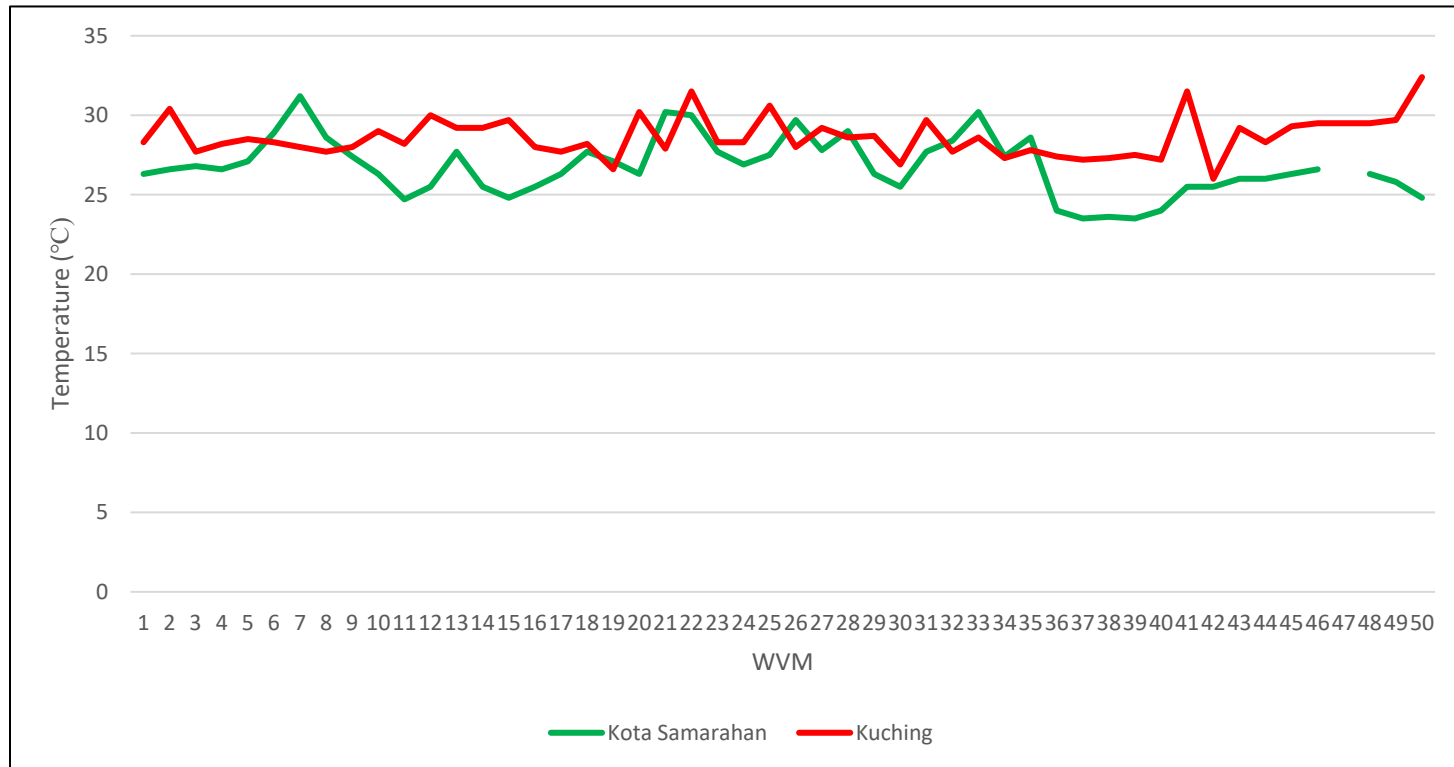


Figure 4.3: Temperature pattern of drinking water from Kota Samarahan and Kuching

4.3 Isolation of *Pseudomonas aeruginosa*

Isolation of *P. aeruginosa* was done in correlation with the most probable (MPN) method. *P. aeruginosa* shows a distinct blue-green coloration or fluorescence under ultraviolet (UV) light as shown in Figure 4.4. Out of a total one hundred (n=100) WVM, forty-one (n=41) WVM was presumptive positive for *P. aeruginosa* with 16 WVM from Kota Samarahan and 25 WVM from Kuching. From the fifty (n=50) WVM from Kota Samarahan, 32% WVM was presumptive positive, while from the fifty (n=50) WVM from Kuching 50% were presumptive positive for the presence of *P. aeruginosa*.



Figure 4.4: Detection of *P. aeruginosa* using asparagine broth through the presence of fluorescent pigment under UV light

Figure 4.5 shows the confirmed positive result in acetamide broth. A confirmed positive result shows a distinct purple colouration. Out of a total of one hundred (n=100) WVM, thirty-one (n=31) WVM was confirmed positive for *P. aeruginosa* with 13% WVM from Kota Samarahan and 18% WVM from Kuching.



Figure 4.5: *P. aeruginosa* growth in acetamide broth

4.4 Most Probable Number of *Pseudomonas aeruginosa*

According to the World Health Organization (WHO) 2011 guidelines for drinking water quality for *P. aeruginosa* in vended water is nil in 100 mL (Abdelrahman et al., 2019). Based on the number of positive tubes from confirmed test, the most probable number (MPN) value was obtained which gives an estimate of the amount of *P. aeruginosa* in drinking water from WVM. Based on the results tabulated in Table 4.4, from the total of one hundred (n=100) WVM, 72% WVM from Kota Samarahan and 64% WVM from Kuching were negative for any presence

of *P. aeruginosa*. Whereas 31% of WVM were positive for the presence of *P. aeruginosa* with a total of 28% of the WVM from Kota Samarahan and 36% of the WVM from Kuching. Based on the MPN result, 32% of the WVM were not in compliance for the bacterial standard for *P. aeruginosa* with 0 in 100 mL. The highest recorded MPN value was from a WVM from Kota Samarahan during with an MPN value of more than 23 MPN/100 mL. Majority of the WVM positive for the presence of *P. aeruginosa* had a low MPN value of 1.1 MPN/100 mL with 35% and 26% with 2.2 MPN/100 mL.

Table 4.4: Number of positive tubes from WVM in Kota Samarahan and its MPN value

No of positive tubes	Kota Samarahan	Kuching	MPN (MPN/100 mL)
0	36	32	-
1	4	7	1.1
2	2	6	2.2
3	1	-	3.6
4	3	1	5.1
5	-	1	6.9
6	-	1	9.2
7	-	-	12
8	-	-	16
9	2	2	23
10	1	-	>23

4.5 Polymerase Chain Reaction (PCR) Analysis

The polymerase chain reaction (PCR) gives further confirmation on the presence of *P. aeruginosa* by targeting the ETA gene which encodes for exotoxin A produced by the bacteria which assist in its ability to cause infection. The DNA amplification will result in a PCR product of 396 bp. A total of 103 isolates from the MPN method including all replicates were analysed for the presence of the ETA gene. Figure 4.7 shows the agarose gel for the PCR results viewed under UV light. Lane L shows the 100 bp ladder (Thermo Fisher GeneRuler), lane 1 to 8 shows the PCR result of the of the isolates, lane 9 shows the negative control and lane 10 shows the positive control (ATTC 15442).

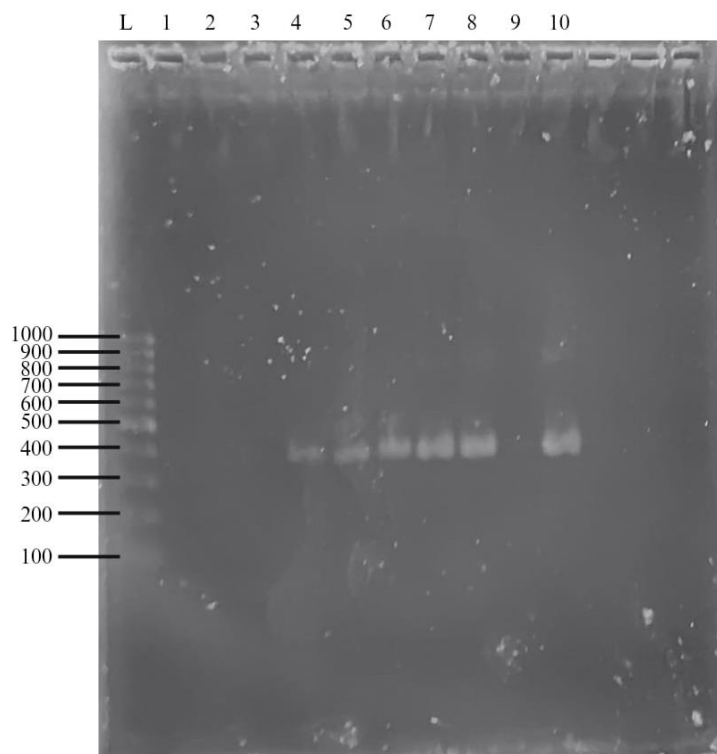


Figure 4.6: PCR result for detection of *P. aeruginosa* ETA gene view on 1.5% agarose gel. L: 100 bp ladder; 1-8: bacterial isolate; 9: negative control (distilled water); 10: positive control (ATTC 15442)

Based on Figure 4.6, the bacterial band formed after AGE shows that the PCR product falls within the expected amplicon size. Out of the 103 isolates, including replicates from the 10 tube single dilution MPN test, a total of 61 were a positive detection of *P. aeruginosa* ETA gene with 27 isolates from Kota Samarahan WVM and 34 isolates from Kuching WVM. This show that 59% of the *P. aeruginosa* isolates have the gene to produce exotoxin A leading to its pathogenicity.

4.6 Antibiotic Resistance Profile

The result tabulated in Table 4.5 shows the result of antibiotic susceptibility test (AST) of *P. aeruginosa* to meropenem (MEM10), ciprofloxacin (CIP5) and Ceftazidime (CAZ30). Based on the Table 4.5, the *P. aeruginosa* isolates were most susceptible to meropenem with 64% susceptible, next is ceftazidime with 7% susceptible and lastly ciprofloxacin with none of the isolated were susceptible to it. The Ciprofloxacin was the least effective antibiotic at preventing the growth of *P. aeruginosa* with the highest resistant at 28%. The most potent antibiotic to prevent *P. aeruginosa* growth was meropenem with highest susceptibility and only 12% resistant.

Table 4.5: Antibiotic profiling of *P. aeruginosa* isolates against selected antibiotics

Antibiotic	Abbreviation	No. and Percentage (%) of <i>P. aeruginosa</i> according to their Susceptible, Intermediate and Resistance Distribution		
		Susceptible (S)	Intermediate (I)	Resistant (R)
Meropenem	MEM10	64	24	12
Ciprofloxacin	CIP5	0	72	28
Ceftazidime	CAZ30	7	83	10

4.7 *Pseudomonas aeruginosa* Biofilm formation in Vitro

The *P. aeruginosa* recorded the highest biofilm forming capacity of an average mean of OD_c: ±0.217. Whereas the lowest was recorded at OD_c: ±0.136. The data indicate optical density of the isolates with standard deviation to the mean optical density value. The OD value is compared to the cutoff value determined from the result for OD of the negative control. The data in Table 4.6 shows that there were no weak or strong positive *P. aeruginosa* biofilm producer when calculated according the formula from Table 3.5. The data from Table 4.6 indicate that *P. aeruginosa* were either non-adherent or a moderate biofilm producer.

Table 4.6: Summary of biofilm-forming capacities of *P. aeruginosa*

<i>P. aeruginosa</i>	OD _c value	Degree of biofilm-forming capacities
<i>P. aeruginosa</i> ATCC 15442	3.00±0.200	Moderate (2OD _c < OD ≤ 4OD)
<i>P. aeruginosa</i> 003	3.00±0.217	Moderate (2OD _c < OD ≤ 4OD)
<i>P. aeruginosa</i> 006	3.00±0.194	Moderate (2OD _c < OD ≤ 4OD)
<i>P. aeruginosa</i> 002	3.00±0.163	Moderate (2OD _c < OD ≤ 4OD)
<i>P. aeruginosa</i> 001	3.00±0.138	Moderate (2OD _c < OD ≤ 4OD)
<i>P. aeruginosa</i> 004	3.00±0.136	Moderate (2OD _c < OD ≤ 4OD)
<i>P. aeruginosa</i> 005	3.00±0.034	Non adherent (OD ≤ ODC)

CHAPTER 5

DISCUSSION

In this study, the working condition of water vending machine (WVM) was assessed in terms overall machine condition, the hygiene level of its surrounding, maintenance regularity and presence of license to operate WVM displayed on the body based on the Food Hygiene Regulations 2009 Part 5 for WVM in the Malaysian Food Act 1983. It was found that a majority of the WVM were in excellent physical condition and regular maintenance schedule. However, some WVM were still observed to have an absence of the dispensing area cover or was in unsanitary condition. According to the Malaysian Food Act 1983, WVM must be equipped with a self-closing door to protect the nozzle from contamination. Some of the WVM either had no cover at all or the cover was broken and does not function as self-closing anymore. This can lead to the drinking water dispensed being compromised as the dispensing nozzle is exposed to contaminants from its surrounding such as dust and wild animals (Ansahrullah & Shafie, 2021).

Less than half (40%) of WVM did not undergo routine maintenance based on the fact that the maintenance sticker was not displayed. Maintenance is crucial as a lack of regular maintenance could lead to proliferation of bacteria and formation of biofilm. A study done by Ang and Tham (2020) on the coliform contamination of the faucets of WVM in Klang valley showed similar result where only 43% of WVM had undergone maintenance. However, it was determined that maintenance was not strongly associated to the total coliform on the WVM faucets.

Only a small portion of WVM were located in suitable environment free from possible sources of contamination such as near drain and nearby garbage bins. Under the Malaysian Food Act 1983, WVM were required to be located at suitable area to prevent any contamination to the drinking water. A previous study done by Ansahrullah and Shafie (2021) on the water quality of WVM in Gombak, Selangor showed that 2 of the highest coliform count came from WVM located near garbage and near water drainage where contamination could occur through cross contamination from pest such as flies present near the garbage to the dispensing nozzle on the WVM. Majority of the WVM were not labelled with the registered name or the address on the WVM body. These WVM were considered as unknown status or unregistered under the Ministry of Health (MOH) (Abdul Fuzi et al., 2021). According to Ansahrullah and Shafie (2021), which water quality of WVM in Gombak, Selangor and comparing the results between registered and unregistered WVM concluded that unregistered WVM had higher potential to breach the standard drinking water quality and overall had poorer WVM conditions and surroundings.

The drinking water property were tested for pH, total dissolved solids and temperature. The Malaysian Food Act 1983 set the standard pH range for drinking water between 6.5 to 8.5. A pH value lower than 6.5 is generally classified as acidic, soft and corrosive. Water acidity indicates that the water contains high levels of metal ions such as iron (Fe), manganese (Mn), cobalt (Co), lead (Pb) and zinc (Zn) which could lead health risks (Tabotabo-Picardal et al., 2018). The drinking water from Kota Samarahan had a range between 7.32 to 8.21, which falls in the standard limit set by the Malaysian Food Act 1983. On the other hand, drinking water from Kuching showed a greater range with 7.22 to 9.29. A total of 5 WVM exceeded the maximum limit of pH. A pH level higher than 8.5 does not pose as a health risk but may alter

the taste (Tabotabo- Picardal et al., 2018). A study done by Hile et al. (2020) on the microbial contamination of drinking water from vending machines of Eastern Coachella Valley, the highest pH value was 9.5 which could lead to metal leaching into the drinking water.

Although the presence of TDS in drinking water may not directly cause harm to humans, it is said that the presence of TDS can affect the taste (Devesa & Dietrich, 2018). A guideline set by World Health Organisation (WHO) under chapter 10 for acceptability aspect, taste odour and appearance showed the preferable level of TDS in drinking water (World Health Organization, 2022). According to this guideline drinking water with less than 600 ppm had a good rating. Comparing the results to this guideline, the low level of TDS in drinking water from WVM shows an excellent quality. However, concentration of TDS too low have been considered to give the drinking water an undesirable flat taste (Safe Drinking Water Foundation, 2023).

Temperature is considered as a contributing factor to the growth of *Pseudomonas aeruginosa*. *Pseudomonas aeruginosa* have an optimum growth temperature between 25 °C to 37°C but is able to tolerate high temperature at 42 °C (Wu et al., 2015). According to a study done by van der Wielen et al. (2023), the temperature between 25 °C to 40 °C demonstrate an optimum growth of *P. aeruginosa* under optimal laboratory conditions. The study concluded that the growth of *P. aeruginosa* increased at temperature of 15 °C to 30 °C. Based on this finding, all the WVM drinking water temperature result fall within this range except for 3 WVM from Kota Samarahan and 6 WVM from Kuching.

This study emphasis the bacteriological contamination of WVM, specifically *P. aeruginosa*. *Pseudomonas aeruginosa* was highlighted in this study as it has been isolated from potable water as well as insufficiently treated water even in the absence of faecal coliforms

(Highsmith & Abshire, 1975). Enumeration of *P. aeruginosa* was done using most probable number (MPN) method. Single dilution MPN test was selected for the enumeration as it is used for the examination of samples with low contamination level such as potable water (da Silva et al., 2018).

Based on the MPN result for *P. aeruginosa* in drinking water from WVM, majority of the WVM complied to the standard set by WHO 2011 guidelines for drinking water quality which is 0 MPN/100 mL with 68% negative for the presence of *P. aeruginosa*. However, this leaves another 32% not in compliance to the bacteriological standard for *P. aeruginosa*. The presence of *P. aeruginosa* in public supply of drinking water is alarming as it is an opportunistic pathogen that can infect immunocompromised individuals. Although it rarely infects healthy individuals, the presence of *P. aeruginosa* in drinking water indicates a failure in the WVM filtration system or maintenance and hygiene practice when handling WVM. This highlights the importance of safety features and regulations for the WVM.

The polymerase chain reaction (PCR) analysis was used to detect the presence of the gene that encode for exotoxin A (ETA) a toxin protein which inhibits protein of host cell produced by majority of *P. aeruginosa* (Lee et al., 2005). Out of 103 isolates of *P. aeruginosa*, including replicate dilution from MPN test, 41% were detected to have the ETA gene which is one of the main factors that contribute to its pathogenicity. *Pseudomonas aeruginosa* antibiotic resistant profile was also studied.

Pseudomonas aeruginosa resistance to meropenem (MEM10), ciprofloxacin (CIP5) and ceftazidime (CAZ30) was studied as these 3 antibiotics were some of the more commonly used antibiotics to treat *P. aeruginosa* infections (Moore et al., 2016). It was examined that resistance to ciprofloxacin was the highest. Whereas meropenem was the most effective in preventing *P.*

aeruginosa growth. The results showed similar findings to a study done in Czech Republic in *P. aeruginosa* from hospital wastewater, where the isolated *P. aeruginosa* showed the highest resistance to ciprofloxacin (Roulová et al., 2022). Ciprofloxacin is the most effective fluoroquinolone and the most widely used against *P. aeruginosa* (Rehman et al., 2018). Its resistance to this antibiotic might be due to its widespread use to treat *P. aeruginosa* infection.

The biofilm forming capacity of 6 random isolates of *P. aeruginosa* isolated from this study was studied. Majority of the of the isolates showed moderate biofilm forming capacity except 1 that was non-adherent. Although the isolates only exhibit moderate strength in biofilm formation, there is still risk of it progressing as biofilm strength is affected by factors such as pH, temperature, as well as influence from already-existing biofilm community (Duoterelo et al., 2017). Biofilm could also create antibiotic tolerant community as shown by Azuma et al. (2013), where biofilm was positively correlated to antibiotic tolerance. *P. aeruginosa* ability to form biofilm could further assist its colonization in WVM.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

Overall, WVM in Kota Samarahan and Kuching showed unsatisfactory physical condition and environmental condition with 30% water vending machine (WVM) with absence of dispensing area cover and 60% situated near water drainage. Although majority of the WVM were well maintained, 40% did not undergo regular maintenance and 78% were unregistered. A total of 32% were not in compliance with the *Pseudomonas aeruginosa* drinking water standard set in the World Health Organization (WHO) guideline 2011. As for the pH and total dissolved solids (TDS), WVM from Kota Samarahan and Kuching were in compliance with the Malaysian Food Act 1983 with only a few WVM from Kuching exceeding the maximum pH limit by 9.29% at most. Both cities showed varied outcome regarding these aspects. Kota Samarahan showing better results for pH value and most probable number (MPN) results whereas Kuching showed better results for physical condition of WVM and regular maintenance. While both cities showed excellent total dissolved solids (TDS) rating but unsatisfactory surrounding conditions as well as presence of a license to operate WVM. These findings highlight the importance of regulations and constant monitoring of WVM as these are available for public use and can lead to health risks.

Pseudomonas aeruginosa isolated were also studied for presence the gene encoding the exotoxin A (ETA), resistance profile and biofilm forming capacities. A total of 41% of the isolates were positive for the ETA gene. The *P. aeruginosa* isolated from WVM also showed the highest resistant to one of the most widely used antibiotic, ciprofloxacin with none susceptible to it. In addition, some of the isolated exhibited moderate strength for biofilm

forming capacity. All these factors contribute to the pathogenicity of *P. aeruginosa*. The findings from this study revealed a potential virulent and hard to treat population which would pose a challenge to control and manage.

In conclusion, the findings in this study provides an understanding to the level of contamination of WVM in Kota Samarahan and Kuching and the potential risk posed by *P. aeruginosa* in public supply of drinking water. It is recommended that regulatory measures for WVM needs to be improved and strengthen with stricter monitoring installation, maintenance and physical condition of the WVM. Regular inspections and regular maintenance schedule is needed to ensure that the WVM comply to the regulations and is safe for public use. The WVM maintenance must also be improved with routine cleaning, disinfection and monitoring of the drinking water parameters. The public should also be highlighted on the potential health risk from WVM and the proper hygiene practice using WVM. It is also recommended that an alternative treatment regimen in treating *P. aeruginosa* infection should be developed.

This study aimed to evaluate the WVM in Kota Samarahan and Kuching as well as to profile the *P. aeruginosa* isolated from WVM, however, there were several limitations in order to fully address the objectives of this study. Firstly, a bigger sample size is needed covering a wider geographical area to provide a more comprehensive understanding into this problem with more physical and chemical parameters to study the drinking water quality. A more detailed analysis on the occurrence of *P. aeruginosa* and its correlation to WVM conditions is needed. A more extensive study on the resistance profile and biofilm forming capacity of *P. aeruginosa* is needed to further assess the potential health risk associated to *P. aeruginosa* from WVM. Moreover, a more comprehensive study design with multiple sampling time points is recommended to monitor the WVM and drinking water quality.

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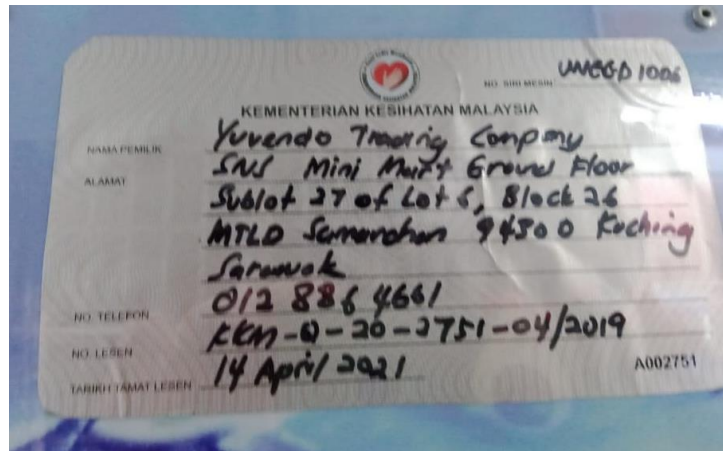
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APPENDICES

Appendix A: Journal Publication

1. Elexson, N., **Sabrina, H.**, Dalene, L., Eddy, B., Nurul, F. R., Nasra, P., Grace, P., Nick, L., Amirah, Z. J., Nur, D. Z., Dayang, N. A. B., Manju, S., & Tunung, R. (2022). Assessment of *Pseudomonas aeruginosa* biofilm-forming capacities from drinking water in water vending machine. *Food Research*, 6(3), 76-83.

Appendix B: WVM data



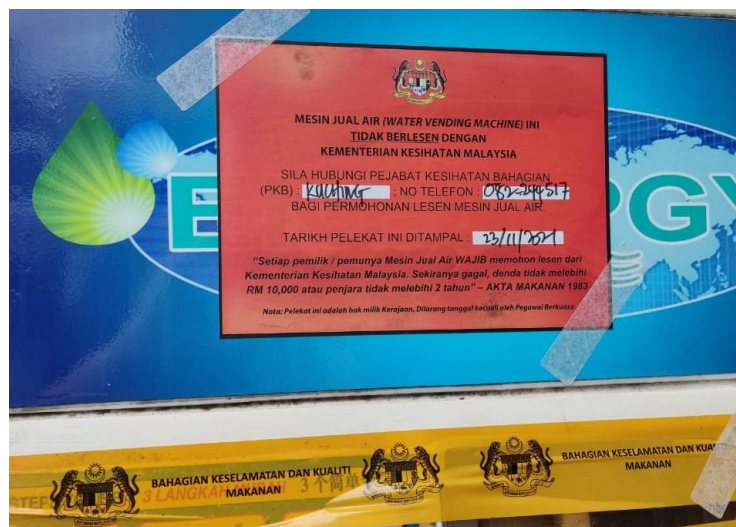
License of WVM displayed



Maintenance sticker of WVM

[illegible]

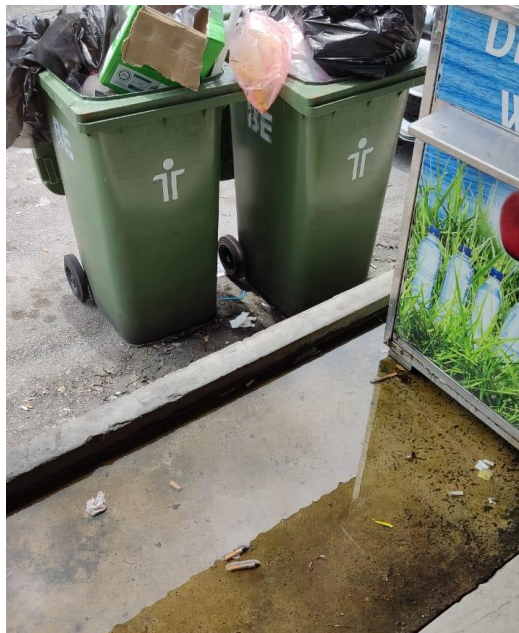
Maintenance checklist of WVM



Unlicensed WVM ceased by MOH



Litter inside dispensing area of WVM



WVM in unsanitary location near puddle of water and next to garbage bin



WVM without dispensing area cover and situated near an open drain