

## Microbiological analysis of drinking water from water vending machines

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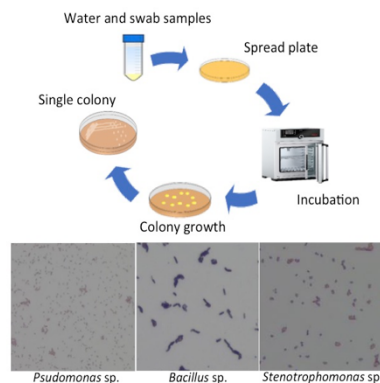
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### Graphical abstract



### Abstract

Commercial water vending machines are gaining popularity nowadays among the general public, probably due to the ease of obtaining clean drinking water. However, improper maintenance of the machines can lead to bacterial contamination. Hence, this study aimed to investigate and determine the microbiological characteristics of drinking water from Water Vending Machines (WVM) by isolating and characterizing culturable bacteria in the water and nozzle swab samples. The samples were obtained from WVM at eight different locations around Johor Bahru, Johor, Malaysia. Several unique bacterial isolates were found, from both Gram-positive and Gram-negative groups. Polymerase chain reaction amplification and 16S rRNA sequence analysis suggested that these isolates are from *Pseudomonas*, *Bacillus*, and *Stenotrophomonas* genera. *In situ* water quality tests which include pH, conductivity, and total dissolved solids were also conducted. Two samples from the inlet source have pH and conductivity values slightly above the reference values stipulated in drinking water regulations. The findings presented here suggest the importance of regular service maintenance of the WVM to ensure that the water samples meet the standard stipulated by the authority.

**Keywords:** Bacterial isolation, drinking water, water vending machines, public health

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## INTRODUCTION

Transmission of waterborne diseases is still a vital issue nowadays, despite multiple efforts worldwide to produce safer drinking water [1]. Low water quality can be due to contamination at the source, human error, and technical failures [2]. Microbial contamination is among the most critical issue as it can directly affect the human health [3]. Drinking water has been reported to contain heterotrophic bacteria, including opportunistic pathogens that are able to cause both nosocomial and community-acquired infections [4]. Up to 663 million people still lack access to good drinking water, and 1.8 billion people still use drinking water that is potentially contaminated [5, 6].

Water vending machines (WVM) are rapidly gaining popularities, especially in Malaysia. These machines typically require service maintenance, that include annual replacement of ultraviolet lamp, frequent change of carbon filters, and replacement of the reverse-osmosis membrane after a year of operation [4]. Nevertheless, water quality from WVM may quickly deteriorate due to day to day activities in the surrounding area [7]. In addition, the machines can be contaminated with heterotrophic bacteria, especially its inner surface or nozzle dispenser [8].

Previously, six out of 14 samples randomly picked from several places in Kuala Lumpur, Malaysia were found to have either coliform or *Escherichia coli* and did not contain free chlorine for disinfection purposes [9]. Similar cases were also reported in Los Angeles [10]

and Dubai [11]. All cases were concluded to be related to the poor maintenance and improper hygienic conditions of the machines. Thus, this study reports on the performance and characteristics of WVM in different locations around Johor Bahru, Malaysia in terms of physical, chemical, and microbiological parameters.

## EXPERIMENTAL

### Sampling

Water samples were collected from eight different locations in the vicinity of Universiti Teknologi Malaysia (UTM), four within UTM and the remaining four were collected from residential areas namely Taman Sri Pulai Perdana (TSP), Taman Universiti (TU), Taman Mutiara Rini (TR), and Taman Tun Aminah (TA) (Table 1).

From each WVM, samples were collected from dispense nozzle of the machines represented as (N), using a sterile cotton swab by gently and firmly swabbing inside the surface. The swab was then kept in a sterile 15 mL Falcon tube containing 3 mL Nutrient Broth (NB) for bacterial cultivation. The other samples taken were outlet sample (O), collected in a 100 mL sterile Falcon tube and kept cold. For the *in situ* measurements, samples were collected in 1 L plastic container from the inlet water (main source) and the outlet (drains). The samples were

immediately transported to the laboratory for bacterial cultivation, and stored at 4 °C prior to use.

**Table 1** Water sample characteristics based on *in situ* analysis from eight WVM.

Sample (Location)	Type	pH	Conductivity (µS/cm)	TDS (mg/L)
S1 (UTM)	Inlet	8.0±0.1	236.3±2.6	51.0±1.1
	Outlet	8.0±0.1	251.0±3.0	52.7±0.7
S2 (UTM)	Inlet	8.0±0.0	244.0±1.1	52.0±0.7
	Outlet	8.0±0.1	249.0±0.7	53.0±0.0
S3 (UTM)	Inlet	8.0±0.0	252.0±1.1	53.0±0.7
	Outlet	7.0±0.0	252.0±0.7	53.0±0.0
S4 (UTM)	Inlet	8.0±0.0	274.0±5.6	53.0±0.7
	Outlet	7.0±0.0	257.0±4.1	52.0±0.7
S5 (TSP)	Inlet	9.0±0.0	264.0±3.3	53.0±0.0
	Outlet	8.0±0.0	280.7±2.4	55.0±0.2
S6 (TU)	Inlet	8.0±0.0	272.0±2.4	53.0±0.7
	Outlet	8.0±0.1	210.0±1.7	39.0±0.7
S7 (TR)	Inlet	8.0±0.0	275.0±4.3	52.0±0.7
	Outlet	7.0±0.1	220.0±6.9	50.0±1.3
S8 (TA)	Inlet	7.0±0.0	1252.0±7.9	241.0±2.0
	Outlet	7.0±0.1	207.0±3.0	48.0±2.4

\*TDS: Total Dissolved Solids

### In Situ analysis

pH was analyzed using HM Digital pH-200, conductivity was analyzed using KEDIDA Pocket Conductivity Meter CT-3031, and total dissolved solids (TDS) were measured using SenION 5 (HACH). The measurements were done in triplicates.

### Bacterial cultivation

Both the collected samples from the swab dispense nozzle and the outlet source were used as culture source. About 0.1 mL of each sample was spread onto Nutrient Agar (NA) and incubated at 37 °C overnight. Distinct single colonies were sub-cultured on a fresh NA plate by serial streaking and incubated overnight at 37 °C. The bacterial isolates were sub-cultured again until a single pure isolate was obtained.

### Bacterial identification

The pure isolates were identified through morphological observation, Gram staining, and 16S rRNA gene analysis. Each single colony was inoculated in NB and incubated at 30 °C overnight with shaking at 200 rpm until the optical density at 600 nm reached 0.7–1.0. Genomic DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega) according to the manufacturer's instruction.

The 16S rRNA gene of the bacterial isolate was amplified using a set of universal primers: forward primer (27F) (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer (1492R) (5'-CGTTACCTTGTTACGACTT 3'). PCR amplification was conducted with a mixture component containing 25 µL total reaction volume. All the other reaction components include 12.5 µL GoTaq Green Master Mix, 0.75 µL forward primer, 0.75 µL reverse primer, 2 µL DNA template, and 25 µL nuclease-free water. Amplification was done in a thermocycler (GeneAmp PCR System 9700) using the following cycle: initial denaturation at 95 °C for 4 min; 30 cycles of denaturation for 1 min at 95 °C, annealing for 30 secs at 55 °C, extension for 2 min at 72 °C; and a final extension at 72 °C for 5 min.

The PCR product was visualized using gel electrophoresis under UV illumination using Gene Flash Gel Documentation System. The amplified 16S rRNA gene was sent to First Base Laboratories (Malaysia) Sdn. Bhd. for sequencing. The resulting sequences were then subjected to Basic Local Alignment Search Tool (BLAST) analysis. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6 [12] and ClustalW.

## RESULTS AND DISCUSSION

The values of the selected water parameters (pH, conductivity, and TDS) for all the samples are shown in Table 1. Several standards were used for comparison, namely the National Water Quality Standards for Malaysia (NWQSM), Malaysian Drinking Water Standard (MDWS), World Health Organisation (WHO), and United States Environmental Protection Agency (US EPA).

The pH of drinking water as recommended by WHO and US EPA lies between 6.5–8.5 [7], which corresponds to that of NWQSM and MDWS. In this study, pH of the water samples is in the range of 7–9. Only one of the samples from inlet source of the WVM exceeds the acceptable limit, namely the S5 inlet water, which may affect its quality [13]. Conductivity varied with values between 200–1260 µS/cm (except for S8 inlet), which are consistent with NQSM, WHO, and US EPA which limit between 10 and 1000 µS/cm. Water conductivity is usually affected by inorganic dissolved solids such as aluminium, cations, calcium, chloride, iron, magnesium, nitrate, sodium, and sulphates [14]. It can also be influenced by organic compounds such as phenols, oil, alcohol, and sugar. Additionally, temperature can also affect water conductivity. The TDS values ranged from around 40–250 mg/L, for all samples. Standards by NQSM, MDWQ, WHO and US EPA state that the TDS value should not exceed 1000 mg/L. TDS is usually comprised of inorganic salts and small amount of organic matter dissolved in the water.

Generally, all water samples from the WVM comply with the standards set, except for one inlet sample. The deviation is probably due to the affected water supply quality in the specific area. Treatments provided by the WVM has also successfully achieved the required physicochemical parameters as outlined in drinking water standards. These parameters are critical, as they may affect bacterial growth on the surface of the machines [15].

A total of 20 isolates from both Gram-negative and Gram-positive groups with different morphological features have been successfully cultivated from the WVM samples. PCR amplification of the 16S rRNA gene and sequencing analysis has successfully been carried out to determine the identity of the isolates, which closely related to three different genera: *Bacillus*, *Pseudomonas*, and *Stenotrophomonas* (Table 2). Half of the isolates are closely-related to *Stenotrophomonas* sp., 40% are *Bacillus* sp., and 10% are *Pseudomonas* sp. While it is difficult to know the effects of the presence of these bacteria in the water samples, it is possible that this is caused by inadequate disinfection [8] or poor maintenance [10].

*Stenotrophomonas* is a Gram-negative bacillus, typically found in soil and water and not part of normal human flora. The genus is found to occur ubiquitously in the environment, exists by living freely or plant-associated. It is also an opportunistic pathogen [16]. *Stenotrophomonas* sp. has been isolated from sponges (*Gelliodes* sp.) at Pahang coastal water [17]. *S. maltophilia* can be found in different environmental niches including soils, plant rhizosphere, surface water, wastewater, drinking water and food, and in contaminated medical care fluids [18-20]. It has the ability to form biofilm by adhering to various biotic and abiotic surfaces that may contribute to contamination in municipal water supplies, taps, and drains, water purification systems, air conditioning and other cooling systems, and medical devices [19]. *S. maltophilia* has also been found in water fountains [21]. It is recognized as an emerging opportunistic pathogen causing nosocomial infections in immune-compromised patients [20, 22, 23]. WHO has declared *S. maltophilia* as one of the top drug-resistant pathogens in hospitals worldwide [24]. In our study, S6-10, S8-10, and S4N are closely-related to *S. maltophilia* with 96%, 82%,

and 86% identity, respectively. This warrants a further investigation to confirm the exact species of the isolates.

**Table 2** Identity of the isolated bacteria from the WVM samples.

Sample	Source	Isolate	Closest Similarity (up to genus level)	Identity (%)
S1	Nozzle	S1N	<i>Pseudomonas</i> sp.	91
		S1-1O	<i>Bacillus</i> sp.	91
	Outlet	S1-2O	<i>Stenotrophomonas</i> sp.	86
S2	Nozzle	S2N	<i>Bacillus</i> sp.	97
		S2-1O	<i>Stenotrophomonas</i> sp.	95
	Outlet	S2-2O	<i>Bacillus</i> sp.	98
S3	Nozzle	S3N	<i>Bacillus</i> sp.	93
	Outlet	S3O	<i>Bacillus</i> sp.	91
S4	Nozzle	S4N	<i>Stenotrophomonas</i> sp.	89
	Outlet	S4O	<i>Bacillus</i> sp.	89
S5	Nozzle	S5N	<i>Stenotrophomonas</i> sp.	86
	Outlet	S5O	<i>Stenotrophomonas</i> sp.	96
S6	Nozzle	S6N	<i>Bacillus</i> sp.	91
		S6-1O	<i>Stenotrophomonas</i> sp.	96
	Outlet	S6-2O	<i>Stenotrophomonas</i> sp.	87
S7	Nozzle	S7N	<i>Stenotrophomonas</i> sp.	91
	Outlet	S7O	<i>Pseudomonas</i> sp.	91
S8	Nozzle	S8N	<i>Stenotrophomonas</i> sp.	87
		S8-1O	<i>Stenotrophomonas</i> sp.	82
	Outlet	S8-2O	<i>Bacillus</i> sp.	91

*Bacillus* sp. is ubiquitous in the environment, especially in soil water and air, with optimum growth at 20–40 °C. However, *B. cereus* is also able to produce toxins responsible for foodborne illnesses, causing vomiting or diarrhoea. It can be found in fresh and marine waters, vegetables, fomites, intestinal tract of invertebrates, and decaying organic matter [25]. Due to the wide distribution of the spore in the environment, especially in soils, they can be easily spread out into water, dust, and food [26-28], potentially leading to contamination of water supply and also WVM. *B. cereus* has the ability to produce biofilm [29], and has been found in vending machines [30,31]. In our study, isolates S2N, S3O, S4O, S2-2O, S3N, and S1-1O are found to be closely-related to *B. cereus*.

*Pseudomonas* is another abundant bacteria in the environment [32]. Most of the *Pseudomonas* sp discovered from water are found to be resistant to antimicrobial agents [1]. *P. aeruginosa* has been reported in water dispensers and vending machines [4,11]. Similarly, a study on drinking water samples (bottled water, water cooler units, and tap water) was found to have 2–3% of *P. aeruginosa* [33]. *P. aeruginosa* has the ability to adhere to the surface of WVM such as buttons and nozzles to form biofilms [34].

On the basis of the results obtained, of the 18 samples examined, bacterial colonies were found to be present in all nozzle and outlet samples. Some of these bacteria have the potential to be nosocomial or opportunistic pathogens. Previous studies have reported that *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes* are commonly found in drinking water networks [32, 35], in polluted environments, and clinical samples [36]. Regardless of the location, we can conclude that regular cleaning and maintenance of the WVM is imperative to maintain the quality of the water supplied. Antibacterial materials can also be incorporated into the WVM components to avoid the formation of bacterial biofilm on the surfaces.

## CONCLUSION

The TDS of all the WVM samples were found within the threshold range, while the pH and conductivity were slightly above the permissible limit stipulated by the various regulatory bodies. Microbiology analysis revealed that the majority of the cultivable bacteria present in the samples are from the phylum *Proteobacteria* and *Firmicutes*. Some bacterial species are potentially the ones associated with contaminated water, although a full identification is necessary to confirm the species. Nevertheless, we can conclude that regular cleaning and maintenance of the WVM is critical. The

placement of the machines should also be considered so that it is not easily exposed to sources of contamination.

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