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Bacterial Strain Identification from Drinking Water in Water Treatment Systems

Mustapha Salisu Muhammad[1,2,3,a], Mohd Hafiz Dzarfan Othman[2,b], Mohd Hafiz Puteh[2,c], Nik Ahmad Nizam Nik Malek[1,d], Abdul Razis Saidin[2,e], Abdulhalim bin Mohd Yusof[2,f], Roziana Kamaludin[2,g], Siti Maryam[2,h], Ojo Samuel[2,i], Liew C.M.[2,j], Parvin A.P.[2,k], Nurul Huda[2,l]

[1]Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia

[2]Advanced Membrane Technology Research Centre (AMTEC), Universiti Teknologi Malaysia, 81310 UTM Skudai, Johor, Malaysia

[3]Department of Biological Science (SOSE), Federal College of Education (Tech), Bichi, P.M.B 3473, Bichi, Kano State, Nigeria

[a]mustysulu@gmail.com, *[b]hafiz@petroleum.utm.my, [c]mhafizputeh@utm.my, [d]niknizam@utm.my, [e]razis@utm.my, [f]halimy.utm@gmail.com, [g]rozianakamaludin7@gmail.com, [h]maryamjasman@gmail.com, [i]femioctober22@gmail.com, [j]c.ming@graduate.utm.my, [k]parvin1999@graduate.utm.my, [l]huda20@graduate.utm.my

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Abstract. The availability of clean and safe water for drinking is essential for human life and existence, which ideally should be suitable for consumption and not contain pathogenic microorganisms, or any contamination leading to pollution. Water treatment systems are integral to modern water purification processes, yet they are frequently challenged by biofouling. Biofouling continues to be a major obstacle in water treatment systems, resulting in decreased efficiency, higher energy usage, and increased operational expenses. Therefore, this study aimed to determine the bacteriological characteristics of drinking water by isolating and identifying bacterial strains from water samples contributing to biofouling. Samples were obtained from water treatment systems (WTS) at different locations in Malaysia. Selected isolates of unique bacterial strains were identified and assigned their accession numbers. Phylogenetic analysis revealed that these isolates were related to *Bacillus cereus*, *Stenotrophomonas maltophilia*, and *Stenotrophomonas pavanii* species, suggesting that deterioration in water quality from the source, human error, and technical failure may cause decline even if the most desirable treatment systems and disinfection procedures applied.

Introduction

Bacterial contamination of water and food is the most pressing issue that leads to infection and diseases in humans. The worsening of water quality generally involves microbial risks, as most recorded health problems in relation to water results from microbial contamination [1, 2]. Nowadays, outbreaks of waterborne diseases have become a serious issue, despite worldwide efforts and newer technologies being used for the production of clean and safe drinking water [3, 4]. The availability of safe drinking water constitutes a global challenge with an effort summoned by the authorities for water quantity assurance and quality [5]. Water is a highly important resource necessary for life sustainment, and safe water for drinking is a basic need for human life [6-8].

In addition, drinking water has been a course of antibiotic-resistant organisms spread out among human and animal populations, with proliferation of resistance genes in natural bacterial ecosystems [9-11]. WTSs have transformed the water purification process with effective and affordable techniques for removing contaminants, including desalination, wastewater treatment, and

portable water production. These processes encompass reverse osmosis (RO), ultrafiltration (UF), nanofiltration (NF), microfiltration (MF) and membrane distillation (MD) which are extensively implemented across various industries for water purification [12]. Despite their versatility, microorganisms predominantly bacteria have a tendency to channel on the filter surface, and other parts by producing extracellular polymeric substances (EPS) forming biofilm. Biofouling leads to increased energy consumption, frequent filter cleaning, reduced filter lifespan, and compromised water quality. Nevertheless, water quality from the WTS may quickly change due to day to day activities within the surrounding arena of the WTS [13, 14]. The filters in WTS can be accumulated by Coliform bacteria, yielding massive concentrations of microorganism in the whole water [15, 16]. Over time, the build-up of biofilm on the filter may lead to permanent harm, requiring regular cleaning and replacement.

However, according to [17], some heterotrophic bacteria can stick fast to the surface of WTS such as buttons, spouts to form biofilms, reducing the filter life span and compromised water quality. The water treatment machine can be contaminated with heterotrophic bacteria from its inner surface or spout dispenser [15]. In light of these repercussions, it is imperative to ascertain and delineate the bacterial strains implicated in biofouling in order to formulate more efficacious control strategies. For this study, the quality of drinking water from WTS was examined through bacteriological analysis in the vicinity of Malaysia. This is aimed to provide a comprehensive assessment of the cleanliness and water quality of these WTS. Moreover, the discovery of this research can be of great importance to public health and humanity.

Experimental

Sampling

The samples were collected from five WTS in different locations of Skudai vicinity sits at the southern tip of the Malay Peninsula. Subsequently, from each WTS1, WTS2, WTS3, WTS4, WTS5 water samples were collected from the spout inside a 200-mL sterile schott bottle and kept inside a box containing ice to optimize the temperature. The collected samples were, immediately transported to the laboratory for microbial analysis. The temperature of the samples was maintained at 30ºC. The samples were stored in an incubator at an optimal temperature of 37ºC prior to use.

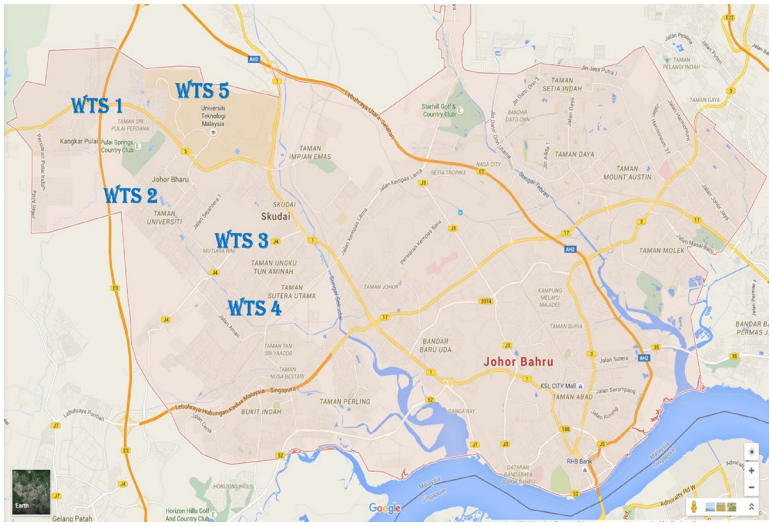


Fig. 1. Geographical location of collected sample.

Bacterial Isolation

Samples from drain spouts were used as the culture source. Then, 0.1 mL of each sample was added to a culture plate containing nutrient agar for inoculation of bacterial isolates over the solid agar surface. Inoculation was performed inside a laminar flow to prevent contamination. The samples were spread on an agar plate under aseptic conditions using a spreader. The plates were sealed and incubated overnight at 37°C for bacterial growth. After overnight culture, colonies were

observed, and prominent single colonies were inoculated on a new agar plate by back and forth streaking using a sterile inoculation loop. The plates were incubated overnight at 37ºC. The bacterial isolates were subculture until a single pure isolate was obtained on an agar plate.

Identification of Isolated Bacteria

The isolated bacterial cultures were identified by morphological observation, Gram staining, and 16S rRNA GENE analysis. Phylogenetic and molecular evolutionary analysis were conducted using MEGA version 6 [18]. Analysis was conducted to study the relationship between the isolate sequence and other known sequences based on their 16S rRNA partial sequences. The 16S rRNA gene sequence was aligned using ClustalW with other sequences from the BLAST results and one outgroup. An isolated bacterial phylogenetic tree was constructed using the neighbor-joining method.

16S rRNA Gene Analysis

The culture was prepared in a 50-mL Falcon tube and incubated at 30°C overnight with shaking at 200 rpm to obtain pure and physiologically stable cells before DNA extraction. Genomic DNA (gDNA) was extracted from bacteria, followed by amplification of the 16S rRNA gene using the polymerase chain reaction protocol with designated primers to produce large amounts of genes. Agarose gel electrophoresis was performed to analyze the outcomes of both DNA extraction and gene amplification for quality and quantity of the gDNA, which was assessed spectrophotometrically using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA).

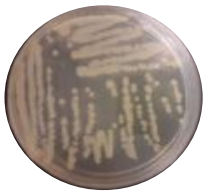
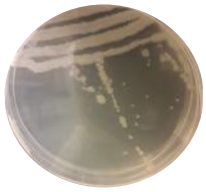
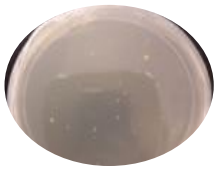
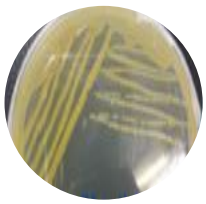
Table 1. DNA concentrations and purity of extracted DNA from 6 selected isolates.

Sample ID	Nucleic Acid Concentration	260/280	260/230	Sample type
S1	159.5 ng/µl	**1.94**	1.94	DNA
S2	274.6 ng/µl	**2.12**	1.93	DNA
S5	278.5 ng/µl	**2.01**	1.69	DNA
S7	329.8 ng/µl	**2.00**	1.71	DNA
S9	273.4 ng/µl	**1.98**	1.66	DNA
S10	563.8 ng/µl	**1.87**	1.51	DNA

Results and Discussion

Based on their individual morphology (Table 2), the isolates were successfully cultivated from the drinking water samples, with six out of the ten being selected for further study. Also, from the Gram staining results, the isolates were identified as Gram-positive and Gram-negative bacteria with rod shape appearance from all strains alongside their morphological identification. From Table 1, for an optimum PCR amplification, the acceptable DNA or relative purity at 260/280 ratio per definition should fall within the limit of 1.8-2.0 [19, 20]. Thus, these values are considered efficient and effective for downstream nucleic acid amplification methods such as PCR, real-time PCR, and DNA sequencing because they fall within the specified limit. The purity of extracted DNA is critical because of its influence on the success of the amplification outcome [21].

Table 1. Morphological characteristics of the bacteria isolated from WVMs.

Sample label	Bacteria Isolate	Morphology	Staining	
			Positive	Negative
S1		Shape : Circular Size : Small Color : Beige Opacity : Opaque Elevation : Convex Margin : Entire Surface : Smooth Appearance: Glister		
S2		Shape : A.Circular Size : Moderate Color : Cream Opacity : Opaque Elevation: Flat Margin : Undulate Surface : Concentric Appearance: Dull		
S5		Shape : Circular Size : Small Color : Cream Opacity : Opaque Elevation : Convex Margin : Entire Surface : Smooth Appearance: Glister		
S7		Shape : Irregular Size : Large Color : Cream Opacity :Transluscent Elevation : Flat Margin : Undulate Surface : Concentric Appearance: Dull		
S9		Shape : Circular Size : Punctiform Color : Yellow Opacity: Transluscent Elevation : Convex Margin : Entire Surface : Smooth Appearance:Glister		
S10		Shape : Circular Size : Moderate Color : Yellow Opacity : Opaque Elevation: Raised Margin : Entire Surface : Smooth Appearance:Glister		

The amplified PCR products were initially confirmed visually by producing light DNA fragment bands of approximately 1500 bp compared with the standard DNA ladder shown in figure 2. The appearance of a light DNA band of approximately 1500 bp on the gel indicated that amplification of the target gene was achieved. In accordance with [19, 22], 16S rRNA gene should have an approximate length of 1500 bp. By amplifying a single copy of DNA, PCR will generate

thousands to millions of copies of DNA. This is an excellent method for detecting nucleic acids, disease identification, eukaryotic species, human identification, forensic science, and pathogens identification [23].

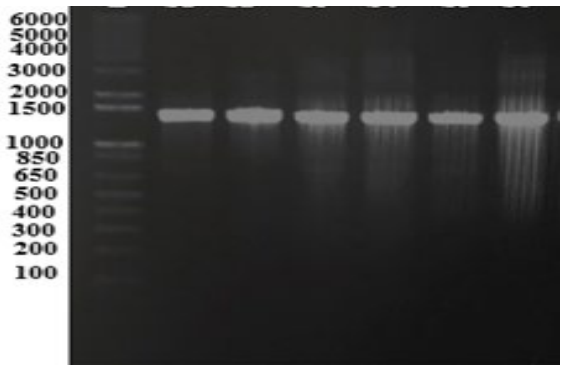


Fig. 2. Gel electrophoresis showing successful PCR amplification of isolated bacteria unknown to drinking water. Lane 1 represents the 1-kb DNA ladder. Other lanes represent amplified 16S rRNA genes of the isolates from water.

After compiling and performing a comparative analysis of the conserved regions of the 16S rRNA from the isolates against the GenBank database. The results from BLAST showed that out of the isolates, S2, and S7, are probably belong to Bacillus genus, S5, S9, S10, and S1 under the genus Stenotrophomonas. The 16S rRNA partial gene sequences of the six species from two genera were then sent for the accession number, as shown in Table 3. However, two of the strains identified from the S1 and S2 isolates had no assigned accession numbers, resulting from low similarity to other 16S ribosomal RNA sequences in the database. In line with [24, 25], a sequence chromatogram always has some noise, such as low-intensity signals arising from baseline or from baseline of nonspecific primer binding. This can reduce the quality and success of the subsequent analyses. Therefore, strains with accession numbers were subjected to phylogenetic analysis.

Table 3. Accession numbers for the isolated strains.

Isolates	Query length	Identity	Accession No
S1	1314	Stenotrophomonas sp. strain S1	Not assigned
S2	1451	Bacillus sp. strain S2	Not assigned
S5	1454	Stenotrophomonas sp. strain S5	MG563676
S7	1499	Bacillus sp strain S7	MG563677
S9	1538	Stenotrophomonas sp strain S9	MG563678
S10	1446	Stenotrophomonas sp strain S10	MG563679

The phylogenetic affiliation and, comparative 16S rRNA gene sequences of 15 consecutive closely related organisms obtained from the GenBank database for each of the four isolated bacterial strains were aligned individually. The bootstrap values are given at the branching points by employing the bootstrap method for the confidence levels of the phylogeny. This was performed to evaluate the tree confidence estimate for branch support. Indications of values above 50% in the phylogenetic tree indicate the reliability of the tree. This is in accordance with a report (inferring evolutionary trees with strong combinatorial evidence) stating that a bootstrap value of less than 50% in a phylogenetic tree has no confidence level and can be considered as unreliable [26-28]. Fujiensis was used as an outgroup species to root a phylogenetic tree. The involvement of outgroups in the phylogenetic tree construction plays a significant role in distinguishing between the two different genera. The bacterial strain S5 was phylogenetically affiliated with the same branch and formed a clade with Stenotrophomonas maltophila (91% similarity), strain S7, and Bacillus cereus (98% similarity), S9 to Stenotrophomonas pavanii (96% similarity), and S10 to Stenotrophomonas maltophilia (96% similarity), with bootstrap values above 50%, providing strong support for the close relationship between the two strains. The bootstrap test assesses the internal consistency of a molecular dataset by determining whether slightly altered alignments support the same phylogenetic clades [29].

In compliance with the comparative analysis of the conserved region of the 16S rRNA gene sequence of all the strains against the GenBank database, the results revealed that the bacterial strains have a relatively good similarity of more than 70% with the known species. This is in line with the report of [30-33] stating that an identity of more than 70% DNA-DNA similarity can be defined as genospecies according to bacterial taxonomists. In addition, it was clarified that strains with less than 95% similarity can be regarded as novel species [34]. Therefore, based on our findings, strains S1 (88%), S2, and S5 (9 1%) could be considered as separate species that may be novel. Then, according to [35-37], to define a bacterial species, a threshold range of 95-97% is generally used. Thus, strains S7, S9, and S10 exhibited 98%, 96%, and 96% gene similarity, respectively, according to the BLAST results. These similarities were shared with strains ATCC 14579, LMG 25348, and ATCC 19861 of B. cereus, S. pavanii, and S. maltophilia.

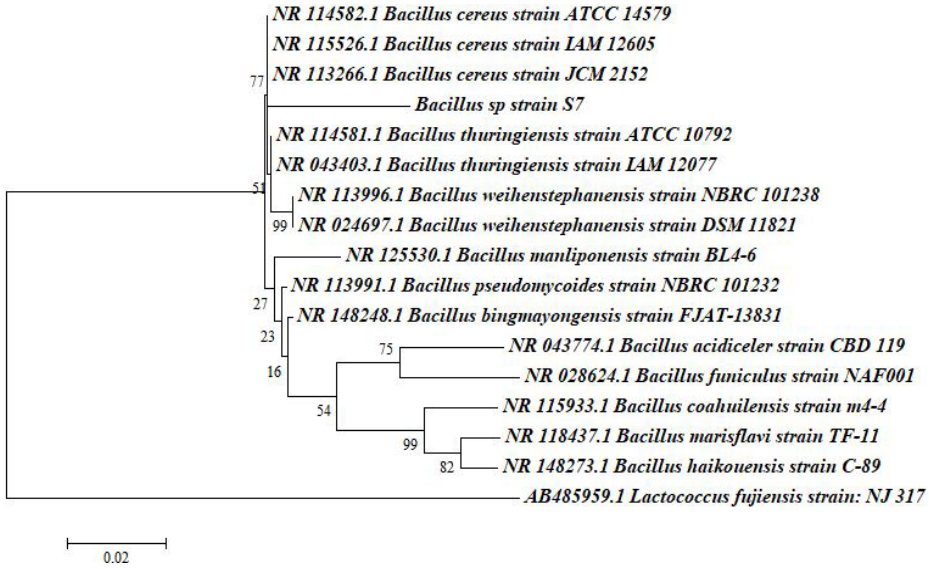


Fig. 3. Phylogenetic tree highlighting the position of Bacillus sp. strain S7 and its close relatives based on the 16S rRNA gene sequence.

The bacterial genus Bacillus encompasses different species that are widely distributed in nature and in soil, water and air, proliferating between 20ºC and 40ºC. Bacillus genus is composed of aerobic or facultatively anaerobic Gram-positive bacilli that create spores, are typically catalase positive, and display motility [38]. The species B. cereus inhabit numerous environments including fresh and marine waters, vegetables and fomites, the intestinal tract of invertebrates, and decaying organic matter [39-42].Owing to the widespread distribution of this bacterium spore in the environment, especially in soils and dust, they can be easily spread out into water and food [39, 42-44]. This might be a major source of contamination in the water treatment system, which can interrupt the microbial quality of drinking water. The presence of this substance in potable water may present significant health hazards due to its capability to induce foodborne illnesses and various other infections. Although food poisoning is typically the main concern with Bacillus cereus, it can also result in more serious infections, particularly in people with weakened immune systems [42]. In a finding by [45] indicated that B. cereus is commonly found in farming areas, where it can percolate into the groundwater during rainfall, suggesting that this vital source of drinking water could also act as a reservoir for Bacillus cereus. Bacillus cereus spores are strong and can withstand treatment methods such as filtration and chlorination, which are frequently employed to clean drinking water [46]. This remarkable durability facilitates their continued presence in drinking water, thereby posing a risk of contamination. Previous reports have indicated that B. cereus is capable of producing biofilms [47-49]. Biofilms, intricate bacterial communities that stick to surfaces and are surrounded by a protective extracellular matrix, have the ability to reintroduce bacteria back into the water treatment system. For instance, Bacillus spp. were found in different water sources, including two drinking water sources, with different levels of spores detected, as demonstrated in a study [50]. Similarly, report from South Africa highlights the

presence of Bacillus strains in drinking water, attributed to human activities [51]. From the short review above, key findings emerge on the isolated sample S7 strain 7 is likely a species of B. cereus present in the WTS samples. This is important finding in the understanding of water quality from treatment systems. Therefore, effective monitoring and risk assessment strategies are necessary to prevent potential health risks associated with the consumption of Bacillus cereus in drinking water [52]

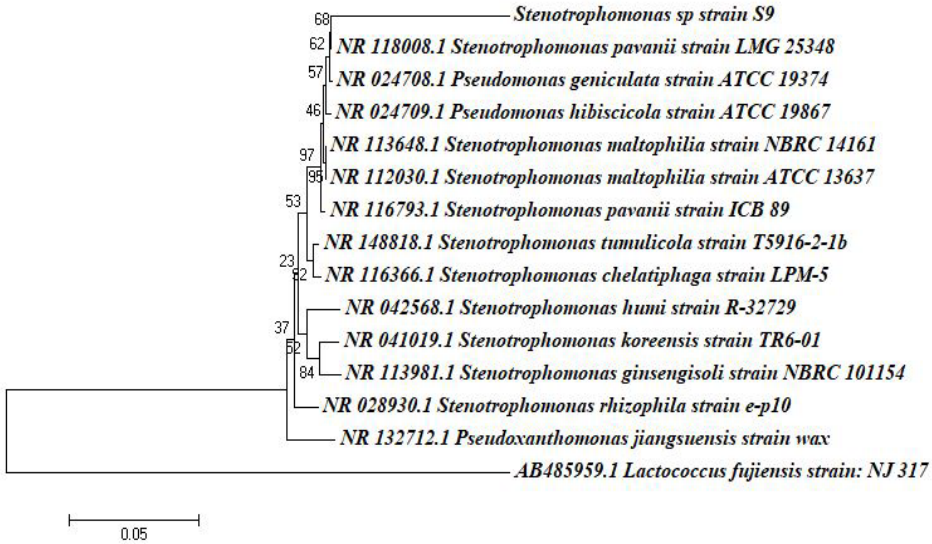


Fig. 4. Phylogenetic tree highlighting the position of Stenotrophomonas sp. strain S9 and its close relatives based on the 16S rRNA gene sequence.

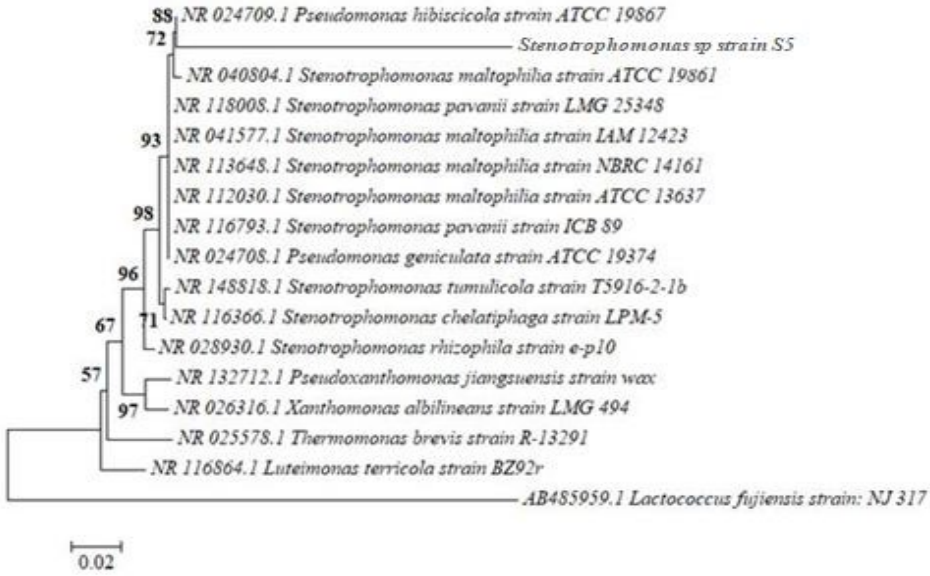


Fig. 5. Phylogenetic tree highlighting the position of Stenotrophomonas sp. strain S5 and its close relative based on the 16S rRNA gene sequence.

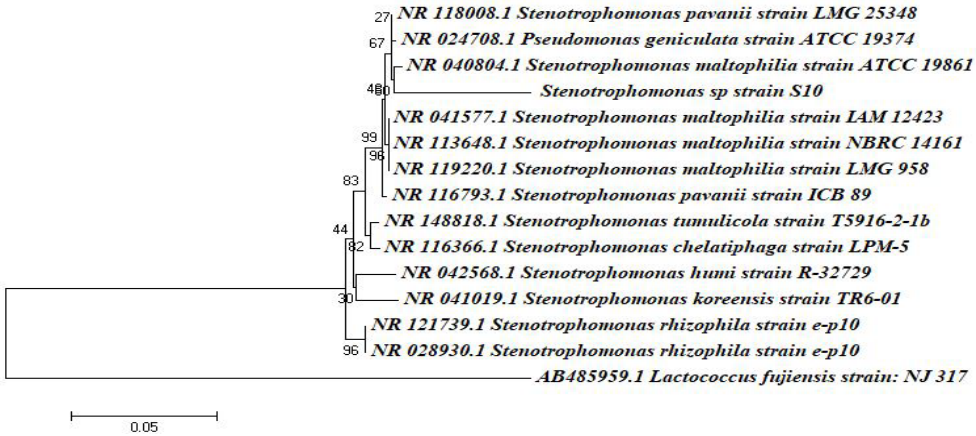


Fig. 6. Phylogenetic tree highlighting the position of Stenotrophomonas sp. strain S10 and its close relatives based on the 16S rRNA gene sequence.

Stenotrophomonas pavanii is a type of bacteria that is rod-shaped, non-spore former and belongs to the Stenotrophomonas genus. Initially, it was discovered in the stalk of sugar cane in Brazil and is known for its ability to fix nitrogen [53]. This organism can form biofilms in water distribution systems, making disinfection efforts more difficult and enabling the survival of bacteria that would likely be eliminated in treatment processes [54]. The risk of drinking water safety is elevated by the ability of S. pavanii to host pathogenic strains within biofilms [55]. These biofilms can attach to internal WTS components, like water tanks, filters and tubing, complicating treatment efforts and enabling bacteria to remain even after post-cleaning. As a result, it is found in a variety of aquatic environments, including treated wastewater and potential drinking water sources. A study revealed that Stenotrophomonas spp. made up a significant portion of the bacterial isolates, with concentrations around 10.2 CFU/mL in treated wastewater and varying levels in natural water sources like ponds and rivers [55]. The discovery of S. pavanii has implications for the safety and quality of water. The discovery of S. pavanii has significant implications for water safety, quality and human health. If infections arise, treatment options can be complicated due to certain strains of S. pavanii developing resistance to various antibiotics [56]. Research has demonstrated that water vending machine can contain different types of bacteria, such as those belonging to the Stenotrophomonas group [57]. The present study from [58] confirmed about S. pavanii can affect water quality in vending machines in several ways, primarily through its ability to form biofilms and its potential as an opportunistic pathogen. In consonance with our findings, the isolate with the sample label S9 strain S9 sourced from WTS drinking water with 96% similarity to Stenotrophomonas pavanii strain LMG 25348 from the GenBank database can probably be characterized as Stenotrophomonas sp. strain S9. Another promising finding was that, [59] isolated the same strain LMG 25348 from sponges (Gelliodes sp.) in Pahang coastal waters. This correlates with [60], who identified same Stenotrophomonas pavanii strain LMG 25348 as an isolate from the body fluid of an insect (butterfly) in England. Based on this review, we hypothesize that the strains have the closest homology. The identification of the identical LMG 25348 strain suggests a noteworthy ecological connection. This results highlights that the organism can be isolated from WTS drinking water resulting from human act and environmental conditions liaising with the report of its adaptability with various places.

Stenotrophomonas maltophilia isolates are diverse species of gamma proteobacteria that can be found in different environmental niches including soils, plant rhizospheres, surface water, wastewater, drinking water, food, or contaminated medical care fluids [61-63]. A statement from the World Health Organization (WHO), in (WHO; Public health importance of antimicrobial resistance), declared Stenotrophomonas maltophilia as one of the top drug-resistant pathogens in hospitals worldwide [64]. The bacteria can inhabit respiratory tracts and might not necessarily indicate active infection but colonization in critically ill patients [65, 66]. S. maltophilia has been found in multiple environmental sources, including water bodies, raising concerns about potential infection transmission, particularly in healthcare facilities where water contamination may

occur [67]. Isolates from both clinical and environmental sources have demonstrated the ability to survive or grow in treated water and non-carbonated mineral water across varying pH levels and temperatures [68, 69]. The bacterium is capable of growing and forming biofilms in potable water distribution systems, posing a potential infection risk for immunocompromised individuals [69]. Extensive results carried out, show that commonly found in drinking water distribution systems, S. maltophilia has been isolated from tap water, bottled water, and treated water samples [65, 70]. Thus, based on the above enumeration, our results of the isolates from sample labels S10 and S5 were characterized as Stenotrophomonas sp strain S10, and S5 can probably be identified as Stenotrophomonas maltophilia. The organism's ability to thrive in diverse environments raises concerns about the potential for contamination of drinking water supplies through environmental pathways [71]. Studies show that S. maltophilia can be present in different water sources, such as treated wastewater, river water, pond water, and tap water. This found evidence from study conducted by [72] in U.S. University, where samples collected were found S. maltophilia. However, the growing recognition of Stenotrophomonas maltophilia as an environmental organism affecting water treatment systems strongly supports our hypothesis.

The presence of resilient bacterial strains like Stenotrophomonas and Bacillus spp. has major implications for the effectiveness of water treatment systems. Their capacity to form dense, resistant biofilms results in higher energy usage, shorter treatment filters lifespan, and increased operational costs. The inhabitance of biofilm bacteria in drinking water is generally harmless, except for species declared as opportunistic pathogens, and under certain conditions may cause disease [1]. Moreover, biofouling can compromise the quality of treated water, as bacteria within biofilms may release endotoxins or other harmful substances into the water supply. This creates a potential public health risk, particularly in systems intended for potable water treatment. In line with the previous studies [73], indicated that the presence of bacterial biofilm population may be due to improper cleaning or contamination rising from users in his study. This can be explained by the fact that humans are a source of WTS contaminating. As such, in view of our observations, the placement sites of the WTS must be considered in addition to regular cleaning and maintenance. This can be compared with the findings of [74] that water treatment systems kept in open public areas with direct sunlight exposure are high in total coliform bacteria as the increase depends on ambient temperature rise. Because some of the bacteria from our findings produce biofilms, this may be an indication of the presence of potential pathogens from the supply of drinking water.

Conclusion

The microbial quality of all samples was observed and identified by 16S rRNA sequencing and phylogenetic analysis, which reveals two bacterial genera. The appearance of these bacteria can be associated with the contamination of the water supply and environmental sources. These isolates were anthropogenically influenced by the environmental isolates. Meanwhile, these bacteria could be easily distributed to WTS due to frequent usage by humans, as well as to the influence of environmental bacterial sources. Drinking water from WTS’s are confirmed as a medium for bacterial accumulation, and possibly can be transmitted to humans, which should be considered. However, regarding public health, we can conclude that WTSs may represent one of the most important sources leading to the transmission of bacteria to humans. This give a highlights that engineering WTS filters to resist bacterial adhesion, such as through the application of antifouling coatings, or surface texturing that target the specific characteristics of the bacterial strains present could prevent the initial attachment of biofilm-forming bacteria. Also, then WTSs should be periodically tested with proper maintenance, particularly when it is constantly used by people. Educative awareness on personal hygiene should be done to people to avoid cross contaminations cause by human activities. As such, this finding can be used to suggest that regular inspections of WTS are important to maintain the hygienic conditions and avoid microbial contamination.

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