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Identification of potentially pathogenic bacteria in a water distribution system in Vanderbijlpark, South Africa

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Abstract

Knowledge of microorganisms in water distribution systems is vital in determining the final quality of potable water. Water quality appears to decline significantly in the distribution pipes, which is primarily attributed to the existence of biofilms. Biofilms harbour many microorganisms, including heterotrophic plate count bacteria (HPC). HPCs are generally used to measure the microbiological quality of drinking water. This study assessed the presence of HPC bacteria in biofilms obtained from four water distribution sites in Vanderbijlpark, a suburb of Gauteng Province in South Africa. Virulence characteristics, including enzyme analysis and antibiotic resistance of the bacteria, were determined. Biochemical and molecular techniques were used to identify the pathogenic organisms. Water samples were also obtained from Rand Water, the leading supplier of potable to the area, and the Vaal River, the ultimate water source. Fifty-two HPC bacteria were isolated on R2A. Initial screening for potential pathogenic isolates indicated that 76% of the isolates demonstrated β-haemolysis, which is the complete breakdown of the red blood cells on horse blood agar and an indication of pathogenicity. None of the isolates from the final treated water at the treatment works demonstrated β-haemolysis. The enzymes associated with the haemolytic isolates included DNase (31%), lecithinase (17%), coagulase (17%), hyaluronidase (14%), proteinase (14%), gelatinase (6%) and lipase (3%). The antibiotic tests showed that the following percentage of HPC isolates were resistant at these sites: SE1 (33%), Sharpeville (25%), SE3 (25%), CE1 (14%) and the Vaal River (23%). Sequencing of rDNA genes showed that potentially pathogenic bacteria, including *Bacillus* spp., *Klebsiella* spp., *Pseudomonas* sp. and *E. coli* were present in the water distribution system in Vanderbijlpark.

1. Introduction

Identifying heterotrophic plate count (HPC) bacteria in water distribution systems is an excellent method of assessing drinking water quality (Amanidaz *et al*., 2015). Water quality changes in drinking water systems are usually due to microbial processes in distribution pipelines (Prest *et al.,* 2016). Although heterotrophic bacteria occur naturally in the human body, some are considered potential health hazards since they may include opportunistic human pathogenic bacteria (Stine *et al.,* 2005). HPC bacteria usually exist as biofilms and change the aesthetic quality of water, including the taste, odour and colour, by producing a sticky/ slimy layer (Amanidaz *et al.,* 2015; Asghari *et al.,* 2018). Therefore, it is necessary to constantly evaluate water distribution systems for microorganisms that may affect human health. The guidelines on water quality in South Africa stipulate that the HPC bacteria in potable water should not exceed 1 000 colony-forming units (CFU/ml) (SABS, 2011). Generally, potable water emanating from water purification plants limits HPC bacteria. But the number of microorganisms in water distribution systems can increase due to bacterial re-growth. Bacteria not detected at entry may multiply in the distribution pipelines (Camper *et al.,* 1999; Lee & Kim, 2003). Many HPC bacteria are associated with biofilms surrounded by a protective exopolymeric substance (EPS) (Rayner *et al.,* 2004). Such bacteria are not affected by disinfectants and other water purifying agents

and could eventually reach the consumer. Some bacteria may even become resistant to water purifying compounds. When HPC bacteria include pathogens, they pose a health risk, especially to those with underdeveloped, compromised or weakened immune systems, including children, the elderly and those living with HIV/AIDS (Pavlov *et al.,* 2004; Horn *et al.,* 2016). Several studies were conducted to assess the potential health risk of HPC bacteria in drinking water. Opinions differ on the health aspects of HPC bacteria. Three studies, one by Calderon and Mood (1991), another by Edberg *et al.* (1996) and one by Edberg and Allen (2004), reported that the presence of HPCs in water poses no health risks. However, Payment *et al.* (1991; 1994) examined filtered and non-filtered tap water and found a link between HPC concentration and gastroenteritis. The findings of the latter study were supported by other studies (Macler and Merkle, 2000; Pavlov *et al.,* 2004; Venter, 2010). Three main factors establish whether a host will develop symptoms after consuming pathogenic bacteria. They include the number of specific microbes the host ingests, the virulence constituents of the microbe, and the host's resistance (Edberg *et al.,* 1996). To fully assess the health effects of HPC bacteria, it is imperative to determine if these microbes have virulence components linked to pathogenesis (Edberg *et al.,* 1996). Some enzymes produced by bacteria include coagulase, elastase, gelatinase, DNAse, lipases and lecithinase (Ehlers *et al.,* 2003). It is usually accepted that the association of these enzymes with pathogenesis is one of the prerequisites for the commencement of an infection (Edberg *et al.,* 1996).

This study aimed to trace the presence of potentially pathogenic heterotrophic microorganisms in drinking water, starting from a water purification plant and ending with its distribution in four suburbs of Vanderbijlpark. The objectives of this study were (i) to obtain biofilms from domestic water pipes from four different sites (SE1, SV, SE2, CE1) in Vanderbijlpark and culture the HPC bacteria, (ii) to identify the potentially pathogenic microorganisms using the blood agar assay and enzyme analysis, (iii) to identify the antibiotic susceptibility/resistance of the bacteria, and (iv) to use biochemical and molecular techniques to identify the potential pathogens.

2. Materials and methods

2.1 Sample collection and isolation of HPC bacteria: This study was conducted in Vanderbijlpark in Gauteng Province, South Africa. Biofilms were obtained from domestic water pipes from 4 different sites SE1: (26°43'00.2''S 27°50'38.3''E), Sharpeville (26°41'08.9''S, 27°52'38.8''E), SE2 (26°43'26.8''S, 27°50'36.8''E), CE1 (26°42'06.8''S, 27° 50'30.0''E) in Vanderbijlpark by unscrewing the taps and scraping the insides of the pipes with a home-made biofilm extractor. The biofilm samples on a 1cm x 1cm device were suspended in 9 ml sterile saline and transported to the laboratory on ice. Alcian blue staining was used to confirm the presence of biofilms according to the method of Barranguet *et al.* (2004), and the slides were examined with a microscope (Motic, Wetzlar, Germany). The biofilm samples were plated on R2A and incubated at 37°C for 24 hours, according to Culotti & Packman (2014). The chlorine concentration was measured in all the water samples according to the method prescribed by APHA (2005). Untreated water samples were collected in sterile 1 L glass bottles from the Vaal River, the primary water source for the area. Potable water was obtained from the water purification facility that supplies Vanderbijlpark potable water. Exactly, 100 ml of both the untreated and treated water samples were filtered through 0.45 µm membrane filters (Lasec, Cape Town, South Africa). The membranes were plated on R2A agar (Acumedia, Lansing, MI) and incubated at 37°C for 24 hr. After incubation, the colonies from the biofilms untreated and treated water samples were selected based on colony morphology. Other physical characteristics such as colour, growth pattern, and pure colonies were obtained by culturing on R2A agar at 37°C for 24 h.

2.2 Pathogenicity testing of HPC isolates: A total of 50 isolates from all the sampling points were purified and inoculated on 5% horse blood agar medium and incubated at 37°C for 24 h to test for pathogenicity. Those colonies that showed haemolysis by producing clear zones around the colonies were preserved in slants on R2A and nutrient agar and were freeze-dried for later identification and testing. The type of haemolysis (α or β) in the different colonies was noted.

2.3 Enzymatic analysis: Tests for proteinase, DNase, lipase, lecithinase, coagulase, hyaluronidase and gelatinase were carried out only on isolates that demonstrated α- and β-haemolysis according to the method described by (Edberg *et al.,* 1996).

2.4 Proteinase: The media for this test was prepared by mixing 250 ml of 3% (w/v) of skim milk (Oxoid), 250 ml of brain heart infusion broth (Merck, Hampshire, UK) and 500 ml agar. The media was autoclaved and poured into plates.

The organisms were aseptically spotted on the solidified plates and incubated at 37°C for 24 h. A clearing around the colonies indicated a positive result.

2.5 DNase: DNase (Biolab, Mulgrave, Australia) was prepared according to the manufacturer's specifications and supplemented with 0.01 toluidine blue O (Sigma Aldrich, St. Louis, MO). The plates were incubated at 37°C for 24 h. The plates were then flooded with 0.1% 1 M HCL solution for 3 min. A positive result was seen by a clearing zone around the bacterial colonies.

2.6 Lipase: Tryptone soy agar (Biolab) was prepared and supplemented with 1% Tween 80 (Sigma Aldrich). Organisms were spotted with a sterile swab, and plates were incubated at 37°C for 24 hours. A zone of clearing around the colonies indicated a positive result.

2.7 Lecithinase: McClung Toabe Agar Base (Fluka, St. Gallen, Switzerland) was prepared according to manufactures specifications and supplemented with 50% egg yolk emulsion (Fluka). A white precipitate around or beneath the colonies after incubation at 37̊C for 24 hours meant that the results were positive.

2.8 Coagulase: Coagulase testing was done using bovine plasma (Sigma Aldrich). Precisely 10 ml of sterile distilled water was added to the plasma to reconstitute it. The plasma was distributed into sterile tubes, and the culture was inoculated. The tubes were incubated in a water bath for 4 h at 37°C. A positive result was indicated when blood clotting occurred, whereas a negative effect showed free-flowing plasma.

2.9 Hyalurodinase :The test for Hyalurodinase was carried out using Brain Heart Infusion broth (Biorad, Berkeley, CA). One gram of Noble agar (Sigma Aldrich) was added to 100 ml of the broth. Minisart filter units filtered an aqueous solution of 5% bovine albumin fraction V (Fluka) and 2 mg/l of hyaluronic acid (Sigma Aldrich). After incubation for 24 hours at 37°C, the plates were flooded with 2 M acetic acid for 10 min. A zone of clearing around the colonies indicated a positive result.

2.10 Gelatinase: Exactly 128 g of gelatin media (Sigma Aldrich) was dissolved in 1000 ml distilled water and distributed into tubes. The tubes were sterilized, and the culture was inoculated by stabbing the gelatin media. The tubes were incubated at 37°C for 24 h, after which the tubes were refrigerated for 30 min. A positive result is indicated by the hydrolyzed gelatin, which cannot solidify and thus remains liquid after refrigeration, while the negative result is a solid gelatin

2.11 Antibiotic susceptibility of HPC bacteria

Selected colonies were grown on Muller-Hinton agar overnight at 37°C and tested for antibiotic sensitivity to the following commonly used antibiotics: ampicillin, bacitracin, cephalothin, colistinsulphate, cotrimoxazole, gentamycin, streptomycin, tetracycline, and vancomycin (Mast Diagnostics, Merseyside, UK) following the protocols in (Jorgensen and Turnidge 2007). Antibiotic discs were placed inside the culture plates and incubated at 37°C for 24 h. The presence and absence of a zone of clearing around the discs were recorded. The tests were repeated three times.

2.12 Biochemical tests: According to the manufacturer's instructions, all the strains found to be resistant to the antibiotics were identified by biochemical means using the API 50CH and API 20E for the Gram-positive and Gram-negative species. The samples were then sent for further identification using the BBL Crystal identification to the Deltamune laboratory (Benoni, South Africa).

2.13 Molecular identification: Pure bacterial isolates were cultured in lactose broth at 37°C for 24 h. DNA was isolated from a 100 ml sample using the Zymo Research ZR: Fungal/ Bacterial DNA kit™ (Irvine, CA) according to the manufacturer's instructions. The primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 907R (5′-CCGTCAATTCCTTTRAGTTT-3′) (Nubel*et al.,* 1996) were used to amplify the 16S rDNA gene of the bacterial isolates. The PCR amplifications were carried out according to Venter (2010). Approximately 3 ul of the PCR products were analyzed by electrophoresis on 1% agarose gel containing ethidium bromide to ascertain the size of the amplicons. The gel fragments were excised, and the DNA was purified using the Wizard SV Gel and PCR clean-up system (Promega, Fitchburg, WI). The DNA was sent for sequencing to Inqaba Laboratories (Pretoria, South Africa).

The sequences were aligned with similar sequences from the GenBank using T-Coffee and MAFFT online alignment programmes. Identification at the species level was defined as a 16S rDNA sequence similarity of 99% with that of the prototype strain sequence in GenBank; identification at the genus level was described as a 16S rDNA sequence similarity of 97% with that of the prototype strain (Drancourt *et al.,* 2000).

3. RESULTS

3.1 Identification of biofilms and isolation of HPC bacteria

Alcian blue staining confirmed the presence of biofilms in the water distribution points (Fig 1).

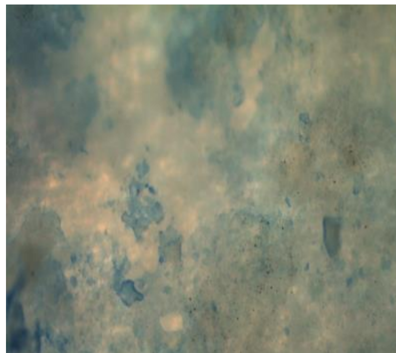

Figure 1. An Alcian Blue stained slide at 100X magnification. The arrows indicate the patchy biofilm areas that retained the blue dye.

The bacterial growth rate, color and shape of the colonies differed in the various samples. The color of the colonies ranged from yellowish, whitish, creamy and orange, while the colony shapes were either round, mounted or spiral. The dominant colonies were whitish and creamy, followed by yellow. The number of microorganisms (cfu/ml) and percentage of morphotypes at each sampling are summarized in Table 1.

Table 1. The average HPC bacterial counts, number and percent of morphotypes found at each sampling point.

Site	**cfu/ml** (average of two counts)	**Morphotypes**	**Percent of different morphotypes in each sampling point**
Vaal River (VR)	90000	15	0.02
Rand Water (RW)	5	2	40.00
Sharpeville (SV)	68	11	16.18
CE1	54	10	18,52
SE1	25	5	20,00
SE3	38	7	18.42

The highest number of microorganisms (90 000 cfu/ml) was found in the water collected from the Vaal River, whereas relatively low cfu's/ml were found in the other samples. The number of morphotypes ranged from 2 to 15, as shown in Table 1.

3.2 Pathogenicity testing (Haemolysin assay)

Exactly 75% of the isolates demonstrated β-haemolysis, which was indicated by the complete clearing around the colonies on blood agar. γ-haemolysis, where no haemolysis occurred, comprised 24% of the isolates. None of the

isolates demonstrated α-haemolysis where a green zone would have been observed. The results are recorded in Table 2.

Table 2. The number of isolates tested and percentages that demonstrated β-haemolysis and γ- haemolysis.

Sampling Site	Number of isolates	β –haemolysis and percentage of isolates	γ- haemolysis and percentage of isolates
VR	15	13 (87%)	2 (13.3%)
RW	2	0	2 (100%)
SV	11	10 (91%)	1 (9.1%)
CE1	10	8 (80%)	2 (20%)
SE 1	5	2 (40%)	3 (60%)
SE 3	7	5 (71%)	2 (28.6%)

3.3 Enzymatic analysis

Of the 50 isolates that were tested for haemolysis, 35 tested positive (β haemolysis) for virulence-associated enzymes (Fig. 2). Most of the isolates produced DNase (31%). Seventeen percent of the isolates produced lecithinase and coagulase, while hyaluronidase and proteinase were produced by 14% of the isolates. Gelatinase and lipase were produced by 6% and 3% of the isolates.

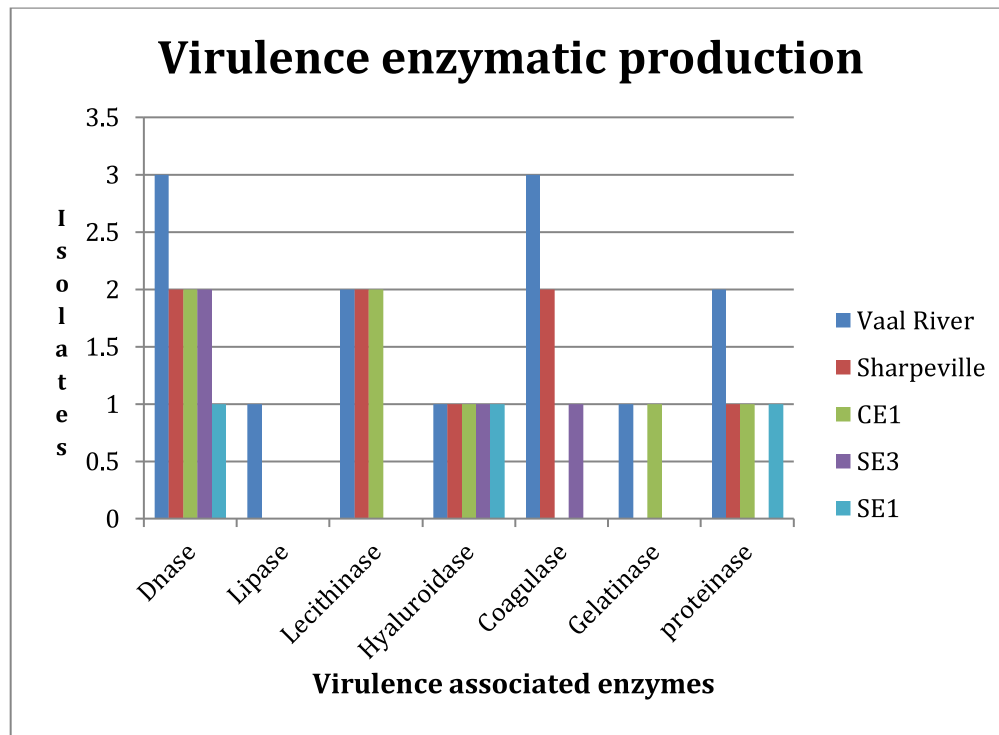

Figure 2. The number of isolates that produced virulence-associated enzymes at the individual sampling sites.

3.4 **Antibiotic susceptibility tests**

Isolates VR3, VR7 and VR11 of the 13 isolates from the Vaal River sample showed resistance to STR, GEN and AMP (Table 3). The isolates, SV4 and SV6, from Sharpeville demonstrated resistance to TET. Isolate 3 from CE1 was resistant to most antibiotics except for GEN and TET. Apart from STR, VAN and AMP isolate 2 from SE3 was resistant to all the antibiotics. The single isolate from SE1 was resistant to six tested antibiotics except for BAC, COL and AMP. Isolates that demonstrated resistance to the commonly used antibiotics were then considered potential pathogens and identified using biochemical and molecular methods.

Table 3. Bacterial isolates that demonstrated resistance to different antibiotics

ISOLATE	ANTIBIOTICS
VR3	STR, GEN, VAC, AMP
VR7	STR, GEN, COT, COL, CEP, AMP
VR11	STR, GEN, AMP, VAN, TET
SV4	STR, COT, TET, COL, CEP, AMP
SV6	GEN, TET, BAC, VAN
CE1 3	STR, COT, VAC, COL, VAN, CEP, AMP
SE3 2	GEN, COT, TET, BAC, COL, CEP
SE1 1	STR, GEN, COT, COL, VAV, CEP

Key: STR= Streptomycin, GEN=Gentamycin, COT=Cotrimoxazole, TET=Tetracycline, BAC=Bacitracin, COL=Colistin sulphate, VAN=Vancomycin, CEP=Cephalothin and AMP=Ampicillin.

3.5 **Identification of HPC isolates using biochemical and molecular methods**

The eight isolates that showed antibiotic resistance were Gram stained to determine the identification kit to be used. The results of the Gram test, API 20E, API 50CH and BBL Crystal identification GP ID system of the eight isolates are summarized in Table 4.

Table 4. Results of the Gram reaction, API 20E, API 50CH and BBL Crystal identification GP ID system of the 8-antibiotic resistant HPC isolates.

Isolate number	Gram reaction	API 20E	API 50CH	BBL Crystal Id kit
1 (VR3)	Neg	*E. coli*	N/A	N/A
2 (VR7)	Neg	*K. pneumonia*	N/A	N/A
3 (VR11)	Neg	*K. pneumonia*	N/A	N/A
4 (SV4)	Pos	N/A	*Bacillus* sp.	*B. megaterium*
5 (SV6)	Pos	N/A	*Bacillus* sp.	*B. cereus*
6 (CE1-3)	Neg	*P. aeruginosa*	N/A	*P. aeruginosa*
7 (SE3-2)	Pos	N/A	*Bacillus* sp.	*B. pimulus*
8 (SE1-1)	Pos	N/A	*Bacillus* sp.	*B. brevis*

The API20E kit was able to identify *Escherichia coli*, *Klebsiella pneumonia and Pseudomonas aeruginosa*, while the API 50CH kit identified *Bacillus* spp. in the 4 Gram-positive isolates. The BBL Crystal identification GP ID system identified *Bacillus megaterium, B. cereus*, *B. pimulus, B.brevis* and *P.aeruginosa.* These isolates were identified with molecular techniques targeting the 16S rDNA region.

3.6 Molecular identification

PCR amplification of the 16SrDNA gene produced a fragment of approximately 900 bp in all the isolates (Fig. 3).

4. DISCUSSION

One of the most critical aspects of this study is that the finished water leaving the treatment plant conformed to South African (<1000 cfu/mL) and international (<500 cfu/mL) water quality standards (Pavlov *et al.,* 2004; WHO, 2017). However, Alcian Blue staining confirmed the presence of biofilms at four sites (SE1, SE3, CE1, and Sharpeville), indicating that the water quality deteriorated as it flowed through the distribution network before reaching the consumer. Biofilms are the primary source of micro-organisms in water distribution systems (Berry *et al.,* 2006; Douterelo *et al.,* 2016).

Finished water of high quality is not necessarily safe at the consumers' taps after it has been transported in pipes for a long distance (Eichler *et al.,* 2006). Biofilms attached to water pipes contain 25 times more bacterial

cells than bulk water (Berry *et al.,* 2006). A biofilm's attached cells can metabolize recalcitrant organic compounds and have an increased resistance to chlorine and other biocides (Berry *et al.,* 2006). Microbial biofilms are also a way in which microorganisms survive environmental conditions since it is reported that cells within a biofilm are more resistant than planktonic ones (Garrett *et al.,* 2008). It is considered that bacteria embedded in the EPS express greater tolerance to antibiotics disinfectants and are harder to remove from surfaces (Stewert and Costerton, 2001). Despite using chlorine and/or chloramines to reduce bacterial regrowth in water distribution systems, the regeneration of HPC bacteria and biofilms has not been controlled completely (Chowdhury, 2012). These factors may account for the presence of biofilms in the Vanderbijlpark water distribution system.

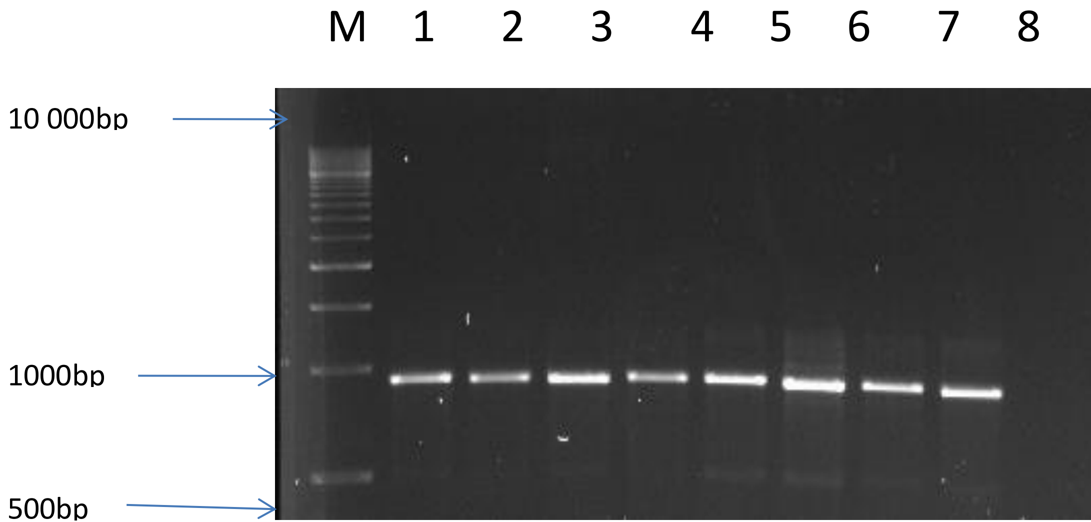

Figure 3. PCR amplification of the 16S rDNA gene. Lane 1, 2 and 3 are samples from Vaal River and 4, 5, 6, 7, and 8 are samples from the distribution points. M is the bp marker.

4.1 **Characteristics of HPC bacteria in the samples**

The Alcian Blue staining confirmed the presence of biofilms in the Vanderbijlpark water distribution system. The staining in Figure 1 suggested that biofilms were present inside the water pipes. Biofilms are produced as continuous, consistently dispersed layers from time to time but can be quite erratic in appearance (Houry *et al.,* 2012). This appeared to be true in the biofilms observed in this study.

The HPC bacteria cultured from the water samples varied greatly in morphology (round, mounted, spiral) and colour (cream, orange, white, and yellow). These results are similar to those of Pavlov *et al.* (2004), who assessed the variation in HPC microorganisms from selected treated and untreated drinking water in South Africa. The morphological and color variation of the colonies suggests a wide range of bacteria living in the Vanderbijlpark water distribution system. The highest microbial load (90 000 cfu/ml) was found in the water sample from the Vaal River. The Vaal River is an open water system. It is a conducive environment for microbial growth since it receives industrial, domestic and agricultural waste, which leads to the accumulation of organic matter. However, there was a drastic decrease in the microbial counts (5 cfu/ml) in the treated water sample from Rand Water, suggesting that effective methods are being used in the water treatment process. However, there appeared to be no relationship between the microbial load and the number of morphotypes in the water samples. For example, although the Vaal River water sample had a very high microbial load, it had the lowest percentage of morphotypes (Table 1). On the contrary, the Rand Water sample had a low bacterial count but had the highest percentage of morphotypes (Table 1). The number of morphotypes detected in the water from the Vaal River may not necessarily suggest low bacterial diversity in this sample.

Since Rand Water is the only water supplier to all the sampling points in this study, the expectation is that the water quality remains uniform throughout the system. Several microorganisms are known to establish themselves even after the water has been successfully treated (Van der Kooij and Zoetemann, 1978; Ridgway and Olson, 1981; Olson, 1982; Nagy and Olson, 1985; Olivieri *et al.,* 1985; Herson *et al.,* 1987; Schindler and Metz, 1991). A water distribution system offers conducive environments for the persistence of microorganisms (Momba, 2000; Douterelo *et al.,* 2016). These conditions include physical (pipe material) (September *et al.,* 2007; Rozej *et al.,* 2015). Such microorganisms may exist in biofilms and can be sheared off from the surfaces of distribution systems and end up in the bulk water. The decomposition of chlorine, a principal constituent of the water treatment process, also contributes to the persistence of microorganisms in the water distribution systems. The average chlorine level in the water leaving the treatment plant was 3 mg/l. The chlorine level decreased as the water flowed through the distribution system, and only 1 mg/l was recorded for Sharpeville. The lower chlorine levels in the distribution system seemed to have contributed to microbial proliferation. These results are similar to those of (Farooq *et al.,* 2008), who also observed a relationship between microbial density and residual chlorine. The chlorination process is supposed to prevent the re-growth of microbes in water systems (Sarbatly and Krishnaiah, 2007). However, this study showed that biofilms were still present in the distribution points despite chlorine treatment of water. It has been reported that opportunistic pathogens may exist in water supplies if there is a chlorination breakdown or if pathogens are chlorine resistant (Kaestli *et al.,* 2019).

4.2 **Presence of pathogenic bacteria in the water samples.**

This study found no opportunistic pathogens in the finished water from the treatment plant. However, such organisms were found in the other sampling points. It is known that β-haemolysis represents an absolute degradation of the haemoglobin of the red blood cells, indicating the potential pathogenicity of microorganisms (Todar, 2009). In this study, 76% of the isolates produced β-haemolysis (Table 2). Pathogenic bacteria have several extracellular substances which act against the host by breaking down the primary and secondary of the host (Todar, 2009). Once inside the body, microorganisms produce virulence-associated enzymes to enable them to survive in hostile environments (Pavlov *et al.,* 2004). It is generally accepted that an organism with more than two extracellular virulence enzymes can be considered a potential pathogen (Edberg *et al.,* 1996). In this study, the isolates tested positive for DNase (31%), lecithinase (17%), coagulase (17%), hyaluronidase (14%), proteinase (14%), gelatinase (6%), and lipase (3%).

4.3 **Antibiotic resistance:** Table 3 shows that 8 isolates were resistant to 4-7 of the 9 antibiotics tested. Antibiotic-resistant heterotrophic bacteria have been reported in many parts of the world (Destiani and Templeton, 2019), including South Africa (Mulamattathil *et al.,* 2014), even after treatment (El-Zanfaly, 2015). Many factors are known to contribute to the antibiotic resistance of microorganisms (Dever and Dermody, 1991; Tenover, 2006). In addition, bacteria in biofilms can withstand high concentrations of antimicrobial agents because of the EPS (Stewart and Osterton, 2001; Stewart, 2002; Fux *et al.,* 2005). The lipid components of the biofilms make it difficult for antibiotics to penetrate the EPS (Tetz *et al.,* 1993, 2004). The survival of bacteria against antibiotics is also influenced by the so-called 'persisters" which are found to be tolerant to multiple drugs (Stewart, 2002; Keren *et al.,* 2004; Harrison *et al.,* 2005; Lewis, 2007). Resistance to antibiotics is a serious health concern, especially to immuno-compromised individuals. A component of the general population at a high risk of being infected with HPC bacteria is increasing worldwide (Pavlov *et al.,* 2004). South Africa is no exception to this phenomenon because of its growing population of immuno-compromised individuals, especially HIV/AIDS positive (Grabow, 1996). It has been reported that 13.1% of the population of South Africa is living with AIDS (Stats SA, 2018).

4.4. Identification of the HPC isolates: Three different methods were used to identify the HPC isolates. The API 20E kit showed that the biofilms harboured *K. pneumonia*, *E. coli* and *P. aeruginosa*. However, the API 50CH kit could not identify any of the isolates to the species level. The BBL Crystal identification kit identified four isolates in *Bacillus* (*B. megaterium, B. cereus, B. pimilus, B. brevis*) and *Pseudomonas aeruginosa*). These microorganisms were reported to be present in the HPC isolates commonly found in drinking water, and some are considered opportunistic pathogens (Rusin *et al.,* 1997; Allen *et al.,* 2004; Horn *et al.,* 2016).

Bacillus spp. are commonly isolated from soil, plants, food and water (Hill *et al.,* 2004). They can withstand nutritional deficiency and harsh environmental conditions because of their ability to produce spores (Bartram *et al.,* 2003). Although *Bacillus* spp. are generally considered harmless, some are pathogenic to humans (Bartram *et al.,*

2003). Some strains can induce diarrhoea after ingestion. For example, *B.cereus* causes bacteraemia in immunocompromised individuals with symptoms such as vomiting and diarrhoea (Bartram *et al.,* 2003). *Bacillus cereus* also produces enterotoxins that cause human gastrointestinal infections (Hansen and Hendrikse, 2001). *Pseudomonas aeruginosa* is usually one of the isolates in HPC assays and is considered an opportunistic pathogen found in various environmental niches, including drinking water distribution systems (Pellet *et al.,* 1983; Khan *et al.,* 2007). Its large genome size (6.3 Mb) and the number of genes enable it to survive in various environments (Stover *et al.,* 2000). *Pseudomonas* spp. are also a dominant microorganism in biofilm formation (Ridgway *et al.,* 1981; Mena and Gerba, 2009); it is, however, a Gram-negative organism, and it is not expected to be found inside water pipes (Mena and Gerba, 2009). Its presence in the water distribution system is probably due to external sources such as pipe replacement and water storage in reservoirs. *Bacillus* spp. were the predominant isolates in the biofilms in the CE1, SE1 and SE3 sampling points in this study. Both *Bacillus* spp. and *Pseudomonas* spp. were identified in Sharpeville. This study's dominant bacteria in HPC isolates are similar to those reported in other studies. A survey of four water distribution systems in Turkey (Bilge *et al.,* 2005) reported that *Bacillus* spp. made up 71% of the HPC microorganisms. Their study also identified *B. cereus*, *Pseudomonas* spp. and *Staphylococcus* spp. as being pathogenic. Similarly, (Rusin *et al.,* 1997) found *Pseudomonas* spp. in drinking water samples in the USA.

4.5 Molecular identification: Sequencing the rDNA genes showed that *E. coli* and *Klebsiella* spp. were present in the Vaal River, *P. aeruginosa* and *B. megaterium* were present in Sharpeville, B. *cereus,* and *B. pumilus* in the samples from CE1, in SE1 and SE3 (Table 5). Identification of the bacteria by sequencing did not correspond exactly to the ones identified by the biochemical tests. The formal identification of bacteria based on phenotypic characteristics is generally not as accurate as identification based on genotypic methods.

Table 5. Molecular identification of the 8 potentially pathogenic samples in this study.

Sample		Accession no.	Highly matched bacteria	% Similarity	Confidence level
Sample 1	VR3	MT 581481.1	*E. coli*	100%	species
Sample 2	VR7	KX 018386.1	*Klebsiella* sp.	100%	species
Sample 3	VR11	MN 481047.1	*K. pneumoniae*	99.50	species
Sample 4	SV4	MN 314664.1	*P. aeruginosa*	100%	species
Sample 5	SV6	MT 588737.1	*Bacillus megatarium*	99.75%	species
Sample 6	CE1-3	MT 588715.1	*B. cereus*	99.87%	species
Sample 7	SE3-2	MT 410639.1	*B.pumilus*	98.49%	genus
Sample 8	SE1-1	MT 410639.1	*B. pumilus*	98.49%	Genus

Escherischia coli in the Vaal River indicates human and animal faecal contamination (Fujioka *et al.,* 1999). *Klebsiella* is considered to be an opportunistic pathogen. Many strains of *Klebsiella* test positive in faecal coliform tests and is of public health significance. The Vaal River is used for recreational purposes, and the presence of *Klebsiella* could represent a serious health risk, especially for people with impaired immune systems. The Vaal River is also a water source for Rand Water, the water distribution system included in this study. It is known that *Klebsiella* can continue to exist in water distribution systems even if the water is chlorinated (Ainsworth, 2004). *Pseudomonas aeruginosa* is widespread in the environment and is an opportunistic pathogen. It is usually recovered in soils and water and can multiply on substrates in contact with water. Therefore, its occurrence should be considered when assessing the general quality of water in distribution systems (Andersson and Bohan, 2001). Its presence in the Sharpeville water system should be a matter of concern for the health authorities.

Bacillus spp. are generally isolated from drinking water supplies, even though the water is treated to acceptable standards (Bartram *et al.,* 2003). *Bacillus cereus* is commonly found in the environment. It is a toxin-producing facultatively anaerobic gram-positive bacteria and can be present in foods. *Bacillus cereus* can cause two types of intestinal illnesses: one is diarrheal, and one leads more to nausea/vomiting. *B. cereus* has also been implicated in infections of the eye, respiratory tract, and wounds. The pathogenicity of *B. cereus*, whether intestinal or non-intestinal, is associated with the production of tissue-destructive exoenzymes (McDowell *et al.,* 2020). *Bacillus pumilus* is found in various soils, plants and environmental surfaces. This bacterium is highly resistant to extreme environmental conditions such as low or no nutrient availability, desiccation, irradiation, H2O2 and chemical disinfection (Nicholson *et al.,* 2000). This is perhaps some of the reasons why *B. pumilus* was able to survive in the water distribution system. Several strains of *B. pumilus* contain the *ces*B-targeted gene that produces cereulide, a small

heat-stable cyclic dodecadepsipeptide produced by some strains of *B. cereus* that have high toxicity to humans (From *et al.,* 2005). The 3 main clusters of the 8 bacterial species (Fig. 4) corresponds with their classification into different families (the *Bacillus* spp. in the family Bacilliaceae; *E.coli* and *Klebsiella* spp in the family Enterobacteriaceae and *Pseudomonas* in the Pseudomonaceae) (Xu and Cote,2003) also showed that *B.pumilus* and *B. megatarium* grouped as Group II while *B. cereus* was differentiated from these species into Group V.

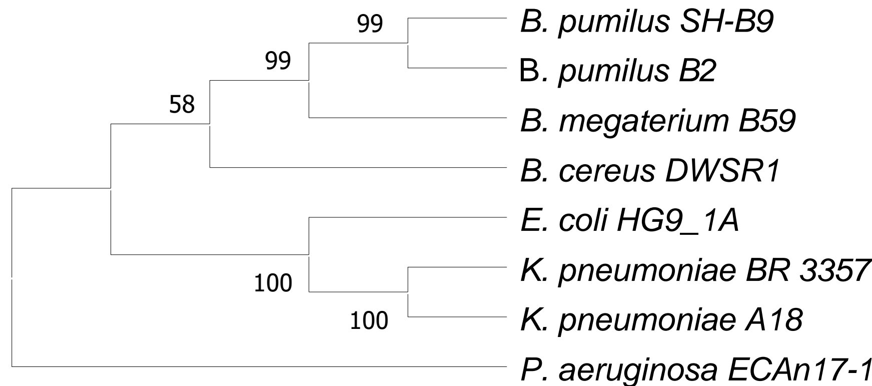

Figure 4. Clustering of bacteria in this study based on 16s rDNA sequences.

5. Conclusion

Biofilms were isolated from water distribution points in Vanderbijlpark. Based on the tests carried out in this study and reports by other researchers, the identified genera in the water pipes can be classified as potential pathogens that can or/ may cause various diseases in people, especially those with a weakened immune system. These microorganisms enter the distribution system either by surviving the treatment process resulting in re-growth (Hashmi *et al.,* 2012) or through external sources. They can adhere to and colonize the internal surfaces of the distribution system to form biofilms, which dissociate from the pipes and end up in the bulk water. Despite disinfection, bacteria can still grow in water distribution systems, and monitoring water quality is essential.

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