

DETERMINATION OF CYTOTOXICITY AND INVASIVENESS OF HETEROTROPHIC PLATE COUNT BACTERIA ISOLATED FROM DRINKING WATER

by

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I, the undersigned, declare that the thesis hereby submitted to the University of Pretoria for the degree
MSc (Med Virology) and the work contained therein is my own original work and has not previously,
in its entirety or in part, been submitted to any university for a degree.

Signed:

 this 27 day of May 2002



“Man is embedded in nature. The biologic science of recent years has been making this a more urgent fact of life. The new, hard problem will be to cope with the dawning, intensifying realization of just how interlocked we are.”

Lewis Thomas

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SUMMARY

Heterotrophic plate counts (HPCs) are commonly used to assess the general microbiological quality of drinking water. Drinking water quality specifications world-wide recommend HPC limits from 100 to 500 cfu.ml⁻¹. However, a number of recent studies revealed evidence that commonly used indicator bacteria may not be as harmless as generally accepted. It appears that immuno-compromised individuals, which represent increasing components of many consumer populations, are particularly at risk. This would include the very young and very old, patients with diseases such as AIDS, and patients on therapy after organ transplantations and cancer treatment.

Since, epidemiological and animal infectivity studies are complex and difficult to control, attempts have been made by researchers to examine HPCs directly in order to assess health risks. These

analyses included: cytotoxicity, invasiveness, enzyme analyses, antibiotic susceptibility and identification.

In this study, 339 bacterial colonies were isolated at random from selected drinking water supplies in South Africa using heterotrophic plate count tests. In a first step to screen for potentially pathogenic properties, 188 (55.5%) of the isolates showed α - and β -haemolysis on human- and horse-blood agar media. Subsequent analysis of the haemolytic isolates for enzymatic properties associated with pathogenicity revealed the presence of chondroitinase in 5.3% of the isolates, coagulase in 16.0%, DNase in 60.6%, elastase in 33.0%, fibrinolysin in 53.7%, gelatinase in 62.2%, hyaluronidase in 21.3%, lecithinase in 47.9%, lipase in 54.8%, and proteinase in 64.4%. Fluorescein and pyocyanin were not produced by any of the isolates.

The Kirby-Bauer quality controlled disc diffusion method was applied in the demonstration of antibiotic resistance by the HPC isolates. Among the haemolytic isolates 77.7% were resistant to oxacillin (1 μ g), 59.6% to penicillin G (2 units), 47.3% to penicillin G (10 units), 54.3% to ampicillin (10 μ g) and 43.1% to ampicillin (25 μ g).

Cell culture studies revealed that 96% of haemolytic isolates were cytotoxic to HEp-2 cells and 98.9% of the 181 cytotoxic isolates adhered to HEp-2 or Caco-2 cells. Gram-negative isolates tended to adhere in larger numbers than gram-positive isolates. The average index of adherence for Gram-negative bacteria was 20-30 bacteria per HEp-2 cell, compared to 3-7 for Gram-positive bacteria.

HEp-2 cells were invaded by 43.6% and Caco-2 cells by 49.7% of the 181 cytotoxic isolates. The invasion index on HEp-2 cells was 1.9×10^{-1} to 8.9×10^{-6} , compared to 7.7×10^{-2} to 8.3×10^{-6} on Caco-2 cells.

The most commonly isolated genera showing potentially pathogenic features were: *Aeromonas*, *Acinetobacter*, *Aureobacterium*, *Bacillus*, *Chryseobacterium*, *Corynebacterium*, *Klebsiella*,

Moraxella, *Pseudomonas*, *Staphylococcus*, *Tsukamurella* and *Vibrio*. All these genera are known to contain opportunistic pathogens.

Our results support earlier findings on potentially pathogenic features of bacteria detected by heterotrophic plate counts on drinking water. These findings seem to be in agreement with some epidemiological studies, which indicated an association between HPCs of drinking water and the incidence of gastroenteritis in consumers. However, the extent of the health risk concerned needs to be defined in detail for meaningful revision of quality guidelines for HPCs in drinking water.

BEPALING VAN SITOTOKSISITEIT EN INDRINGING VAN HETEROTROFIESE PLAATTELLING BAKTERIEË GEÏSOLEER VANUIT DRINKWATER

deur

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OPSOMMING

Heterotrofiese plaattellings (HPTs) word meestal gebruik om die algemene mikrobiologiese kwaliteit van drinkwater te bepaal. 'n Limiet van 100 tot 500 kve.ml⁻¹ vir HPTs word wêreldwyd deur drinkwater kwaliteit spesifikasies aanbeveel. Onlangse studies het aangetoon dat hierdie algemeen gebruikte indikator bakterieë gevaarliker blyk te wees as wat aanvanklik gedink is. Dit blyk dat daar 'n groter risiko is vir immuungekompromeerde individue, wat 'n toenemende komponent van die verbruikers verteenwoordig. Hierdie groep sluit jong kinders en bejaardes in asook pasiënte met siekte toestande soos VIGS sowel as persone wat orgaanoorplantings en kankerbehandeling ondergaan het.

Epidemiologiese en diere infektieweits studies is ingewikkeld en moeilik om te kontroleer daarom het verskeie navorsers sedertdien probeer om HPTs direk te bestudeer om sodoende die gesondheidsrisiko te bepaal. Hierdie analyses het die volgende ingesluit: sitotoksiteit, indringing,

ensiem-analises, antibiotika-weerstandbiedendheid en identifikasie van hierdie bakterieë.

In hierdie studie is 'n totaal van 339 bakterieë kolonies ewekansig geïsoleer deur gebruik te maak van roetine HPT toetse wat voorsien is deur geselekteerde drinkwater verskaffers in Suid Afrika.

Gedurende 'n eerste stap evaluerings proses vir potensiële patogeniese eienskappe het 188 (55.5%) van die isolate α - of β - hemolise op mens- en perde-bloed agar media getoon. Hierdie hemolitiese isolate is verder onderwerp aan ensiematiese analises, wat geassosieer word met patogenisiteit, die volgende resultate is verkry: chondroïtienase in 5.3% van die isolate, koagulase in 16%, DNase in 60.6%, elastase in 33%, fibrinolisien in 53.7%, gelatienase in 62.2%, hialuronidase in 21.3%, lesitienase in 47.9%, lipase in 54.8% en proteïenase in 64.4%. Fluoresseëen en piosianien was nie deur enige van die isolate geproduseer nie.

Deur gebruik te maak van die Kirby-Bauer kwaliteits kontrole skyf diffusie metode is die bestandheid van hierdie hemolitiese isolate teen antibiotika bepaal: 77.7% was bestand teen oxasillien (1 μ g), 59.6% teen penisillien G (2 eenhede), 47.3% teen penisillien G (10 eenhede), 54.3% teen ampisillien (10 μ g) en 43.1% teen ampisillien (25 μ g).

Selkultuur studies het aangetoon dat 96% van die hemolitiese isolate sitotoksies was vir HEp-2 selle en 98.9% van die 181 sitotoksiese isolate het aan die HEp-2 en Caco-2 selle geheg. Gram-negatiewe isolate het in groter getalle geheg as Gram-positiewe isolate. Die gemiddelde indeks vir aanhegting vir Gram-negatiewe bakterieë was 20-30 bakterieë per HEp-2 sel in vergelyking met 3-7 Gram-positiewe bakterieë per sel.

HEp-2 selle is binne-gedring deur 43.6% en Caco-2 selle deur 49.7% van die 181 sitotoksiese isolate. Die binne-drings indeks vir HEp-2 selle was 1.9×10^{-1} tot 8.9×10^{-6} , in vergelyking met 7.7×10^{-2} tot 8.3×10^{-6} vir Caco-2 selle.

Die mees algemene genera wat geïsoleer is en wat potensiële patogeniese eienskappe getoon het was: *Aeromonas*, *Acinetobacter*, *Aureobacterium*, *Bacillus*, *Chryseobacterium*, *Corynebacterium*,

Klebsiella, Moraxella, Pseudomonas, Staphylococcus, Tsukamurella en *Vibrio*. Dit is bekend dat al hierdie genera opportunistiese patogene in sluit.

Die resultate verkry in hierdie studie stem ooreen met vorige studies met betrekking tot potensiële patogeniese eienskappe van bakterieë wat geïsoleer is deur roetine heterotrofiese plaattellings vanuit drinkwater. Hierdie bevindings stem ook ooreen met sommige epidemiologiese studies wat 'n verwantskap aangetoon het tussen HPTs van drinkwater en die voorkoms van gastroënteritis in verbruikers. Dit is egter nodig dat die omvang van die gesondheidsrisiko geassosieer met HPTs verder ondersoek moet word om meer detail vir die hersiening van kwaliteits riglyne daar te stel.



LIST OF ABBREVIATIONS:

| | |
|-----------------------------|--|
| Caco-2: | Human adenocarcinoma cell line |
| cfu.ml⁻¹: | Colony forming units per millilitre |
| DNA: | Deoxyribonucleic acid |
| EDTA: | Ethylenediaminetetraacetate |
| ESBL: | Extended-spectrum β -lactamase |
| FCS: | Fetal calf serum |
| GN: | Gram-negative microplates |
| GP: | Gram-positive microplates |
| HEp-2: | Human epithelial carcinoma |
| HPCs: | Heterotrophic plate counts |
| ID₅₀: | Infectious dose |
| LRF: | Large restriction fragment |
| MAC: | <i>Mycobacterium avium</i> complex |
| MEM: | Minimum essential medium |
| MIC: | Minimum inhibitory concentration |
| MRSA: | Methicillin resistant <i>Staphylococcus aureus</i> |
| NSF: | National Scientific Foundation |
| PBS: | Phosphate-buffered saline |
| PCA: | Plate count agar |
| PCR: | Polymerase chain reaction |
| PFGE: | Pulsed-field gel electrophoresis |
| TSA-SB: | Sheep blood agar |
| VERO: | African green monkey kidney cells |
| WHO: | World Health Organisation |
| Y-1: | Mouse adrenal cells |
| YXA: | Yeast extract agar |

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LIST OF PUBLICATIONS AND CONFERENCE CONTRIBUTIONS

Publications:

1. Determination of cytotoxicity and invasiveness of heterotrophic plate count bacteria isolated from drinking water by Pavlov, D.N., de Wet, C.M.E., Grabow, W.O.K., Ehlers, M.M. Accepted for publication in *Water Science and Technology*.
2. Identification of potentially pathogenic heterotrophic plate count bacteria isolated from selected drinking water supplies in South Africa by Pavlov, D.N., de Wet, C.M.E., Grabow, W.O.K., Ehlers, M.M. Submitted for publication in *Water Research*.
3. Determination of cytotoxicity and invasiveness of heterotrophic plate count bacteria isolated from drinking water by Pavlov, D.N., de Wet, C.M.E., Grabow, W.O.K., Ehlers, M.M. Report to the Water Research Commission of South Africa.
4. Potentially pathogenic features of heterotrophic plate count bacteria isolated from drinking water by Pavlov, D.N., de Wet, C.M.E., Grabow, W.O.K., Ehlers, M.M. Article published at the conference proceedings of the first international NSF International / WHO symposium on heterotrophic plate count (HPC) bacteria in drinking water "*HPC Bacteria in Drinking Water-Public Health Implications?*" Geneva, Switzerland (22-24 April 2002).

National presentations:

1. Assessment of potentially pathogenic features of heterotrophic plate count bacteria isolated from drinking water by Pavlov, D.N., de Wet, C.M.E., Grabow, W.O.K., Ehlers, M.M. Poster on Faculty Day of the Faculty of Health Sciences, University of Pretoria on 22 April 2001.

2. Assessment of potentially pathogenic features of heterotrophic plate count bacteria isolated from drinking water by Pavlov, D.N., de Wet, C.M.E., Grabow, W.O.K., Ehlers, M.M. Oral presentation at the 12th Biennial congress of the Southern African Society for Microbiology, Bloemfontein, South Africa, 2-5 April 2002.

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CHAPTER 1

INTRODUCTION

Heterotrophic bacteria are widely used as indicators of drinking water quality (Grabow, 1996). Counts of these organisms are generally referred to as heterotrophic, standard or total plate counts (Edberg *et al.*, 1996; Grabow, 1996). The organisms are detected by propagation on non-selective media rich in nutrients to support the multiplication of the widest possible range of bacteria, which may also include other organisms such as yeasts (Grabow, 1996). Plates are generally incubated for 24 h or 48 h, at temperatures ranging from 20°C to 37°C (Grabow, 1996). The heterotrophic plate count (HPC) is obtained by counting all bacterial colonies visible to the naked eye under the specified conditions and are used to assess the quality of treated drinking water supplies (Grabow, 1996). Details of the HPC and its application in water quality assessment and monitoring have been described by Grabow (1990, 1996), Reasoner (1990), States and Sykora (1995), and WHO (1996, 1997, 2001).

In view of the above features the HPC is included in water quality specifications and recommendations world-wide. The South African Bureau of Standards specifies a HPC limit of 100 cfu.ml⁻¹ for drinking water (SABS, 1999; SABS, 2001). This limit is endorsed by the Department of Health and Water Affairs, and is in line with specifications and recommendations in the rest of the world (WHO, 2001).

However, the application of HPC specifications is a contentious issue all over the world. Reasons are basically that under circumstances largely depending on the quality of raw water sources, intensive and expensive treatment is required to produce drinking water which meets HPC specifications. In addition, the general perception of the HPC is that it determines harmless organisms and reflects no meaningful health risk.

However, evidence has been presented that heterotrophic plate counts may include bacteria which are not altogether as harmless as the definition tends to indicate. Many of these pathogenic, or potentially pathogenic microorganisms are associated with secondary infections in patients where defence mechanisms have been weakened by primary infections caused by more virulent pathogens (Rusin *et al.*, 1997). The very young with underdeveloped immune systems, the elderly with weakened immune systems and individuals with compromised immune systems are at high risk to these infections (Grabow, 1996; Rusin *et al.*, 1997; Barbeau *et al.*, 1998). The latter include patients with immune-compromising infections such as AIDS and patients under medical treatment for various forms of cancer and organ transplant patients (Grabow, 1996; Rusin *et al.*, 1997; Barbeau *et al.*, 1998).

Heterotrophic bacteria of the following genera have been associated with opportunistic infections: *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Klebsiella*, *Legionella*, *Moraxella*, *Mycobacterium*, *Serratia*, *Pseudomonas*, *Xanthomonas* (Rusin *et al.*, 1997; Kudinha *et al.*, 2000). The risk of infection by heterotrophic bacteria in drinking water has been estimated at levels as low as 7.3 per billion for low exposures to *Aeromonas*, to 98 per 100 for patients on antibiotic treatment exposed to high levels of *Pseudomonas* (Rusin *et al.*, 1997).

Several epidemiological studies have been conducted to examine the potential health risk of HPC bacteria found in drinking water. In two separate studies, Calderon and Mood (1988; 1991) found no association of HPC concentrations with point-of-entry devices and gastroenteritis (Edberg *et al.*, 1996). However, according to a study on filtered and non-filtered tap water conducted by Payment and colleagues (1991a; 1994), an association was found between HPC concentrations and gastroenteritis. Epidemiological and animal infectivity studies are complex and difficult to control, therefore attempts have been made by researchers such as Janda and Bottone (1981), Lye and Dufour (1991), Edberg *et al.* (1996, 1997) to examine HPC directly in order to assess health risks. These analyses included: cytotoxicity, invasiveness, enzyme analyses, antibiotic susceptibility and identification.

The component of the general population which is at risk of being infected by HPC bacteria is increasing world-wide. South Africa is no exception to this rule and the population in total may in fact be at higher risk to infection by HPC bacteria than populations in many other countries, because of the increasing number of immuno-compromised persons such as HIV positive patients (Grabow, 1996). It is, therefore, evident that bacteria detected by heterotrophic plate counts and the composition of bacteria covered by these counts, warrant urgent attention in water quality monitoring and the assessment of health risks associated with drinking water supplies.

The objectives of this study were:

- To establish the most suitable plate technique, cost effective growth medium and optimal incubation conditions for the isolation of HPC bacteria.
- To characterize representative numbers of HPC bacteria isolated randomly from selected water distribution systems.
- To investigate the growth and haemolysis of HPC isolates on horse- and human-blood agar media.
- To examine the production of possible virulence factors by HPC bacteria using various enzymatic tests.
- To determine the susceptibility of HPC bacteria to natural and synthetic antibiotics.
- To evaluate the cytotoxicity, adherence and invasiveness of HPC isolates using cell cultures.
- To identify the isolated HPC bacteria displaying potentially pathogenic properties.
- To assess the potential health implications and re-evaluate current HPC limits for South African conditions based on these results.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

It was believed that with the start of the new millennium the provision of safe drinking water would rely on the choice of treatment and on extensive water quality surveillance systems to detect epidemics of gastroenteritis (Grabow, 1996; Hellard *et al.*, 1997). Biological treatment successfully removes micro-pollutants, reduces the chlorine demand and improves the quality of drinking water (Norton *et al.*, 2000). However, plant failure, secondary contamination of treated water supplies and human negligence have led to outbreaks of waterborne diseases (Grabow, 1996).

Little information is available on waterborne diseases in South Africa due to the lack of appropriate methods for detecting and recording of these infections. In South Africa, as in the rest of the world, waterborne diseases are of interest to public health practitioners and regulators who consider the potential of bacteria as tools to evaluate water quality and treatment (Grabow, 1996; WHO, 2001). Water quality monitoring programmes are based on tests for indicator organisms such as total coliform bacteria and faecal coliforms (Grabow, 1996). Nevertheless, these tests can not assure the safety of the water from viruses, protozoa and opportunistic heterotrophic plate count (HPC) pathogens (Ptak and Ginsburg, 1977; Hellard *et al.*, 1997).

Heterotrophic bacteria are found in tap, bottled as well as other sources of potable water and require simple organic compounds rather than carbon dioxide as a source of carbon (LeChevallier *et al.*, 1980; Rusin *et al.*, 1997). In drinking water distribution systems they show bacterial regrowth, aftergrowth and biofilm formation (Grabow, 1996). Some of the heterotrophic bacteria identified as opportunistic pathogens include: *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Klebsiella*, *Legionella*, *Moraxella*, *Mycobacterium*, *Serratia*, *Pseudomonas* and *Xanthomonas* (Rusin *et al.*, 1997; Kudinha *et al.*, 2000). Opportunistic pathogens are naturally present in the environment and the consumption

or exposure to water containing large numbers of HPC organisms can lead to diseases such as gastroenteritis, skin and mucous membrane infections particularly in persons whose immune systems are compromised by AIDS, organ transplantation or chemotherapy (WHO, 1996; Rusin *et al.*, 1997; WHO, 1997; Brandi *et al.*, 1999).

The heterotrophic plate count method, referred to as standard or total plate count, determines bacterial quality changes in water during treatment, storage and distribution (Reasoner, 1990). The HPC test, which is based on the ability of the organisms to grow and multiply in or on appropriate media under certain incubation conditions, detects a wide variety of bacteria such as species of faecal origin, water inhabitants and bacteria producing resistant spores (Grabow, 1996; WHO, 2001). Due to possible suppression of coliform detection, the heterotrophic bacterial counts in drinking water should not exceed 500 colony-forming units per milliliter (cfu.ml⁻¹) according to regulations set in Canada (USEPA, 1976; USEPA, 1989). In South Africa the recommended limit for heterotrophic plate counts is 100 cfu.ml⁻¹, but is disregarded due to difficulties in producing and delivering water which conforms to such a limit (Grabow, 1990; SABS, 2001).

Research showed that a small percentage of heterotrophic bacteria isolated from drinking water are cytotoxic and can directly damage human cells in tissue culture (Lye and Dufour, 1991; Payment *et al.*, 1994). The assessment of potential health risks involves direct examination of HPC bacteria because epidemiological and animal infectivity studies are complex or difficult to control (Lye and Dufour, 1991). The analyses for cytotoxic HPC bacteria include haemolysis, production of extracellular enzymes, cytotoxicity, adherence and invasiveness, and these features are used as potential virulence factors (Edberg *et al.*, 1996).

Specific members of HPC bacteria may cause hospital- and community-acquired infections such as pneumonia, meningitis and septicaemia (Rusin *et al.*, 1997). Pneumonia is caused mainly by *Aeromonas* spp., *Legionella pneumophila*, *Pseudomonas aeruginosa* and *Xanthomonas maltophilia* (Rusin *et al.*, 1997). *Acinetobacter* spp. have been associated with cases of meningitis whereas *Mycobacterium avium* complex and gram-positive bacteria are responsible for septicaemia (Rusin *et*

al., 1997). Therefore, opportunistic pathogens pose a threat to the immuno-compromised population and in South Africa with its high number of HIV positive cases their importance increases.

The HPC method does not detect all culturable micro-organisms in water. Among the bacteria which fail to produce visible colonies under the conditions concerned is the large group of mycobacteria (Grabow, 1996; Covert *et al.*, 1999). This group includes a component known as the *Mycobacterium avium* complex (MAC) (Grabow, 1996; Covert *et al.*, 1999). The MAC group is of increasing concern because it includes known pathogens exceptionally resistant to water treatment and disinfection processes, and these pathogens are able to proliferate in raw water sources and drinking water distribution systems (Grabow, 1996; Covert *et al.*, 1999). The MAC group, and many other heterotrophic organisms in water with potentially pathogenic properties, are not addressed in this study.

The objective of this project is to determine the potential health risk of HPC bacteria isolated from selected drinking water supplies in South Africa. The intention is to focus on HPC bacteria with special emphasis on virulence factors such as cytotoxicity, adherence and invasiveness.

2.2 Microbiology of drinking water

Disinfection of drinking water does not imply sterilisation, since there will remain some level of residual microbial risk (Haas *et al.*, 1993; Emmerson, 2001). It is not practical to obtain sterile water because of the high costs involved and a major proportion of the South African population, who are underprivileged, will not be able to afford the water. The microbiological quality of water can deteriorate due to bacteria growing on residual nutrients or because of the entrance of microorganisms in the distribution systems through cross-connections, repairs, breaks and loss of pressure (Emmerson, 2001; WHO, 2001). Many outbreaks of infection or pseudoinfection occur through lack of prevention measures and ignorance of the source resulting in transmission of pathogens (Emmerson, 2001). In order to reduce the level of organic matter and to ensure proper disinfection, treatment processes such as flocculation, settling, filtration and chlorination are applied

to drinking water (Payment and Franco, 1993).

The natural bacterial content in drinking water would not disappear even in the presence of a residual chlorine (Edberg *et al.*, 1997). In tap water distribution systems the disinfectant may dissipate and these naturally occurring bacteria multiply (LeChevallier *et al.*, 1980; Edberg *et al.*, 1996). Thus, an inverse proportionality exists between the incidence of heterotrophic plate count bacteria and chlorine concentration (Reasoner, 1990). However, no correlation exists between chlorine concentration and the occurrence of *Mycobacteria* species, which are members of the HPC bacteria, even at low pH (Covert *et al.*, 1999).

The presence of chlorine in water would inactivate indicator bacteria such as *E. coli* and thermotolerant coliforms, but it would leave the most resistant pathogens such as *Giardia* cysts, *Cryptosporidium* oocysts, *Mycobacterium avium* complex and human enteric viruses unaffected (Payment, 1999). *Pseudomonas aeruginosa* evades chlorine deactivation by the production of an extracellular protective capsule (WHO, 2001). Although unable to multiply in drinking water, enteroviruses, reoviruses, rotaviruses, adenoviruses and the resting stages of parasites (cysts, oocysts, ova) resist chlorination and can cause waterborne gastroenteritis (Haas *et al.*, 1993; Grabow, 1996; WHO, 1996; WHO, 1997).

Giardia and *Cryptosporidium* species are protozoan parasites forming cysts and oocysts (Casemore, 1991; Jakubowski *et al.*, 1991; Bellamy *et al.*, 1993; Barwick *et al.*, 2000). These structures are able to resist heat, physical and chemical agents, and because of their low numbers in water are not readily detectable (Casemore, 1991; Jakubowski *et al.*, 1991; Bellamy *et al.*, 1993; Barwick *et al.*, 2000). Chlorine dissolved in water is unable to deactivate these organisms and their ingestion may lead to diarrhoea attacks (LeChevallier, 1999; Barwick *et al.*, 2000).

The resistance of nontuberculous *Mycobacteria* to disinfection helps them survive in drinking water distribution systems (Covert *et al.*, 1999). Moreover, research showed that zinc may contribute to the persistence of *Mycobacterium avium* complex organisms in these distribution systems (Falkinham,

1996). This indicates that MAC organisms may be ubiquitous in drinking water and pose a potential health threat (Covert *et al.*, 1999).

A wide variety of bacteria in potable water are poorly described because of the disarray in taxonomy and the lack of research (Ward *et al.*, 1986). Known gram-negative obligate aerobic, non-fermentative bacteria such as *Acinetobacter*, *Aeromonas*, *Flavobacterium* and *Pseudomonas*, constitute the major proportion of the heterotrophic bacterial population (LeChevallier *et al.*, 1980). These HPC bacteria produce water-soluble and -insoluble pigments and have an optimum growth temperature within the mesophilic range (20°C to 37°C) which enables them to survive in drinking water (Ward *et al.*, 1986). Research showed, that the thick peptidoglycan layer of the cell walls of Gram-positive bacteria and the mycolic acid of *Mycobacterium* species enable them to survive and to proliferate in high numbers in chlorinated drinking water (LeChevallier *et al.*, 1980).

2.3 Biofilms

A biofilm represents a thin layer of actively growing microorganisms that attach to pipes or sediment deposits and produce exopolysaccharides, which may provide protection against environmental conditions such as high temperature, low pH and chemicals (Davey and O'toole, 2000; Momba *et al.*, 2000). Other functions include: initial attachment of microorganisms to surfaces; maintenance of microcolonies and biofilm structures; resistance to environmental stress, antimicrobial agents, protozoan predation and retention of nutrients within the biofilm (Atlas, 1997). The microbial communities inside the biofilm consist of multiple species of microorganisms that interact with each other and their environment (Davey and O'toole, 2000). Fast-growing microorganisms dominate the biofilm whereas the slow-growing organisms increase in numbers over time (Norton and LeChevallier, 2000).

Formation of biofilms associated with regrowth or aftergrowth of organisms is responsible for the deterioration of the bacteriological quality of drinking water (Momba *et al.*, 2000). Regrowth organisms are derived from viable cells of disinfectant injured bacteria, whereas aftergrowth involves

the growth of microorganisms native to a water distribution system (Reasoner, 1990; Momba *et al.*, 2000). Bacterial numbers in the biofilm may increase after viable bacteria survive the disinfection process and thus multiply in the distribution system (Momba *et al.*, 2000).

Heterotrophic plate count bacteria, pathogenic and toxigenic microbiological agents, such as *Acinetobacter* spp., *Campylobacter* spp., *Helicobacter pylori* and *Cryptosporidium parvum*, have been isolated from biofilms in chlorinated and non-disinfected water systems (Colbourne *et al.*, 1988; Momba *et al.*, 2000). The number of these micro-organisms may increase after dissipation from the biofilm and exposure to environmental stress (Norton and LeChevallier, 2000). It was found that the *P. aeruginosa* ATCC 27853 biofilm was susceptible to the antibiotics tobramycin and amikacin but was resistant to gentamicin (Ceri *et al.*, 1999). Research showed, that the aggregation of *Acinetobacter* strains EB22 in the exopolysaccharide layer of the biofilm increased their resistance to disinfection (WHO, 2001). Monochloramine or hydrogen peroxide, that are less reactive but more persistent than free chlorine, can control the biofilm formation by maintaining a longer disinfectant residual throughout the distribution system (Momba *et al.*, 2000). However, the aggregation of bacteria in biofilms caused a 2.3 fold increase of resistance to monochloramine and a 100 fold increase in resistance to hypochlorous acid (WHO, 2001).

Biofilms have been responsible for implant failure and for limitation of the lifetimes of indwelling medical devices (Anderl *et al.*, 2000). Biofilms constitute a protected mode of growth allowing survival of the organisms embedded in them in a hostile environment and thus causing environmental problems (Costerton *et al.*, 1999). Once in the biofilm, the organisms are protected from host defence mechanisms such as opsonisation and phagocytosis with the help of polymeric substances (Anderl *et al.*, 2000). Studies showed, that biofilm-grown sessile microorganisms are resistant to antibiotics, whereas planktonic cultures of the same organisms are susceptible (Costerton *et al.*, 1995; Kumon *et al.*, 1995; Yassien *et al.*, 1995; Stewart, 1996). Formation of biofilms and their resistance to antibiotics are at the root of many persistent and chronic infections such as gastro-enteritis (Costerton *et al.*, 1999; Momba *et al.*, 2000). These infections may be due to a single species or to a mixture of bacterial or fungal species found in the biofilms (Costerton *et al.*, 1999). The antibiotic

resistance is lost once the biofilm is dispersed (Costerton *et al.*, 1995). The difference in antibiotic susceptibility between planktonic and sessile populations of the same organism may be due to the diffusion of antibiotics or changes in the microbial physiology of the biofilm (Costerton *et al.*, 1995; Stewart, 1996).

Modern microbiology is focussing on bacteria found in natural, clinical and industrial settings that persist in association with surfaces (Davey and O'toole, 2000). Biofilm research led to exciting progress in the development of new technologies for studying these communities, advanced the understanding of the ecological significance of surface-attached bacteria and provided new insights into the molecular genetic basis of biofilm development (Davey and O'toole, 2000).

2.4 Indicator organisms

Little information exists on the relationship between indicator bacteria and possible health effects due to consumption of water meeting current quality guidelines (WHO, 2001). While acknowledging the beneficences of traditional water quality measurements based on coliform bacteria to the protection of public health, there exists inadequacy of this approach to assessing risks from other pathogens (CRCWQT, 2001). Nevertheless, monitoring the hygienic quality of drinking water requires the detection of indicator bacteria (WHO, 2001). Indicator organisms should be present whenever pathogens are present and should be detectable by simple, rapid and inexpensive methods (Grabow, 1990; WHO, 1993). Total and faecal coliform indicators demonstrate faecal pollution in water as an indirect health risk (Payment and Franco, 1993). However, coliforms are inadequate to indicate the presence of pathogens such as viruses and protozoan parasites (Payment and Armon, 1989; WHO, 2001).

Coliform organisms indicate drinking water quality because of their easy detection and enumeration in water (WHO, 2001). The group includes members of the following genera *Escherichia*, *Citrobacter*, *Enterobacter* and *Klebsiella* (WHO, 2001). *Budvicia* and *Rahnella* are coliforms never found in mammalian faeces (WHO, 2001). The detection of these organisms in treated water would

imply inadequate treatment, post-treatment contamination or aftergrowth on residual nutrients within the distribution system (WHO, 2001). The chlorine sensitive nature of the coliform group could result in a misleading assurance of safety for chlorinated supplies that are prone to contamination by more resistant organisms such as *Legionella* (CRCWQT, 2001).

Water quality guidelines and standards set by various authorities and countries differ (Grabow, 1996). However, they do specify that drinking water should rarely if ever contain total coliform bacteria, and never faecal coliforms or *E. coli* (Grabow, 1996). In addition, drinking water should be free of pathogens such as viruses and protozoa (*Standard Methods*, 1995; WHO, 1996).

TABLE 2.1: Microbiological requirements of drinking water (SABS, 2001)

| 1 | 2 | 3 | 4 | 5 |
|---|----------------------------|---|---------|---------|
| Determinants | Units | Allowable compliance contribution ¹⁾ | | |
| | | 95% min. | 4% max. | 1% max. |
| | | Upper limits | | |
| Heterotrophic plate count | count. ml ⁻¹ | 100 | 1 000 | 10 000 |
| Total coliform | count.100 ml ⁻¹ | not detected | 10 | 100 |
| Faecal coliform | count.100 ml ⁻¹ | not detected | 1 | 10 |
| Somatic coliphages | count.10 ml ⁻¹ | not detected | 1 | 10 |
| Enteric viruses | count.100 l ⁻¹ | not detected | 1 | 10 |
| Protozoan parasites (<i>Giardia/Crypto sporidium</i>) | count.100 l ⁻¹ | not detected | 1 | 10 |
| ¹⁾ The allowable compliance contribution shall be at least 95% to the limits indicated in column 3, with a maximum of 4% and 1%, respectively, to the limits indicated in columns 4 and 5. The objective of disinfection should, nevertheless, be to attain 100% compliance to the limits indicated in column 3. | | | | |

Other indicators employed in the water industry include: *Candida albicans*, *Staphylococcus aureus*, *Clostridium perfringens*, acid-fast bacteria, protozoan parasites, viruses and bacteriophages (Grabow, 1996). The presence of bacteriophages after treatment indicates that viruses have not been removed,

whereas spores of sulphite-reducing clostridia demonstrate the presence of persistent microorganisms (WHO, 2001). Enterococci referred to as faecal streptococci are associated with faecal pollution because they do not readily multiply in water environments (Grabow, 1996).

Regular analyses of heterotrophs and indicator bacteria determine water quality after storage (WHO, 2001). The heterotrophic plate count method detects bacteria and bacterial spores, which may result in aesthetic problems or opportunistic infections (Grabow, 1996). However, the method can not differentiate bacterial spores from vegetative cells and is therefore of limited value as an indicator (WHO, 2001). Heterotrophic plate counts are useful in regards of post-treatment contamination and growth within distribution systems (WHO, 2001). Heterotrophic bacteria such as *Aeromonas* and *Pseudomonas* serve as indicators of bacterial regrowth and thus provide useful information regarding sanitation (WHO, 2001).

2.5 Heterotrophic plate counts

One way of evaluating the general microbiological quality of finally treated and disinfected drinking water is the use of heterotrophic plate counts (Grabow, 1996). The standard HPC method is approved by the U.S. Environmental Protection Agency (USEPA) for reporting heterotrophic plate counts when residual disinfectant levels are less than 0.2 mg.L⁻¹ (Jackson *et al.*, 2000). The method assesses changes in finished water quality by measuring the bacterial regrowth or aftergrowth during distribution and storage (Reasoner, 1990). Microbial growth on corrosion materials and changes of the microbial population following a change in the type of disinfectant are determined using the HPC method (Reasoner, 1990).

The HPC is a useful tool for water quality assessment because it includes organisms such as spore-formers (mainly *Bacillus* species), which are exceptionally resistant to water treatment and disinfection processes, as well as bacteria which are able to proliferate in drinking water distribution networks and in stored water supplies including bottled water (Grabow, 1996). The isolated microorganisms assess the efficiency of coagulation, filtration and disinfection, which are designed

to keep microbial levels low (WHO, 2001). The test is widely used because it can be carried out at low costs in laboratories with basic facilities and expertise, and it yields results within a relatively short period of time. Details of the HPC method and its application in water quality assessment and monitoring have been described by Grabow (1990, 1996), Reasoner (1990), States and Sykora (1995) and WHO (1996, 1997, 2001).

In view of the above features the HPC is included in water quality specifications and recommendations world-wide. The South African Bureau of Standards specifies a HPC limit of 100 cfu.ml⁻¹ for drinking water (SABS, 1999; SABS, 2001). This limit is endorsed by the Departments of Health and Water Affairs, and is in line with specifications in the rest of the world (WHO, 2001). In the case of bottled water a maximum HPC of 50 cfu.ml⁻¹ is generally specified (WHO, 1997).

HPCs exceeding specified limits tend not to be perceived as a serious violation of water quality specifications and are widely accepted. This issue is growing in concern and importance particularly in the face of accumulating evidence that HPCs may actually detect organisms of meaningful health significance (Ptak and Ginsburg, 1977; Payment *et al.*, 1994; Edberg *et al.*, 1996; Hellard *et al.*, 1997; Rusin *et al.*, 1997).

Uncertainty exists regarding the human health effects resulting from exposure to HPC organisms following ingestion or inhalation of aerosols (Reasoner, 1990). In water meeting current quality standards, none of the microbial parameters provides adequate information on possible health risks to the population (WHO, 2001). Epidemiological investigations have failed to confirm the link between waterborne diseases and the presence of HPC organisms in drinking water (WHO, 2001). Factors which increase the urgency of questions concerning HPC specifications include the escalating component of highly vulnerable individuals among consumers of bulk drinking water supplies (Grabow, 1996). These individuals include the very young and very old, as well as individuals with compromised immune systems due to diseases such as AIDS, and medical interventions with immune systems as part of procedures such as organ transplantation and cancer therapy (Grabow, 1996). These developments imply that drinking water suppliers expose themselves to offenses with far-

reaching legal implications or high costs to produce drinking water supplies which strictly adhere to HPC specifications (Grabow, 1996).

2.5.1 Bacteria associated with heterotrophic plate counts

The microbiological quality of drinking water can deteriorate due to nuisance bacteria growing on residual nutrients such as assimilable organic carbon or ammonia (WHO, 2001). Results indicated, that environmental heterotrophic plate count bacteria show higher resistance to disinfection than laboratory grown organisms such as *Escherichia coli* and if adequate residual levels of disinfectant are not maintained, these microorganisms may multiply in the distribution system (Reasoner and Geldreich, 1985; WHO, 2001). According to research, nearly all HPC bacteria surviving chlorine disinfection consist of sporeforming gram-positive and acid fast organisms of the genera *Bacillus* and *Mycobacterium*, respectively (WHO, 2001). It has been suggested, that prechlorination with chlorine could lead to a shift in the composition of the HPC population to gram positive bacteria (Norton and LeChevallier, 2000).

Microbiological analyses demonstrated, that enumeration of HPC bacteria is useful to indicate the presence of opportunistic pathogens, the effectiveness of disinfection and the potential for coliform suppression by coliform antagonists (LeChevallier *et al.*, 1980). In a study conducted to identify bacteria in distribution water, over 30% of the total bacterial population were opportunistic pathogens (LeChevallier *et al.*, 1980). *Aeromonas* spp. were the most prevalent opportunistic bacterial organisms in chlorinated distribution water (9.5%) and the most common species in raw water (15.9%) (LeChevallier *et al.*, 1980). *Acinetobacter* spp., *Flavobacterium* spp., *Pseudomonas* spp. comprised 25% of the HPC bacterial population (LeChevallier *et al.*, 1980). Pigmented bacteria are a constituent part of the HPC organisms (Reasoner, 1990). The percentage occurrence of yellow, orange and pink bacteria in treated water was found to be seasonally related (Reasoner, 1990).

HPC bacteria and those poorly described or not yet studied bacteria present in water are adopted to their environment by competing successfully for nutrient sources (Edberg *et al.*, 1996). The number

of these microorganisms may increase or decrease because of interaction of water with the biofilm on the inside of distribution pipes (Edberg *et al.*, 1996). Higher heterotrophic plate counts were observed at higher water temperatures with greater species diversity in the warmer summer periods than during the cold winter months (LeChevallier *et al.*, 1980; Rattray and Logan, 1993). LeChevallier *et al.* (1980) found, that the dominant population during the cooler months were gram-positive bacteria. No correlation could be found between pH levels in treated waters and heterotrophic activities (Rattray and Logan, 1993).

According to research, nontuberculous *Mycobacteria* have been isolated from 38% of drinking water supplies examined (Covert *et al.*, 1999). *Mycobacterium avium complex* (MAC) organisms were present in 19% of the water samples, however other mycobacterial opportunistic pathogens such as *M. kansasii*, *M. mucogenicum* and *M. peregrenium* were present (Covert *et al.*, 1999). These organisms were isolated from well-operated and maintained drinking water utilities with HPC levels of ≤ 500 cfu.ml⁻¹ and chlorine residuals of up to 2.8 mg.L⁻¹ (Covert *et al.*, 1999). This implies that exposure to drinking water containing nontuberculous *Mycobacteria* could pose a health risk to immuno-compromised hosts and to a lesser extent to immuno-competent individuals (Covert *et al.*, 1999).

HPC bacteria may form populations of stressed cells when left for prolonged incubation periods in sterile water (Bogosian *et al.*, 1998; Bogosian *et al.*, 2000). These cells are in a viable state but can not be cultured on standard microbiological media (Bogosian *et al.*, 1998; Bogosian *et al.*, 2000). However, the addition of nutrients to stressed cells may result in their resuscitation and the appearance of populations of culturable cells (Bogosian *et al.*, 2000). Viable but nonculturable cells may also be present in water and can be detected using various molecular techniques (Bogosian *et al.*, 2000).

2.5.1.1 *Acinetobacter*

Acinetobacter species are ubiquitous in water and may act as opportunistic pathogens causing nosocomial urinary tract infections, pneumonia, bacteremia, secondary meningitis and wound infections (Bergogne-Berezin, 1994; Jellison *et al.*, 2001). These diseases are predisposed by factors such as malignancy, burns and major surgery (Rusin *et al.*, 1997; Jellison *et al.*, 2001). Due to difficulties of naming individual species and biovars, the term *Acinetobacter calcoaceticus baumanii* complex is used in API references to cover any subgroups of this species (Palleroni, 1984).

Acinetobacter calcoaceticus baumanii complex is multidrug resistant and is therefore difficult to treat (Jellison *et al.*, 2001). Some strains are susceptible to β -lactams and quinolones, with imipenem-cilastatin or ampicillin-sulbactam as the most effective agents against these bacteria (Jellison *et al.*, 2001).

2.5.1.2 *Aeromonas, Plesiomonas and Pseudomonas*

Aeromonas and *Plesiomonas* spp. have been grouped together traditionally in spite of recent genetic evidence, which suggests that these two genera are actually not closely related (Atlas, 1997; Vitovec *et al.*, 2001). They are found in various habitats and may act as opportunistic pathogens (Atlas, 1997; Vitovec *et al.*, 2001). *Plesiomonas* spp., such as *P. shigelloides*, are well known opportunistic human and animal pathogens, causing diarrhea and extraintestinal infections (Atlas, 1997; Vitovec *et al.*, 2001).

Aeromonas and *Pseudomonas* species have become increasingly recognised as enteric pathogens although their etiological role remains to be documented in animal models or studies on human volunteers (Pin *et al.*, 1997). These organisms occur in soil, fresh water and sewage, and have been isolated from drinking water even after chlorination (Brandi *et al.*, 1999; Kudinha *et al.*, 2000; WHO, 2001). Their detection is accomplished by simple, inexpensive and rapid methods applicable in a basic routine bacteriological laboratory (WHO, 2001). These bacteria do not indicate faecal contamination

but demonstrate regrowth in distribution systems and are associated with unpleasant taste, odour and turbidity (Brandi *et al.*, 1999; WHO, 2001).

Unlike most other gram-negative organisms, *Pseudomonas* and *Aeromonas* produce virulence factors (Table 2.2) such as cholera related cytotoxin, enterotoxins, haemagglutinin, α - and β -haemolysins and the ability to bind and invade epithelial cells (Majeed and Macrae, 1994; Ashbolt *et al.*, 1995; Pin *et al.*, 1997; Brandi *et al.*, 1999; Kudinha *et al.*, 2000). These opportunistic pathogens have been involved in human infections, such as gastroenteritis, peritonitis, endocarditis, meningitis, septicemia as well as in urinary tract and wound infections (Pin *et al.*, 1997).

According to research, the density of *Aeromonads* in aquatic environments was found to correlate to eutrophication and to vary with seasonal trends (Ashbolt *et al.*, 1995). There is no dispute over the emergence of *Aeromonas* as an important human pathogen (Kudinha *et al.*, 2000).

Aeromonas is comprised mainly of three motile mesophilic species, *A. hydrophila*, *A. sobria* and *A. caviae* (Ashbolt *et al.*, 1995; Graf, 1999). Studies showed, that *A. veronii* biotype *sobria* isolated from freshwater are the most prolific producers of exotoxins (15 out of 17 isolates of *A. veronii* biotype *sobria* tested positive for haemolysin, cytotoxin and enterotoxin as illustrated in Table 2.2) (Ashbolt *et al.*, 1995). Eighty eight percent of *A. veronii* biotype *sobria* isolated from sources such as: marine, fresh river, storm waters, tertiary and primary effluents, tested positive for the three exotoxins (haemolysin, cytotoxin and enterotoxin) (Table 2.3) (Ashbolt *et al.*, 1995). *A. hydrophila* is also considered a significant exotoxin-producer followed by other *Aeromonas* species (Ashbolt *et al.*, 1995). These *Aeromonas* species are similar and have been associated with human infections such as diarrhoea (McGowan and del Rio, 1990; Rusin *et al.*, 1997; Brandi *et al.*, 1999).

TABLE 2.2: Percentage of exotoxin-producing aeromonads isolated from different water sources (Ashbolt *et al.*, 1995)

| Source | Haemolysin (H) | Cytotoxin (C) | Enterotoxin (E) | Number of strains tested | | |
|-------------------|-------------------|------------------|--------------------|--------------------------|-----|-----|
| | | | | (H) | (C) | (E) |
| Marine | 12.5 | 22 | 16.7 | 64 | 59 | 6 |
| Freshwater | 64.7 | 27.2 | 84.3 | 139 | 136 | 51 |
| Stormwater | 42.1 | 58.1 | 70 | 38 | 31 | 10 |
| Tertiary Effluent | 21.7 | 33.3 | 100 | 23 | 21 | 3 |
| 1° Eff. or Sewage | 13.2 | 24.3 | 33.3 | 38 | 37 | 3 |
| No strains tested | 302 | 284 | 73 | 302 | 284 | 73 |

TABLE 2.3: Distribution of toxigenic characteristics of aeromonads by phenotype or PCR identification (Ashbolt *et al.*, 1995)

| Group | Haemolysin (H) | Cytotoxin (C) | Enterotoxin (E) | % of Strains with ² : | |
|------------------------------------|-------------------|------------------|--------------------|----------------------------------|-------|
| | | | | (H+C+E) | (H+C) |
| <i>A. hydrophila</i> | 65/88 | 63/80 | 30/41 | 63 | 75 |
| <i>A. veronii-sobria</i> | 17/24 | 20/23 | 17/17 | 88 | 74 |
| <i>A. veronii-veronii</i> | 37018 | 37018 | 36891 | 0 | 63 |
| <i>A. caviae</i> | 36907 | 36997 | 36891 | 36891 | 6 |
| <i>A. schubertii</i> | 36902 | 36900 | 36891 | - | 0 |
| <i>A. trota</i> | 36892 | 36892 | 36891 | - | - |
| Atypical | 35/151 | 42/144 | 37084 | 31 | 22 |
| No. of strains | 302 | 284 | 73 | | |
| <i>A. spp.</i> by PCR ¹ | 46/100 | 32/116 | 37216 | 36.4 | 24.1 |
| Not <i>A. spp.</i> by PCR | 4/38 | 7/35 | 36892 | -50 | 11.4 |

¹ Not including *A. hydrophila* or *A. veronii* isolates.

² Only based on the common isolates for the exotoxins concern.

Pseudomonas spp. have not been considered human pathogens, however several members of this group are involved in human and animal opportunistic infections (Foght *et al.*, 1996). *P. aeruginosa* may cause superficial infections due to contaminated water but is not responsible for enteric infections following ingestion (WHO, 2001). *P. aeruginosa* is the most frequent source of infection of burn patients, the second leading cause of nosocomial pneumonia, the third most common cause of nosocomial urinary tract infections and the fourth leading cause of surgical wound infections (Rusin *et al.*, 1997). *P. aeruginosa* is responsible for septicaemia, meningitis and infections of drainage sites resulting from trauma (Janda and Bottone, 1981; Wiedmann *et al.*, 2000). Cystic fibrosis and immuno-compromised patients are prone to colonisation with mucoid morphotypes of *P. aeruginosa*, which have been associated with progressive pulmonary infections in these individuals (Janda and Bottone, 1981; Wiedmann *et al.*, 2000). Due to this, the legislation in several countries requires that bottled water products must be free of *P. aeruginosa* (Foght *et al.*, 1996; Wiedmann *et al.*, 2000). The lack of robust identification tools for these organisms can lead to the misidentification of nonpathogenic *Pseudomonas* spp. as pathogenic, causing costly and unnecessary food recalls (Foght *et al.*, 1996; Wiedmann *et al.*, 2000).

2.5.1.3 *Bacillus and Staphylococcus*

Bacillus and *Staphylococcus* spp. are found in water distribution systems (Atlas, 1997). *Bacillus* spp. produce resistant endospores which help these bacteria to survive chlorination, exposure to high temperature, low pH and other environmental conditions (Atlas, 1997). *Bacillus cereus* spores are found in many foods, especially rice, pulses vegetables and in tap water distribution systems (Edberg *et al.*, 1996; Mims *et al.*, 1998). Infection is acquired by ingestion of organisms or toxins (Mims *et al.*, 1998). *B. cereus* causes food poisoning, the commonest association being with reheated cooked rice and pulses (Mims *et al.*, 1998). This organism induces bacteremia in immuno-compromised hosts as well as symptoms such as vomiting and diarrhoea (Mims *et al.*, 1998).

S. aureus is present in the nose and skin of humans, and has been isolated from water (LeChevallier *et al.*, 1980). Colonies of *S. aureus* are yellow coloured due to carotenoids, which are not produced



in the presence of glucose during anaerobic growth or in liquid culture (Atlas, 1997). Infections caused by staphylococcal species include deep and superficial abscesses, endocarditis, wound infections, mastitis, osteomyelitis, pneumonia, meningitis and sepsis (Atlas, 1997). In addition, *S. aureus* produces several toxins that cause diseases such as staphylococcal food poisoning and toxic shock syndrome (Atlas, 1997). The emergence of methicillin resistant strains of *S. aureus* (MRSA) and vancomycin resistant staphylococci represents a problem in medicine today (Atlas, 1997).

2.5.1.4 *Chromobacterium* and *Flavobacterium*

Chromobacterium and *Flavobacterium* species are found in water environments (Atlas, 1997). *Chromobacterium violaceum* causes pyogenic infections in animals and humans (Atlas, 1997). *Flavobacterium* produces yellow to orange pigmented colonies (Palleroni, 1984; Atlas, 1997). *Flavobacterium breve* and *Flavobacterium meningosepticum* are the opportunistic pathogens present in this genus (Palleroni, 1984; Atlas, 1997).

2.5.1.5 *Klebsiella*

Klebsiella colonizes the mucosal surfaces of mammals such as humans, horses or swine (Duncan, 1988; Podschun and Ullmann, 1998). *Klebsiella* spp. have been detected in surface water used for human consumption despite adequate disinfecting concentrations of chlorine (Duncan, 1988; Podschun and Ullmann, 1998). These organisms give a positive faecal coliform test that may be interpreted as faecal contamination of the water supply (Duncan, 1988). The pathogenicity of *Klebsiella* is associated with the production of cytotoxins, enterotoxins and haemolysins (Minami *et al.*, 1994; Podschun and Ullmann, 1998). *Klebsiella* causes community-acquired bacterial pneumonia, occurring especially in chronic alcoholics and shows characteristic radiographic abnormalities due to a severe pyogenic infection which has a high fatality rate if untreated (Podschun and Ullmann, 1998). Members of this genus are involved in septicemia and meningitis in newborns in neonatal intensive care units (Podschun and Ullmann, 1998). As opportunistic pathogens, *Klebsiella* spp. attack immunocompromised individuals who are suffering from diseases such as

diabetes mellitus or chronic pulmonary obstruction (Podschun and Ullmann, 1998). Evidence indicated, that 8% of all nosocomial bacterial infections in the United States and Europe, are due to *Klebsiella* spp. thus placing them among the eight most significant infectious pathogens in hospitals (Podschun and Ullmann, 1998). Nosocomial infections due to this organism are associated with the medically most important species of the genus- *Klebsiella pneumoniae* (Podschun and Ullmann, 1998). As a cause of nosocomial gram-negative bacteremia, *K. pneumoniae* is second only to *Escherichia coli* (Yinnon *et al.*, 1996). *K. pneumoniae* is associated with nosocomial infections of the genito-urinary, respiratory tracts and septicaemic foci in debilitated or chronically ill patients (Selden *et al.*, 1971; Podschun and Ullmann, 1998). *K. terrigena* and *K. planticola*, formerly regarded as environmental *Klebsiella* species, are considered as pathogens and have been isolated from human clinical specimens (Podschun and Ullmann, 1998). The spread of antibiotic-resistant strains, especially of extended-spectrum β -lactamase (ESBL)-producing strains, renewed interest in *Klebsiella* infections (Podschun and Ullmann, 1998).

2.5.1.6 *Legionella*

Legionellae thrive in stagnant water at ambient temperatures and may survive chlorination by residing in sludge and scale or inside protozoa (Emmerson, 2001). After isolation of *Legionella* species from the air-conditioning system during the first outbreak in Philadelphia, the bacteria have been isolated from various sources in the environment especially from aquatic ones (Kwaik *et al.*, 1998). In the environment, *Legionella* spp. can not multiply extracellularly and act as parasites of protozoa such as *Acanthamoeba*, *Hartmannella* and *Tetrahymena* (Kwaik *et al.*, 1998; Emmerson, 2001). *Legionellae* remain the only bacterial species that are prolific in their intracellular replication within amoebae (Kwaik *et al.*, 1998). This host-parasite interaction is central to the pathogenesis and ecology of these bacteria (Kwaik *et al.*, 1998).

Legionella spp. are heterotrophic bacteria found in domestic water sources and are associated with cases of pneumonia (Rusin *et al.*, 1997). The low nutrient content in cooling tower systems may favor the occurrence of legionellae but in water with high nutrient content and low water temperature,

legionellae can not compete with other bacteria (Kusnetsov *et al.*, 1993). Reports demonstrate concentrations of 1.4×10^4 to 1.7×10^5 cells.ml⁻¹ of *Legionella* spp. in raw drinking water sources, detected using direct fluorescent antibody techniques (Rusin *et al.*, 1997). *L. pneumophila* is the major pathogen in the genus responsible for Pontiac fever and legionnaires' disease (Alary and Joly, 1991; Rusin *et al.*, 1997; Kwaik *et al.*, 1998). All *Legionella* spp. are considered potentially pathogenic for humans (Rusin *et al.*, 1997).

2.5.1.7 *Moraxella* and *Serratia*

Moraxella and *Serratia* spp. are present in groundwater (Rusin *et al.*, 1997). *Moraxella* are well established parasites of humans with good adaptation to the host and can act as extranasal invaders (Palleroni, 1984; Rusin *et al.*, 1997). *M. nonliquefaciens* and *M. osloensis* are responsible for septicaemia, bone and joint infections, urinary tract infections, meningitis and empyema (Rusin *et al.*, 1997). *M. nonliquefaciens* are known to cause upper respiratory and eye infections (Graham *et al.*, 1990; Rusin *et al.*, 1997). Although considered to be harmless, *M. osloensis* can induce significant health problems such as meningitis and septicaemia (Palleroni, 1984; Rusin *et al.*, 1997). *Serratia marcescens* may act as opportunistic pathogens causing urinary tract infections in hospitalized patients (Palleroni, 1984; Rusin *et al.*, 1997). Nonpigmented biogroups A5/8 and TCT of *S. marcescens* are confined to hospitalized patients (Palleroni, 1984). However, pigmented biogroups A1 and A2/6 of *S. marcescens* are found in the natural environment and occasionally in human patients (Palleroni, 1984).

2.5.1.8 *Mycobacterium avium*

The *Mycobacterium avium* complex (MAC), which consists of *M. avium* and *M. intracellulare*, is of increasing concern because it includes known pathogens exceptionally resistant to water treatment and disinfection processes (Grabow, 1996; Taylor *et al.*, 2000). Members of MAC have been found in drinking water and because there is no evidence of person-to-person transmission, the environment is considered as a possible source of infection (Rusin *et al.*, 1997; Covert *et al.*, 1999). MAC is

responsible for human and animal diseases including infections of the lungs, lymph nodes, skin, bones, gastrointestinal and genitourinary tract (Mazurek *et al.*, 1993; Norton and LeChevallier, 2000). MAC can cause pulmonary disease, osteomyelitis and septic arthritis in people with no known predisposing factors (Jones *et al.*, 1995). These bacteria are well known as a major cause of disseminated opportunistic infections in immuno-compromised patients (Covert *et al.*, 1999). The incidence of MAC infections in HIV positive patients has increased exponentially over the past years and is the second most common cause of death (Singh and Yu, 1994). In a study conducted by Kulski and colleagues (1995), it was found that the detection rate of MAC at autopsy has been as high as 53% in the examined AIDS patients.

Research showed, that serotypes of MAC associated with human infections correlate with serovars found in water (Von Reyn *et al.*, 1994). Water can therefore act as a reservoir and as a potential source of infection (Von Reyn *et al.*, 1994). Covert and colleagues (1999) detected MAC organisms in 54% of ice samples and in 35% of the public drinking water samples investigated, and suggested possible health implications. These bacteria were isolated from well operated, well-maintained drinking water utilities with acceptable HPC levels of $<500 \text{ cfu.ml}^{-1}$ and chlorine residuals of up to 2.8 mg.L^{-1} (Covert *et al.*, 1999).

In drinking water, the occurrence of MAC appears to be infrequent and sporadic due to the formation of clumps of nontuberculous mycobacteria (Falcao *et al.*, 1993; Falkinham, 1996; LeChevallier, 1999). High levels of MAC's may be associated with distribution system events that dislodge biofilms, such as flushing or flow reversals (LeChevallier, 1999). Little is known regarding the risk of MAC infection in water. The data suggest that one-third of HIV-infected humans exposed to $10\text{--}500 \text{ cfu.100 ml}^{-1}$ for long periods might develop MAC infection (LeChevallier, 1999). Determination of growth requirements, antibiotic and heavy metal susceptibilities, serotype, bacteriophage type, plasmid profile, multilocus enzyme electrophoretic type, and species by DNA probe analysis can provide insight into MAC infections (Mazurek *et al.*, 1993).

It is evident that *Mycobacterium avium* complex poses a threat to the immuno-compromised population and to a lesser degree to immuno-competent individuals with no known predisposing factors (Jones *et al.*, 1995). It is therefore important to determine the incidence of MAC especially in South Africa with its increasing numbers of HIV positive patients. However, MAC's do not form part of the current study.

2.5.1.9 *Xanthomonas* and *Sphingomonas*

Xanthomonas spp. are found in distribution water supplies (LeChevallier *et al.*, 1980). *Xanthomonas* are capable of colonising the human body and thus causing disease (Rusin *et al.*, 1997). Risk factors for patients include stays in intensive care units, mechanical ventilation, antibiotic treatment and cancer (Rusin *et al.*, 1997). *X. maltophilia* causes opportunistic infections such as septicaemia, pneumonia, wound infections, meningitis and endocarditis (Von Graevenitz, 1995).

Sphingomonas species are isolated from biofilms in drinking water distribution systems (Koskinen *et al.*, 2000). *Sphingomonas aromaticivorans*, *S. subterranea*, *S. xenophaga* and *S. stygia* are some of the species included in this genus (Koskinen *et al.*, 2000). Considering the potentially pathogenic features of *Sphingomonas* spp. such as respiratory infections, their presence in drinking water is not desirable (Koskinen *et al.*, 2000).

2.5.1.10 *Shewanella*

Shewanella putrefaciens (“*Pseudomonas putrefaciens*”) has been recovered from nonhuman sources, including aquatic reservoirs (marine, freshwater and sewage), oil, gas, dairy, soil and has been implicated as a human pathogen (Khashe and Janda, 1998). This bacterium is responsible for a number of microbial illnesses such as bacteremia, soft tissue infections and otitis media (Butt *et al.*, 1997; Chen *et al.*, 1997). According to research, *S. alga* appears to be the more virulent species, due to the production of a haemolytic substance (Khashe and Janda, 1998). Significant differences exist between *S. putrefaciens* and *S. alga* regarding resistance to antimicrobial agents, pathogenicity and

the ability to adhere to epithelial cells (Khashe and Janda, 1998).

2.5.1.11 *Burkholderia pseudomallei*

Burkholderia pseudomallei is widely distributed in soil and water of certain regions (Hunter, 1997). This species was until recently classified as belonging to the genus *Pseudomonas* (Hunter, 1997). This organism was isolated from environmental specimens one year after an outbreak of acute melioidosis in a remote coastal community in northwestern Australia (Inglis *et al.*, 2000). Melioidosis, due to infection by *B. pseudomallei*, leads to purulent abscesses, which can affect several body systems (Hunter, 1997). Septicemic invasion may occur as well (Hunter, 1997). Environmental sampling during the outbreak investigation identified the potable water supply as a possible source of *B. pseudomallei* (Inglis *et al.*, 2000).

2.5.1.12 *Tsukamurella* and *Rhodococcus*

Tsukamurella spp. are gram-positive, weakly or variably acid-fast, non-motile, obligate aerobic bacilli that exist primarily as saprophytes in soil and foam (thick stable scum on aeration vessels and sedimentation tanks) of activated sludge (Goodfellow *et al.*, 1998; Larkin *et al.*, 1999). Moreover, they are actinomycetes related to *Rhodococcus*, *Nocardia* and *Mycobacteria* (Larkin *et al.*, 1999). *Tsukamurella* spp. are opportunistic pathogens, causing mainly diseases in immuno-compromised individuals (Medical Bacteriology, 2001). Infections with these microorganisms have been associated with chronic lung diseases, immuno-suppression, indwelling foreign bodies and postoperative wound infections (Larkin *et al.*, 1999). *Tsukamurella* were reported in four cases of catheter-related bacteremia and in individual cases including a chronic lung infection, necrotizing tenosynovitis with subcutaneous abscesses, cutaneous and bone infections, meningitis and peritonitis (Larkin *et al.*, 1999).

Rhodococcus spp. are pleomorphic, weakly acid-fast, gram-positive coccobacilli that grow well aerobically on non-selective media (Medical Bacteriology, 2001). *Rhodococcus equi* is ubiquitous

in soil and in activated sludge foam, and is the causative agent of rhodococcosis in humans (Medical Bacteriology, 2001). Incidence of infection is increasing in immuno-compromised populations particularly AIDS patients (Medical Bacteriology, 2001). Virulence factors characteristic of these pathogens include: capsular polysaccharide, cholesterol oxidase which lyses cell membranes, mycolic acids that stimulate granuloma formation and factors that promote survival in macrophages (Medical Bacteriology, 2001). *Rhodococcus spp.* cause abscesses in lungs, lymph nodes, meninges, pericardium and skin (Medical Bacteriology, 2001).

2.5.2 Waterborne outbreaks associated with HPC bacteria

The collection of waterborne disease surveillance data is useful in assuring the safety of drinking and recreational water (Barwick *et al.*, 2000). However, the data may underestimate the incidence of outbreaks caused by different etiologic agents (Barwick *et al.*, 2000). *P. aeruginosa*, *Serratia marcescens*, *Klebsiella pneumoniae* and other bacterial agents were all isolated from 11 patients in Missouri suffering with fever and upper respiratory symptoms associated with vaporizer and whirlpool use (Barwick *et al.*, 2000). Determination of the epidemiology of individual cases of illnesses associated with water depends on several factors such as the public awareness, the extent of laboratory testing and the investigative activities of health and environmental agencies (Barwick *et al.*, 2000).

A few studies gave an indication of the relative importance of opportunistic HPC bacteria in causing human infections (Rusin *et al.*, 1997). A study conducted by Burke and colleagues (1984) indicated that gastrointestinal illness resulted from consuming aeromonads in drinking water. Individuals, such as children and adults, affected by aeromonad-induced diarrhoea harbor enterotoxigenic strains (Pin *et al.*, 1997). A known relationship exists between pathogenicity and phenotypic markers, such as haemolysin, production of lysine decarboxylase, Voges-Proskauer reaction or fermentation of arabinose, salicin or glucitol (Pin *et al.*, 1997). In addition, there is a link between the production of haemolysin or cytotoxin and the enterotoxigenicity of *Aeromonas* isolates (Pin *et al.*, 1997).

Aeromonas species are associated with wound and systemic infections (Ashbolt *et al.*, 1995). HPC bacteria that cause community- and hospital-acquired pneumonia include *Aeromonas* spp. and *Xanthomonas maltophilia* (Rusin *et al.*, 1997). *Acinetobacter* spp., *Legionella pneumophila* and *Pseudomonas aeruginosa* are other HPC organisms responsible for serious cases of pneumonia (Marrie, 1994; Rusin *et al.*, 1997). *Mycobacterium avium* complex is the etiological agent for 0.6% of the pneumonia cases in the U.S. and causes severe pulmonary or gastrointestinal diseases in AIDS patients (Marrie, 1994; Rusin *et al.*, 1997).

The estimated annual number of cases of meningitis is about 15 900 per year caused mainly by *Acinetobacter* and *Pseudomonas* spp. (Schlech, 1992). Other HPC bacteria are rarely associated with meningitis (Rusin *et al.*, 1997).

Septicaemia is caused by *Pseudomonas aeruginosa*, *Acinetobacter* spp. and *Mycobacterium avium* complex (Tilley and Roberts, 1994; Rusin *et al.*, 1997). AIDS patients are particularly affected by septicaemia due to infection of *Mycobacterium avium* complex (Rusin *et al.*, 1997). Other HPC bacteria have never been documented as a cause of septicaemia or were implicated in rare cases (Rusin *et al.*, 1997).

2.6 Methodology to detect HPC organisms and the production of potential virulence factors

Although bacteria grown on artificial media give useful information on the bacterial population in the sample, the data is incomplete since only a few species can be cultivated (WHO, 2001). Environmental microbiology applies these cultivable organisms in the improvement of water sanitation and public health (WHO, 2001). The analysis of HPC bacteria requires time-consuming preparation of media and can be difficult to interpret (Jackson *et al.*, 2000). The heterotrophic plate count (HPC) is commonly used to assess the general microbiological quality of drinking water (Grabow, 1996). The plate counts are carried out by inoculating samples of water (using spread or pour plate technique) onto a rich plate count agar growth medium without any selective agents to

allow the growth of a wide spectrum of heterotrophic microorganisms (Grabow, 1996). Plates are generally incubated for 24 h or 48 h, at temperatures ranging from 20°C to 37°C (Grabow, 1996). Organisms which produce colonies under these conditions consist predominantly of many species of bacteria while some fast-growing fungi (yeasts and moulds) may develop visible colonies (Grabow, 1996). The HPC is obtained by counting all colonies visible to the naked eye under the specified conditions (Grabow, 1996).

2.6.1 Isolation of HPC bacteria

Pure cultures of HPC bacteria are isolated from drinking water samples using the streak plate technique (Atlas, 1997). In this method, a loopful of bacterial cells is streaked across the surface of a sterile solidified agar medium in a Petri plate maintaining aseptic techniques (Atlas, 1997). Different streaking patterns can be used to separate individual bacterial cells on the agar surface (Atlas, 1997). The plates are then incubated under favorable conditions to permit the growth of the HPC organisms (Atlas, 1997). Each isolated colony is assumed to arise from a single bacterium and therefore to represent a clone of a pure culture (Atlas, 1997). The purity of the isolates is maintained by re-streaking them onto fresh media and by freezing them in 50% glycerol (Atlas, 1997). Glycerol is employed as an antifreeze agent to prevent damage due to ice crystals and to ensure the ability to recover viable microorganisms when frozen cultures are thawed (Atlas, 1997).

The purity of the bacterial isolates is ascertained by application of the Gram-staining technique (Atlas, 1997). This Gram-staining procedure begins with the primary stain crystal violet, which stains bacterial cells blue-purple, followed by application of the mordant Gram's iodine (a substance that fixes the primary stain in the bacterial cells) and decolourisation with acetone-alcohol to remove the primary stain (Atlas, 1997). Counterstaining is achieved with the application of the stain safranin, which stains the bacteria that were decolourised in the previous step (Atlas, 1997). Gram-positive bacteria appear blue-purple whereas Gram-negative bacteria appear red-pink (Atlas, 1997). The ability to distinguish between Gram-negative and Gram-positive bacteria is due to differences in their cell wall structures, thus Gram-positive bacteria with their thick cell walls retain the primary stain

when acetone-alcohol is applied (Atlas, 1997).

2.6.2 Culturing of HPC bacteria

Culture techniques include liquid cultivation techniques and methods based on solid media for the detection of colony formation such as pour plate, spread plate and membrane filtration techniques (WHO, 2001). Recently, the SimPlate total plate count method for determining the most probable number (MPN) of microorganisms was developed as an alternative test method for HPCs in water (Jackson *et al.*, 2000). It was established by IDEXX Laboratories, Westbrook, Maine and approved by the Association of Official Analytical Chemists (AOAC) International Research Institute (Jackson *et al.*, 2000).

The application of the HPC analysis requires the comparison of HPC methods to determine which method provides the highest HPC numbers (Reasoner, 1990). These techniques are selective since a single medium composition, a single incubation temperature and aerobic or anaerobic atmosphere can be tested (WHO, 2001).

2.6.2.1 Pour plate method

The pour plate technique is simple and easy to use (Reasoner, 1990; Jackson *et al.*, 2000). In this method, a volume of 0.1-1.0 ml of culture is pipetted into a sterile Petri plate (Madigan *et al.*, 1997; Jackson *et al.*, 2000). Melted agar medium is added and mixed by swirling the plate on the table top (Madigan *et al.*, 1997; Jackson *et al.*, 2000). The method is not suitable for enumeration of heat-sensitive and nutritionally stressed heterotrophic bacteria in water (Reasoner, 1990; Jackson *et al.*, 2000). As a result of this, the pour plate technique yields lower bacterial counts than does the spread plate method (Reasoner, 1990; Jackson *et al.*, 2000). Furthermore, colony morphology characteristics can not be observed because bacteria are submerged in the agar (Reasoner, 1990; Jackson *et al.*, 2000). However, the pour plate method with incubation at 35°C for 48 h has been extensively used for bacterial enumeration in drinking water and is generally preferred (Standard

Methods, 1998).

2.6.2.2 Spread plate method

In the spread plate method, 0.1 ml volume of diluted culture is spread over the surface of an agar plate using a sterile glass spreader (Madigan *et al.*, 1997). The procedure is considered more tedious than the pour plate method (Reasoner, 1990; Madigan *et al.*, 1997). However, the spread plate method provides more suitable conditions for growth of HPC bacteria in water than the pour plate method (Reasoner, 1990; Madigan *et al.*, 1997). It is easy to observe differences in colony shape, texture, pigmentation, size and other characteristics because bacterial growth is not constrained by the surrounding agar (Reasoner, 1990; Madigan *et al.*, 1997).

2.6.2.3 Membrane filter method

The membrane filter method allows the analysis of sample volumes larger than 1.0 ml (Reasoner, 1990; Madigan *et al.*, 1997). It enumerates low concentrations of bacteria in finished drinking water and in this way brings greater flexibility to the HPC analysis (Reasoner, 1990; Madigan *et al.*, 1997). Research showed that the spread plate method correlates with the membrane filter test (Reasoner, 1990; Madigan *et al.*, 1997).

2.6.2.4 SimPlate HPC method

The SimPlate HPC method uses a medium containing substrates that are hydrolysed by microbial enzymes to release 4-methylumbelliferone, which fluoresces blue under a long-wavelength 6-W UV light after incubation for 48 h at 35°C (Jackson *et al.*, 2000). The bacteria are detected as fluorescent wells on the SimPlate (Jackson *et al.*, 2000). The bacterial density of a water sample is determined by noting the number of positive wells and by using the MPN table provided for SimPlate (Jackson *et al.*, 2000). The SimPlate HPC method was found to be equivalent to the pour plate method by regression analysis (Jackson *et al.*, 2000). This test is easy to use and does not require preparation

of media or sterilization (Jackson *et al.*, 2000). Counting of positive fluorescent wells is easy and takes less time than counting colonies on the standard HPC plate (Jackson *et al.*, 2000).

Incubation temperatures and nutrient media are important in maximizing the bacterial count (Reasoner, 1990). In general, the longer the incubation period, the higher the HPC becomes (Reasoner, 1990). The media used for the detection of heterotrophic bacteria promote the growth of a large number of organisms, including those that have been stressed or have adapted to a low nutrient environment (Payment *et al.*, 1994). Total plate count and R₂A media are applied in the enumeration of HPC bacteria (Payment *et al.*, 1994). Plate Count Agar and incubation at 37°C indicate the presence of rapid-growing pathogenic bacteria (Reasoner, 1990). However, no single colony formation method and set of conditions exists to permit enumeration of all bacteria present in water (Reasoner, 1990).

2.6.3 Pathogenicity of HPC bacteria

The assessment of health risk for each microbe involves the nature of its pathogenesis and the ability of the host to respond (Edberg *et al.*, 1996). Pathogenicity depends upon the number of microbes with which the host makes contact, the virulence components of the microbe and the resistance of the challenged target organ system of the host (Edberg *et al.*, 1996). Therefore, pathogenicity involves biochemical mechanisms whereby microorganisms overcome nonspecific and specific host immune defenses in order to cause disease (Finlay and Falkow, 1989; Duncan and Edberg, 1995).

Although normal host defenses control organisms of low virulence, immunocompromised patients can be infected by relatively nonpathogenic organisms (Sparling, 1983; Finlay and Falkow, 1989; Edberg *et al.*, 1996). According to studies any microorganism which sustains itself in humans would infect compromised individuals and act as opportunistic pathogen (Finlay and Falkow, 1989). For example, *Pseudomonas aeruginosa* infects compromised patients and not those with intact host defenses (Finlay and Falkow, 1989; WHO, 2001). *Bacillus* spp., which are present in drinking water, have been implicated in opportunistic infections as well as gastrointestinal illnesses (Finegold and

Baron, 1986; Atlas, 1997). Little information is available on the association of *Bacillus* spp. with subacute diseases such as endemic gastrointestinal illnesses (Finegold and Baron, 1986; Atlas, 1997).

The likelihood of a successful challenge by a pathogen, resulting in infection, depends upon its invasiveness and virulence (WHO, 1996; WHO, 1997; WHO, 2001). Some microbes cause disease by the toxins they produce or are cytotoxic on invasion of the host cell, while others cause damage to the host by eliciting immune response (Duncan and Edberg, 1995). The infective dose of HPC pathogens can not be predicted from their average concentration in water because of their adherence to suspended solids (WHO, 1997; WHO, 2001). Therefore, treated drinking water must comply with national standards and guidelines regarding HPC bacteria or other agents potentially pathogenic to humans (WHO, 1997; WHO, 2001).

2.6.4 Enzymatic analyses

The virulence of some microorganisms is partly due to the production of extracellular enzymes (Pelczar *et al.*, 1993). In order to assess the health risks from naturally occurring HPC bacteria, the presence or absence of virulence components associated with pathogenesis should be determined (Finlay and Falkow, 1989; Edberg *et al.*, 1996). One of the mechanisms whereby extracellular enzymes are capable of enhancing the bacterial virulence involves the destruction of host-protective macromolecules such as mucus, lipoprotein membranes, hyaluronic acid and immunoglobulins (Weinberg, 1985). In this manner, enzymes may enable the pathogens to invade body cells as well as tissues and to interfere with normal mammalian functions (Atlas, 1997).

There are several extracellular enzymes involved with bacterial pathogenesis (Table 2.4), which include chondroitinase, coagulase, DNase, elastase, haemolysin, hyaluronidase, gelatinase, lecithinase, lipase and proteinase (Finlay and Falkow, 1989; Ewald, 1991; Mekalanos, 1992; Edberg *et al.*, 1996). In fact, *Yersinia ruckeri* produces proteinase, lipase and haemolysin as extracellular factors, which lead to the appearance of symptoms associated with pathogenicity (Secades and Guijarro, 1999).

Proteinases, lipases and lecithinases produced by *Pseudomonas* spp. are responsible for the spoilage of foods such as milk and various other dairy products (Shah, 1994). The production of pyocyanin and fluorescein by *P. aeruginosa* is determined utilizing P and F agars as substrates (Edberg *et al.*, 1996). These two kinds of agars are used for detecting and differentiating *P. aeruginosa* from other pseudomonads based on the appearance of pyocyanin and fluorescein, respectively (King *et al.*, 1954; Janda and Bottone, 1981; Edberg *et al.*, 1996).

2.6.4.1 Hyaluronidase and Chondroitinase

Microbial hyaluronidase and chondroitin sulfatase have been studied because of possible association between these enzymes and their mechanisms of pathogenicity (Smith and Willett, 1968; Atlas, 1997). Hyaluronidase may help the pathogens to penetrate the tissues of the host by hydrolyzing hyaluronic acid, the substance that holds together the cells of connective tissues (Pelczar *et al.*, 1993; Atlas, 1997). Hyaluronidase activity is known among coagulase-positive staphylococci (*Staphylococcus aureus*) and group-A haemolytic streptococci (*Streptococcus pyogenes*) (Smith and Willett, 1968; Atlas, 1997). Chondroitinase is an enzyme that attacks chondroitin sulfate (Smith and Willett, 1968; Atlas, 1997). Pathogens producing hyaluronidase and chondroitinase spread through body tissues, therefore these enzymes are known as spreading factors (Atlas, 1997). *Corynebacterium acnes* and *Propionibacterium acnes* are known to produce chondroitinase enzymes (Smith and Willett, 1968; Atlas, 1997).

TABLE 2.4: Frequency of production of extracellular enzymes by *Pseudomonas aeruginosa* isolated from clinical and environmental sources (Janda and Bottone, 1981)

| Enzyme | Frequency among clinical isolates (n = 54) | | | | | Frequency among environmental isolates (n = 22) |
|----------------|--|-----------------|---------------------|-------------------|----------------|---|
| | Total | Sputum (n = 20) | Urogenital (n = 16) | Systemic (n = 15) | Facial (n = 3) | |
| Haemolysin | 53 (98%) | 19 (95%) | 16 (100%) | 15 (100%) | 3 (100%) | 2 (9%) |
| Proteinase | 53 (98%) | 19 (95%) | 16 (100%) | 15 (100%) | 3 (100%) | 12 (55%) |
| Gelatinase | 51 (94%) | 19 (95%) | 15 (94%) | 14 (93%) | 3 (100%) | 16 (73%) |
| Pyocyanin | 50 (93%) | 18 (90%) | 15 (94%) | 15 (100%) | 1 (33%) | 0 |
| Fibrinolysin | 44 (82%) | 16 (80%) | 11 (69%) | 14 (93%) | 3 (100%) | 1 (5%) |
| Lipase | 43 (81%) | 16 (80%) | 13 (81%) | 11 (73%) | 3 (100%) | 2 (9%) |
| Coagulase | 41 (76%) | 16 (80%) | 10 (63%) | 14 (93%) | 2 (67%) | 0 |
| Lecithinase | 31 (57%) | 9 (45%) | 10 (63%) | 9 (60%) | 3 (100%) | 14 (64%) |
| Elastase | 20 (37%) | 4 (20%) | 5 (31%) | 10 (67%) | 1 (33%) | 0 |
| DNase | 9 (16%) | 1 (5%) | 3 (18%) | 5 (33%) | 0 | 0 |
| Chondroitinase | 0 | ND | ND | ND | ND | 0 |
| Hyaluronidase | 0 | ND | ND | ND | ND | 0 |

n= number of samples

ND= not done

2.6.4.2 Coagulase and Fibrinolysin

Coagulase is capable of clotting plasma by converting fibrinogen to fibrin thus enhancing the bacterial virulence (invasiveness) (Atlas, 1997). Deposition of fibrin around the bacterial cells protects them against the circulatory defense mechanisms of the host (Atlas, 1997). Coagulase-negative strains of *S. aureus* still have been found to be virulent pathogens (Atlas, 1997). It is thus difficult to associate virulence with the activity of a single enzyme, even though this enzyme may be involved in the pathogenesis of various bacteria (Atlas, 1997). Fibrinolysin catalyzes the lysis of fibrin clots and in this way pathogens are free to spread to surrounding areas (Atlas, 1997). Fibrinolysins enhance the

invasiveness of pathogenic strains of *Staphylococcus* and *Streptococcus* by preventing fibrin in the host from walling off the area of bacterial infection (Atlas, 1997).

2.6.4.3 DNase

DNases are a class of extracellular endonucleases capable of hydrolysing polymerised DNA into smaller oligonucleotides (MacFaddin, 1980; Edberg *et al.*, 1996). The depolymerization of DNA results in changes in the physical properties of the nucleic acid molecule, such as a decrease in the viscosity of a DNA solution and an increase in absorption with UV light (MacFaddin, 1980). DNase may inactivate the genetic machinery of phagocytes once they have engulfed the DNase-producing bacteria (Janda and Bottone, 1981). The enzyme may co-serve with elastase production as a genetic marker for disseminating strains (Janda and Bottone, 1981).

2.6.4.4 Elastase

Elastase has been classified as a zinc metalloprotease similar to several other bacterial proteases (Galloway, 1991; Edberg *et al.*, 1996). Elastase is capable of degrading elastin (cellular glue) and is distinguishable from alkaline proteinase, another proteolytic enzyme secreted by *P. aeruginosa* (Galloway, 1991). Elastolysis is of concern in pathogenesis since several tissues, such as lung and vascular tissues, are composed of elastin and require elastic properties for carrying out their assigned functions within the organism (Galloway, 1991). Elastase is the substance responsible for haemorrhagic skin lesions and necrosis (Pollack, 1984; Galloway, 1991). It has been implicated in the destructive vascular lesions (ecthyma gangrenosum) produced by *P. aeruginosa* in association with septicaemic infections (Mull and Callahan, 1965; Galloway, 1991). Elastase is responsible for the dissolution of the elastic lamina of blood vessels, which is an important pathologic characteristic of these lesions (Pollack, 1984; Galloway, 1991). The enzyme is involved in pulmonary infections other than the chronic infections associated with cystic fibrosis (Galloway, 1991).

2.6.4.5 Haemolysin

Haemolysins are enzymes causing the lysis of erythrocytes (Atlas, 1997). A haemolysin is a type of cytolysin or cell-killing toxin (Atlas, 1997). When haemolysin-producing bacteria are grown on blood agar plates, zones of clearing may be seen around colonies due to destruction of red blood cells (Atlas, 1997). A complete zone of clearing around the bacterial colony is referred to as β -haemolysis and a partial zone of clearing due to the incomplete destruction of red blood cells is referred to as α -haemolysis (Atlas, 1997). Alpha haemolysis involves the conversion of haemoglobin to methemoglobin, seen as a zone of green discolouration with partial clearing around the colony (Atlas, 1997).

P. aeruginosa produces at least two distinct hemolysins: phospholipase C and heat-stable glycolipid (Berka and Vasil, 1982). These two hemolysins work in conjunction with phosphatase to break down lipids and lecithin (Liu, 1979). Hemolysin produced during *P. aeruginosa* infections of the lung may degrade surfactants leading to atelectasis and other pathological alterations (Liu, 1979).

2.6.4.6 Lecithinase

Lecithinase, also known as phospholipase C or phosphatidylcholine phosphohydrolase, hydrolyses lecithin, which is a lipid component of eukaryotic membranes (Atlas, 1997). This enzyme destroys the integrity of cytoplasmic membranes of many cells (Atlas, 1997). Lecithinase acts as a hemolysin, causing lysis of red blood cells in addition to destroying cells of other tissues (Atlas, 1997). The release of iron from the lysed blood cells allows the pathogen to grow in an environment that has a low concentration of this essential growth nutrient (Atlas, 1997).

2.6.4.7 Lipase and Gelatinase

Lipase and gelatinase are enzymes that enhance the invasiveness of HPC bacteria (Edberg *et al.*, 1996). Lipase hydrolyses long-chain fatty acid esters, whereas gelatinase breaks down proteins

(gelatin) into smaller peptides and amino acids used for cellular transport as well as metabolism (Sierra, 1957; MacFaddin, 1980; Atlas, 1997). Polyoxyethylene sorbitan monooleate (Tween 80) is hydrolysed by lipase, releasing oleic acid (Sierra, 1957; Atlas, 1997). Oleic acid released from the hydrolysis of Tween 80 binds with the calcium in the medium, forming microcrystals of calcium oleate inside the agar plate (Sierra, 1957; Atlas, 1997).

2.6.4.8 Proteinases

Proteinases are a class of enzymes that attack the peptide bonds of protein molecules, forming small peptides (Atlas, 1997). These enzymes might provide bacterial nutrients in the host by degrading mucosal or luminal proteins and casein (Schneider and Parker, 1978; Secades and Guijarro, 1999). Therefore, skim-milk agar is used for identifying *P. aeruginosa* proteinase producers and for differentiating *P. aeruginosa* from other species of *Pseudomonas* (Wretling *et al.*, 1977; Secades and Guijarro, 1999). Evidence suggests that elastase, a metalloprotease, is required for maximal virulence of *P. aeruginosa* (Secades and Guijarro, 1999).

The production of bacterial proteinases could contribute to the pathogenesis of infections and therefore they could be considered virulence factors (Secades and Guijarro, 1999). Extracellular proteinases promote the invasiveness of *P. aeruginosa* (Pollack, 1984; Secades and Guijarro, 1999). The rapidity and degree to which proteinases cause necrosis of local tissue and disturb the integrity of blood vessels suggests a mechanism by which the organism effects the breakdown of physical barriers to penetrate (Pollack, 1984; Secades and Guijarro, 1999). Proteinases are not highly cytotoxic but they destroy connections between cells, such as the proteoglycan ground substance of the cornea and supporting structures composed of fibrin or elastin (Mull and Callahan, 1965; Wretling and Pavlovskis, 1981; Secades and Guijarro, 1999). The enzyme may alter host defenses through the inactivation of complement factors and the cleavage of IgG (Fick *et al.*, 1982). Proteinases produce overt disease at local sites of infection and contribute to subsequent bacterial disinfection (Janda and Bottone, 1981).

The production of these enzymes by HPC bacteria is a requirement for the development of an infection (Edberg *et al.*, 1996). However, microorganisms can be exceptionally equipped to cause infection and not disease, because disease is the unfavorable outcome of the microbial infection (Finlay and Falkow, 1989). Thus, infection and disease depend on both the host and the microorganism (Finlay and Falkow, 1989).

2.6.5 Antibiotic susceptibility of HPC organisms

The discovery and use of antibiotics have revolutionised medical practice in the twentieth century (Atlas, 1997). Antibiotics represent a major class of antimicrobial agents that inhibit the growth of or kill microorganisms by binding selectively to proteins or cellular organelles in order to disrupt their essential functions (Atlas, 1997). The selection of a particular antibiotic necessary for the treatment of a specific disease depends on several factors such as the sensitivity of the infecting microorganism to the antibiotic, the side effects of the antimicrobial agent, the biotransformations and the chemical properties that determine its distribution within the body (Atlas, 1997).

As a result of the increasing use of antibiotics in agriculture and medicine, antibiotic-resistant bacteria and antibiotics can be found in the environment (Urriza *et al.*, 2000). Rivers obtain these pollutants through the sewage of urban effluents and agricultural run-off (Urriza *et al.*, 2000). As river water is used for human and animal consumption, pollution in the water contributes to the spread of bacterial antibiotic resistance (Urriza *et al.*, 2000). Research showed, that 100% of *A. hydrophila* strains isolated from rural groundwater supplies have been found resistant to at least two antibiotics (MacKeon *et al.*, 1995). *A. hydrophila* was naturally resistant to ampicillin and cephalothin (Walsh *et al.*, 1995). Quinolone-resistant strains of *Aeromonas* originate from discharges of quinolone compounds into the river (Urriza *et al.*, 2000).

Acquired resistance to antibiotics are encoded by plasmids or transposable elements (Rice and Bonomo, 1996). According to research, resistance in *Aeromonas* is governed by chromosomal genes (Urriza *et al.*, 2000). In addition, these microorganisms may produce chromosomal beta-lactamases

thus making them resistant to beta-lactams (Walsh *et al.*, 1995).

Determination of the MIC (Minimum Inhibitory Concentration), based on the activities of antibiotics against planktonic bacteria, is the standard assay for antibiotic susceptibility testing (Ceri *et al.*, 1999). The MIC measures the actions of antibiotics against microorganisms and serves as an important reference in the treatment of many acute infections (Ceri *et al.*, 1999). However, the application of MICs in the treatment of chronic infections involving bacterial biofilms is ineffective, because a reaction-diffusion barrier limits the transport of the antibiotic into the biofilm (Costerton *et al.*, 1995). Since antibiotics are unable to penetrate biofilms, the heterotrophic plate count bacteria embedded in them are protected and may be responsible for environmental problems (Anderl *et al.*, 2000). This antibiotic resistance is due to regions of slow growth or starvation within the biofilm, because slow growing bacteria are less susceptible to antimicrobial agents (Anderl *et al.*, 2000). The Calgary Biofilm Device (CBD) proved to be important in the development of new antibiotics selected for their efficacies against biofilms (Ceri *et al.*, 1999). The CBD is described as a new technology for the rapid and reproducible assay of biofilm susceptibilities to antibiotics (Ceri *et al.*, 1999).

Antibiotic susceptibility testing requires observation of antibiotics inhibiting the growth of microbial cultures *in vitro* in order to select the appropriate agent for treating a disease (Atlas, 1997). The Kirby-Bauer test is a standardised antimicrobial susceptibility method in which the bacterial suspension is inoculated uniformly onto the surface of a Mueller-Hinton agar and filter-paper disks impregnated with known concentrations of chemotherapeutic agents are placed on the solidified agar surface (Tortora *et al.*, 1992; Atlas, 1997). During incubation, the antibiotics diffuse from the disks into the agar (Tortora *et al.*, 1992; Atlas, 1997). The appearance of a clear zone around the antibiotic disk indicates inhibition of microbial growth and the effectiveness of the chemotherapeutic agent (Tortora *et al.*, 1992; Atlas, 1997). The diameter of the zone is compared to a standard table for the specific drug and concentration, and the results determine whether the microorganism is sensitive, intermediately sensitive or resistant to the particular antibiotic (Tortora *et al.*, 1992; Atlas, 1997). The test is simple, inexpensive and valuable in medical practice (Tortora *et al.*, 1992). Clinical laboratories use light scattering or equivalent automated liquid diffusion methods for antibiotic

sensitivity testing, because the disk-diffusion method does not determine if a drug is bactericidal and not just bacteriostatic (Tortora *et al.*, 1992; Atlas, 1997).

One of the greatest triumphs of medical science is the development of antibiotics, although they can be associated with various problems and disadvantages (Tortora *et al.*, 1992). An increasing concern, for example, is the misuse of antibiotics that leads to the emergence of resistant strains of pathogens (Tortora *et al.*, 1992).

2.6.6 Cytotoxicity, Adherence and Invasiveness associated with HPC bacteria

One of the approaches for the detection of bacterial isolates that could have a potential public health risk is to search for virulence factors (Payment *et al.*, 1994). Bacteria must attach to, invade and multiply in epithelial cells or produce toxins that will disrupt the normal growth of epithelial cells in order to produce disease (Payment *et al.*, 1994). The demonstration of attachment to and invasiveness of the cell, its subsequent destruction (cytolysis) and the detection of toxins or enzymes are methods used in clinical microbiology (Payment *et al.*, 1994).

Cytotoxic activity assays of heterotrophic plate count bacteria are laborious, time consuming and often unsuccessful because the characteristics which contribute to the pathogenicity of many bacteria are highly variable (Lye and Dufour, 1991). The frequency of isolation of cytotoxic bacteria from drinking water is dependent upon the primary medium used and the density of bacteria present (Lye and Dufour, 1991). The release of cytotoxic and cytotoxic products from the bacterial colonies is recognized by zones of cellular lysis (Lye and Dufour, 1991). Cell adherence and invasion are determined by calculating the index of adherence and invasion, respectively (Payment *et al.*, 1994).

The majority of cytotoxic strains isolated from drinking water are found to express protease (95%) and haemolytic activity (70%) (Lye and Dufour, 1991). All strains of *Aeromonas hydrophila* and *Aeromonas sobria* are capable of producing haemolysins and cytotoxins (Lye and Dufour, 1991; Majeed and Macrae, 1994). The production of cytotoxins by *Aeromonas* species is illustrated in

Figure 2.1 (Majeed and Macrae, 1994). Cytotoxins can cause cell damage or death thus producing dysentery-like illness (Majeed and Macrae, 1994). Optimal detection of cytotoxic activity in the HPC bacteria is accomplished using blood agar medium (Lye and Dufour, 1991). Research showed that VERO and HEp-2 tissue culture cell lines are more sensitive to cytotoxins than the mouse adrenal Y-1 cells (Lye and Dufour, 1991). As shown in Table 2.5, human epithelial carcinoma (HEp-2) cells detected 6% more cytotoxin-producing strains than African Green Monkey kidney (VERO) cells and 17% more than Y-1 cells (Lye and Dufour, 1991). The HEp-2 cell line is more sensitive in detecting those bacterial colonies causing cytotoxic effects (rounding up of cells) and is recommended as the tissue culture cell line for detecting cytotoxin-producing bacteria (Lye and Dufour, 1991).

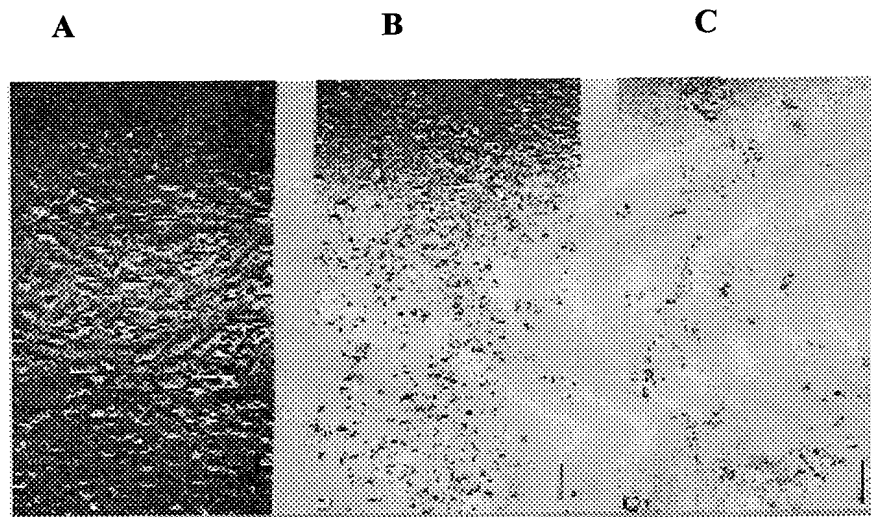


Figure 2.1: Vero cell assay for *Aeromonas* cytotoxin: (A) negative control; (B) cytotoxin response elicited by *A. hydrophila* strain after incubation for 4 h at 37° C; (C) after 8 h (Majeed and Macrae, 1994).

TABLE 2.5: The effect of tissue culture cell line on the detection of cytotoxicity in known bacterial strains using the membrane filter transfer method (Lye and Dufour, 1991)

| Strain | Number tested | Number of strains expressing cytotoxicity on cell cultures | | |
|-------------------------------|---------------|--|------|-------|
| | | Y-1 | Vero | HEp-2 |
| <i>Aeromonas hydrophila</i> | 12 | 11 | 11 | 11 |
| <i>A. sobria</i> | 5 | 5 | 5 | 5 |
| <i>A. caviae</i> | 4 | 0 | 0 | 0 |
| <i>A. salmonicida</i> | 2 | 0 | 0 | 0 |
| <i>Escherichia coli</i> | 3 | 0 | 2 | 3 |
| <i>Pseudomonas aeruginosa</i> | 4 | 3 | 4 | 4 |
| <i>P. cepacia</i> | 3 | 2 | 3 | 3 |
| <i>P. maltophilia</i> | 2 | 0 | 0 | 1 |
| <i>P. paucimobilis</i> | 1 | 1 | 1 | 1 |
| Totals | 36 | 22 | 26 | 28 |
| (%) | 100 | 61 | 72 | 78 |

HEp-2 and Caco-2 cell line cultures are applied in the cytotoxicity analysis (Whitaker, 1972; Bird and Forrester, 1981; Payment *et al.*, 1994; Edberg *et al.*, 1996; Edberg *et al.*, 1997). Cytotoxic characteristics displayed by HPC bacteria are determined using HEp-2 cells, whereas Caco-2 cells are applied in the demonstration of adherence and invasiveness of HPC bacteria (Payment *et al.*, 1994; Edberg *et al.*, 1996; Edberg *et al.*, 1997). These assays determine the production of cytotoxins as well as other factors by HPC bacteria in drinking water and the potential health hazards associated with them (Payment *et al.*, 1994; Edberg *et al.*, 1996; Edberg *et al.*, 1997).

The percentage of cytotoxic heterotrophic plate count bacteria present in drinking water is higher at 35°C, as suggested by Payment *et al.* (1994). HPC bacteria, such as *Aeromonas* spp., are capable of producing cytotoxins and exert other virulence properties such as adherence and invasiveness in

order to cause disease (Majeed and Macrae, 1994). Their attachment to the intestinal mucosa leads to gastrointestinal infections (Majeed and Macrae, 1994). Experimental evidence is lacking for the unidentifiable strains of HPC bacteria commonly found in drinking water systems (Lye and Dufour, 1991).

During the process of invasiveness, microorganisms invade human cells and tissues to establish an infection within the body (Atlas, 1997). Therefore, invasive microorganisms may destroy the tissues they infect (Atlas, 1997). The ability to adhere to intestinal mucosa is the first step in the colonisation and development of disease for many bacterial enteropathogens (Krovacek *et al.*, 1994). *Aeromonas* strains adhere to and invade epithelial cells grown in culture (Figure 2.2) (Krovacek *et al.*, 1994). According to research, the majority of clinical isolates of *Aeromonas* strains showed localized adhesion to intestinal cells in vitro assay, whereas environmental isolates showed diffused adhesion (Fig. 2.2) (Krovacek *et al.*, 1994).

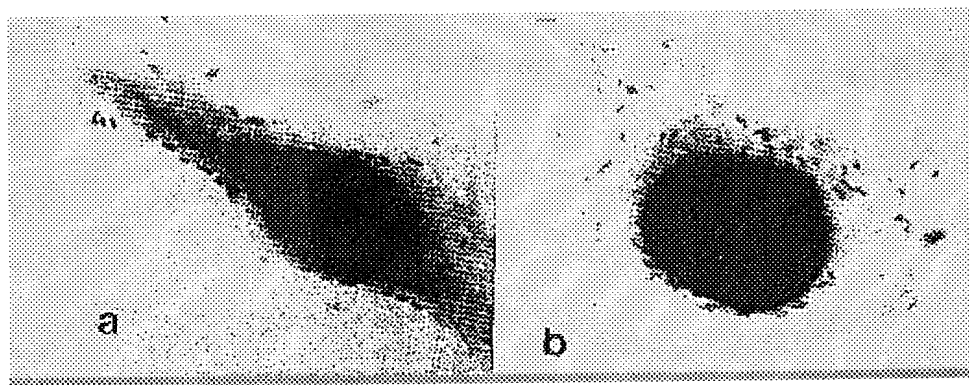


Figure 2.2: Adhesion of *A. hydrophila* to human intestinal 407 cells. (a) Localised adhesion of *A. hydrophila* 2H, isolated from a patient with diarrhoea. Note bacteria adhering along the cells. (b) Diffused adhesion of *A. hydrophila* 6 SS, isolated from sea sediment. Note bacterial adhesion around the cells and on the cell matrix (Krovacek *et al.*, 1994).

It is apparent from these studies, that *Aeromonas* spp. and other HPC bacteria are capable of adhering to HEp-2 cells (Majeed and Macrae, 1994). *Aeromonas* spp. isolated from human diarrhoea bind

to HEp-2 cells in higher numbers than the environmental isolates (Krovacek *et al.*, 1994). Adhesins are specific virulence factors produced by these HPC microorganisms, which help them attach to the surfaces of mammalian cells (Atlas, 1997). Capsules, slime layers and pili are surface layer components that may contribute to the ability of specific pathogenic HPC bacteria to attach to particular host cells or tissues (Atlas, 1997).

There is much debate about the importance of haemolysins, proteases, cytotoxins and other factors produced by bacteria during the establishment of infection (Lye and Dufour, 1991). Examination of environmental signals controlling virulence is one step in the process of deducing strategies that microbes have adopted to become successful commensals and pathogenic organisms (Mekalanos, 1992). One of these regulatory strategies includes physical-chemical parameters such as iron, calcium, temperature, osmolarity, anaerobiosis, stress and catabolite repression (Mekalanos, 1992). The next step is to determine the significance of the bacterial products in drinking water, such as haemolysins, proteases, cytotoxins, and their relation to potential health hazards (Lye and Dufour, 1991).

2.6.7 Identification of HPC bacteria

Several microscopic, metabolic, serologic and gene probe procedures are used for the identification of clinical and environmental microbial isolates (Atlas, 1997). The selection of a specific identification method depends on its accuracy, reliability and speed (Atlas, 1997). The method is based on the observation of colony morphology and other growth characteristics on the primary isolation medium, and on the microscopic observation of stained specimens (Atlas, 1997).

Various phenotypic and molecular methods have been developed and used for subtyping bacterial isolates (Wiedmann *et al.*, 2000). Biochemical characterisation (biotyping), bacteriocin typing, bacterial fatty acids profiling and multilocus enzyme electrophoresis pertain to the phenotypic subtyping methods (Wiedmann *et al.*, 2000). Molecular subtyping methods include pulsed-field gel electrophoresis (PFGE), PCR-based typing methods, DNA sequence-based typing and ribotyping

(Wiedmann *et al.*, 2000). Ribotyping consists of digestion of bacterial chromosomal DNA, followed by Southern hybridisation with a ribosomal operon probe (Wiedmann *et al.*, 2000).

2.6.7.1 Metabolic identification of HPC bacteria

Bacteria are identified by the substrates they utilise and the products of their metabolism (Atlas, 1997). The pattern of test results obtained in these systems, such as the API 20-E, is converted to a numerical code that can be used to determine the identity of the isolate (Atlas, 1997). The API 20-E system employs twenty miniature capsule reaction chambers in which twenty metabolic characteristics are determined (Atlas, 1997). A suspension of bacteria is inoculated into each of the reaction chambers and a seven-digit biotype number is received from which a computer-assisted identification can be made (Atlas, 1997). Using this system non-fermentative Gram-negative rods and anaerobic bacteria can be identified, however the system was developed for clinical isolates and therefore is not reliable for environmental isolates (Atlas, 1997).

2.6.7.2 Identification of HPC bacteria using Biolog microplates

Biolog microplates provide a standardised micromethod using 95 biochemical tests to identify a broad range of Gram-positive and enteric, non-enteric and fastidious Gram-negative bacteria (Smalla *et al.*, 1998; Biolog, 1999; Gamo and Shoji, 1999). Biolog's MicroLog 1, MicroLog 2 or MicroLog 3 software is used to identify the bacterium from its metabolic pattern in the GN2 (Gram-negative) and GP2 (Gram-positive) MicroPlate (Biolog, 1999). Biolog MicroPlates test the ability of a microorganism to utilise or oxidise a pre-selected panel of different carbon sources (Smalla *et al.*, 1998; Gamo and Shoji, 1999). The test yields a characteristic pattern of purple wells, which constitutes a metabolic fingerprint of the capacities of the inoculated organism (Gamo and Shoji, 1999).

All necessary nutrients and biochemicals are pre-filled and dried into the 96 wells of the plate (Smalla *et al.*, 1998; Biolog, 1999). Tetrazolium violet is used as a redox dye to colourimetrically indicate

the utilisation of the carbon sources (Smalla *et al.*, 1998; Biolog, 1999). During the test the isolate to be identified is suspended in a special gelling inoculating fluid at the recommended cell density (Smalla *et al.*, 1998; Biolog, 1999). The cell suspension is inoculated into the MicroPlate, 150 µl per well (Smalla *et al.*, 1998; Biolog, 1999). In wells that contain a chemical that can be oxidised, there is a burst of respiration and the cells reduce the tetrazolium dye forming a purple colour (Smalla *et al.*, 1998; Biolog, 1999). Other wells remain colourless, as does the reference well A-1 with no carbon source (Smalla *et al.*, 1998; Biolog, 1999).

The MicroPlates are incubated for either 4-6 hours or 16-24 hours to allow the pattern to form (Smalla *et al.*, 1998; Biolog, 1999). The plates are read with the Flow Titertek Multiskan Plus (Labsystems, Finland). The pattern of purple wells is then keyed into Biolog's MicroLog computer program, which automatically cross-references the pattern to an extensive library of species (Smalla *et al.*, 1998; Biolog, 1999). If an adequate match is found, an identification is made (Smalla *et al.*, 1998; Biolog, 1999).

Interpretation of the substrate utilization profiles obtained by the Biolog assay is unique for each isolate and is performed carefully (Gamo and Shoji, 1999). Colour development in response to a certain carbon substrate can be affected by factors such as initial density of the inoculum, growth rates of the utilisers and incubation period (Gamo and Shoji, 1999). A low inoculum density may increase the lag time in colour development and can not contribute to the resulting substrate utilisation profile (Gamo and Shoji, 1999).

2.6.7.3 Identification of HPC bacteria using the VITEK 32 analyzer

The VITEK Automated Microbiology System was designed to provide an accurate automated system for identifying a large variety of bacteria (VITEK Senior/Junior procedures manual, BioMerieux VITEK, Inc.). The system uses a 3 mm thick plastic card containing a series of chambers, which are linked by a series of channels to an inlet port on the side of the card (VITEK Senior/Junior procedures manual, BioMerieux VITEK, Inc.). Each chamber contains a biochemical appropriate

for the identification of a group of bacteria (VITEK Senior/Junior procedures manual, BioMerieux VITEK, Inc.). The cards are designed to be inserted into an incubator/reader (VITEK Senior/Junior procedures manual, BioMerieux VITEK, Inc.). The optical density of each chamber is read automatically and the information is transmitted to the computer, which displays the results in a detailed report (VITEK Senior/Junior procedures manual, BioMerieux VITEK, Inc.).

2.6.8 Molecular techniques in detection of HPC bacteria

The scientific community is searching for new alternative techniques to assess microbial water quality (WHO, 2001). The survival of many bacteria in natural environments could affect consumer health (Reasoner, 1990). Since bacteria are able to enter a state in which they are viable but non-culturable other methods of investigation are required (Reasoner, 1990). Viable but non-culturable organisms can be detected using molecular methods such as hybridisation, amplification, cloning and sequencing that target nucleic acids (WHO, 2001). Gene probe detection systems do not require cultures of these bacteria and they can be identified directly from clinical samples (Atlas, 1997). Speed and accuracy are two advantages of these molecular techniques (Atlas, 1997).

The polymerase chain reaction permits the production of sufficient quantities of DNA segments within a short period of time so that the presence or absence of specific genes can be made (Atlas, 1997). The PCR reaction consists of DNA denaturation, primer annealing and chain elongation by the action of taq or another thermostable DNA polymerase (Atlas, 1997). By repetitive cycling of these steps, the target DNA sequence is amplified (Atlas, 1997).

Radioactivity or DNA probes can be neglected when large restriction fragment (LRF) patterns are used to compare specific strains of *Mycobacterium avium* complex (Mazurek *et al.*, 1993). LRF pattern is produced by digesting genomic DNA with infrequently cutting endonucleases and separating the LRFs by pulsed-field gel electrophoresis (Mazurek *et al.*, 1993). These patterns are useful and reliable epidemiologic markers for future studies of MAC infections (Mazurek *et al.*, 1993).

Research confirms the long-term potential for molecular subtyping methods to complement and replace phenotypic characterisation methods (Wiedmann *et al.*, 2000). Molecular methods can be used not only to track pathogens and spoilage organisms but to predict phenotypic characteristics and species identification (Wiedmann *et al.*, 2000). Molecular groupings may replace current taxonomic concepts in bacteriology (Wiedmann *et al.*, 2000). To date, however, applications of molecular subtyping have focused on differentiation of medically important bacterial species (Wiedmann *et al.*, 2000).

2.7 Risk assessment regarding HPC bacteria

In microbial risk assessment, the dose-response relationship enables estimates to be made on the ability of the microorganism to colonize the body (Rusin *et al.*, 1997). The infectious doses (ID_{50}) for some HPC bacteria are quite high, as shown in Table 2.6 (Rusin *et al.*, 1997). These bacteria were found in <1% to as high as 50% of the samples tested by Rusin and colleagues (1997).

TABLE 2.6: Infectious dose studies associated with various heterotrophic bacteria found in drinking water (Bergogne-Berezin, 1994; Rusin *et al.*, 1997)

| Bacterium | Animal | Route of exposure | Median dose | Data appropriate for model |
|-------------------------------|--------------------|-------------------|-----------------|----------------------------|
| <i>Acinetobacter</i> | Mice | Intraperitoneal | 10^6 - 10^8 | No |
| <i>Aeromonas hydrophila</i> | Humans | Oral | 10^7 - 10^8 | Yes |
| <i>Legionella pneumophila</i> | Guinea pigs | Aerosol | $<10^2$ | No |
| <i>Mycobacterium avium</i> | Mice | Oral | 10^8 | No |
| <i>Pseudomonas aeruginosa</i> | Mice | Oral | 10^8 | Yes |
| | Mice: antibiotics | Oral | 10^4 - 10^8 | |
| | Humans | Oral | 10^{10} | |
| | Humans: ampicillin | Oral | 10^7 | Yes |

TABLE 2.7: Daily risks of colonisation by *Aeromonas hydrophila* or *Pseudomonas aeruginosa* ingested in drinking water (Rusin *et al.*, 1997)

| Bacterium | Colony-forming units.2L ⁻¹ , daily exposure | Daily risk of colonisation for single exposure (cfu.ml ⁻¹) |
|--|--|--|
| <i>Aeromonas hydrophila</i> | 200 | 7.3x10 ⁻⁹ |
| | 38 000 | 1.4x10 ⁻⁶ |
| <i>Pseudomonas aeruginosa</i> | 20 | 1.7x10 ⁻⁸ |
| | 4 600 000 | 3.8x10 ⁻³ |
| <i>Pseudomonas aeruginosa</i> : ampicillin | 20 | 4.1x10 ⁻⁷ |
| | 4 600 000 | 9.0x10 ⁻² |

The risk for colonisation without disease ranged from 7.3x10⁻⁹ cfu.ml⁻¹ for low exposures to *Aeromonas* to higher risks predicted at the higher levels of exposure to *Pseudomonas* of 9.0x10⁻² cfu.ml⁻¹ with antibiotic pretreatment (Table 2.7) (Rusin *et al.*, 1997). According to this research, 90% of the time the risk would be less than 6.8 cases of *Aeromonas* infections per 10 000 people exposed and that 95% of the time it would be less than 1 case per 1 000 people, on the basis of any single day of exposure (Rusin *et al.*, 1997). An estimate of the annual risk could not be done because of the uncertainty surrounding the prevalence and concentrations of the HPC bacteria throughout the year (Rusin *et al.*, 1997). Overall, the risk is seen as a transient colonisation, the probability of infection is low and the chance of disease outcome is associated with immuno-compromised individuals (Rusin *et al.*, 1997).

2.8 Established national water quality guidelines

The primary aim of the *Guidelines for drinking water quality* is the protection of public health (WHO, 1993). The guideline values recommended are not mandatory limits and must be considered in the context of local or national environmental, social, economic and cultural conditions (WHO, 1993). The standards that individual countries will develop can thus be influenced by national priorities and economic factors (WHO, 1993).

The Department of Health (South Africa) has a health risk range for HPC bacteria of: no risk for counts less than 100 cfu.ml⁻¹ and greater risk for counts above 10 000 cfu.ml⁻¹ (Table 2.1). Thus, the South African Bureau of Standards (SABS) specifies a HPC limit of 100 cfu.ml⁻¹ for drinking water (SABS, 2001). This limit is endorsed by the Departments of Health and Water Affairs, and is in line with specifications and recommendations in the rest of the world (WHO, 1997; WHO, 2001). In the case of bottled water a maximum HPC of 50 cfu.ml⁻¹ is generally specified (WHO, 1997).

2.9 Summary

Heterotrophic bacteria are found in tap, bottled as well as other sources of potable water and require simple organic compounds rather than carbon dioxide for growth (Rusin *et al.*, 1997). In drinking water distribution systems they show bacterial regrowth, aftergrowth and biofilm formation (Grabow, 1996).

HPCs exceeding specified limits tend not to be perceived as a serious violation of water quality specifications and are widely accepted. However, evidence has been presented that heterotrophic plate counts may include bacteria which are not as harmless as generally perceived. These bacteria are naturally present in the environment and the consumption or exposure to water containing large numbers of these organisms can lead to opportunistic infections (WHO, 1996; Rusin *et al.*, 1997; WHO, 1997; Brandi *et al.*, 1999). Factors which increase the urgency of questions concerning HPCs include the escalating component of highly vulnerable individuals among consumers of bulk drinking water supplies (Grabow, 1996). These individuals include the very young and very old, as well as individuals with compromised immune systems due to diseases such as AIDS, and medical interventions with immune systems as part of procedures such as organ transplantation and cancer therapy (Grabow, 1996). Therefore, bacteria detected by heterotrophic plate counts warrant urgent attention in water quality monitoring and the assessment of health risks associated with drinking water supplies.

In order to assess the health risks from naturally occurring HPC bacteria, the presence or absence of virulence components associated with pathogenesis should be determined (Finlay and Falkow, 1989; Edberg *et al.*, 1996). Research showed that a small percentage of heterotrophic bacteria isolated from drinking water are cytotoxic and can directly damage human cells in tissue culture (Lye and Dufour, 1991; Payment *et al.*, 1994). The assessment of potential health risks involves direct examination of HPC bacteria because epidemiological and animal infectivity studies are complex or difficult to control (Lye and Dufour, 1991). The analyses for cytotoxic HPC bacteria include haemolyses, antibiotic susceptibility, enzyme and cytotoxic analyses (Edberg *et al.*, 1996). Although no single extracellular enzyme has been proved to be the sole factor responsible for virulence, there is no doubt that such enzymes do play a role in the pathogenic process (Pelczar *et al.*, 1993). The ability of pathogenic bacteria to penetrate tissues, or invasiveness, is due to some of these enzymes, since chondroitinase attacks the chondroitin sulphate and acts as a spreading agent and coagulase is capable of clotting plasma (Edberg *et al.*, 1996). Elastase dissolves cellular glue, whereas fibrinolysin catalyzes the lysis of fibrin clots and thus enhances the invasiveness of pathogens (Edberg *et al.*, 1996). Proteinase disrupts the body's proteins and gelatinase breaks down proteins (gelatin) into smaller peptides and amino acids (Edberg *et al.*, 1996). Haemolysin destroys red blood cells and hyaluronidase breaks down hyaluronic acid, the substance that holds together the cells of connective tissue (Edberg *et al.*, 1996). DNase destroys the nucleic acid of cells and lipase dissolves lipids (Edberg *et al.*, 1996). Lecithinase hydrolyses lecithin, which is a lipid component of eukaryotic membranes (Edberg *et al.*, 1996). Pyocyanin and fluorescein demonstrate the presence of *Pseudomonas aeruginosa* (Edberg *et al.*, 1996).

Future developments will depend on better definition of the biochemistry, genetics and immunology of the bacterial factors involved in pathogenesis (Sparling, 1983; Casadevall and Pirofski, 1999). Moreover, host-pathogen interactions can be analysed using host damage to characterise microbial pathogenicity (Casadevall and Pirofski, 1999). Since infection depends on specific interactions between bacterial adhesins and eukaryotic receptors, the chemistry of each of these structures and the nature of their interactions should be studied (Sparling, 1983). Thus, results of such future work will be interesting and of considerable practical benefit (Sparling, 1983; Casadevall and Pirofski, 1999).

It is, therefore, evident from the literature that HPC bacteria may indeed pose a potential public health risk. Therefore, the aim of the study was to examine in detail the production of haemolysis, extracellular enzymes involved in bacterial pathogenesis, antibiotic susceptibility, cytotoxins, adherence and invasiveness by HPC bacteria isolated from selected drinking water supplies in South Africa.

CHAPTER 3

MATERIALS AND METHODS

3.1 PILOT STUDY

A pilot study was conducted prior to the initiation of the project in order to determine the most appropriate plate method, incubation condition and growth medium for the isolation of representative numbers of heterotrophic plate count (HPC) bacteria from selected water samples in South Africa. The pour plate and the spread plate techniques were compared with the implementation of the following incubation conditions: 20°C, 28°C, 37°C for 24 h, 48 h, 72 h and 120 h. Serial dilutions of tap water were made using Ringer's solution (Merck). Four different media were utilized: R₂A (Oxoid LTD, Basingstoke, Hampshire, England), R₃A (Oxoid), Yeast Extract Agar (YXA) (Oxoid) and Plate Count Agar (PCA) (Merck, Darmstadt, Germany). Media were prepared according to the manufacturer's instructions. The medium designated as R₃A was double-strength R₂A medium that proved useful for subculturing of aquatic bacterial isolates (Reasoner and Geldreich, 1985). The experiments were performed in triplicate.

3.1.1 Pour plate method

The pour plate method was performed according to procedures described by Tortora *et al.* (1992); Atlas, (1997); WHO, (2001) (Appendix I). PCA, R₂A, R₃A and YXA media were used and the inoculated plates were incubated at 20°C, 28°C, 37°C for 120 h (Atlas, 1997). Bacterial colonies were counted (between 25-250 colonies) after 24 h, 48 h, 72 h and 120 h (Atlas, 1997). The average number of colony forming units per millilitre (cfu.ml⁻¹) was calculated and recorded.

3.1.2 Spread plate method

The spread plate method was performed according to procedures described by Atlas, (1997); WHO, (2001) (Appendix I). Inoculated agar plates, containing PCA, R₂A, R₃A and YXA media, were turned upside down and incubated at 20°C, 28°C, 37°C for 120 h. Bacterial colonies (between 25-250) were counted after 24 h, 48 h, 72 h and 120 h. The average number of colony forming units per millilitre (cfu.ml⁻¹) was calculated and recorded.

3.2 ISOLATION OF HETEROTROPHIC PLATE COUNT BACTERIA

Plate Count Agar plates (exceeding the 100 cfu.ml⁻¹ HPC guideline set by the SABS) were sent to the Department of Medical Virology, University of Pretoria by the laboratories of a water treatment facility on a weekly basis during the period of 2000/02/25 to 2000/06/15 for the selection and purification of specific bacterial colonies. The samples were collected from four points of a pipeline. Heterotrophic plate count assays were carried out on 256 bacterial isolates from the water treatment facility and on 83 bacterial isolates from other areas such as Soshanguve, Botshabelo and Umgeni Water. Representative numbers of HPC bacteria were randomly selected from samples of the water distribution systems and purified by the streak plate technique (incubation at 37°C for 24h), Gram-stained and stored in 50% glycerol at -20°C for future analysis.

3.2.1 Purification of the HPC isolates

Single bacterial colonies were randomly selected from water samples and their morphology described (Atlas, 1997). Each bacterial colony was streaked aseptically on the surface of a sterile solidified PC agar plate and incubated upside down at 37°C for 24 h (Atlas, 1997). A single bacterial colony was picked from each plate and re-streaked to obtain a pure culture of the HPC isolate (Atlas, 1997). The same procedure was repeated three to four times before application of the Gram-staining technique, which determined the Gram-reaction and purity of the bacterial isolates.

3.2.2 Gram-staining method

Gram-staining was performed on the isolated HPC colonies in order to determine their Gram-reaction, cell shape and purity (Tortora *et al.*, 1992; Atlas, 1997). Gram-staining was performed on 24 h old bacterial colonies (Appendix I). Gram-positive and Gram-negative bacteria were distinguished (Tortora *et al.*, 1992; Atlas, 1997).

3.2.3 Maintaining and preserving pure HPC cultures

Twenty four hour old pure bacterial cultures were inoculated aseptically into sterile bottles containing 9.0 ml of sterile nutrient broth (Atlas, 1997). Each suspension was incubated at 37°C for 24 h (Atlas, 1997). Sterile 50% glycerol solutions (Sigma) were prepared in separate test tubes (Atlas, 1997). Aliquots of 0.9 ml of the glycerol solutions and 0.9 ml of the pure bacterial suspensions were added aseptically into sterile cryogenic vials (Corning Costar Corporation, Cambridge, MA, Canada). After thorough mixing, the bacterial isolates were frozen at -20°C (Atlas, 1997). The function of the glycerol was to prevent damage due to ice crystals and to ensure the recovery of viable microorganisms when the frozen bacterial cultures are thawed (Atlas, 1997). Prior to initiation of each test, the bacterial isolates were streaked, Gram-stained and subcultured on PCA media for several times to ascertain their purity.

3.2.4 Growth of HPC bacteria on blood agars

The first step in screening the HPC isolates for potentially pathogenic features consisted of testing their ability to grow on human- and horse-blood agar media, which were obtained from the Department of Medical Microbiology, University of Pretoria, RSA. Pure 24 h bacterial cultures were streaked aseptically on blood agar plates and incubated at 37°C for 24 h (Atlas, 1997). The observation of clear zones around the bacterial colonies indicated β -haemolysis, whereas green zones around the colonies suggested α -haemolysis and no haemolysis was referred to as γ -haemolysis (Atlas, 1997).

3.3 ENZYMATIC ANALYSES

In order to cause an infection, microorganisms should possess virulence factors associated with pathogenesis (Edberg *et al.*, 1996). Therefore, HPC isolates displaying α - or β -haemolysis were used to examine the production of the following extracellular enzymes.

3.3.1 Chondroitinase

The basic medium consisted of 100 ml of Brain Heart Infusion broth (Oxoid) and 1 g of Noble agar (Difco Laboratories, Detroit, MI, USA) (Smith and Willett, 1968; Janda and Bottone, 1981; Edberg *et al.*, 1996). The medium was autoclaved at 121°C for 15 min and cooled to 50°C (Smith and Willett, 1968; Edberg *et al.*, 1996). Aqueous solutions consisting of 4 mg.ml⁻¹ of chondroitin sulphate A from bovine trachea (Sigma Chemical Co., St. Louis, MO, USA) and 5% bovine albumin fraction V (Sigma) were filter sterilized using 0.20 μ m Minisart filter units (Sartorius AG, Goettingen, Germany) (Smith and Willett, 1968; Edberg *et al.*, 1996). The chondroitin sulphate A was added to the cooled media to give a final concentration of 400 mg.ml⁻¹ (Smith and Willett, 1968; Edberg *et al.*, 1996). The bovine albumin fraction V was added with constant stirring to give a final concentration of 1% (Smith and Willett, 1968; Edberg *et al.*, 1996). The medium was poured to a 3- 4 mm depth in 60 mm Petri dishes. The pH of the medium was adjusted to pH 6.8 (Smith and Willett, 1968; Edberg *et al.*, 1996). Pure 24 h bacterial cultures were inoculated aseptically on the surface of the agar plates and incubated for 48 h at 37°C (Smith and Willett, 1968; Edberg *et al.*, 1996). After incubation, plates were flooded with 2 M acetic acid (BDH Laboratory Supplies, UK) for 10 min (Smith and Willett, 1968; Edberg *et al.*, 1996). The appearance of a clear zone around the bacterial colonies represented a positive test (Smith and Willett, 1968; Edberg *et al.*, 1996) (Appendix I).

Positive control: *Propionibacterium acnes* (11828)

Negative control: *Bacillus subtilis* ATCC 6683

3.3.2 Coagulase

Rabbit coagulase plasma (Pro-Lab Diagnostics, UK) containing EDTA (ethylenediaminetetraacetate) was dispensed in 0.5 ml aliquots in 12 x 75 mm test tubes according to the manufacturer's instructions (Pro-Lab Diagnostics) (Edberg *et al.*, 1996). One loopful of a 24 h pure bacterial culture was added aseptically to each test tube to produce a turbid suspension (Pro-Lab Diagnostics) (Edberg *et al.*, 1996). The tubes were examined periodically for coagulation by gently tipping the tubes (without shaking or agitating) after the first hour and once every hour thereafter (Pro-Lab Diagnostics) (Edberg *et al.*, 1996). A positive coagulase test was represented by any degree of clotting (from a loose clot suspended in plasma to a solid clot) (Pro-Lab Diagnostics) (Edberg *et al.*, 1996).

Positive control: *Staphylococcus aureus* ATCC 29213

Negative control: *Bacillus subtilis* ATCC 6683

3.3.3 DNase

DNase agar (Difco Laboratories) supplemented with 0.01% toluidine blue O (Sigma) was employed as the substrate (Janda and Bottone, 1981; Edberg *et al.*, 1996). The medium was autoclaved at 121°C for 15 min and cooled to 50°C (Edberg *et al.*, 1996). Toluidine blue O was filter sterilized (0.20 µm Minisart filter units) before adding it to the cooled agar medium (Edberg *et al.*, 1996). The medium was poured aseptically into 60 mm Petri dishes to a depth of 3 to 4 mm (Edberg *et al.*, 1996). Pure 24 h bacterial cultures were inoculated aseptically on the surface of the DNase agar plates and incubated for 72 h at 37°C (Edberg *et al.*, 1996). The plates were flooded with 0.1% of a 1 M HCl (Merck) and the development of a pink halo or a zone of clearing around the bacterial colonies indicated a positive test (Edberg *et al.*, 1996) (Appendix I).

Positive control: *Staphylococcus aureus* ATCC 29213

Negative control: *Bacillus subtilis* ATCC 6683

3.3.4 Elastase

Elastin powder from bovine neck ligament (Sigma) was prepared as a 1% suspension in nutrient agar (Difco Laboratories) (Sbarra *et al.*, 1960; Janda and Bottone, 1981; Edberg *et al.*, 1996). The medium was autoclaved at 121 °C for 15 min and poured aseptically into 60 mm Petri dishes after cooling to 50 °C (Sbarra *et al.*, 1960; Edberg *et al.*, 1996). Pure 24 h bacterial cultures were inoculated aseptically on the surface of the agar plates and incubated for 24 h at 37 °C (Sbarra *et al.*, 1960; Edberg *et al.*, 1996). After incubation, the plates were held at room temperature (25 °C) for up to 5 days (Sbarra *et al.*, 1960; Edberg *et al.*, 1996). Positive tests were observed by the clearing of the opaque medium around the inoculum spot (Sbarra *et al.*, 1960; Edberg *et al.*, 1996).

Positive control: *Pseudomonas aeruginosa* ATCC 49189

Negative control: *Bacillus subtilis* ATCC 6683

3.3.5 Fibrinolysin

Fibrinolysis was determined by using fibrinogen type III: from human plasma (Sigma) (Janda and Bottone, 1981; Edberg *et al.*, 1996). Nutrient agar medium (Difco Laboratories) was autoclaved separately at 121 °C for 15 min (Edberg *et al.*, 1996). The fibrinogen (280 mg per 100 ml of media) was filter sterilized according to the manufacturer's instructions using 0.20 µm Minisart filter units (Sartorius AG, Goettingen, Germany) before adding it aseptically to the cooled medium (Edberg *et al.*, 1996). The medium was poured aseptically into 60 mm Petri dishes to a depth of 3 - 4 mm (Edberg *et al.*, 1996). Pure 24 h bacterial cultures were inoculated aseptically on the surface of the agar plates and incubated for 48 h at 37 °C (Edberg *et al.*, 1996). Clear zones (diameter of more than 2 mm) around the inoculum spot indicated lysis of human fibrinogen and represented a positive test (Edberg *et al.*, 1996).

Positive control: *Pseudomonas aeruginosa* ATCC 49189

Negative control: *Bacillus subtilis* ATCC 6683

3.3.6 Gelatinase

Chemiluminescent Detection Film (Boehringer, Mannheim, Germany) served as the gelatin substrate support (Edberg *et al.*, 1976; Edberg *et al.*, 1996). A turbid 24 h bacterial suspension was prepared in 1.0 ml of sterile distilled water (Edberg *et al.*, 1976; Edberg *et al.*, 1996). The film was cut into 3 x 2 mm strips, which were placed aseptically into the bacterial suspension (Edberg *et al.*, 1976; Edberg *et al.*, 1996). The substrate film was incubated for 48 h at 37°C (Edberg *et al.*, 1976; Edberg *et al.*, 1996). The removal of the gelatin coating from the surface of the X-ray film represented a positive test (Edberg *et al.*, 1976; Edberg *et al.*, 1996). When gelatin hydrolysis occurred, the gelatin coating dissolved exposing the blue photographic film base. With the negative test, the film color remained green (Edberg *et al.*, 1976; Edberg *et al.*, 1996).

Positive control: *Pseudomonas aeruginosa* ATCC 49189

Negative control: *Bacillus subtilis* ATCC 6683

3.3.7 Hyaluronidase

One gram of Noble agar (Difco Laboratories) was added to 100 ml of Brain Heart Infusion Broth (Oxoid) (Smith and Willett, 1968; Edberg *et al.*, 1996). The medium was autoclaved at 121°C for 15 min and cooled to 50°C (Smith and Willett, 1968; Janda and Bottone, 1981; Edberg *et al.*, 1996). An aqueous solution of 2 mg of hyaluronic acid (Sigma) per ml and 5% bovine albumin fraction V (Sigma) were filter sterilized using 0.20 µm Minisart filter units (Sartorius AG, Goettingen, Germany) (Smith and Willett, 1968; Edberg *et al.*, 1996). The hyaluronic acid solution was added to the cooled media to give a final concentration of 400 mg.ml⁻¹ (Smith and Willett, 1968; Edberg *et al.*, 1996). The bovine albumin fraction V was added with constant stirring to give a final concentration of 1% (Smith and Willett, 1968; Edberg *et al.*, 1996). The final pH of the medium was adjusted to 6.8 (Smith and Willett, 1968; Edberg *et al.*, 1996). The agar medium was poured aseptically to a depth of 3 to 4 mm in 60 mm Petri dishes (Smith and Willett, 1968; Edberg *et al.*, 1996). Pure 24 h bacterial cultures were inoculated aseptically on the surface of the agar plates and incubated for 48

h at 37°C (Smith and Willett, 1968; Edberg *et al.*, 1996). After incubation, the plates were flooded with 2 M acetic acid (BDH Laboratory Supplies, UK) for 10 min (Smith and Willett, 1968; Edberg *et al.*, 1996). The non-degraded substrate precipitated as a conjugate with the albumin, leaving a clear zone around those colonies which produced soluble enzymes that attacked the hyaluronidate (Smith and Willett, 1968; Edberg *et al.*, 1996) (Appendix I).

Positive control: *Staphylococcus aureus* ATCC 29213

Negative control: *Bacillus subtilis* ATCC 6683

3.3.8 Lecithinase

Lecithinase was determined by utilising an egg yolk (50%) agar base (Difco Laboratories) (Edberg *et al.*, 1996). Ten millilitres of the 50% egg yolk enrichment were added to 90 ml of sterilized Bacto McClung Toabe Agar Base (Difco Laboratories) (Edberg *et al.*, 1996). The medium was poured aseptically into 60 mm Petri dishes to a depth of 3 - 4 mm (Edberg *et al.*, 1996). Pure 24 h bacterial cultures were inoculated aseptically on the surface of the agar plates and incubated for 72 h at 37°C (Edberg *et al.*, 1996). A white precipitate around or beneath the inoculum spot was taken as a positive test (Edberg *et al.*, 1996).

Positive control: *Clostridium perfringens* (Department of Medical Microbiology, University of Pretoria)

Negative control: *Bacillus subtilis* ATCC 6683

3.3.9 Lipase

Trypticase soy agar (Difco Laboratories) supplemented with 1% Tween 80 (polyoxyethylene sorbitan monooleate) (Duchefa, Netherlands) served as the substrate (Edberg *et al.*, 1996). The medium was autoclaved at 121°C for 15 min and cooled to 50°C (Edberg *et al.*, 1996). The medium was poured aseptically into 60 mm Petri dishes to a depth of 3 - 4 mm (Edberg *et al.*, 1996). Pure 24 h bacterial

cultures were inoculated aseptically on the surface of the agar plates and incubated for 72 h at 37°C (Edberg *et al.*, 1996). The appearance of a turbid halo around the inoculum spot was taken as a positive test (Edberg *et al.*, 1996).

Positive control: *Clostridium perfringens* (Department of Medical Microbiology, University of Pretoria)

Negative control: *Bacillus subtilis* ATCC 6683

3.3.10 Proteinases

Equal volumes of 3% (wt/vol) of skim milk (Difco Laboratories) and Brain Heart Infusion Broth (Oxoid) with the addition of 1.5 g agar per 100 ml were prepared separately (Burke *et al.*, 1991; Edberg *et al.*, 1996). Both solutions were autoclaved separately at 121°C for 15 min (Burke *et al.*, 1991; Edberg *et al.*, 1996). Equal volumes of the two sterile solutions were mixed at 60°C and the medium was dispensed into 60 mm Petri dishes (Burke *et al.*, 1991; Edberg *et al.*, 1996). Pure 24 h bacterial cultures were inoculated aseptically on the surface of the agar plates and incubated for 48-72 h at 37°C (Burke *et al.*, 1991; Edberg *et al.*, 1996). A zone of clearing around the inoculum spot was regarded as a positive test (Burke *et al.*, 1991; Edberg *et al.*, 1996).

Positive control: *Pseudomonas aeruginosa* ATCC 49189

Negative control: *Bacillus subtilis* ATCC 6683

3.3.11 Pyocyanin

The presence of pyocyanin was determined by the utilisation of Bacto Pseudomonas Agar P (Difco Laboratories) as the substrate (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996). P agar was suspended in 1 liter of distilled water containing 10 g of glycerol (Sigma) and autoclaved at 121°C for 15 min (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996). The inoculum was obtained from a pure 24 h bacterial culture and the colonies were spot inoculated aseptically on the

90 mm P agar dishes (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996). The plates were incubated for 24 h at 37°C (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996). Bacterial colonies were examined under ultraviolet light (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996). Positive results were indicated by a blue pigment that diffused into the agar (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996).

Positive control: *Pseudomonas aeruginosa* ATCC 49189

Negative control: *Bacillus subtilis* ATCC 6683

3.3.12 Fluorescein

The presence of fluorescein was determined by the utilisation of Bacto Pseudomonas Agar F (Difco Laboratories) as the substrate (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996). F agar was suspended in 1 liter of distilled water containing 10 g of glycerol and autoclaved at 121°C for 15 min (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996). The inoculum was obtained from a pure 24 h bacterial culture and the colonies were spot inoculated aseptically on the 90 mm F agar dishes (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996). The plates were incubated for 24 h at 37°C (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996). Bacterial colonies were examined under ultraviolet light (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996). Positive results were indicated by a light, bright greenish-yellow color diffusing into the agar with a fluorescein zone surrounding the growth (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996).

Positive control: *Pseudomonas aeruginosa* ATCC 49189

Negative control: *Bacillus subtilis* ATCC 6683

3.4 ANTIBIOTIC SUSCEPTIBILITY TESTING OF HPC BACTERIA

The antibiotic susceptibility of the bacterial isolates was tested using the Kirby-Bauer quality controlled disk diffusion method (Raphael *et al.*, 1983; Atlas, 1997). Nutrient Agar No. 2 medium (Difco Laboratories) was prepared in accordance with the manufacturer's instructions. This medium allowed the rapid cultivation of the large variety of HPC bacteria found in drinking water, which were previously isolated on PCA medium. The medium was poured in 90 mm Petri dishes to a depth of 3 - 4 mm (Raphael *et al.*, 1983; Atlas, 1997). Five pure colonies of the organism were touched aseptically with a loop and seeded in 1 ml of sterile 0.9% saline solution which was prepared by dissolving 0.9 g of NaCl per 100 ml of distilled water (Raphael *et al.*, 1983; Atlas, 1997). The optical density of the turbid bacterial suspension was set in the range of an absorbance of 0.080 to 0.100 at 625 nm wavelength using the digital spectrophotometer Spectro 22 (LaboMed, Inc., USA) (Raphael *et al.*, 1983; Atlas, 1997). A wooden-stick cotton swab was inserted into the test tube and the bacterial suspension was smeared on the surface of the agar plate (Raphael *et al.*, 1983; Atlas, 1997). When the inoculum has dried, the antibiotic discs (Mast Diagnostics, Mast group Ltd, Merseyside, U.K.) were placed on the plate with sterile forceps (Raphael *et al.*, 1983; Atlas, 1997). Plates were incubated aerobically and the inhibition zone diameters were measured after 16 -18 h (including the 6 mm of discs) (Raphael *et al.*, 1983; Atlas, 1997). The end point was taken as the complete inhibition of growth as it appeared to the unaided eye (Raphael *et al.*, 1983; Atlas, 1997). Organisms were reported as either resistant, intermediate or sensitive to each antimicrobial tested (Raphael *et al.*, 1983; Atlas, 1997).

Two basic classes of antibiotics were tested. The natural and first generation antibiotics (Mast Diagnostics) included: Penicillin G 2 units, Penicillin G 10 units, Ampicillin 10 µg, Ampicillin 25 µg, Streptomycin 10 µg, Streptomycin 25 µg, Erythromycin 10 µg, Erythromycin 15 µg, Kanamycin 30 µg (Edberg *et al.*, 1996). The synthetic and later generation antibiotics (Mast Diagnostics) included: Ciprofloxacin 1 µg, Ciprofloxacin 5 µg, Piperacillin 75 µg, Gentamycin 10 µg, Gentamycin 100 µg, Cefoxitin 30 µg, Oxacillin 1 µg (Edberg *et al.*, 1996).

3.5 IDENTIFICATION OF THE ISOLATED HPC BACTERIA

Several microscopic, metabolic, serologic and gene probe procedures are used for the identification of clinical and environmental microbial isolates (Atlas, 1997). The selection of a specific identification method depends on its accuracy, reliability and speed (Atlas, 1997).

Biolog microplates provide a standardised micromethod using 95 biochemical tests to identify a broad range of Gram-positive and enteric, non-enteric and fastidious Gram-negative bacteria (Smalla *et al.*, 1998; Biolog, 1999; Gamo and Shoji, 1999). The VITEK Automated Microbiology System was designed to provide an accurate automated system for identifying a large variety of bacteria (VITEK Senior/Junior procedures manual, BioMerieux VITEK, Inc.).

3.5.1 VITEK 32 analyser

A VITEK 32 analyser (BioMerieux Vitek, Inc. USA) was used to identify the Gram-negative HPC isolates. The VITEK 32 consisted of incubator/reader, computer with screen, printer and UPS (BioMerieux Vitek, Inc.). Different identification cards were available to optimise results obtained from the VITEK 32 analyser (BioMerieux Vitek, Inc.). Organisms were separated into different identification groups based upon their Gram-reaction (BioMerieux Vitek, Inc.). Gram-negative and Gram-positive identification cards are available and oxidase, catalase or indole tests are performed before identification.

3.5.1.1 Catalase test

1. A pure 24 h bacterial colony was transferred aseptically to the surface of a glass slide.
2. One to two drops of 3% H₂O₂ (BDH Laboratory Supplies, UK) were added on top of the bacterial colony.
3. The presence of catalase in the culture was indicated by effervescence, caused by the liberation of free oxygen as gas bubbles (Cown, 1981).

Positive result: Rapid appearance and sustained production of gas bubbles or effervescence.

Negative result: Delayed or no bubble formation.

Positive control: *Staphylococcus aureus* ATCC 29213

Negative control: *Streptococcus faecalis* ATCC 29212

3.5.1.2 Oxidase test

a) Indirect paper strip technique

This test was performed with sticks impregnated with the oxidase reagent (Bactident Oxidase, Merck). A pure 24 h bacterial colony was taken with a sterile disposable wooden stick and spread onto the impregnated filter strips (Cown, 1981).

Positive reaction: Development of a dark purple colour after approximately 1 minute.

Negative reaction: No colour change.

Positive control: *P. aeruginosa* ATCC 49189

Negative control: *E. coli* ATCC 11775

b) Wet filter paper method

A strip of filter paper was moistened with 1-2 drops of oxidase reagent. A pure 24 h bacterial colony was touched with the end of a sterile disposable wooden stick. The bacterial colony was smeared onto the impregnated filter paper.

Positive result: Development of a dark purple colour within 10-30 sec.

Negative result: No colour change or light purple.

Positive control: *P. aeruginosa* ATCC 49189

Negative control: *E. coli* ATCC 11775

Oxidase reagent was prepared by mixing equal amounts of naphthyl-1-amine (Sigma) and p-aminodimethylaniline (Sigma) in an 1 ml syringe.

3.5.1.3 Preparing the bacteria for identification

The purity of each bacterial isolate was ascertained by streaking the bacteria for single colonies and performing a gram stain on these colonies (BioMerieux Vitek, Inc.). The bacterial isolates were inoculated onto slants of Nutrient Agar No.2 growth medium and incubated at 37°C (BioMerieux Vitek, Inc.).

3.5.1.4 Marking the identification card

Each card was allowed to warm to room temperature in its foil pouch (BioMerieux Vitek, Inc.). The card was removed from its foil pouch and was placed horizontally on a table (BioMerieux Vitek, Inc.). The card should not be scratched as it would influence its readability (BioMerieux Vitek, Inc.). The card was marked, including the mark for the result of the external test (BioMerieux Vitek, Inc.). The system would scan this part of the card for the VITEK ID (BioMerieux Vitek, Inc.). The numbers should be machine-readable and written in dark squared-off numbers (BioMerieux Vitek, Inc.). Each number was written as one stroke, not lifting up the pen for any segment (BioMerieux Vitek, Inc.). The system read missing digits as "0" (zero) and questionable digits as "?" (questionable marks) (BioMerieux Vitek, Inc.). The number 1 was written on the right hand side of the figure eight (BioMerieux Vitek, Inc.).

3.5.1.5 Calibrating the colorimeter

After switching the colorimeter on, a test tube was filled with 0.45% saline solution and was inserted

in the slot on the colorimeter (BioMerieux Vitek, Inc.). The transmission was set to 100% and the test tube was removed (BioMerieux Vitek, Inc.). The back of a marking pen was inserted into the slot (BioMerieux Vitek, Inc.). The transmission was set to 0% and the marking pen was removed (BioMerieux Vitek, Inc.). The colorimeter was thus calibrated and ready for use (BioMerieux Vitek, Inc.). The colorimeter was calibrated after every fourth sample (BioMerieux Vitek, Inc.).

3.5.1.6 Diluting the sample and inoculating the card

Samples had to be diluted with 0.45% saline solution to ensure proper growth conditions in the test cards (BioMerieux Vitek, Inc.). Approximately 1.8 ml of saline were dispensed into the test tube and inoculated with the bacterial isolate (BioMerieux Vitek, Inc.). After thorough mixing, the optical density of the bacterial solution was measured with the colorimeter (BioMerieux Vitek, Inc.). The density could be lowered by adding more inoculating fluid or raised by adding more of the bacterial inoculum (BioMerieux Vitek, Inc.). The correct densities for specific cards were displayed inside the lid of the colorimeter (BioMerieux Vitek, Inc.). The test tube was inserted in the slot provided on the filling stand (BioMerieux Vitek, Inc.). One end of the bent tube was inserted into the filling port of the card and the other was inside the test tube containing the organism (BioMerieux Vitek, Inc.). The card was placed in the Filler chamber in which a vacuum formed (BioMerieux Vitek, Inc.). The pressure was released, drawing sample into the test card and thus filling it (BioMerieux Vitek, Inc.). This cycle was repeated for the remaining cards (BioMerieux Vitek, Inc.). The bent tube was removed from the card and a sealer was inserted into the port (BioMerieux Vitek, Inc.). The wells were inspected for air bubbles and the card was incubated (BioMerieux Vitek, Inc.).

3.5.1.7 Incubating and reading the cards

After opening the reader menu, the cards were inserted into the incubator/reader in the tray (BioMerieux Vitek, Inc.). Cards should not remain inside the incubator/reader for more than fifteen minutes if unread for the first time (BioMerieux Vitek, Inc.). The instrument conducted the remainder of the identification (BioMerieux Vitek, Inc.). The results were displayed on the status menu and the

cards were removed (BioMerieux Vitek, Inc.).

3.5.2 Biolog GN2 and GP2 MicroPlates

Biolog GP2 and GN2 MicroPlates (Biolog, Inc., Hayward, CA, USA) were employed in the identification of Gram-positive and Gram-negative HPC bacteria unidentifiable by the VITEK 32 analyser (Biolog, 1999). The GP2 and GN2 MicroPlate test panels provided a standardised micromethod using 95 biochemical tests to identify a broad range of gram-positive and Gram-negative bacteria, respectively (Biolog, 1999). Biolog's MicroLog 1, MicroLog 2 or MicroLog 3 software can be used to identify the bacterium according to its metabolic pattern in the GP2 or in the GN 2 MicroPlate (Biolog, 1999).

Gram-stain reaction was performed on the HPC isolates to verify their purity and Gram identity (Biolog, 1999). The test was initiated by removing aseptically 24 h bacterial colonies from the agar plates with sterile inoculation loop and suspending the cells in sterile test tubes containing 18 ml of 0.9% saline solution (Biolog, 1999). After thorough mixing, the optical density of the bacterial suspension was measured and adjusted in the range of an absorbance of 0.400 to 0.800 at 625 nm wavelength using the Spectro 22 digital spectrophotometer (Biolog, 1999). The density could be lowered by adding more inoculating fluid or raised by adding more bacterial colonies (Biolog, 1999).

The bacterial suspension was poured into the sterile multichannel pipette reservoir (Biolog, 1999). Sterile tips were used for the 10-Channel Repeating Pipetter (Biolog, 1999). The tips were primed by dispensing the first delivery back into the reservoir (Biolog, 1999). The wells were filled with 150 µl of the bacterial suspension (Biolog, 1999). The inoculating fluid formed a soft gel in the wells, the MicroPlates were covered and incubated at 37°C for 24 h (Biolog, 1999).

After 24 h incubation, each plate was read with the MicroPlate reader-the Flow Titerk Multiscan Plus (Labsystems, Finland) (Biolog, 1999). The MicroPlate lid was removed and the plate was placed into the drawer of the Flow Titerk Multiscan Plus reader with the negative control well, A-1, in the upper

left corner (Biolog, 1999). The reader performed a dual wavelength read at 590 nm (for purple colour) and at 750 nm (for background reference)(Biolog, 1999). The similarity index was at least 0.500 in order to be considered acceptable (Biolog, 1999). Biolog MicroLog 14.01A software determined the identity of each of the bacterium examined (Biolog, 1999).

A positive reaction was indicated by the appearance of a purple colour in the wells compared to the negative well A-1 (Biolog, 1999). False positive colour was defined as purple colour forming in the control well A-1 (Biolog, 1999). This was typically caused by utilization by capsulated strains of their extracellular polysaccharides and was commonly observed with *Klebsiella*, *Enterobacter* and *Serratia* strains (Biolog, 1999).

3.6 ANALYSIS OF CYTOTOXICITY EXHIBITED BY HPC BACTERIA

HEp-2 cells CCL-23 (American Type Culture Collection, VA, USA) were grown in 250 ml tissue culture flasks (Cellstar, Greiner Labortechnik) and incubated at 37°C in a 5% CO₂ incubator (Galaxy CO₂ Incubator- Biotech, Northants, England) (Lye and Dufour, 1991; Payment *et al.*, 1994; Edberg *et al.*, 1996). Eagle's Minimum Essential Medium (MEM) (Highveld Biological, Lyndhurst, RSA) supplemented with 5% fetal bovine serum (Delta Bioproducts, Kempton Park, RSA) served as the growth medium (Lye and Dufour, 1991; Payment *et al.*, 1994).

Cytotoxicity of HPC isolates was determined using pure 24 h bacterial cultures. The bacterial colonies were aseptically removed from the agar plates with a sterile inoculation loop and suspended in sterile test tubes containing 5 ml of sterile distilled water. The suspension was thoroughly mixed and the number of bacteria was adjusted to 10⁸ cells per ml (i.e., an optical density of 0.300 at 660 nm) (Lye and Dufour, 1991; Payment *et al.*, 1994). One millilitre of each of the bacterial suspensions was passed through 0.45 µm Cellulose Nitrate Filters (Sartorius AG, Germany) (Payment *et al.*, 1994). To increase the expression of cytotoxins, each of the membrane filters containing bacteria was placed on human blood agar plates for 24 h at 37°C prior to initiation of the analysis (Payment *et al.*, 1994).

HEp-2 cells were seeded in 92 mm Nunclon tissue culture plates (Nalge Nunc, Denmark) at a concentration of 2×10^5 cells per ml and incubated at 37°C in a 5% CO₂ incubator (Galaxy CO₂ Incubator- Biotech, Northants, England) (Lye and Dufour, 1991; Payment *et al.*, 1994). After 48 h of incubation, the medium was discarded aseptically and a confluent HEp-2 cell monolayer was observed at the bottom of each tissue culture plate (Lye and Dufour, 1991; Payment *et al.*, 1994). Overlay medium stock was prepared using filter sterilised double-strength Eagle's Minimum Essential Medium, consisting of 96% MEM and 4% fetal bovine serum (Lye and Dufour, 1991; Payment *et al.*, 1994). A 2% Sea Kem ME Agarose solution (FMC Bioproducts, ME, USA), consisting of 2 g agar in 100 ml phosphate-buffered saline pH 7.2 (Sigma), was prepared separately and autoclaved at 121°C for 15 min (Lye and Dufour, 1991; Payment *et al.*, 1994). Equal amounts of the double-strength MEM and the agar were mixed at 50°C to give a final concentration of 1% agar (Lye and Dufour, 1991; Payment *et al.*, 1994). The overlay medium was poured aseptically on top of each tissue culture plate containing the formed HEp-2 cell monolayer (Lye and Dufour, 1991; Payment *et al.*, 1994). After a short exposure to absorbent cellulose pads (Millipore Corporation, Bedford, MA) wetted with phosphate-buffered saline (pH 7.2), each of the membranes containing bacteria was placed on the agar overlay covering the HEp-2 cell monolayer (Lye and Dufour, 1991; Payment *et al.*, 1994).

Agar overlays were removed by inverting the tissue culture plates and removing the agars with a spatula in one smooth motion (Lye and Dufour, 1991; Payment *et al.*, 1994). Non-affected cells remained attached to the bottom of the tissue culture plate (Lye and Dufour, 1991; Payment *et al.*, 1994). Each cell monolayer was air dried and stained for 5 min with Crystal violet-formaldehyde stain (Merck) (Lye and Dufour, 1991; Payment *et al.*, 1994). The presence of areas of plaques of cytolysis after removal of the agar overlays indicated a positive test (Lye and Dufour, 1991; Payment *et al.*, 1994) (Appendix I).

Positive test: *Staphylococcus aureus* ATCC 29213

Negative test: *Bacillus subtilis* ATCC 6683

3.7 ANALYSIS OF CELL ADHERENCE DISPLAYED BY HPC BACTERIA

Caco-2 HTB-37 (American Type Culture Collection, VA, USA) and HEp-2 cells were employed to evaluate the adherence potential of HPC bacteria (Darfeuille-Michaud *et al.*, 1990; Payment *et al.*, 1994). After a 10 day incubation period, Caco-2 (human adenocarcinoma cell line) cells had the characteristics of human enterocytes (Darfeuille-Michaud *et al.*, 1990; Payment *et al.*, 1994). Caco-2 cells were grown in 250 ml tissue culture flasks (Cellstar) and incubated at 37°C in a 5% CO₂ incubator (Galaxy CO₂ Incubator- Biotech, Northants, England) (Payment *et al.*, 1994). Eagle's Minimum Essential Medium (Highveld Biological) supplemented with 5% fetal calf serum (Delta Bioproducts) was used as the maintenance medium (Darfeuille-Michaud *et al.*, 1990; Payment *et al.*, 1994). Growth medium was MEM supplemented with 15-20% fetal calf serum (Darfeuille-Michaud *et al.*, 1990; Payment *et al.*, 1994). Caco-2 cells (8×10^4 cells per ml) were prepared in 16-well Lab-Tek chambers (Nalge Nunc) and incubated at 37°C in a 5% CO₂ incubator (Galaxy CO₂ Incubator- Biotech, Northants, England) (Darfeuille-Michaud *et al.*, 1990; Payment *et al.*, 1994).

Pure 24 h cytolytic HPC isolates were suspended in sterile test tubes containing 10 ml of sterile tryptic soy broth (Difco Laboratories) (Payment *et al.*, 1994). Each bacterial suspension was incubated at 37°C for 24 h with light agitation (Payment *et al.*, 1994). After 24 h, the number of bacteria was adjusted to 10^8 cells per ml for a final bacterium-to-cell ratio of 100:1 (i.e., an optical density of 0.300 at 660 nm) (Payment *et al.*, 1994). The optical density could be lowered by adding more tryptic soy broth or raised by adding more bacterial cells. Each bacterial suspension was introduced into Lab-Tek chambers containing the Caco-2 monolayers for 3 h at 37°C in a 5% CO₂ atmosphere incubator (Galaxy CO₂ Incubator- Biotech, Northants, England) (Payment *et al.*, 1994). The monolayers were washed three times with phosphate-buffered saline (Sigma) (Payment *et al.*, 1994). The Caco-2 monolayers were fixed with 100% methanol (BDH Laboratory Supplies, UK) for 30 min (Payment *et al.*, 1994). After discarding the methanol, Caco-2 monolayers were stained with 0.01% acridine orange (Merck) in 0.5 M acetate buffer pH 3.5 (Sigma) for 15 min and examined under UV fluorescence by using a 100X oil immersion objective (Payment *et al.*, 1994). The index of adherence was defined as the average number of bacteria per cell and was determined by counting the number

of bacteria adhering to 100 Caco-2 cells (Darfeuille-Michaud *et al.*, 1990; Payment *et al.*, 1994). Experiments were performed in triplicate.

The same procedure was followed when studying the adherence of HPC bacteria using HEp-2 cells. Cells were grown in 16-well Lab-Tek chambers using 5% MEM and incubated for 24 h at 37°C in a 5% CO₂ incubator (Galaxy CO₂ Incubator- Biotech, Northants, England).

The index of adherence was calculated as follows:

Index of adherence = (number of bacteria adhering to 100 cells)/100

Positive test: *Staphylococcus aureus* ATCC 29213

Negative test: *Bacillus subtilis* ATCC 6683

3.8 ANALYSIS OF CELL INVASION DISPLAYED BY HPC BACTERIA

Caco-2 cells HTB-37 (American Type Culture Collection, VA, USA) were grown in 250 ml tissue culture flasks (Cellstar) and incubated at 37°C in a 5% CO₂ incubator (Galaxy CO₂ Incubator- Biotech, Northants, England) (Payment *et al.*, 1994). Eagle's Minimum Essential Medium (Highveld Biological) supplemented with 5% fetal calf serum (Delta Bioproducts) served as the maintenance medium (Payment *et al.*, 1994). Growth medium was MEM supplemented with 15-20% fetal calf serum (Payment *et al.*, 1994). To evaluate the invasive potential of adhering HPC bacteria, a 1 ml sample of Caco-2 cells at a density of 8×10^4 cells per ml was seeded in 24-well tissue culture plates (Corning Costar Corporation) and grown for 10 days (Payment *et al.*, 1994).

Pure 24 h cytolytic HPC isolates were suspended in sterile test tubes containing 10 ml of sterile tryptic soy broth (Difco Laboratories) (Payment *et al.*, 1994). Bacterial suspensions were incubated at 37°C for 24 h with light agitation (Payment *et al.*, 1994). After 24 h, the number of bacteria was adjusted to 10^8 cells per ml (serial dilutions) (Payment *et al.*, 1994).

The 24-well tissue culture plates containing Caco-2 monolayers were drained of their medium (Payment *et al.*, 1994). One millilitre of each bacterial suspension was added to the single wells (Payment *et al.*, 1994). After incubation for 3 h at 37°C in a 5% CO₂ incubator (Galaxy CO₂ Incubator- Biotech, Northants, England), the cells were washed three times with phosphate-buffered saline (Sigma) and incubated for 1 h in minimum essential medium (Highveld Biological) containing 100 µg of gentamicin (Boehringer Mannheim GmbH, Germany) per ml to kill non-invasive bacteria (Payment *et al.*, 1994). Each monolayer was washed with phosphate-buffered saline and lysed with 1 ml of 1% Triton X -100 (Whittaker M.A. Bioproducts, Maryland, USA) for 20 min to extract bacteria that had penetrated the cells (Payment *et al.*, 1994). Surviving bacteria were counted by a plate dilution method using Plate Count Agar medium (Merck) (Payment *et al.*, 1994) (Appendix I).

The same procedure was followed when studying the invasiveness of HPC bacteria using HEp-2 cells. HEp-2 cells were grown in 24-well tissue culture plates using 5% MEM and incubated for 24 h at 37°C in a 5% CO₂ incubator (Galaxy CO₂ Incubator- Biotech, Northants, England).

The invasion index was calculated as follows (Payment *et al.*, 1994):

Invasion index = (number of colonies obtained / number of inoculated bacteria) x 100

Positive test: *Pseudomonas aeruginosa* ATCC 49189

Negative test: *Bacillus subtilis* ATCC 6683

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Background

The heterotrophic plate count method proved to be useful in determining the microbiological quality of water during treatment, storage and distribution (Reasoner, 1990; Grabow, 1996; WHO, 2001). As the designation “heterotrophic” indicates, bacteria detected by the HPC test readily multiply in water supplies and distribution systems, which contain basic organic nutrients in the absence of sufficient levels of disinfectants to suppress their multiplication. These HPC bacteria are typically associated with aftergrowth and biofilms in drinking water distribution systems, and do not find the conditions of the human host congenial (Edberg *et al.*, 1996; Grabow, 1996). Moreover, microorganisms detected by the HPC method may include a variety of bacteria associated with unacceptable aesthetic conditions of drinking water supplies, such as taste, odour, turbidity and appearance (Edberg *et al.*, 1996; Grabow, 1996).

Drinking water quality specifications used world-wide allow HPCs of 100 cfu.ml⁻¹ (WHO, 1997; SABS, 2001; WHO, 2001) and in some cases as high as 500 cfu.ml⁻¹ (LeChevalier *et al.*, 1980; Rusin *et al.*, 1997). HPC bacteria are generally regarded as harmless organisms, which constitute no meaningful health risk. Consequently, HPCs exceeding specified limits tend not to be perceived as a serious violation of water quality specifications and are widely accepted. However, there is growing concern about accumulating evidence suggesting that HPCs may actually include organisms which are not as harmless as generally perceived (Ptak and Ginsburg, 1977; Payment *et al.*, 1994; Edberg *et al.*, 1996; Hellard *et al.*, 1997; Rusin *et al.*, 1997). It is evident that bacteria detected by heterotrophic plate counts and the composition of bacteria covered by these counts, warrant urgent attention in water quality monitoring and the assessment of health risks associated with drinking water supplies.

The objective of this project was primarily to study bacteria detected by the HPC method using selected drinking water supplies in South Africa and to identify bacteria which may constitute a

potential health risk. The intention was to focus on HPC bacteria showing potential virulent properties such as cytotoxicity, adherence and invasiveness.

4.2 Pilot study

According to the U.S. Environmental Protection Agency's Surface Water Treatment Rule (40 CFR 141.74), the pour plate method with incubation at 35°C for 48 h has been extensively used for bacterial enumeration in drinking water (Reasoner, 1990; Standard Methods, 1998). However, it is evident from a variety of studies that the pour plate method may reduce bacterial counts in potable water due to heat stress (Reasoner *et al.*, 1985; Reasoner, 1990). As an alternative the spread plate or the membrane filter methods can be applied (WHO, 2001). The spread plate and the membrane filter methods have not been applied in the past, because they were not included in *Standard Methods* until the 16th edition (1985) (Reasoner, 1990). The media most commonly employed to isolate heterotrophic bacteria from water samples included R₂A, Plate Count Agar (PCA) and Sheep Blood Agar (TSA-SB) media (Reasoner, 1990; Carter *et al.*, 2000).

In order to select a suitable isolation technique and incubation conditions, a pilot study was conducted. Experiments were conducted in triplicate. The pour plate and the spread plate techniques were compared with implementation of the following incubation conditions: 20°C, 28°C, 37°C for 24 h, 48 h, 72 h and 120 h. Results obtained from the pilot study are listed in Table 4.1 and Table 4.2. The average number of colony forming units per millilitre was calculated and recorded for the three plates of each dilution.

Plate count agar is the recommended medium for heterotrophic plate counts (35°C, 48 h incubation) of water and wastewater (Reasoner *et al.*, 1985). R₂A medium contains lower nutrients than the PC agar medium and is well suited for enumeration of bacteria that grow well in low-nutrient environments such as drinking water (Rusin *et al.*, 1997). However, PC agar or other media with high nutrient contents are more suitable for the isolation of human bacterial pathogens (Rusin *et al.*, 1997). R₂A medium is similar to modified Henrici medium and casein-peptone-starch-medium but does not contain glycerol or iron as FeCl₃ or FeSO₄ (Reasoner *et al.*, 1985). R₃A medium is basically double-strength R₂A medium, which in some studies proved useful in the subculturing of aquatic bacterial isolates (Reasoner *et al.*, 1985).

TABLE 4.1 Microbiological analysis of drinking water using the pour plate technique, different nutrient media and incubation conditions

| Pour plate method | | | | | |
|-------------------|------|-----------------------------|-----------------------------|--|--|
| Time | T/°C | YXA (cfu.ml ⁻¹) | PCA (cfu.ml ⁻¹) | R ₂ A (cfu.ml ⁻¹) | R ₃ A (cfu.ml ⁻¹) |
| 24 h | 20 | 1.1x10 ² ± 0.58 | 1.8x10 ² ± 3.2 | *1.6x10 ¹ ± 1 | 1.1x10 ² ± 10 |
| 24 h | 28 | 1.1x10 ³ ± 165 | 6.5x10 ² ± 137 | 3.6x10 ² ± 23 | 7.9x10 ¹ ± 3 |
| 24 h | 37 | 4.7x10 ² ± 115 | *4.0x10 ² ± 100 | 4.6x10 ² ± 123 | *2.7x10 ¹ ± 21 |
| 48 h | 20 | 1.6x10 ³ ± 147 | 3.5x10 ³ ± 451 | 9.1x10 ² ± 61 | 1.5x10 ² ± 6 |
| 48 h | 28 | *2.1x10 ³ ± 566 | 3.6x10 ³ ± 300 | 3.1x10 ³ ± 781 | *1.1x10 ² ± 78 |
| 48 h | 37 | *1.0x10 ³ | *4.7x10 ³ ± 1154 | *1.1x10 ³ ± 208 | *4.3x10 ³ ± 2887 |
| 72 h | 20 | 2.9x10 ³ | 4.9x10 ³ ± 361 | *2.2x10 ³ ± 379 | *7.0x10 ¹ |
| 72 h | 28 | 6.0x10 ³ ± 212 | 5.8x10 ³ ± 833 | 5.9x10 ³ ± 1850 | *2.0x10 ² |
| 72 h | 37 | *1.7x10 ³ ± 577 | *5.0x10 ³ ± 1000 | 8.8x10 ³ ± 5940 | >300 |
| 120 h | 20 | 6.0x10 ³ ± 866 | 1.1x10 ⁴ ± 1007 | 6.0x10 ³ ± 917 | *1.3x10 ² ± 17 |
| 120 h | 28 | 1.3x10 ⁴ ± 3747 | *1.6x10 ⁴ ± 2828 | 10.0x10 ³ ± 1629 | *3.0x10 ² ± 100 |
| 120 h | 37 | *3.7x10 ³ ± 2081 | *6.3x10 ³ ± 1155 | 6.6x10 ³ | >300 |

* = indicates that the average number of cfu.ml⁻¹ is computed for plates, which had less than 30 colonies.

N= experiments were performed in triplicate.

Note: Counts were made between 30 to 300 colonies.

At 37°C and 48 h of incubation, the PCA medium gave the highest bacterial counts (4.7x10³ cfu.ml⁻¹) with the pour plate method (Table 4.1). However, after 72 h incubation and 28°C high bacterial counts were observed with the YXA medium (Table 4.1). The R₂A medium gave low counts at 20°C after 24 h of incubation with both methods (Table 4.1 and Table 4.2). R₂A medium showed results, which differed slightly from those of the PCA and YXA media using either of the two methods (Table 4.1 and Table 4.2). Low results were recorded for the R₃A medium at 28°C and 37°C for 24 h incubation with the pour plate technique (Table 4.1).

According to these results, the PCA medium and incubation at 28°C for 120 h gave the highest bacterial counts with the pour plate method (Table 4.1). However, bacterial pathogens causing infections in humans, are well known to be active at 37°C. Therefore, at 37°C and 48 h of incubation, the highest bacterial counts (4.7x10³ cfu.ml⁻¹) were obtained with the PCA medium

and thus, showing to be the most suitable nutrient medium for the enumeration of HPC bacteria.

TABLE 4.2 **Microbiological analysis of drinking water using the spread plate technique, different nutrient media and incubation conditions**

| Spread plate method | | | | | |
|---------------------|------|-----------------------------|-----------------------------|--|--|
| Time | T/°C | YXA (cfu.ml ⁻¹) | PCA (cfu.ml ⁻¹) | R ₂ A (cfu.ml ⁻¹) | R ₃ A (cfu.ml ⁻¹) |
| 24 h | 20 | *2.5x10 ² ± 42 | 6.3x10 ² ± 104 | 5.0x10 ¹ ± 44 | 5.3x10 ² ± 166 |
| 24 h | 28 | 1.9x10 ³ ± 241 | 1.6x10 ³ ± 42 | 1.9x10 ³ ± 106 | 1.3x10 ³ ± 71 |
| 24 h | 37 | *1.3x10 ³ ± 577 | *1.5x10 ⁴ ± 4583 | 1.0x10 ³ ± 231 | 9.1x10 ³ ± 231 |
| 48 h | 20 | *2.0x10 ³ | *5.0x10 ³ | 3.6x10 ³ ± 153 | 5.3x10 ³ ± 495 |
| 48 h | 28 | *3.0x10 ³ | *9.0x10 ³ ± 7071 | 9.8x10 ³ | *3.0x10 ³ |
| 48 h | 37 | *3.0x10 ³ | *1.8x10 ⁴ ± 7778 | 3.9x10 ³ ± 2193 | >300 |
| 72 h | 20 | *7.0x10 ³ | >300 | 2.2x10 ⁴ ± 29838 | *1.8x10 ⁴ |
| 72 h | 28 | *3.0x10 ³ | *7.0x10 ³ | *4.0x10 ³ | *1.0x10 ⁴ |
| 72 h | 37 | *3.0x10 ³ | *2.3x10 ⁴ | 6.1x10 ³ ± 141 | >300 |
| 120 h | 20 | *7.0x10 ³ | >300 | 4.2x10 ⁴ ± 46808 | >300 |
| 120 h | 28 | *6.0x10 ³ | *7.0x10 ³ | 3.0x10 ⁴ | 1.9x10 ⁵ |
| 120 h | 37 | *4.0x10 ³ | *2.3x10 ⁴ | >300 | >300 |

* = indicates that the average number of cfu.ml⁻¹ is computed for plates, which had less than 30 colonies.

N= experiments were performed in triplicate.

Note: Counts were made between 30 to 300 colonies.

After 24 h of incubation at 37°C, the PCA medium showed the highest bacterial counts (1.5x10⁴ cfu.ml⁻¹) using the spread plate method (Table 4.2). Similar tendency was noted at 20°C (Table 4.2). YXA and R₂A media yielded bacterial counts of 1.9x10³ cfu.ml⁻¹ at 28°C for the same incubation period (24 h) and spread plate technique (Table 4.2).

At 37°C and 48 h of incubation, the PCA medium gave the highest bacterial counts (1.8x10⁴ cfu.ml⁻¹) with the spread plate method (Table 4.2). However, at 20°C and 28°C the highest bacterial counts were observed for the R₂A and R₃A media (Table 4.2).

After 72 h incubation at 37°C, the highest bacterial counts were obtained with the PCA medium

using the spread plate method (Table 4.2). At 20°C and 28°C, the R₂A and R₃A turned out to be the most effective media (Table 4.2).

After 120 h at 20°C, the highest colony counts were recorded for the R₂A medium using the spread plate method (Table 4.2). At 28°C, bacterial counts of 1.9×10^5 cfu.ml⁻¹ were obtained with the R₃A medium (Table 4.2). However, PCA medium showed the highest bacterial counts at 37°C with the spread plate method (Table 4.2).

According to these results it can be concluded that at 37°C and 48 h of incubation, the spread plate method using PCA medium gave the highest bacterial counts (1.8×10^4 cfu.ml⁻¹). Similar tendency was observed for 24 h of incubation (Table 4.2). Thus, with the spread plate technique the PCA medium appeared once more to be the most suitable growth medium.

In conclusion, the PCA medium and incubation at temperature of 37°C for a period of 24 - 48 h was most suitable for the enumeration of HPC bacteria isolated from drinking water. Moreover, 37°C temperature is known to correspond to the internal temperature of the human body and was, therefore, selective for HPC bacteria with potential pathogenic features. The spread plate method turned out to be the better technique, since it gave the highest bacterial counts (1.8×10^4 cfu.ml⁻¹ compared to the 4.7×10^3 cfu.ml⁻¹ obtained with the pour plate method) at the above mentioned incubation conditions. In agreement with Reasoner and colleagues (1985), meaningful results were obtained with R₂A and R₃A media at 28°C with longer incubation periods.

The pour plate method had several disadvantages such as the production of compact, slow growing bacterial colonies which were difficult to transfer and significant numbers of the bacterial colonies were killed due to the transient exposure of the sample to 50°C previously melted agar (Atlas, 1997; WHO, 2001). Limitations of the spread plate method arose due to the small sample volume or diluted sample that could be absorbed by the agar and the overgrowth by one specific bacterial species (Atlas, 1997). However, the spread plate in comparison to the pour plate method caused no heat shock and all colonies were on the agar surface where they could readily be distinguished from particles and bubbles (Atlas, 1997). Bacterial colonies could be transferred quickly and colony morphology could easily be discerned and compared to published descriptions (Atlas, 1997).

4.3 Isolation of heterotrophic plate count bacteria

Traditionally, the pour plate procedure with the use of PCA medium has been applied in the enumeration of HPC bacteria found in drinking water (APHA, 1996). However, recently spread plate procedures using high nutrient 5% sheep blood agar (TSA-SB) were employed in determination of the presence of certain virulence factors in HPC bacteria isolated from drinking water (Payment *et al.*, 1994; Carter *et al.*, 2000). According to researchers such as Reasoner *et al.* (1985) and Reasoner (1990) no culture method or set of conditions would guarantee enumeration of all bacteria that may be found in water. Thus, given the choice of methods and media available, the user should evaluate their effectiveness for the types of water tested (Reasoner, 1990).

Heterotrophic plate counts, using the pour plate method, PCA medium and incubation at 37°C for 24 h (SABS, 2001), were carried out on samples (exceeding the 100 cfu.ml⁻¹ guideline set by the SABS) from selected drinking water supplies in three different areas of South Africa during the period 2000-02-25 to 2000-06-15. Although our pilot study recommended the use of spread plates in the isolation of HPC bacteria from drinking water, 153 pour plate samples, performed at the laboratories of the water suppliers, were obtained for microbiological analyses. The spread plate method, PCA medium and incubation at 37°C for 24 h were applied to 20 river water samples.

A total of 339 HPC bacteria were randomly isolated, purified and Gram-stained. Different colony morphologies were observed among the HPC isolates such as flat, convex, smooth, circular, irregular, finely or coarsely granulated, pinhead or with larger dimensions. Some of the HPC isolates produced orange and yellow pigments, whereas red and pink bacteria constituted a small number (4.1%) of the pigmented isolates. Similar findings were reported by other researchers such as Carter *et al.* (2000) and were important for interpreting changes in the microbiological quality of finished water (Geldreich and Reasoner, 1990; Rusin *et al.*, 1997).

After streaking the purified bacterial isolates on human and horse blood agar plates, a total of 188 (55.5%) HPC isolates were α - or β -haemolytic (Figure 4.1) and consisted of 56.4% Gram-negative and 43.6% Gram-positive isolates (Table 4.4). Alpha haemolysis was indicated by 26%

(48 isolates) of the HPC isolates, of which 19.7% (37 isolates) were Gram-negative and 5.9% (11 isolates) Gram-positive bacteria (Table 4.4). Beta-haemolysis was observed for 74% (140 isolates) of the HPC isolates, of which 36.7% (69 isolates) were Gram-negative and 37.8% (71 isolates) Gram-positive bacteria (Table 4.4).

Gram-negative haemolytic isolates, such as *Aeromonas veronii* biovar *sobria* showed the highest percentage occurrence in the drinking water samples (18.1%), followed by *Pseudomonas* species (4.8%) and *Aeromonas hydrophila* (3.7%) (Figure 4.2). Dominant haemolytic Gram-positive isolates included: *Tsukamurella inchoensis* (13.3%), *Staphylococcus* species (6.9%) and *Corynebacterium* species (3.7%) (Figure 4.2). Similar HPC bacteria (except *Tsukamurella inchoensis*) were isolated from drinking water by other researchers such as Payment *et al.* (1994), Ashbolt *et al.* (1995) and Edberg *et al.* (1996).

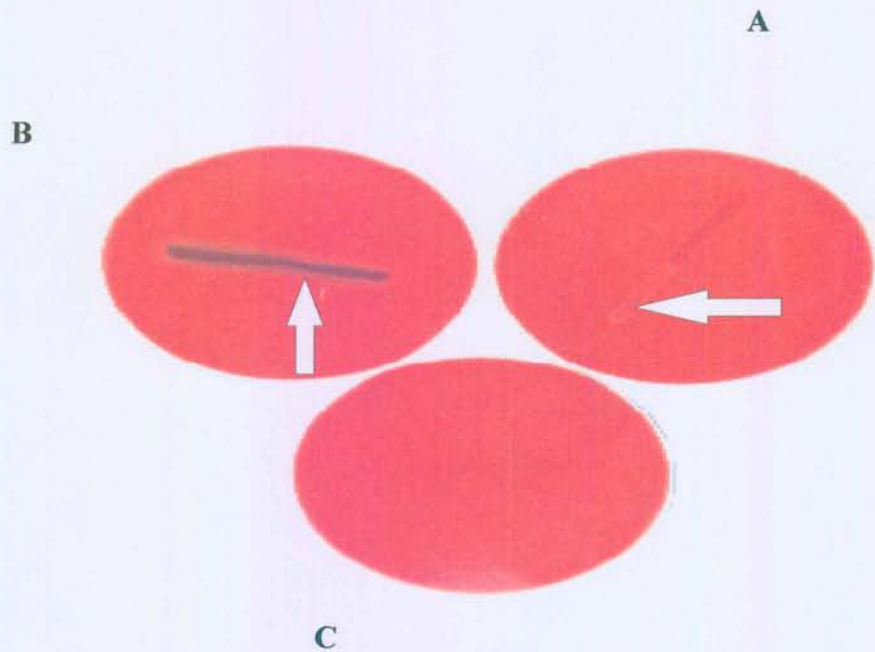


Figure 4.1: Demonstration of α -, β - and γ -haemolysis by HPC bacteria isolated randomly from selected drinking water supplies in South Africa. (A) Clear zones around the bacterial colonies indicating β -haemolysis, whereas green zones around the colonies suggested α -haemolysis (B) and no haemolysis was referred to as γ -haemolysis (C) (Atlas, 1997).

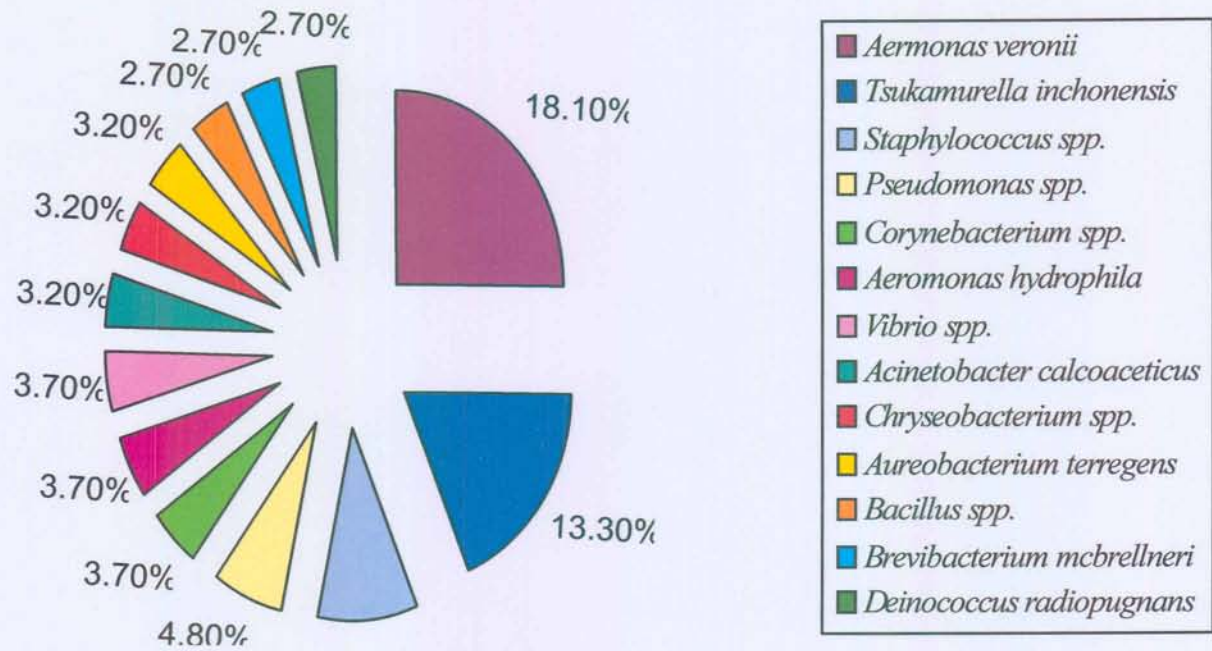


Figure 4.2: Percentage representation of the most commonly occurring haemolytic HPC bacteria isolated from selected drinking water supplies in South Africa.

4.4 Extracellular enzymes related to pathogenicity

There is a delicate balance in the intestine between the number of microbes and their virulence characteristics versus the immune status of the host (Duncan *et al.*, 1995). Microorganisms have developed a vast array of techniques in order to evade host defences (Duncan *et al.*, 1995). These techniques help them associate with the epithelium of the intestine and from there to exert their effect (Duncan *et al.*, 1995).

A wide variety of microbes are found in the food and water consumed daily (Duncan *et al.*, 1995). The production of sterile water and food is not possible (Duncan *et al.*, 1995). Moreover, the over-regulation of the microbial content of food and water could lead to serious toxicological effects because many of the processes used to reduce the number of microorganisms yield toxic byproducts (Duncan *et al.*, 1995). For example, the interaction of chlorine with natural organic matter can produce trihalomethanes that are carcinogenic (Duncan *et al.*, 1995).

In order for an infection to occur, microorganisms must possess specific virulence factors that allow them to escape the host's nonspecific defence mechanisms and to attach to a particular site (Duncan *et al.*, 1995; Edberg *et al.*, 1996). Subsequently, microorganisms must have additional series of virulence factors to allow them to cause disease (Duncan *et al.*, 1995; Edberg *et al.*, 1996). Therefore, this study examined the production of extracellular enzymes (chondroitinase, coagulase, DNase, elastase, fibrinolysin, fluorescein, gelatinase, haemolysin, hyaluronidase, lecithinase, lipase, proteinase and pyocyanin) by the HPC isolates.

Environmental strains can be relatively inert enzymatically (Janda and Bottone, 1981). Some bacterial species utilize citrate in their metabolism and may yield false-positive reactions for coagulase activity (Pro-Lab Diagnostics). Evaluating results of the coagulase test tubes should be observed hourly during the first four hours of incubation. Some strains of *S. aureus* produce fibrinolysin which may lyse clots formed earlier (Atlas, 1997). Occasionally, a *Pseudomonas* culture is encountered that will produce small amounts of pigment in the medium (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996). The formation of nonpigmented colonies does not completely rule out a *Pseudomonas aeruginosa* isolate (The United States Pharmacopeia, 1995; Edberg *et al.*, 1996). All tests depended upon the growth of a small bacterial inoculum in

which the amount of the enzyme increased as the culture grew (Edberg *et al.*, 1976).

After analyzing the 188 haemolytic HPC isolates against a panel of 12 enzymes the following positive results were obtained (Figure 4.3; Table 4.4): DNase (60.6%) (Figure 4.4), gelatinase (62.2%) (Figure 4.5), proteinase (64.4%) (Figure 4.6), chondroitinase (5.3%) (Figure 4.7), hyaluronidase (21.3%) (Figure 4.7), coagulase (16.0%) (Figure 4.8), elastase (33.0%) (Figure 4.9), fibrinolysin (53.7%) (Figure 4.9), lecithinase (47.9%) (Figure 4.10), lipase (54.8%) (Figure 4.11). None of the HPC isolates produced fluorescein (Figure 4.12) and pyocyanin (Figure 4.13), which are characteristic of *Pseudomonas aeruginosa*. Different *Pseudomonas* spp. were isolated in this study, however, no *Pseudomonas aeruginosa* was identified in the drinking water samples. DNases, gelatinases and proteinases were the most commonly produced enzymes. These enzymes are known to destroy cell components, such as nucleic acids and proteins (Atlas, 1997).

Isolates which produced more than two extracellular enzymes associated with pathogenesis consisted of: *Aeromonas* spp., *Acinetobacter* spp., *Actinobacillus ureae*, *Aureobacterium terregens*, *Bacillus* spp., *Brevibacterium mcbrellneri*, *Brochothrix campestris*, *Burkholderia cocovenenans*, *Cellulomonas cellasea*, *Chromobacterium violaceum*, *Chryseobacterium* (*Flavobacterium*) spp., *Chryseomonas luteola*, *Clavibacter michiganense*, *Corynebacterium* spp., *Curtobacterium citreum*, *Dermabacter hominis*, *Deinococcus radiopugnans*, *Eikenella corrodens*, *Flavimonas oryzihabitans*, *Rathayibacter tritici*, *Rhodococcus equi*, *Pseudomonas* spp., *Serratia* spp., *Staphylococcus* spp., *Tsukamurella inchoensis* and *Vibrio* spp. (Table 4.4).

It is generally considered necessary to contain more than one extracellular enzyme in order for a microbe to be virulent (Edberg *et al.*, 1996). Although no single extracellular enzyme has been proved to be the sole factor responsible for bacterial virulence, there is no doubt that such enzymes do play a role in their pathogenic process (Edberg *et al.*, 1996).

One of the mechanisms whereby extracellular enzymes are capable of enhancing the bacterial virulence involves the destruction of host-protective macromolecules such as mucus, lipoprotein membranes, hyaluronic acid and immunoglobulins (Edberg *et al.*, 1996). In this manner, enzymes may enable the pathogens to invade body cells as well as tissues and to interfere with normal cell functions (Edberg *et al.*, 1996). Approximately 24 HPC bacteria produced only one virulence factor (Table 4.4). Some of the HPC isolates (in total 12 isolates), such as *Acinetobacter iwoffii*,

Actinobacillus ureae, *Aureobacterium terregens*, *Deinococcus radiopugnans* (2 isolates), *Klebsiella* spp. (3 isolates), *Micrococcus luteus*, *Staphylococcus* spp. (2 isolates) and *Vibrio metschnikovii* did not produced any of the extracellular enzymes (Table 4.4).

According to our results, HPC isolates such as *Aeromonas* spp., *Acinetobacter* spp., *Aureobacterium terregens*, *Bacillus* spp., *Chryseobacterium* (*Flavobacterium*) spp., *Corynebacterium* spp., *Rhodococcus equi*, *Pseudomonas* spp., *Serratia* spp., *Staphylococcus* spp., *Tsukamurella inchoensis* and *Vibrio* spp. were the most potentially virulent among all of the HPC bacteria isolated from the selected drinking water supplies in South Africa. These isolates were found to produce extracellular enzymes involved in bacterial pathogenesis such as proteinase, gelatinase, DNase, lipase and fibrinolysin.

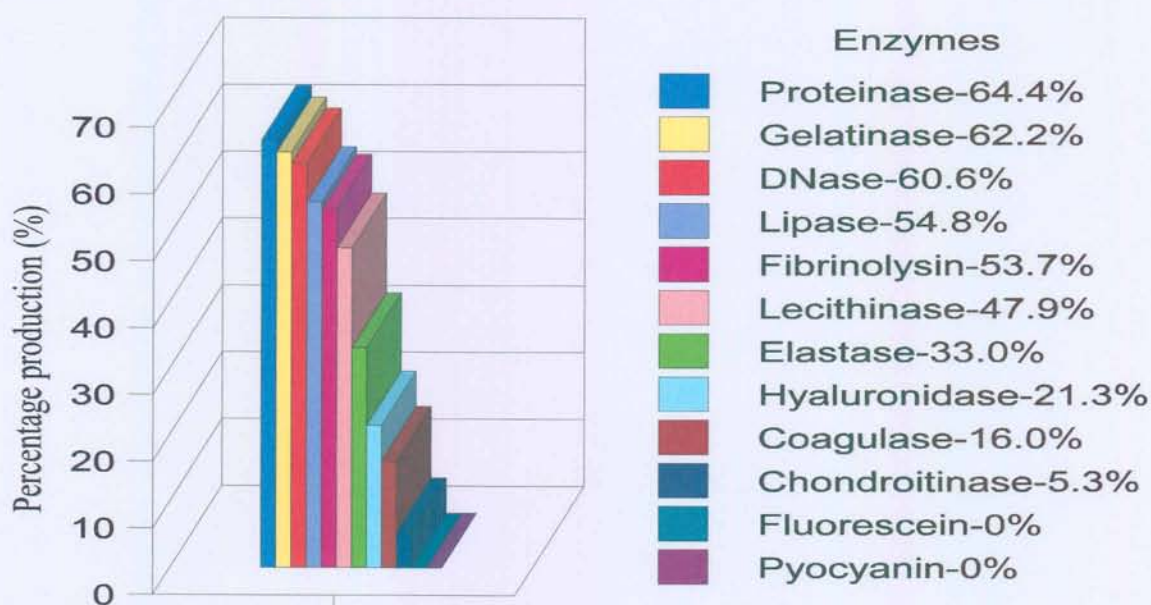


Figure 4.3: Enzymatic analyses of the 188 haemolytic HPC bacteria isolated at random from selected drinking water supplies in South Africa.

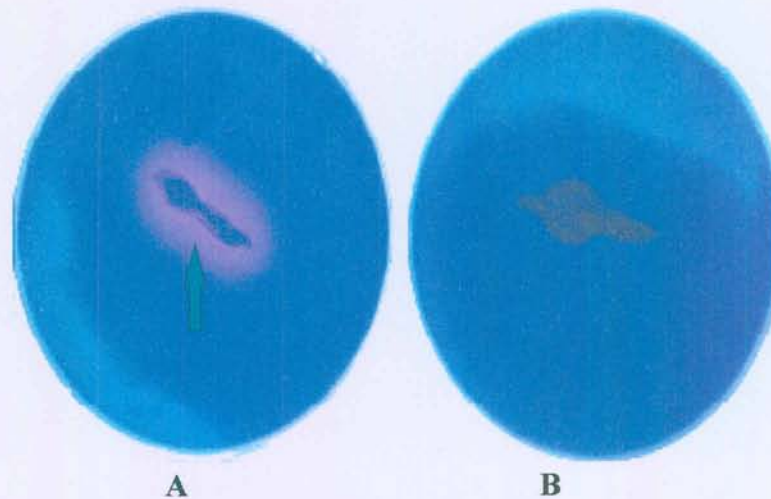


Figure 4.4: Demonstration of DNase activity by the HPC isolates. (A) Positive control: development of a pink or a clear zone after flooding the plate with 0.1% 1 M HCl. (B) Negative control.

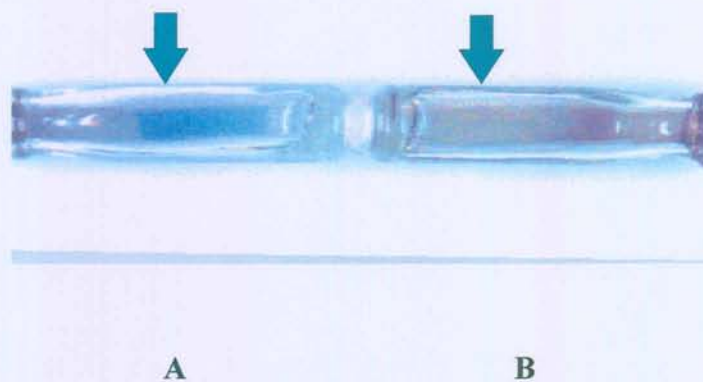


Figure 4.5: Manifestation of gelatinase activity by the HPC isolates. (A) Positive control: the gelatin coating dissolved exposing the blue photographic film base. (B) Negative test: the film colour remained green.

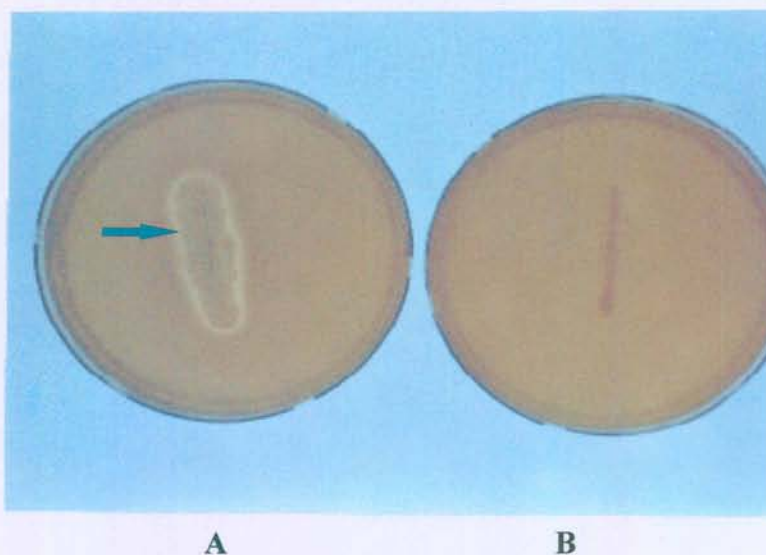


Figure 4.6: Production of proteinase by the HPC isolates. (A) Positive test: clear zone around the inoculum spot. (B) Negative test.

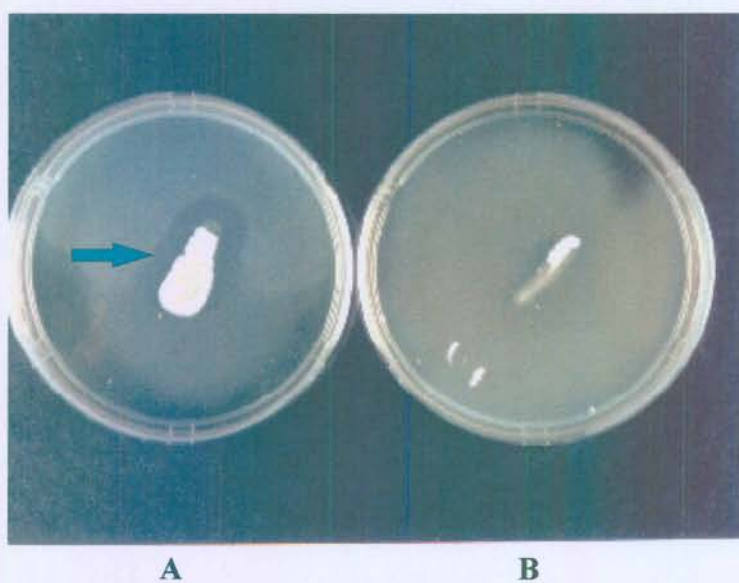


Figure 4.7: Demonstration of hyaluronidase and chondroitinase activity by the HPC isolates. (A) Positive test: appearance of a clear zone around the inoculum spot after flooding the plates with 2 M acetic acid for 10 min. (B) Negative result.

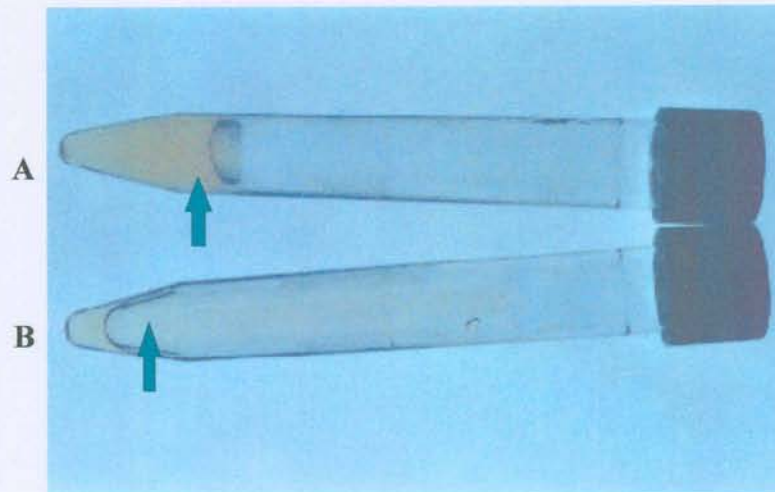


Figure 4.8: Coagulase activity demonstrated by the HPC isolates. (A) Positive result: any degree of clotting of the plasma. (B) Negative result: the plasma remained in a liquid state.

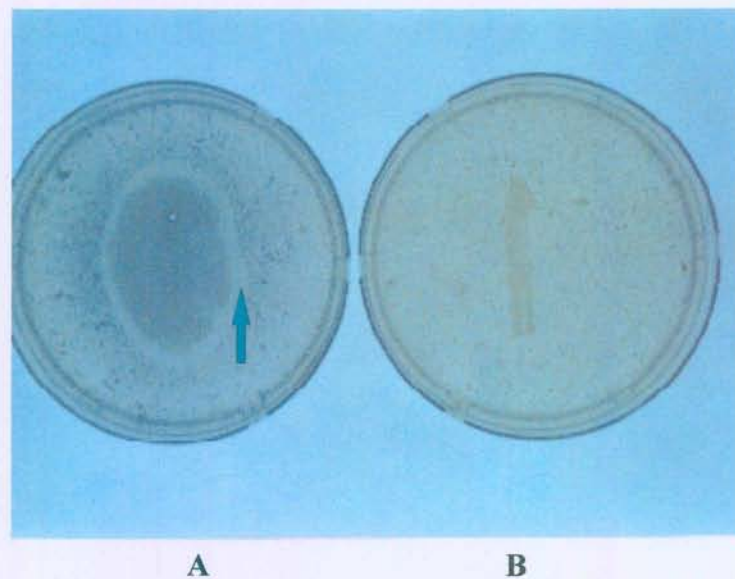


Figure 4.9: Production of elastase and fibrinolysin by the HPC isolates. (A) Positive test: clear zone greater than 2 mm around the inoculum spot. (B) Negative test.

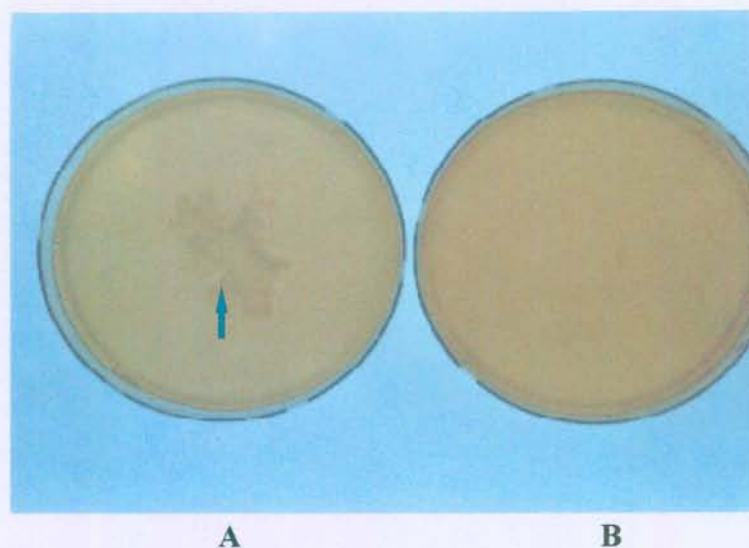


Figure 4.10: Demonstration of lecithinase activity by the HPC isolates. (A) Positive result: white precipitate around or beneath the inoculum spot. (B) The negative test showed no white precipitation.

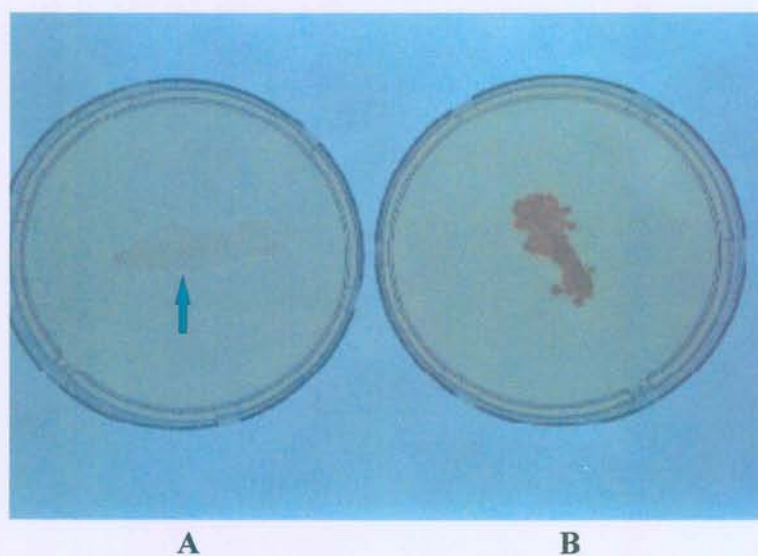


Figure 4.11: Lipase production by the HPC isolates. (A) Positive test: appearance of a turbid halo around or beneath the inoculum spot. (B) Negative test.

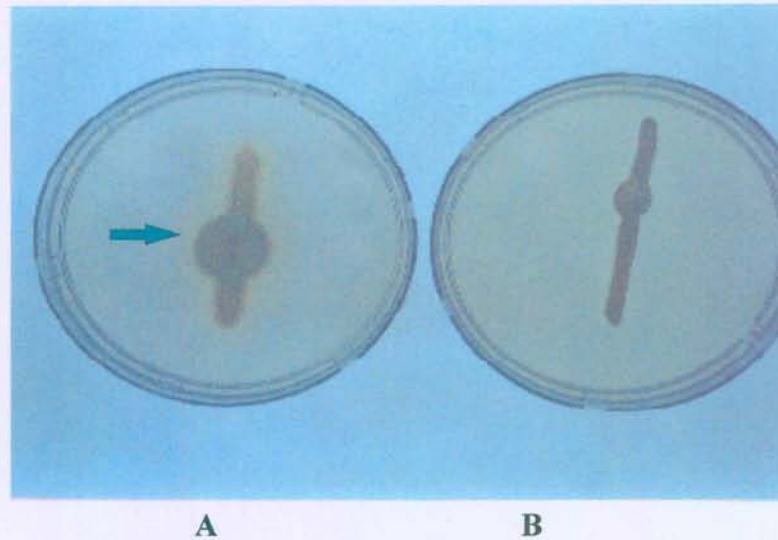


Figure 4.12: Production of fluorescein by *Pseudomonas aeruginosa*. (A) Positive test was indicated by a light, bright greenish-yellow colour diffusing into the agar with a fluorescent zone surrounding the growth. (B) Negative test.

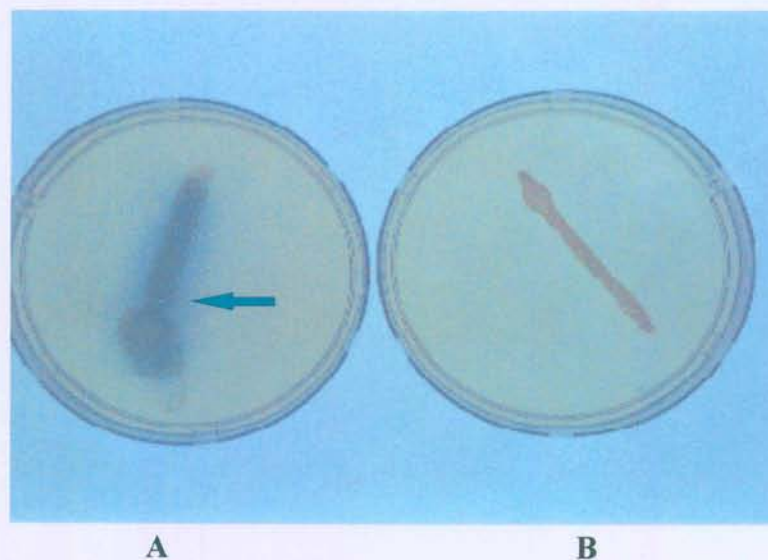


Figure 4.13: Production of pyocyanin by *Pseudomonas aeruginosa*. (A) Positive result was indicated by a blue pigment that diffused into the agar. (B) Negative result.

4.5 Antibiotic susceptibility of the HPC isolates

The antibiotic susceptibility of the bacterial isolates was tested using the Kirby-Bauer quality controlled disk diffusion method (Raphael *et al.*, 1983; Atlas, 1997). Although the method is designed to challenge an organism with a level of antimicrobial activity that it may meet in a therapeutic setting in the tissues, it can not completely simulate the *in vivo* situation (Raphael *et al.*, 1983). The method is exclusively used for organisms that grow rapidly (Raphael *et al.*, 1983). Standardised methods for slow-growers and fastidious organisms have not yet been developed (Raphael *et al.*, 1983). Two basic classes of antibiotics were tested: natural and synthetic antibiotics (Edberg *et al.*, 1996).

Since environmental bacteria are constantly exposed to antibiotics in their natural habitat through sewage of urban effluents, agricultural run-off or direct fecal contamination, the risk of these HPC bacteria obtaining antibiotic resistance increases. The fact that environmental isolates carry antibiotic resistance is important because of their ability to pass it on to opportunistic pathogens. Therefore, the purpose of this test was to inspect whether these HPC bacteria isolated from drinking water displayed resistance to various antibiotics commonly occurring in the environment.

Table 4.3 shows, that the highest incidence of antibiotic resistance among haemolytic HPC isolates was recorded against natural antibiotics such as penicillin G 2 units (59.6%), penicillin G 10 units (47.3%), ampicillin 10 µg (54.3%) and ampicillin 25 µg (43.1%) than to synthetic agents (Figure 4.14). Synthetic antibiotics are active against a broad range of bacteria, including heterotrophic plate count bacteria, because these antibiotics were developed from chemical modifications of the natural antibiotics (Edberg *et al.*, 1996). However, an exception was observed for the synthetic antibiotic oxacillin 1 µg, since 77.7% of the HPC isolates were resistant to this antibiotic. All isolates were susceptible to ciprofloxacin 5 µg and gentamicin 100 µg (Figure 4.14). In total, 51 (27.1%) of the 188 haemolytic HPC isolates were sensitive to all of the antibiotics or displayed resistance to only one antibiotic (Table 4.4). The data on antibiotic resistance of HPC bacteria were in agreement with those of Edberg and colleagues (1996).

Table 4.3: The resistance of HPC bacteria isolated from selected drinking water supplies to synthetic and natural antibiotics

| Natural antibiotics | Percentage of bacteria resistant | Synthetic antibiotics | Percentage of bacteria resistant |
|-----------------------|----------------------------------|-----------------------|----------------------------------|
| penicillin G 2 units | 59.6% | oxacillin 1 µg | 77.7% |
| ampicillin 10 µg | 54.3% | cefoxitin 30 µg | 17.0% |
| penicillin G 10 units | 47.3% | piperacillin 75 µg | 3.2% |
| ampicillin 25 µg | 43.1% | gentamicin 10 µg | 2.7% |
| erythromycin 10 µg | 13.8% | ciprofloxacin 1 µg | 2.1% |
| erythromycin 15 µg | 10.6% | ciprofloxacin 5 µg | 0% |
| streptomycin 10 µg | 10.6% | gentamicin 100 µg | 0% |
| kanamycin 30 µg | 6.9% | | |
| streptomycin 25 µg | 5.3% | | |

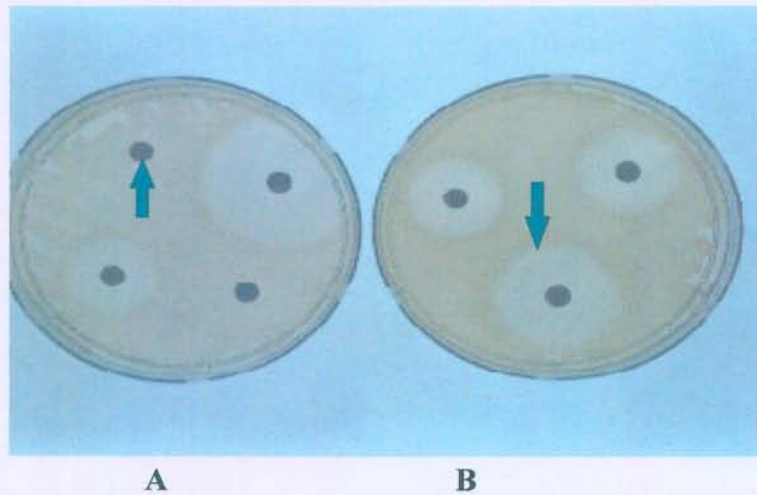


Figure 4.14: Demonstration of antibiotic resistance of HPC isolates to various natural and synthetic antibiotics. (A) Resistance to penicillin G2 units. (B) Susceptibility to gentamicin 100 µg.

4.6 Cytotoxicity analysis of the HPC isolates

Cytotoxicity analysis was carried out on a HEp-2 cell line CCL-23 (human epithelial cells). The test revealed that a total of 181 (96%) of the 188 haemolytic HPC bacteria were cytotoxic to HEp-2 cells (Table 4.4). As shown in Figure 4.15 (A), the positive control *Pseudomonas aeruginosa* completely destroyed the HEp-2 cell monolayer, whereas in Figure 4.15 (B) the cell layer remained intact.

The release of cytotoxic and cytotoxic products from the bacterial colonies was recognized by zones of cellular lysis (Lye and Dufour, 1991). Cytotoxins could cause cell damage or death thus producing dysentery-like illness in the host (Majeed and Macrae, 1994). Optimal detection of cytotoxic activity in the HPC bacteria was accomplished using blood agar medium (Lye and Dufour, 1991).

The HEp-2 cell line was sensitive in detecting those bacterial colonies causing cytotoxic effects (rounding up of cells) and was, therefore, recommended as the tissue culture cell line for detecting cytotoxin-producing bacteria (Lye and Dufour, 1991). Cytotoxic characteristics were not displayed by the following isolates: *Francisella philomiragia* (1 out of 2 isolates), *Vibrio tubiashii* (1 out of 3 isolates), *Serratia fonticola* (2 isolates), *Achromobacter cholinophagum* (1 out of 2 isolates), *Xanthomonas campestris PV juglandis* (1 out of 2 isolates) and *Morganella morganii* (1 out of 1 isolate) (Table 4.4).

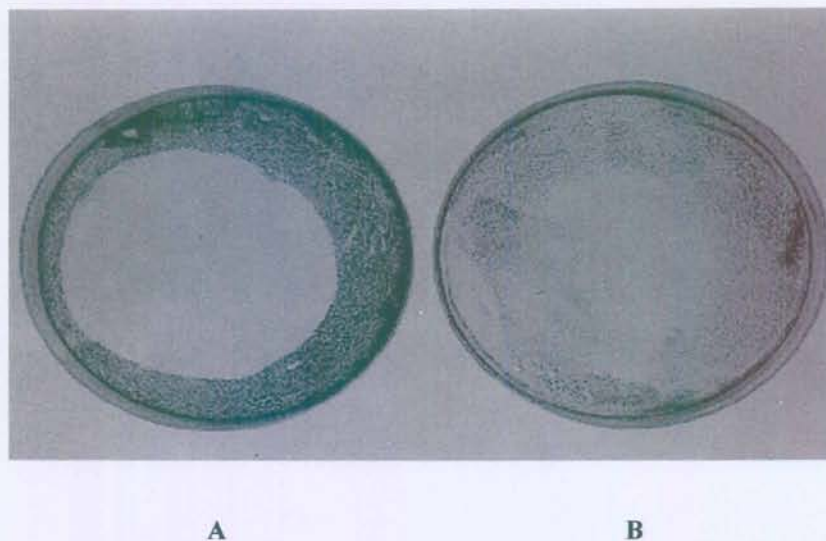


Figure 4.15: Cytotoxicity displayed by haemolytic HPC isolates. (A) Positive control (*Pseudomonas aeruginosa* ATCC 49189) completely destroying the HEp-2 cell monolayer. (B) Negative control (*Bacillus subtilis* ATCC 6683) HEp-2 cells remained unaffected.

4.7 Adherence analyses

Adherence analyses were carried out on HEP-2 and Caco-2 HTB-37 (human colorectal adenocarcinoma) cells. In these tests, 179 (98.9%) of the 181 cytotoxic HPC isolates were adherent to the HEP-2 and Caco-2 cells (Table 4.4). An index of adherence was defined as the average number of bacteria per cell. The average index of adherence for Gram-negative bacteria was between 20 - 30 bacteria per HEP-2 cell, compared to 3 - 7 Gram-positive bacteria per HEP-2 cell (Figure 4.16). Gram-negative isolates such as *Aeromonas*, *Acinetobacter* and *Pseudomonas* spp. adhered to the cells in larger numbers than Gram-positive bacteria, except for *Staphylococcus* and *Micrococcus* species for which the adherence index was as high as 30 - 40 bacteria per HEP-2 cell because of their grouping in pairs or tetrads of cocci (Figure 4.16). However, Gram-positive bacilli adhered in smaller numbers possibly due to their cell morphology and formation of chains of single, thick bacilli [Figure 4.16 (A)], compared to Gram-negative bacilli which were relatively small in size [Figure 4.16 (B)].

The adherence index for Gram-positive bacteria on Caco-2 cells was higher than for HEP-2 cells, ranging between 5 - 12 bacteria per cell (Figure 4.17). A similar tendency was observed with the Gram-negative isolates (Table 4.4). Caco-2 cells appeared to be the more suitable cell line investigating the adhesive capacity of HPC bacteria, possibly due to their ability to obtain the characteristics of human enterocytes after a 10-day incubation period, when the cell monolayers were postconfluent (Darfeuille-Michaud *et al.*, 1990). In a study, conducted by Darfeuille-Michaud and colleagues (1990) it was noticed that 15 day-old confluent cultures of Caco-2 cells were covered by typical brush border microvilli that projected out perpendicular to the cell surface. The surface of the Caco-2 monolayer was irregular and formed domes where bacteria preferentially adhered to, even when these bacteria did not possess any ability to adhere and could not be eliminated by the different washes with saline (Darfeuille-Michaud *et al.*, 1990). Adherence of HPC bacteria such as *Aeromonas*, *Bacillus* to the intestinal mucosa followed by invasion is, therefore, essential for the development of gastrointestinal infections such as diarrhoea (Majeed and Macrae, 1994).

The results indicated that haemolytic HPC isolates such as *Aeromonas*, *Acinetobacter*, *Micrococcus*, *Pseudomonas* and *Staphylococcus* spp. were some of the most adhering bacteria

to the cell lines. It was found that generally Gram-negative HPC bacteria such as *Aeromonas*, *Acinetobacter* and *Pseudomonas* spp. adhered to the cells in larger numbers compared to the Gram-positive isolates such as *Bacillus* and *Tsukamurella* spp. This can possibly be explained by the formation of single chains of Gram-positive bacilli, thus resulting in low numbers of attached bacteria. However, an exception was observed for Gram-positive cocci such as *Micrococcus* and *Staphylococcus* spp., which formed clusters of cells and adhered in large numbers.

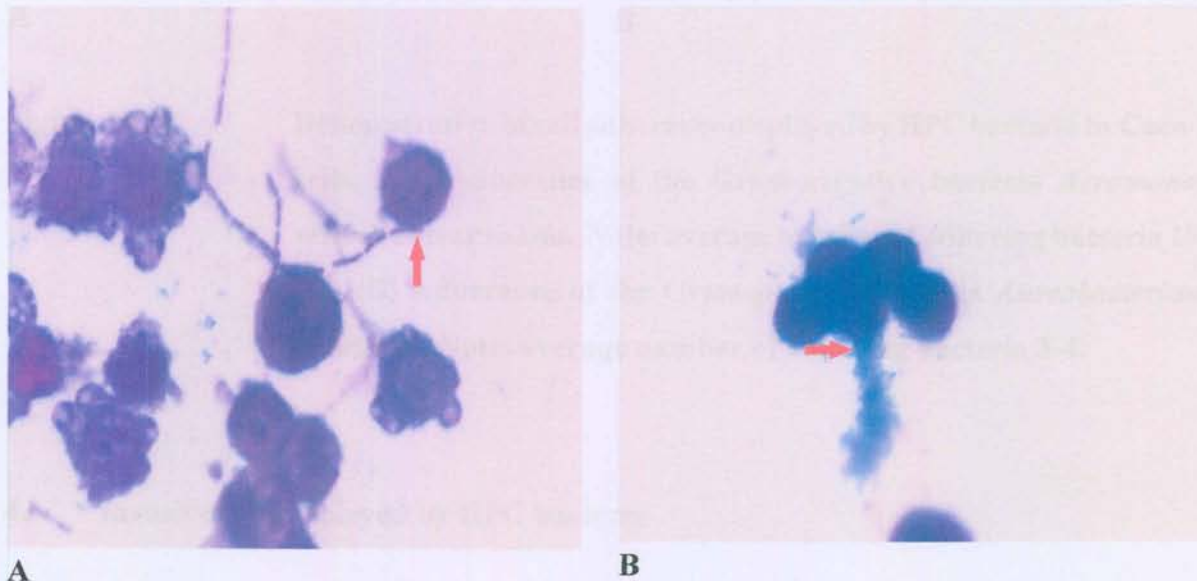


Figure 4.16: Demonstration of cell adherence displayed by the HPC isolates. (A) Adherence of the Gram-positive isolate *Tsukamurella inchoensis* to HEp-2 cells. Note: average number of adhering bacteria 2-3. (B) Adherence of the Gram-negative isolate *Acidovorax temperans* to Hep-2 cells. Note: average number of adhering bacteria 4-10.

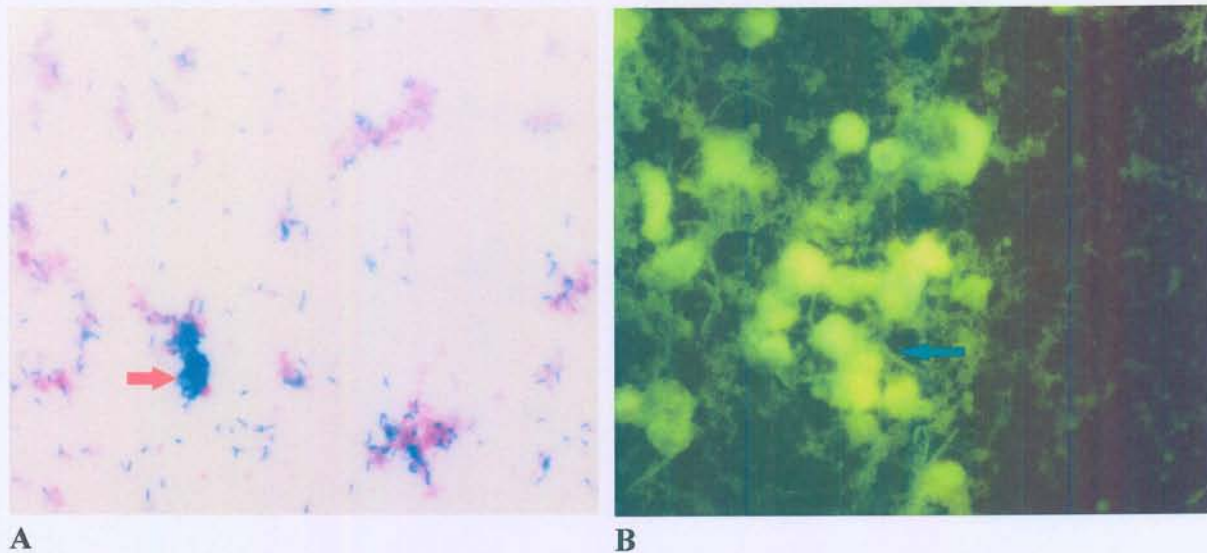


Figure 4.17: Demonstration of cell adherence displayed by HPC bacteria to Caco-2 cells. (A) Adherence of the Gram-negative bacteria *Aeromonas veronii* biovar *sobria*. Note: average number of adhering bacteria 10-20. (B) Adherence of the Gram-positive bacteria *Aureobacterium terregens*. Note: average number of adhering bacteria 3-4.

4.8 Invasiveness displayed by HPC bacteria

Invasiveness of HPC bacteria was determined using HEp-2 and Caco-2 cells. In these tests, the cell lines were inoculated for a period of 3 h with HPC isolates and non-invasive bacteria were later destroyed by the addition of the antibiotic gentamicin. In order to release invasive HPC bacteria, HEp-2 and Caco-2 cells were ruptured with Triton X-100 and the bacterial concentration was determined by the spread plate dilution method.

Analyses of invasiveness revealed that 79 (43.6%) of the 181 cytotoxic HPC isolates invaded HEp-2 cells (Table 4.4). These 79 invasive HPC isolates were divided into 42 (53.2%) Gram-positive and 37 (46.8%) Gram-negative bacteria (Table 4.4). Some of the HPC isolates involved with potential invasiveness on HEp-2 cells included: 9.4% *Tsukamurella inchoensis*, 4.4% *Acinetobacter* spp., 3.9% *Aeromonas* spp., 3.3% *Staphylococcus* spp., 2.2% *Corynebacterium diphtheriae*, 2.2% *Vibrio* spp., 1.6% *Pseudomonas* spp. and 1.6% *Rhodococcus equi* (Table 4.4).

A higher number of cytotoxic HPC isolates were invasive on Caco-2 cells, possibly due to their ability to obtain the characteristics of human enterocytes after a 10-day incubation period (Darfeuille-Michaud *et al.*, 1990). In total 90 (49.7%) of the 181 cytotoxic HPC isolates invaded the Caco-2 monolayer, of which 49 (54.4%) were Gram-positive isolates, compared to 41 (45.6%) were Gram-negative (Table 4.4). Invasive HPC isolates on Caco-2 cells included: 8.8% *Aeromonas* spp., 8.3% *Tsukamurella inchoensis*, 5.0% *Staphylococcus* spp., 3.3% *Pseudomonas* spp., 2.8% *Acinetobacter* spp., 2.8% *Corynebacterium diphtheriae*, 2.2% *Chryseobacterium* spp., 1.7% *Bacillus* spp., 1.7% *Vibrio* spp. (Table 4.4).

The following invasion index was established: (number of colonies obtained/number of inoculated bacteria) x100. The invasion index on Hep-2 cells ranged from 1.9×10^{-1} to 8.9×10^{-6} , whereas the invasion index on Caco-2 cells varied between 7.7×10^{-2} to 8.3×10^{-6} (Table 4.4). The highest invasion index was recorded for *Actinobacillus ureae* at 1.9×10^{-1} on HEp-2 cells.

In conclusion, the HPC bacteria isolated from drinking water supplies were invasive on both cell lines. This showed that HPC bacteria had the potential of causing disease, particularly in immunocompromised patients, by evading their immune defense mechanisms and invading epithelial cells thus disrupting their normal metabolism. However, there is no clear-cut evidence that HPC bacteria as a whole pose a public health risk (Rusin *et al.*, 1997).

4.9 Identification of the haemolytic HPC isolates

Several microscopic, metabolic, serologic and gene probe procedures are available for identification of clinical and environmental microbial isolates (Atlas, 1997). In this study, the VITEK Automated Microbiology System and Biolog MicroPlates were used for determining the identity of haemolytic HPC isolates found in the selected drinking water samples. These systems shared some similarities, since both of them were based on biochemical reactions. The VITEK 32 analyser was designed to provide an accurate miniaturised automated system for identifying a large variety of bacteria based on different biochemical reactions (VITEK Senior/Junior procedures manual, BioMerieux VITEK, Inc.). Each chamber of the VITEK ID card contained a biochemical appropriate for the identification of the specific HPC isolate. The VITEK 32 analyser could identify Gram-negative or Gram-positive aerobic and anaerobic bacteria, however

in this study it was primarily used to identify the Gram-negative HPC isolates, because it is known that the system does not provide reliable results for Gram-positive bacteria such as *Bacillus* and *Tsukamurella* spp.

Biolog GP2 and GN2 MicroPlates were employed in the identification of Gram-positive and Gram-negative HPC bacteria unidentifiable by the VITEK 32 analyser (Biolog, 1999). The GN2 and GP2 Microplate performance characteristics have been determined by establishing a database from a large collection of clinical and environmental stock microorganisms (Figure 4.18) (Biolog, 1999). The database was designed to give identifications of all species in the database, in accordance with current standards of classical identification methods and current taxonomic nomenclature (Biolog, 1999). The GN2 MicroPlate test panel provided a standardized micromethod using 95 biochemical tests to identify a broad range of enteric, non-enteric and fastidious Gram-negative bacteria. Biolog MicroPlates tested the ability of each microorganism to utilise or oxidise a pre-selected panel of different carbon sources (Smalla *et al.*, 1998; Gamo and Shoji, 1999). The test yielded a characteristic pattern of purple wells, which constituted a unique metabolic fingerprint of the capacities of the inoculated organism and therefore, was used to identify the isolate (Gamo and Shoji, 1999). The GP2 MicroPlates were used with Gram-positive bacteria, however two Gram-positive isolates (bacilli; catalase positive) were not identifiable.

Some bacterial isolates were tested with both identification systems in order to check the accuracy of these systems. The isolate marked J₁₀ (Table 4.4) was identified as *Acinetobacter calcoaceticus baumannii* complex by both systems (VITEK 32 analyser and GN2 MicroPlate) and the level of certainty was higher with the VITEK 32 analyser (99%). The isolate marked C392/01A₁₀ was identified as *Pseudomonas synxantha* by the GN2 MicroPlate (80% certainty), however the same isolate was identified as *Chryseobacterium (Flavobacterium) indologenes* with the VITEK 32 analyser (60% certainty). Therefore, the isolate was identified as *Pseudomonas synxantha*. Two gram-positive isolates, namely J₁₆ and M₇, could not be identified with neither of the systems and were therefore defined as unidentifiable.

In this study, HPC bacteria mostly isolated from drinking water included species of the following genera: *Aeromonas*, *Acinetobacter*, *Aureobacterium*, *Bacillus*, *Chryseobacterium*, *Corynebacterium*, *Klebsiella*, *Moraxella*, *Pseudomonas*, *Staphylococcus*, *Tsukamurella* and *Vibrio*. In agreement with reports by other researchers, all these genera contain species that are opportunistic pathogens, which may cause various diseases such as gastroenteritis, meningitis, pneumonia and septicemia (Rusin *et al.*, 1997).

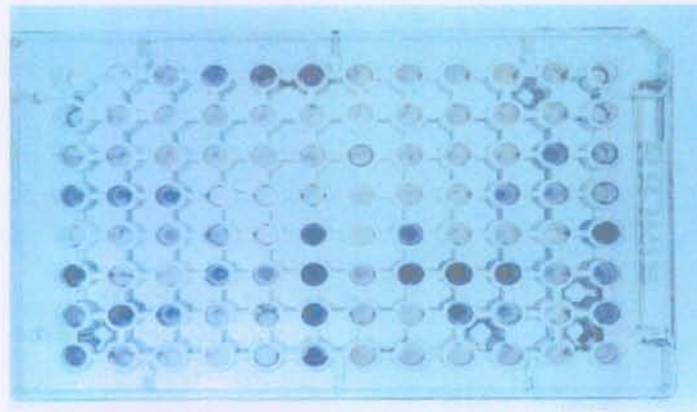


Figure 4.18: A photo of the Biolog MicroPlates used in the identification of haemolytic HPC bacteria isolated from drinking water.

Our findings are in agreement with earlier observations that heterotrophic bacteria detected by commonly used HPC tests may indeed include substantial numbers of bacteria which constitute a potential health risk in terms of hospital- and community-acquired infections (Rusin *et al.*, 1997). Based on these results, it can be concluded that HPC bacteria present in drinking water may constitute a potential health risk to immuno-compromised individuals and particularly in South Africa with its high number of HIV positive cases the importance of HPC bacteria increases.

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|--|--|--------------|----------------------|----------------------|--------------------|--------|------------------------------------|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| A ₁ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, proteinase | Penicillin G 2 units Oxacillin 1 µg | + | - | - | 1.6 | 1.3 | <i>Tsukamurella inchoensis</i> |
| A ₂ | (+)bacilli | β | β | proteinase | Ciprofloxacin 1µg | + | - | - | 3.1 | 7.7 | <i>Tsukamurella inchoensis</i> |
| A ₄ | (+)bacilli | β | β | gelatinase, proteinase | Piperacillin 75 µg Oxacillin 1 µg | + | 2.1x10 ⁻⁴ | 7.5x10 ⁻⁵ | 1.4 | 1.67 | <i>Aureobacterium terregens</i> |
| A ₉ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lecithinase, lipase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 3.0x10 ⁻⁵ | 0.2 | 3.3 | <i>Corynebacterium diphtheriae</i> |
| A ₁₀ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 1.2x10 ⁻³ | 2.6x10 ⁻⁴ | 0.2 | 11.7 | <i>Tsukamurella inchoensis</i> |
| A ₁₁ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lecithinase, | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 2.5x10 ⁻⁴ | 6.0x10 ⁻⁴ | 2.5 | 5.3 | <i>Tsukamurella inchoensis</i> |

Note: (-) indicates negative result

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|-------------------|------------|-------|--|---|--------------|----------------------|----------------------|--------------------|--------|-----------------------------------|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| A ₁₂ | (-) cocco bacilli | α. | α | none | Penicillin G 2 units Streptomycin 10 µg Streptomycin 25 µg Oxacillin 1 µg | + | 1.5x10 ⁻⁶ | - | 4.5 | 7.7 | <i>Klebsiella ozaenae</i> |
| A ₁₃ | (+)bacilli | β | β | DNase, gelatinase, lecithinase, proteinase | Oxacillin 1 µg | + | 6.3x10 ⁻⁴ | 7.7x10 ⁻⁶ | 0.2 | 0.3 | <i>Rhodococcus equi</i> |
| A ₁₇ | (-)bacilli | β | β | none | Penicillin G 2 units Streptomycin 10 µg Streptomycin 25 µg Oxacillin 1 µg | + | 4.6x10 ⁻⁴ | 3.3x10 ⁻⁴ | 2 | 21.3 | <i>Klebsiella pneumoniae</i> |
| A ₁₉ | (+)bacilli | β | β | DNase, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Ciprofloxacin 1µg Cefoxitin 30 µg Oxacillin 1 µg | + | 1.4x10 ⁻⁶ | - | 3.5 | 7.3 | <i>Tsukamurella inchonensis</i> |
| A ₂₀ | (-)cocco bacilli | β | β | DNase, gelatinase, lipase, proteinase | none | + | 3.8x10 ⁻⁴ | 1.8x10 ⁻⁴ | 7.3 | 3.7 | <i>Flavimonas oryzihabitans</i> |
| A ₂₁ | (-)bacilli | β | β | lipase, proteinase | Erythromycin 10 µg Erythromycin 25 µg | + | - | 2.7x10 ⁻⁴ | 32.5 | 32.3 | <i>Klebsiella pneumoniae</i> |
| A ₂₂ | (+)bacilli | β | β | DNase, elastase, gelatinase, lecithinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 1.1x10 ⁻³ | 9.0x10 ⁻⁴ | 4.5 | 8 | <i>Brevibacterium mcbrellneri</i> |

TABLE 4.4 Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------------|------------|-------|---|---|--------------|----------------------|----------------------|--------------------|--------|---|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| A ₂₃ | (-)bacilli | β | β | DNase, fibrinolysin, gelatinase, lipase, hyaluronidase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Streptomycin 10 µg Erythromycin 10 µg Oxacillin 1 µg | + | 5.0x10 ⁻⁶ | - | 69 | 9.7 | <i>Aeromonas veronii</i> biovar sobria |
| A ₂₁ | (-)cocco bacilli | α | α | coagulase | Penicillin G 2 units Streptomycin 10 µg Streptomycin 25 µg Erythromycin 10 µg Oxacillin 1 µg | + | 3.4x10 ⁻⁴ | 2.2x10 ⁻⁶ | 25 | 26.3 | <i>Acinetobacter</i> <i>Iwoffii</i> / <i>junii</i> |
| B ₁ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lecithinase | Oxacillin 1 µg | + | - | 1.3x10 ⁻⁵ | 4.35 | 6 | <i>Dermabacter</i> <i>hominis</i> |
| B ₃ | (+) cocci | β | β | none | none | + | 5.3x10 ⁻³ | 7.3x10 ⁻⁴ | 70 | 7.3 | <i>Staphylococcus</i> <i>saprophyticus</i> |
| B ₅ | (+)bacilli | β | β | coagulase, DNase, fibrinolysin, gelatinase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | - | - | 7 | 2 | <i>Brevibacterium</i> <i>mcbrellneri</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|-------------|------------|-------|---|---|--------------|----------------|----------------------|--------------------|--------|---|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| B ₆ | (-) bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Erythromycin 10 µg Erythromycin 15 µg Oxacillin 1 µg | + | - | - | 9.6 | 12.7 | <i>Aeromonas hydrophila</i> / <i>caviae</i> |
| B ₁₁ | (+) cocci | β | β | coagulase, DNase, lipase, hyaluronidase, proteinase | none | + | - | 1.1x10 ⁻⁶ | 44 | 19 | <i>Staphylococcus xylosus</i> |
| B ₁₂ | (-) bacilli | β | β | coagulase, DNase, fibrinolysin, gelatinase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 1.6x10 ⁻⁵ | 65 | 21.7 | <i>Aeromonas veronii</i> biovar <i>sobria</i> |
| B ₁₅ | (-) bacilli | β | β | coagulase, DNase, elastase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Oxacillin 1 µg | + | - | 1.2x10 ⁻⁵ | 55 | 16 | <i>Aeromonas veronii</i> biovar <i>sobria</i> |
| B ₁₆ | (-) bacilli | β | β | DNase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 1.9x10 ⁻⁵ | 45 | 37.7 | <i>Aeromonas veronii</i> biovar <i>sobria</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|--|--|--------------|----------------------|----------------------|--------------------|--------|---|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| B ₁₇ | (-)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Oxacillin 1 µg | + | - | 1.1x10 ⁻⁴ | 25.5 | 18 | <i>Aeromonas veronii</i> biovar <i>sobria</i> |
| B ₁₈ | (-)bacilli | β | β | chondroitinase, DNase, elastase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Oxacillin 1 µg | + | - | - | 55 | 12.7 | <i>Aeromonas veronii</i> biovar <i>sobria</i> |
| B ₁₉ | (-)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lipase, hyaluronidase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Kanamycin 30 µg Oxacillin 1 µg | + | - | 1.4x10 ⁻⁵ | 28.5 | 14.7 | <i>Aeromonas veronii</i> biovar <i>sobria</i> |
| B ₂₀ | (-)bacilli | β | β | chondroitinase, DNase, elastase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Oxacillin 1 µg | + | - | 1.3x10 ⁻⁵ | 65 | 20 | <i>Aeromonas veronii</i> biovar <i>sobria</i> |
| C ₃ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, hyaluronidase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Oxacillin 1 µg | + | 7.7x10 ⁻⁵ | - | 0.7 | 4.3 | <i>Clavibacter michiganense</i> ss <i>tessellarius</i> |
| C ₄ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lecithinase, | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Oxacillin 1 µg | + | 1.3x10 ⁻⁵ | - | 1.8 | 1.8 | <i>Corynebacterium diphtheriae</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|---|--|--------------|----------------------|----------------------|--------------------|--------|------------------------------------|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| C ₆ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, hyaluronidase, lecithinase, proteinase | Penicillin G 2 units Ampicillin 10 µg Oxacillin 1 µg | + | 3.3x10 ⁻³ | - | 7 | 5 | <i>Rhodococcus equi</i> |
| C ₁₀ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lecithinase, | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 1.5 | 2.3 | <i>Tsukamurella inchonensis</i> |
| C ₁₁ | (+)bacilli | β | β | DNase, fibrinolysin, gelatinase, hyaluronidase, lecithinase, | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | - | - | 2 | 6.7 | <i>Tsukamurella inchonensis</i> |
| C ₁₃ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, hyaluronidase, lecithinase, | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 4.9x10 ⁻⁴ | 5.5 | 9.7 | <i>Aureobacterium terregens</i> |
| C ₁₆ | (+)bacilli | β | β | DNase, elastase, gelatinase, hyaluronidase, lecithinase, | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 7.1x10 ⁻⁶ | 5 | 9 | <i>Corynebacterium diphtheriae</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|--|---|--------------|----------------------|----------------------|--------------------|--------|---|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| C ₁₇ | (-)bacilli | β | β | coagulase, fibrinolysin, gelatinase, lipase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 10 | 26.3 | <i>Aeromonas veronii</i> biovar sobria |
| C ₁₈ | (+)bacilli | β | β | chondroitinase, DNase, elastase, fibrinolysin, gelatinase, lecithinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 1.6x10 ⁻³ | 7.1x10 ⁻⁴ | 3 | 5.7 | <i>Corynebacterium hoagii</i> |
| C ₁₉ | (-)bacilli | β | β | coagulase, DNase, fibrinolysin, lipase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 1.3x10 ⁻⁶ | 30.5 | 1.9 | <i>Aeromonas hydrophila</i> / <i>caviae</i> |
| C ₂₃ | (-)bacilli | β | β | DNase, fibrinolysin, lipase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 21 | 5.9 | <i>Aeromonas veronii</i> biovar sobria |
| C ₂₄ | (-)bacilli | β | γ | DNase, elastase, fibrinolysin, gelatinase, lipase, hyaluronidase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 31 | 30.3 | <i>Aeromonas veronii</i> biovar sobria |

TABLE 4.4 Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|---|---|--------------|----------------------|----------------------|--------------------|--------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| D ₁ | (+)bacilli | β | β | coagulase, DNase, elastase, fibrinolysin, gelatinase, lipase, hyaluronidase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 1.0x10 ⁻⁴ | 0.1 | 2.7 | <i>Brevibacterium mcbrellneri</i> |
| D ₂ | (+)cocci | β | β | chondroitinase, hyaluronidase | Ampicillin 10 µg, | + | - | - | 0.3 | 11.3 | <i>Deinococcus radiopugnans</i> |
| D ₃ | (+)bacilli | β | β | DNase, fibrinolysin, gelatinase, hyaluronidase, proteinase | Penicillin G 2 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 2.0x10 ⁻⁴ | 2.5 | 2.3 | <i>Aureobacterium terregens</i> |
| D ₅ | (+)bacilli | β | β | chondroitinase, elastase, fibrinolysin, gelatinase, hyaluronidase | Penicillin G 2 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 3.2x10 ⁻⁴ | 5.0x10 ⁻⁵ | 3.5 | 2.3 | <i>Aureobacterium terregens</i> |
| D ₇ | (+)bacilli | β | β | elastase, proteinase | Erythromycin 10 µg Erythromycin 15 µg | + | 1.8x10 ⁻⁴ | 1.6x10 ⁻³ | 0.7 | 0.1 | <i>Bacillus licheniformis</i> |
| D ₁₀ | (-)bacilli | β | β | DNase, elastase, fibrinolysin, lipase, lecithinase, proteinase | Oxacillin 1 µg | + | 4.7x10 ⁻⁶ | 1.5x10 ⁻⁵ | 7.5 | 7 | <i>Vibrio tubiashii</i> |
| D ₁₁ | (-)bacilli | β | β | coagulase, chondroitinase, DNase, fibrinolysin, lipase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 1.0x10 ⁻⁵ | 6.0x10 ⁻⁵ | 11.5 | 15 | <i>Aeromonas veronii</i> biovar sobria |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|---|--|--------------|----------------------|----------------------|--------------------|--------|---|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| D ₁₃ | (-)bacilli | α | α | lipase, hyaluronidase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Erythromycin 10 µg Erythromycin 15 µg Oxacillin 1 µg | + | 5.3x10 ⁻⁴ | 1.7x10 ⁻⁴ | 29 | 14.7 | <i>Pseudomonas alcaligenes</i> |
| D ₁₄ | (-)bacilli | β | β | chondroitinase, DNase, elastase, fibrinolysin, gelatinase, lipase, hyaluronidase, lecithinase, proteinase | Penicillin G 2 units Erythromycin 10 µg Erythromycin 15 µg Kanamycin 30 µg Oxacillin 1 µg | + | 3.7x10 ⁻⁵ | 8.3x10 ⁻⁶ | 12.5 | 6 | <i>Aeromonas veronii</i> biovar sobria |
| D ₁₅ | (-)bacilli | β | β | coagulase, DNase, fibrinolysin, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 1.0x10 ⁻⁴ | 9 | 9.7 | <i>Aeromonas veronii</i> biovar sobria |
| D ₁₇ | (-)bacilli | β | γ | DNase, fibrinolysin, lipase, hyaluronidase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Erythromycin 10 µg Oxacillin 1 µg | + | - | - | 8 | 12.3 | <i>Aeromonas hydrophila</i> / <i>caviae</i> |
| E ₁ | (+)bacilli | β | β | coagulase, DNase, gelatinase, lipase, proteinase | none | + | 5.2x10 ⁻³ | - | 0.9 | 4.3 | <i>Tsukamurella inchonensis</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|----------------|------------|------------|-------|--|--|--------------|----------------------|----------------------|--------------------|--------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| E ₃ | (+)bacilli | β | β | gelatinase, lecithinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Piperacillin 75 µg Cefoxitin 30 µg Oxacillin 1 µg | + | - | 4.4x10 ⁻⁴ | 4.6 | 2.3 | <i>Tsukamurella inchoensis</i> |
| E ₅ | (+)bacilli | β | β | gelatinase, lecithinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Piperacillin 75 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 3.4x10 ⁻⁴ | 4.8x10 ⁻⁵ | 1.4 | 3.7 | <i>Corynebacterium diphtheriae</i> |
| E ₈ | (-)bacilli | β | γ | none | Penicillin G 2 units Oxacillin 1 µg | + | - | - | 9.5 | 9.3 | <i>Acinetobacter Iwoffii / junii</i> |
| E ₉ | (-)bacilli | β | β | elastase, fibrinolysin, gelatinase, hyaluronidase, lecithinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Piperacillin 75 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 1.3x10 ⁻³ | 4.4x10 ⁻⁴ | 5 | 9.3 | <i>Chryseobacterium (Flavobacterium) indologenes</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|--|--|--------------|----------------------|----------------------|--------------------|--------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| E ₁₁ | (+)bacilli | β | β | DNase, gelatinase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Piperacillin 75 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 5.0x10 ⁻⁴ | 1.3x10 ⁻³ | 2.5 | 6.7 | <i>Tsukamurella inchoensis</i> |
| E ₁₃ | (-)bacilli | β | β | coagulase, DNase, fibrinolysin, gelatinase, lipase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 2.8x10 ⁻⁶ | - | 4.55 | 8.3 | <i>Aeromonas veronii</i> biovar sobria |
| E ₁₄ | (-)bacilli | β | β | coagulase, DNase, elastase, fibrinolysin, gelatinase, lipase, hyaluronidase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Oxacillin 1 µg | + | - | - | 8.5 | 14 | <i>Aeromonas veronii</i> biovar sobria |
| E ₁₅ | (-)bacilli | β | β | coagulase, DNase, elastase, fibrinolysin, gelatinase, lipase, hyaluronidase, lecithinase, proteinase | Erythromycin 10 µg Erythromycin 15 µg | + | - | - | 19.5 | 15 | <i>Aeromonas veronii</i> biovar sobria |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|---|---|--------------|----------------------|----------------------|--------------------|--------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| E ₁₆ | (-)bacilli | β | β | lipase, hyaluronidase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Erythromycin 10 µg Erythromycin 15 µg Oxacillin 1 µg | + | 1.5x10 ⁻⁴ | 5.5x10 ⁻⁵ | 32 | 33.7 | <i>Chromobacterium violaceum</i> |
| E ₁₈ | (-)bacilli | β | β | coagulase, DNase, elastase, fibrinolysin, gelatinase, lipase, hyaluronidase, proteinase | Oxacillin 1 µg | + | - | - | 31 | 30 | <i>Aeromonas veronii</i> biovar sobria |
| F ₃ | (+)bacilli | β | β | coagulase, chondroitinase, DNase, fibrinolysin, gelatinase, lecithinase, proteinase | Penicillin G 2 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 2.3x10 ⁻³ | 5.0x10 ⁻⁴ | 2.1 | 3 | <i>Tsukamurella inchoensis</i> |
| F ₄ | (-)bacilli | α | α | elastase, fibrinolysin, gelatinase, hyaluronidase, proteinase | Penicillin G 2 units Ampicillin 10 µg Kanamycin 30 µg Oxacillin 1 µg | + | 8.0x10 ⁻⁵ | 7.9x10 ⁻⁵ | 6.5 | 6 | <i>Chryseobacterium (Flavobacterium) indologenes</i> |
| F ₈ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase | none | + | 3.7x10 ⁻⁴ | 1.1x10 ⁻³ | 10.5 | 4 | <i>Brevibacterium mcbrellneri</i> |
| F ₉ | (-)bacilli | β | γ | DNase, elastase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Oxacillin 1 µg | + | - | 3.6x10 ⁻⁴ | 1 | 2.3 | <i>Aeromonas veronii</i> biovar sobria |

TABLE 4.4 **Determination of potentially pathogenic features of haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotox icity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|--|---|------------------|----------------|--------|--------------------|--------|---|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| F ₁₀ | (-)bacilli | α | α | lipase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 3.5 | 17.7 | <i>Pseudomonas stutzeri</i> |
| F ₁₂ | (-)bacilli | β | β | coagulase, DNase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 6.5 | 7.7 | <i>Aeromonas veronii</i> biovar sobria |
| F ₁₆ | (+)cocci | β | β | lipase | Penicillin G 2 units Cefoxitin 30 µg Erythromycin 10 µg Erythromycin 15 µg Oxacillin 1 µg | + | - | - | 33 | 30.3 | <i>Staphylococcus cohnii</i> |
| F ₁₇ | (-)bacilli | β | β | DNase, fibrinolysin, gelatinase, lipase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Streptomycin 10 µg Oxacillin 1 µg | + | - | - | 12 | 8.3 | <i>Aeromonas veronii</i> biovar sobria |
| G ₄ | (+)bacilli | β | β | elastase, fibrinolysin, gelatinase | Oxacillin 1 µg | + | - | - | 2.5 | 2 | <i>Rathayibacter tritici</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------------|------------|-------|---|---|--------------|-----------------------|----------------------|--------------------|--------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| G ₅ | (-)bacilli | β | β | coagulase, elastase, fibrinolysin, gelatinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 11.5 | 8.7 | <i>Vibrio parahaemolyticus</i> |
| G ₆ | (-)cocco bacilli | α | α | proteinase | Streptomycin 10 µg Kanamycin 30 µg Oxacillin 1 µg | + | - | - | 28.5 | 14.7 | <i>Sphingomonas paucimobilis A</i> |
| G ₈ | (+)bacilli | β | β | fibrinolysin, gelatinase, hyaluronidase, proteinase | Penicillin G 2 units Ampicillin 10 µg Oxacillin 1 µg | + | 3.10x10 ⁻⁴ | - | 5.5 | 7.3 | <i>Rhodococcus equi</i> |
| G ₉ | (+)bacilli | β | β | DNase, fibrinolysin, gelatinase, proteinase | Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 1.9x10 ⁻² | 8.5x10 ⁻³ | 2 | 2.3 | <i>Tsukamurella inehonensis</i> |
| G ₁₀ | (-)bacilli | β | β | coagulase, DNase, fibrinolysin, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 1.9x10 ⁻⁵ | 6.1x10 ⁻⁶ | 10 | 11.3 | <i>Aeromonas veronii</i> biovar sobria |
| G ₁₂ | (-)bacilli | β | β | coagulase, DNase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 9.4x10 ⁻⁵ | 3.5 | 5 | <i>Aeromonas veronii</i> biovar sobria |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|---|--|--------------|----------------------|----------------------|--------------------|--------|---|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| G ₁₄ | (-)bacilli | β | β | coagulase, DNase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 4.5 | 6 | <i>Aeromonas veronii</i> biovar sobria |
| G ₁₅ | (+)bacilli | β | β | DNase, fibrinolysin, gelatinase, hyaluronidase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 4.8x10 ⁻³ | 2.0x10 ⁻⁴ | 3.5 | 4 | <i>Tsukamurella inchoensis</i> |
| G ₁₆ | (-)bacilli | β | β | DNase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 1.1x10 ⁻⁵ | 10.5 | 8.7 | <i>Aeromonas veronii</i> biovar sobria |
| G ₁₈ | (-)bacilli | β | γ | DNase, elastase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Oxacillin 1 µg | + | - | - | 15.5 | 11 | <i>Aeromonas veronii</i> biovar sobria |
| G ₁₉ | (-)bacilli | β | β | DNase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 12 | 12 | <i>Aeromonas veronii</i> biovar sobria |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|---|--|--------------|----------------------|----------------------|--------------------|--------|--------------------------------|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| H ₃ | (+)bacilli | β | β | DNase, fibrinolysin, gelatinase, hyaluronidase, lecithinase, | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 3.6x10 ⁻⁵ | 3.2x10 ⁻⁴ | 3 | 5 | <i>Tsukamurella inchoensis</i> |
| H ₅ | (+)bacilli | β | β | coagulase, DNase, fibrinolysin, gelatinase, lipase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 2.0x10 ⁻⁴ | - | 3.5 | 2.5 | <i>Dermabacter hominis</i> |
| H ₇ | (+)bacilli | β | β | elastase, fibrinolysin, gelatinase, lipase, lecithinase | none | + | - | 4.4x10 ⁻⁵ | 2 | 9.3 | <i>Cellulomonas cellasea</i> |
| H ₈ | (+)bacilli | β | β | coagulase, DNase, fibrinolysin, gelatinase, lecithinase, proteinase | Penicillin G 2 units Ampicillin 10 µg Oxacillin 1 µg | + | 5.0x10 ⁻³ | 1.5x10 ⁻³ | 0 | 0 | <i>Tsukamurella inchoensis</i> |
| H ₁₀ | (+)bacilli | β | β | DNase, fibrinolysin, gelatinase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 4.0x10 ⁻³ | - | 2.5 | 7.3 | <i>Tsukamurella inchoensis</i> |

TABLE

TABLE 4.4 Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)

| Isolate | Isolate | Gram-stain | Haemolysin human horse | | Enzyme analyses | Antibiotic resistance | Cytotox icity | Invasion index HEp-2 Caco-2 | | Index of adherence HEp-2 Caco-2 | | Identity |
|-----------------|-----------------|-----------------|------------------------------|---|--|---|------------------|-----------------------------------|----------------------|---------------------------------------|------|--|
| I ₄ | H ₁₂ | (+)bacilli | β | β | fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 1.4x10 ⁻⁵ | - | 4.5 | 2.3 | <i>Tsukamurella inchoensis</i> |
| I ₉ | H ₁₅ | (-)bacilli | β | β | elastase, fibrinolysin, gelatinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 18.5 | 16.7 | <i>Aeromonas hydrophila / caviae</i> |
| I ₁₃ | H ₁₇ | (-)bacilli | β | β | DNase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 17.5 | 18 | <i>Aeromonas veronii</i> biovar sobria |
| I ₁₄ | | | | | | | | | | | | |
| I ₁₅ | H ₁₈ | (-)bacilli | α | α | lipase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 1.1x10 ⁻⁵ | 16 | 13.7 | <i>Pseudomonas stutzeri</i> |
| I ₁₆ | | | | | | | | | | | | |
| I ₁₉ | I ₃ | (+) diplo cocci | α | α | none | Streptomycin 10 µg Erythromycin 10 µg Erythromycin 15 µg Kanamycin 30 µg | + | - | - | 27 | 8.7 | <i>Deinococcus radiopugnans</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|-------------------|------------|-------|---|---|--------------|----------------------|----------------------|--------------------|--------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| I ₄ | (+) cocco bacilli | α | α | none | Ciprofloxacin 1 µg Streptomycin 10 µg Erythromycin 10 µg Erythromycin 15 µg Kanamycin 30 µg | + | - | - | 16.5 | 18.3 | <i>Deinococcus radiopugnans</i> |
| I ₉ | (+) bacilli | β | β | DNase, fibrinolysin, gelatinase, hyaluronidase, lecithinase | Penicillin G 2 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 2.00 | 7.3 | <i>Tsukamurella inchonensis</i> |
| I ₁₃ | (-) cocco bacilli | β | β | fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Oxacillin 1 µg | + | 8.1x10 ⁻⁵ | 1.8x10 ⁻⁵ | 11.5 | 7.3 | <i>Acinetobacter calcoaceticus baumannii</i> complex |
| I ₁₄ | (+) bacilli | γ | α | none | Oxacillin 1 µg | + | - | - | 7 | 7.3 | <i>Aureobacterium terregens</i> |
| I ₁₅ | (-) cocco bacilli | β | β | fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Oxacillin 1 µg | + | 1.5x10 ⁻⁴ | 1.8x10 ⁻⁵ | 17 | 12.7 | <i>Acinetobacter calcoaceticus baumannii</i> complex |
| I ₁₆ | (-) cocco bacilli | β | β | DNase, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Kanamycin 30 µg Oxacillin 1 µg | + | 1.5x10 ⁻⁴ | 3.0x10 ⁻⁶ | 28 | 15.7 | <i>Acinetobacter calcoaceticus baumannii</i> complex |
| I ₁₉ | (+) cocci | γ | β | none | none | + | 1.1x10 ⁻⁴ | 1.3x10 ⁻⁵ | 29 | 16.3 | <i>Staphylococcus warneri</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|--------------------------------|------------|------------|-------|---|--|--------------|-----------------------|----------------------|--------------------|------------|-----------------------------------|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| R ₅₃ A ₅ | (-)bacilli | β | β | elastase, fibrinolysin, gelatinase, lipase, hyaluronidase, proteinase | none | + | 1.71x10 ⁻⁵ | 4.2x10 ⁻⁴ | 5.5 | 11.5 | <i>Vibrio harveyi</i> |
| R ₅₃ B ₁ | (+)bacilli | β | γ | fibrinolysin, gelatinase, hyaluronidase | none | + | - | 1.3x10 ⁻³ | 2.5 | 3 | <i>Brochothrix campestris</i> |
| R ₅₃ B ₆ | (+)bacilli | β | β | gelatinase, lecithinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 3.25x10 ⁻⁴ | 6.2x10 ⁻⁴ | 3 | 3 | <i>Tsukamurella inchoensis</i> |
| BP ₃ B ₅ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, proteinase | none | + | 3.0x10 ⁻⁵ | 5.2x10 ⁻⁴ | 5.5 | 6 | <i>Exiguobacterium acetylicum</i> |
| J ₃ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lecithinase, proteinase | Penicillin G 2 units Ampicillin 10 µg Oxacillin 1 µg | + | - | - | 1.5 | 4.33 | <i>Listeria monocytogenes</i> |
| J ₆ | (-)bacilli | β | γ | gelatinase, lipase | Streptomycin 10 µg Streptomycin 25 µg Oxacillin 1 µg | - | not tested | not tested | not tested | not tested | <i>Francisella philomiragia</i> |
| J ₇ | (-)bacilli | β | γ | lipase | Streptomycin 10 µg Streptomycin 25 µg Oxacillin 1 µg | + | - | - | 9.5 | 14 | <i>Francisella philomiragia</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|--|---|--------------|----------------------|----------------------|--------------------|--------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| J ₁₀ | (-)bacilli | α | α | fibrinolysin, lipase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 2.0x10 ⁻⁵ | - | 21 | 18 | <i>Acinetobacter calcoaceticus baumannii</i> complex |
| J ₁₁ | (+)bacilli | β | β | elastase, fibrinolysin, gelatinase, proteinase | Penicillin G 2 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 1.0x10 ⁻⁵ | 1.2 | 2.7 | <i>Tsukamurella inchoensis</i> |
| J ₁₂ | (-)bacilli | β | β | DNase, fibrinolysin, gelatinase, lipase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 1.0x10 ⁻⁵ | - | 7.5 | 18 | <i>Aeromonas veronii</i> biovar sobria |
| J ₁₃ | (-)bacilli | γ | α | lipase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 1.1x10 ⁻⁵ | 4.5 | 7.3 | <i>Pseudomonas stutzeri</i> |
| J ₁₄ | (+)bacilli | β | γ | DNase, fibrinolysin, gelatinase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Erythromycin 10 µg Erythromycin 15 µg Oxacillin 1 µg | + | 5.5x10 ⁻⁴ | 1.4x10 ⁻³ | 3.1 | 3.7 | <i>Tsukamurella inchoensis</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------------|------------|-------|--|---|--------------|----------------------|----------------------|--------------------|--------|--------------------------------------|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| J ₁₅ | (+)bacilli | β | β | fibrinolysin, gelatinase, lipase, proteinase | Ampicillin 10 µg Oxacillin 1 µg | + | 1.7x10 ⁻³ | - | 1.2 | 1.7 | <i>Curtobacterium citreum</i> |
| J ₁₆ | (+)bacilli | β | β | elastase, fibrinolysin, gelatinase, hyaluronidase | Erythromycin 10 µg Erythromycin 15 µg | + | - | 8.3x10 ⁻⁴ | 2 | 2.3 | non-identifiable |
| J ₁₇ | (-)bacilli | α | α | elastase, fibrinolysin, proteinase | Oxacillin 1 µg | + | - | - | 6.5 | 6 | <i>Acinetobacter lwoffii / junii</i> |
| J ₁₈ | (-)bacilli | γ | β | DNase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Erythromycin 10 µg Oxacillin 1 µg | + | 3.5x10 ⁻⁵ | 2.9x10 ⁻⁶ | 13.5 | 6.7 | <i>Serratia marcescens</i> |
| J ₁₉ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, proteinase | Penicillin G 2 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 5.0x10 ⁻⁴ | 1.0x10 ⁻⁴ | 0.9 | 1 | <i>Tsukamurella inchonensis</i> |
| K ₃ | (-)cocco bacilli | α | α | proteinase | Oxacillin 1 µg | + | - | - | 0 | 0 | <i>Achromobacter cholinophagum</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|------------|------------|-------|---|--|--------------|----------------------|----------------------|--------------------|--------|---|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| K ₅ | (+)bacilli | β | β | DNase, gelatinase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 2.1x10 ⁻³ | 3.2x10 ⁻⁴ | 10 | 8.7 | <i>Tsukamurella inchoensis</i> |
| K ₁₂ | (-)bacilli | γ | α | DNase | Erythromycin 10 µg Erythromycin 15 µg | + | 1.9x10 ⁻¹ | - | 16 | 17.7 | <i>Actinobacillus ureae</i> |
| K ₁₃ | (-)bacilli | β | γ | lipase, lecithinase | Oxacillin 1 µg | + | - | - | 10 | 9.7 | <i>Shewanella putrefaciens</i> (<i>Alteromonas putrefaciens</i>) |
| K ₁₇ | (+)bacilli | β | β | DNase, gelatinase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | - | 8.4x10 ⁻⁴ | 2 | 3.3 | <i>Bacillus cereus</i> / <i>thuringiensis</i> |
| K ₁₉ | (-)bacilli | β | β | DNase, fibrinolysin, gelatinase, lipase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 15 | 14.3 | <i>Aeromonas veronii</i> biovar <i>sobria</i> |
| L ₂ | (-)bacilli | α | α | lipase, proteinase | Oxacillin 1 µg | + | - | - | 2.5 | 6 | <i>Pasteurella haemolytica</i> |

TABLE 4.4 Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|-------------------|------------|-------|--|---|--------------|----------------------|----------------------|--------------------|--------|---|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| L ₃ | (+)bacilli | β | β | DNase, elastase, gelatinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 0.4 | 1.3 | <i>Tsukamurella inchonensis</i> |
| L ₇ | (+)bacilli | β | β | DNase, elastase, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | 2.1x10 ⁻³ | - | 3.5 | 2 | <i>Corynebacterium diphtheriae</i> |
| L ₈ | (-)bacilli | β | β | coagulase, elastase, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 10.5 | 10.7 | <i>Aeromonas veronii</i> biovar sobria |
| L ₉ | (-)bacilli | β | β | DNase, gelatinase, lipase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 5.4x10 ⁻⁵ | 10 | 11.3 | <i>Aeromonas veronii</i> biovar sobria |
| L ₁₀ | (-)filam. bacilli | α | α | lipase | none | + | - | - | 13.5 | 11.7 | <i>Xanthomonas campestris</i> PV <i>malvacearum</i> |
| L ₁₁ | (-)bacilli | β | β | elastase, hyaluronidase, proteinase | Oxacillin 1 µg | + | 9.9x10 ⁻⁵ | - | 1 | 4.7 | <i>Acinetobacter johnsonii</i> / genospecies 7 |

TABLE 4.4 Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|----------------|------------|-------|--|--|--------------|----------------------|----------------------|--------------------|--------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| L ₁₃ | (-)bacilli | γ | β | none | Streptomycin 10 µg Streptomycin 25 µg | + | 1.6x10 ⁻⁴ | - | 3.5 | 4.7 | <i>Vibrio metschnikovii</i> |
| L ₁₅ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lecithinase, | Oxacillin 1 µg | + | - | 1.3x10 ⁻⁴ | 5.5 | 5.3 | <i>Corynebacterium ulcerans</i> |
| M ₁ | (+)diplo cocci | α | α | proteinase | Erythromycin 10 µg Erythromycin 15 µg | + | - | - | 17 | 18.3 | <i>Staphylococcus simulans</i> |
| M ₂ | (+)diplo cocci | α | α | none | Ciprofloxacin 1 µg Oxacillin 1 µg | + | - | - | 20 | 26.7 | <i>Micrococcus luteus</i> |
| M ₅ | (+)bacilli | β | β | DNase, elastase, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | - | 5.5x10 ⁻⁴ | 4.5 | 3.7 | <i>Bacillus cereus / thuringiensis</i> |
| M ₆ | (-)bacilli | α | α | proteinase | Streptomycin 10 µg Streptomycin 25 µg Oxacillin 1 µg | + | - | - | 9.5 | 6 | <i>Rahnella aquaticus</i> |
| M ₇ | (+)bacilli | β | β | DNase, elastase, gelatinase, lipase, lecithinase, proteinase | Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | 3.0x10 ⁻⁴ | 1.6 | 2.3 | non-identifiable |
| M ₁₀ | (-)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | none | + | - | - | 9 | 6.7 | <i>Chryseomonas luteola</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------|-----------------|------------|----------|-------------------------------|--|--------------|----------------------|----------------------|--------------------|--------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| M ₁₁ | (+)cocci | α | α | proteinase | none | + | - | 3.3×10^{-5} | 13.5 | 14.7 | <i>Micrococcus diversus</i> |
| M ₁₂ | (-)bacilli | β | β | DNase, lipase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 μ g Ampicillin 25 μ g Oxacillin 1 μ g | + | - | - | 10.5 | 10 | <i>Aeromonas veronii</i> biovar sobria |
| M ₁₃ | (-)bacilli | α | α | DNase, lipase, | none | + | - | - | 5 | 6.33 | <i>Actinobacillus ureae</i> |
| M ₁₄ | (+)cocci | β | β | DNase, gelatinase, proteinase | none | + | 3.1×10^{-5} | 1.4×10^{-5} | 18 | 22.7 | <i>Staphylococcus equorum</i> |
| M ₁₅ | (-)bacilli | α | α | DNase, gelatinase | none | + | - | - | 4.5 | 6.3 | <i>Acinetobacter johnsonii</i> / genospecies 7 |
| M ₁₆ | (+)streptococci | γ | α | proteinase | none | + | - | - | 14 | 15 | <i>Deinococcus radiopugnans</i> |
| M ₁₇ | (-)bacilli | α | α | lipase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 μ g Ampicillin 25 μ g Cefoxitin 30 μ g Oxacillin 1 μ g | + | 1.9×10^{-5} | 1.9×10^{-6} | 8 | 6.3 | <i>Acinetobacter calcoaceticus baumannii</i> complex |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-------------------|------------|------------|-------|--|---|--------------|----------------------|--------|--------------------|--------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| M ₁₈ | (-)bacilli | α | α | fibrinolysin, lipase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 8.9x10 ⁻⁶ | - | 14 | 5.3 | <i>Acinetobacter calcoaceticus baumannii</i> complex |
| M ₁₉ | (-)bacilli | β | β | DNase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Streptomycin 10 µg Erythromycin 10 µg Oxacillin 1 µg | + | - | - | 9 | 6 | <i>Aeromonas veronii</i> biovar <i>sobria</i> |
| 312A ₁ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 6.0x10 ⁻⁴ | - | 3.5 | 3.7 | <i>Bacillus cereus</i> / <i>thuringiensis</i> |
| 352A ₃ | (+)bacilli | β | β | DNase, elastase, fibrinolysin, gelatinase, lipase, lecithinase, | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 6.4x10 ⁻⁴ | - | 3.5 | 3.7 | <i>Bacillus cereus</i> / <i>thuringiensis</i> |
| 372A ₄ | (-)bacilli | γ | α | lipase | Oxacillin 1 µg | + | - | - | 19 | 12 | <i>Chryseobacterium (Flavobacterium) indologenes</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|------------------------|-------------|------------|-------|---|---|--------------|----------------------|----------------------|--------------------|--------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| 486A ₅ | (+)cocci | β | β | DNase, hyaluronidase, lecithinase, proteinase | none | + | - | 1.5x10 ⁻³ | 30 | 31.3 | <i>Staphylococcus epidermidis</i> |
| 460.01 A ₆ | (+) bacilli | γ | α | DNase, fibrinolysin, hyaluronidase, lipase, lecithinase, proteinase | none | + | - | 7.7x10 ⁻² | 15 | 12 | <i>Aureobacterium terregens</i> |
| C391/01A ₇ | (-)bacilli | α | α | DNase, elastase, fibrinolysin, gelatinase, hyaluronidase, lipase, proteinase, | Piperacillin 75 µg Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Gentamycin 10 µg Streptomycin 10 µg Erythromycin 10 µg Erythromycin 15 µg Kanamycin 30 µg Oxacillin 1 µg | + | 9.7x10 ⁻⁴ | 2.0x10 ⁻⁴ | 6 | 5 | <i>Chryseobacterium (Flavobacterium) gleum</i> |
| C391/01A ₈ | (-)bacilli | α | α | DNase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Cefoxitin 30 µg Oxacillin 1 µg | + | - | 2.0x10 ⁻⁵ | 6.5 | 6 | <i>Chryseomonas luteola</i> |
| C392/01A ₁₀ | (-)bacilli | α | α | DNase, elastase, fibrinolysin, gelatinase, lipase, proteinase, | Penicillin G 2 units Ampicillin 10 µg Kanamycin 30 µg Oxacillin 1 µg | + | 1.4x10 ⁻⁴ | 2.0x10 ⁻⁵ | 10.5 | 11 | <i>Pseudomonas synxantha</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|------------------------|-------------------|------------|-------|---|--|--------------|----------------------|------------|--------------------|------------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| C392/01A ₁₁ | (-)bacilli | β | β | proteinase | Oxacillin 1 µg | - | not tested | not tested | not tested | not tested | <i>Vibrio tubiashii</i> |
| C399/01A ₁₃ | (-)cocco bacilli | β | β | DNase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase, | none | + | - | - | 12 | 6.3 | <i>Serratia marcescens</i> |
| 384A ₁₄ | (-)bacilli | α | α | DNase, elastase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase, | Penicillin G 2 units Ampicillin 10 µg Gentamycin 10 µg Kanamycin 30 µg Oxacillin 1 µg | + | 2.3x10 ⁻³ | - | 11 | 17 | <i>Chryseobacterium (Flavobacterium) indologenes</i> |
| 425A ₁₆ | (+)cocci clusters | α | α | fibrinolysin, gelatinase, lipase, proteinase, | none | + | negative | - | 8 | 4.3 | <i>Deinococcus radiopugnans</i> |
| 425A ₁₇ | (-)bacilli | β | β | none | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 1.8x10 ⁻⁴ | - | 4 | 3.3 | <i>Actinobacillus ureae</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------------|------------|------------|-------|---|---|--------------|----------------------|----------------------|--------------------|------------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| 318A ₂₀ | (-)bacilli | α | α | DNase, elastase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase, | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Gentamycin 10 µg Streptomycin 10 µg Kanamycin 30 µg Erythromycin 10 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 9.4x10 ⁻⁵ | 3.9x10 ⁻⁴ | 7 | 9.3 | <i>Chryseobacterium (Flavobacterium) gleum</i> |
| 886 A ₁ | (-)bacilli | γ | α | DNase, fibrinolysin, gelatinase, | none | + | - | - | 13 | 12.3 | <i>Pseudomonas paucimobilis</i> |
| 886 A ₂ | (-)bacilli | β | β | elastase, fibrinolysin, gelatinase, lipase, proteinase, | Streptomycin 10 µg Streptomycin 25 µg Erythromycin 10 µg Erythromycin 15 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 1.7x10 ⁻⁴ | 4.7x10 ⁻⁵ | 25 | 11.3 | <i>Vibrio tubiashii</i> |
| mm 147 A ₃ | (-)bacilli | β | γ | DNase, gelatinase | Oxacillin 1 µg | - | not tested | not tested | not tested | not tested | <i>Serratia fonticola</i> |
| mm 147 A ₆ | (-)bacilli | α | α | DNase, elastase, gelatinase, lipase, proteinase, | none | + | - | - | 1.5 | 2 | <i>Eikenella corrodens</i> |
| mm 218 A ₇ | (-)bacilli | α | α | lipase, lecithinase | Gentamycin 10 µg Oxacillin 1 µg | + | 1.2x10 ⁻⁵ | - | 8 | 6 | <i>Moraxella catarrhalis</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------------|------------|------------|-------|--|--|--------------|----------------------|----------------------|--------------------|------------|--------------------------------------|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| mm 218 A ₈ | (-)bacilli | α | α | lipase, lecithinase | Oxacillin 1 µg | + | - | - | 3.5 | 6 | <i>Moraxella catarrhalis</i> |
| mm 218 A ₉ | (-)bacilli | γ | α | lipase, lecithinase | Oxacillin 1 µg | + | 2.2x10 ⁻³ | 1.2x10 ⁻⁶ | 2.5 | 7.3 | <i>Acidovorax temperans</i> |
| mm 255A ₁₂ | (-)bacilli | β | γ | DNase, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 6.2x10 ⁻⁶ | 2.0x10 ⁻⁴ | 13 | 8.7 | <i>Aeromonas hydrophila / caviae</i> |
| mm 369A ₁₃ | (-)bacilli | β | β | coagulase, DNase, elastase, gelatinase, fibrinolysin, lipase, hyaluronidase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 7 | 17 | <i>Aeromonas hydrophila / caviae</i> |
| mm 369A ₁₄ | (+)bacilli | γ | α | coagulase, chondroitinase, DNase, lipase, hyaluronidase, lecithinase, proteinase | none | + | 1.4x10 ⁻⁵ | 2.2x10 ⁻⁴ | 19 | 12.3 | <i>Brevibacterium mcbrellneri</i> |
| mm 369A ₁₅ | (-)bacilli | β | γ | DNase | Oxacillin 1 µg | - | not tested | not tested | not tested | not tested | <i>Serratia fonticola</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------------|------------|------------|-------|--|---|--------------|----------------------|----------------------|--------------------|------------|--------------------------------------|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| mm 369A ₁₆ | (+)bacilli | β | β | DNase, lipase, hyaluronidase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Cefoxitin 30 µg Oxacillin 1 µg | + | 2.5x10 ⁻⁴ | 1.8x10 ⁻⁴ | 24.5 | 25 | <i>Tsukamurella inehonensis</i> |
| mm 378A ₁₈ | (-)bacilli | γ | α | none | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Erythromycin 10 µg Erythromycin 15 µg Oxacillin 1 µg | + | - | - | 7.5 | 16.3 | <i>Klebsiella pneumoniae</i> |
| mm 378A ₁₉ | (-)bacilli | β | β | DNase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Oxacillin 1 µg | + | - | - | 10.5 | 11 | <i>Aeromonas hydrophila / caviae</i> |
| mm 147B ₁ | (+)cocci | β | β | coagulase, DNase, lipase, hyaluronidase, lecithinase, proteinase | Streptomycin 10 µg | + | 9.5x10 ⁻⁵ | 2.9x10 ⁻⁴ | 35 | 31 | <i>Staphylococcus aureus</i> |
| mm 147B ₄ | (-)bacilli | γ | α | DNase, elastase, gelatinase, lipase, proteinase | Penicillin G 2 units Oxacillin 1 µg | + | 2.1x10 ⁻⁶ | - | 2 | 4.3 | <i>Burkholderia cocovenenans</i> |
| mm 218B ₅ | (-)bacilli | α | α | lipase | Streptomycin 10 µg Streptomycin 25 µg Oxacillin 1 µg | - | not tested | not tested | not tested | not tested | <i>Achromobacter cholinophagum</i> |

TABLE 4.4 **Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)**

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------------|------------|------------|-------|--|--|--------------|----------------|----------------------|--------------------|------------|--|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| mm 218B ₆ | (+)cocci | β | β | coagulase, DNase, lipase, hyaluronidase, lecithinase, proteinase | none | + | - | - | 30 | 16.7 | <i>Staphylococcus aureus</i> |
| mm 218B ₈ | (-)bacilli | β | β | chondroitinase, DNase, fibrinolysin, gelatinase, lipase, lecithinase, proteinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Oxacillin 1 µg | + | - | - | 1 | 18.7 | <i>Vibrio fluvialis</i> |
| mm 255B ₉ | (-)bacilli | γ | α | lipase, lecithinase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Erythromycin 10 µg Erythromycin 15 µg Oxacillin 1 µg Cefoxitin 30 µg | + | - | - | 11.5 | 9.3 | <i>Pseudomonas mendocina</i> |
| mm 255B ₁₀ | (-)bacilli | α | α | lipase | Penicillin G 2 units Penicillin G 10 units Ampicillin 10 µg Ampicillin 25 µg Erythromycin 10 µg Erythromycin 15 µg Oxacillin 1 µg Cefoxitin 30 µg | + | - | 6.7x10 ⁻⁶ | 5.5 | 9.3 | <i>Pseudomonas stutzeri</i> |
| mm 255B ₁₁ | (-)bacilli | β | γ | DNase, gelatinase | Kanamycin 30 µg Oxacillin 1 µg | - | not tested | not tested | not tested | not tested | <i>Xanthomonas campestris PV juglandis</i> |

TABLE 4.4 Summary of the potentially pathogenic features and identification of the haemolytic HPC bacteria isolated randomly from selected drinking water supplies in South Africa (continued)

| Isolate | Gram-stain | Haemolysin | | Enzyme analyses | Antibiotic resistance | Cytotoxicity | Invasion index | | Index of adherence | | Identity |
|-----------------------|----------------|------------|-------|--|--|--------------|----------------------|----------------------|--------------------|------------|-----------------------------------|
| | | human | horse | | | | HEp-2 | Caco-2 | HEp-2 | Caco-2 | |
| mm 369B ₁₂ | (+)cocci | β | β | lipase, lecithinase | Erythromycin 10 µg Erythromycin 15 µg | + | 7.6x10 ⁻³ | 1.1x10 ⁻³ | 19 | 11.3 | <i>Staphylococcus lugdunensis</i> |
| mm 369B ₁₃ | (+)diplo cocci | γ | α | DNase | Gentamycin 10 µg Kanamycin 30 µg | + | 2.4x10 ⁻² | 2.3x10 ⁻⁴ | 13 | 16 | <i>Staphylococcus warneri</i> |
| mm 369B ₁₄ | (+)cocci | β | β | coagulase, DNase, lipase, hyaluronidase, lecithinase, proteinase | none | + | - | - | 17 | 14 | <i>Staphylococcus lugdunensis</i> |
| mm 378B ₁₆ | (+)cocci | β | β | coagulase, DNase, lipase, hyaluronidase, lecithinase, proteinase | Streptomycin 10 µg | + | - | 5.4x10 ⁻⁵ | 14 | 14 | <i>Staphylococcus lugdunensis</i> |
| mm 378B ₁₇ | (-)bacilli | α | α | lipase | Streptomycin 10 µg Streptomycin 25 µg Oxacillin 1 µg | - | not tested | not tested | not tested | not tested | <i>Morganella morganii</i> |
| mm 378B ₁₈ | (-)bacilli | β | γ | fibrinolysin, gelatinase, proteinase | Oxacillin 1 µg | + | - | - | 18 | 19.3 | <i>Actinobacillus ureae</i> |
| mm 378B ₁₉ | (-)bacilli | γ | α | DNase, fibrinolysin, proteinase | none | + | 1.5x10 ⁻⁵ | 5.7x10 ⁻⁵ | 18 | 15.3 | <i>Pseudomonas paucimobilis</i> |

Note: (-) indicates negative result

4.10 Drinking water quality guidelines

In the past decade, there has been growing concern about the safety of public water supplies (Payment *et al.*, 1991b). Water disinfected with chlorine and with a 0.5 mg.l^{-1} of free residual chlorine for 30 min at a pH less than 8, with turbidity of less than 1 NTU would constitute minimal health risk to consumers (Payment *et al.*, 1991b). It was assumed that current water quality standards were sufficient to protect the public against the risk of gastrointestinal disease (Payment *et al.*, 1991a). However, there is non-trivial endemic level of unreported gastrointestinal diseases due to the consumption of tap water (Payment *et al.*, 1991a). In a study, conducted by Payment and colleagues (1991a), 35% of gastrointestinal illness was attributed to the consumption of drinking water meeting current water quality guidelines.

In view of the above features HPCs should continue to be included in water quality specifications and recommendations world-wide. The South African Bureau of Standards specifies a HPC limit of 100 cfu.ml^{-1} for drinking water (SABS, 1999; SABS, 2001). This limit is endorsed by the Departments of Health and Water Affairs, and is in line with specifications and recommendations in the rest of the world (WHO, 1997; WHO, 2001). In the case of bottled water a maximum HPC of 50 cfu.ml^{-1} is generally specified (WHO, 1997).

However, the application of HPC specifications is a contentious issue all over the world. Reasons are basically that under circumstances largely depending on the quality of raw water sources, intensive and expensive treatment is required to produce drinking water which meets HPC specifications (Grabow, 1996). In addition, the general perception of HPCs is that it detects harmless organisms and reflects no meaningful health risk. In South Africa the recommended limit for HPCs is disregarded due to difficulties in producing and delivering water which conforms to such a limit (Grabow, 1990; SABS, 2001). According to this some countries allow HPCs as high as 500 cfu.ml^{-1} in drinking water (LeChevalier *et al.*, 1980). These countries attach little importance to HPCs and use them merely as a water quality guideline (USEPA, 1989; Reasoner, 1990; Standard Methods, 1998).

The Department of Health (South Africa) has a health risk range for HPC bacteria of:

- no risk for counts less than 100 cfu.ml⁻¹
- insignificant risk for counts between 100 cfu.ml⁻¹ and 1000 cfu.ml⁻¹
- low risk for counts between 1000 cfu.ml⁻¹ and 10 000 cfu.ml⁻¹
- greater risk for counts above 10 000 cfu.ml⁻¹

Nonetheless, HPCs exceeding specified limits tend not to be perceived as a serious violation of water quality specifications and are widely accepted. There is a growing concern because of accumulating evidence suggesting that HPCs may detect organisms of meaningful health significance (Ptak and Ginsburg, 1977; Payment *et al.*, 1994; Edberg *et al.*, 1996; Grabow, 1996; Hellard *et al.*, 1997; Rusin *et al.*, 1997). Factors which increase the urgency of questions concerning HPC specifications include the escalating component of highly vulnerable individuals among consumers of bulk drinking water supplies (Grabow, 1996; Barbeau *et al.*, 1998). These individuals include the very young and very old, as well as individuals with compromised immune systems due to diseases such as AIDS, and medical interventions with immune systems as part of procedures such as organ transplantation and cancer therapy (Grabow, 1996; Barbeau *et al.*, 1998). These developments imply that drinking water suppliers expose themselves to offenses with far-reaching legal implications or high costs to produce drinking water supplies which strictly adhere to HPC specifications. Furthermore, it would be quite unfeasible to produce sterile water and in developing countries such as South Africa, the population would not be simply in the position to afford it.

Several reported outbreaks of viral and parasitic diseases of waterborne origin urged a review of the safety of current standards (Hayes *et al.*, 1989; USEPA, 1976; Payment *et al.*, 1991). These standards are based on the absence of faecal and total coliform bacteria in drinking water, faecal coliform bacteria serving as indicators of faecal pollution and the risk of the presence of pathogenic microorganisms (Payment *et al.*, 1991b).

Australia has adopted the National Health and Medical Research Council (NHMRC) drinking water guidelines very successfully (Padiglione *et al.*, 1997). According to these guidelines, good quality drinking water is defined by a series of values (Padiglione *et al.*, 1997). However, water may be of acceptable drinking quality even if not all “barriers” (e.g., filtration) are in place or if

not all values are met (Padiglione *et al.*, 1997).

In contrast, the United States relied on legally enforceable and non-negotiable standards to ensure a safe water supply (Padiglione *et al.*, 1997). However, this expensive system may not provide better protection of public health (Padiglione *et al.*, 1997). During the 1993 outbreak of *Cryptosporidium* in Milwaukee, turbidity levels fluctuated above the usual readings, but corrective action was delayed as the readings did not reach the stipulated action level (Padiglione *et al.*, 1997). Therefore, deviation from usual parameters should signal a need for investigation (Padiglione *et al.*, 1997).

It is, therefore, vital to maintain a quality of water that is acceptable to the consumer and at the same time the costs associated with water quality surveillance and control must be carefully evaluated before developing national standards (WHO, 1993). “*HPC Bacteria in Drinking Water-Public Health Implications?*” marks the first international symposium on heterotrophic plate count (HPC) bacteria in drinking water and its public-health implications, which takes place in Geneva, Switzerland (2002). According to Stan Hazan, Executive Director- NSF Center for Public Health Education, the timing for this symposium is of global importance and organizations around the world are currently focusing on drinking- water safety. Various organizations around the world such as the NSF, WHO and European Member States are aiming as well at achieving scientifically based consensus regarding the appropriate interpretations of HPC measurements in drinking water and at adopting new drinking water directives.

The water treatment industry should be particularly concerned about opportunistic pathogens such as the *Mycobacterium avium* complex, because they are highly resistant to disinfectants compared to indicator bacteria such as coliforms (Reynolds, 2001). The levels of chlorine routinely applied in drinking water treatment are not effective against MAC organisms and may account for them being found in distribution systems (Fact Sheet No 7, 1997).

The total number of HPC bacteria in drinking water can not be used as the sole indicator of health risk, since it is not an adequate measure of the disease-causing potential of water (Barbeau *et al.*, 1998). Therefore, more research is needed to assess the risk of infection associated with HPC bacteria (Barbeau *et al.*, 1998). Future drinking water regulation revisions and changes to the

treatment processes should therefore be directed towards the elimination of specific pathogens and to the prevention of exogenous sources of contamination rather than the elimination of natural water biota, which can never be accomplished (Edberg et al., 1997). New technologies based on molecular techniques would provide more reliable methods of assessing human health risks, better early warning systems for hazardous events contaminating water supplies and improve ways of identifying the contamination source should be incorporated (CRCWQT, 2001).

CHAPTER 5

CONCLUSIONS

Water is essential to sustain life and a satisfactory supply must be provided to consumers (WHO, 1993). An enormous effort should be made to achieve a drinking water quality as high as practicable (WHO, 1993). Failure to provide adequate protection and effective treatment of drinking water will expose the community to the risk of outbreaks of intestinal and other infectious diseases (WHO, 1993).

Previously, HPC bacteria were considered harmless however several epidemiological studies conducted in countries such as Canada and USA suggested the potential health risk associated with HPC bacteria present in treated drinking water supplies which comply to water quality standards (Payment *et al.*, 1994; Ashbolt, 1995; Edberg *et al.*, 1996; Grabow, 1996; Hellard *et al.*, 1997; Rusin *et al.*, 1997). According to these studies, some members of the HPC bacteria produce virulence factors and therefore, may act as opportunistic pathogens. The component of the community that are specifically at risk of HPC infections include the very young and the elderly with weakened immune systems, as well as individuals with immuno-compromising diseases such as AIDS and patients that underwent organ transplantation or chemotherapy. South Africa with its high number of HIV positive individuals is no exception to this rule.

Although, it is known that high concentrations of HPC bacteria can develop in favourable locations in a distribution system, there is paucity of data on human health effects resulting from exposure to these organisms following ingestion or inhalation (aerosols) (Reasoner, 1990; Edberg *et al.*, 1997). However, information is available about aerosol exposure to waterborne pathogens such as *Legionella* spp. and about skin infections due to *Pseudomonas aeruginosa* or some *Mycobacteria* spp. acquired by exposure to contaminated bathing water, but documented reports of adverse health effects due to high numbers of HPCs in drinking water are scarce to nonexistent (Reasoner, 1990).

To determine if HPC isolates can be potentially pathogenic, analyses were performed to examine the production of various potential virulence factors by these bacteria. Thus, a total of 339 HPC bacteria were randomly isolated from selected drinking water supplies in South Africa meeting current water quality guidelines. These isolates were then subjected to a battery of screening tests, namely: haemolysis, enzymatic analyses, antibiotic susceptibility, cytotoxicity, adherence and invasiveness. Finally, the most potentially virulent HPC isolates were identified using the VITEK 32 analyser and Biolog MicroPlates.

In this study, 188 (55.5%) bacteria of the original 339 HPC isolates showed α - or β -haemolysis. Enzymatic analyses were then conducted and HPC isolates capable of producing more than two extracellular enzymes associated with bacterial pathogenesis included: *Aeromonas*, *Acinetobacter*, *Aureobacterium*, *Bacillus*, *Chryseobacterium*, *Corynebacterium*, *Klebsiella*, *Moraxella*, *Pseudomonas*, *Staphylococcus*, *Tsukamurella*, *Vibrio*. The most commonly produced enzymes by these isolates were proteinase, gelatinase, DNase, lipase and fibrinolysin. Antibiotic analysis showed that HPC isolates were more resistant to natural (ampicillin, erythromycin, kanamycin, penicillin, streptomycin) than to synthetic antibiotics (cefoxitin, ciprofloxacin, gentamicin, oxacillin, piperacillin).

Haemolytic HPC bacteria were grown for 24 h on blood agar media as a pretreatment step before performing the cytotoxicity analysis. A total of 181 HPC isolates (53.4% from the initial 339 isolates) displayed cytotoxic characteristics. However, Lye and Dufour (1991) used nutrient-poor R₂A medium and found that only 1.2% of the HPC bacteria showed cytotoxicity. In a separate study, Payment and colleagues (1994) indicated that blood agar medium enhanced the production of extracellular enzymes and cytotoxins. According to their research, 25% of the HPC bacteria isolated on blood agar medium at 35°C were cytotoxic and had extracellular enzymes associated with pathogenesis. This explains the high percentage of HPC isolates detected in this study. Adherence analyses showed that 98.9% of the 181 cytotoxic HPC isolates were adherent to the HEp-2 and Caco-2 cells. Analyses of invasiveness revealed that 43.6% and 49.7% of the 181 cytotoxic HPC isolates invaded HEp-2 and Caco-2 cells, respectively.

Aeromonas spp. were the most commonly isolated bacteria (18.10%) and have been associated with various infections in humans, such as diarrhoea, peritonitis, endocarditis, meningitis,

septicemia, urinary tract and wound infections (Pin *et al.*, 1997). The second most frequently isolated bacterium in this study was *Tsukamurella* (13.30%). None of the previous studies have identified this bacterium possibly due to the lack of an appropriate identification system. *Tsukamurella* spp. have been implicated in chronic lung diseases, immuno-suppression, indwelling foreign bodies and postoperative infections (Larkin *et al.*, 1999).

According to Rusin and colleagues (1997) the overall risk of infection due to HPC bacteria such as *Aeromonas* and *Pseudomonas* would be less than 6.8 cases of infection per 10 000 people exposed and that 95% of the time it would be less than 1/1000, on the basis of any single day of exposure. Therefore, the probability of infection is low for healthy people (Rusin *et al.*, 1997). However, the probability of infection for those consumers with weakened immune systems is not known (Rusin *et al.*, 1997).

Countries, such as South Africa that are developing national drinking-water limits or standards should take into account the costs and benefits associated with the control of aesthetic and organoleptic quality (WHO, 1993). Various countries with severely limited resources should prioritize and consider the impact on health in each case (WHO, 1993). Source water that is aesthetically unsatisfactory may discourage the consumer from using otherwise safe water supply and its colour, taste, odour may be an indication of potential health hazards (WHO, 1993). Therefore, many parameters must be considered in the assessment of water quality, such as source protection, treatment efficiency and reliability, and protection of the distribution system (WHO, 1993). The costs involved in water quality surveillance must also be investigated before applying water quality guidelines (WHO, 1993).

Future research should include the following: determination of the seasonal fluctuations of HPC bacteria in drinking water, evaluating the health risks for an individual with multiple exposures to opportunistic pathogens, estimating the increase in host susceptibility conferred to antibiotic use or immuno-suppression (Rusin *et al.*, 1997). The MAC group, which is not detectable by HPCs, is of increasing concern because it includes known pathogens that are exceptionally resistant to water treatment and disinfection processes (Grabow, 1996; Covert *et al.*, 1999). These pathogens are able to proliferate in raw water sources and drinking water distribution systems (Grabow, 1996; Covert *et al.*, 1999). The MAC group, and many other heterotrophic organisms in water

with potentially pathogenic properties, were not addressed in this study. However, it is strongly recommended that this group be thoroughly investigated, because the incidence of MAC infections in HIV positive patients has increased exponentially over the past years and is the second most common cause of death worldwide (Singh *et al.*, 1994).

In water meeting current quality standards, none of the microbial parameters provides adequate information on possible health risks to the population (WHO, 2001). With the aging of the population, opportunistic infections particularly those caused by organisms thought to be nonpathogenic, have been on the rise in recent years and this suggests that more research in this area is needed (Reasoner, 1990; Barbeau *et al.*, 1998). Potentially virulent HPC bacteria are naturally present in the environment and the consumption or exposure to water containing large numbers of HPC organisms can lead to diseases such as gastroenteritis, skin and mucous membrane infections particularly in persons whose immune systems are compromised by AIDS, organ transplantation or chemotherapy (WHO, 1996; Rusin *et al.*, 1997; WHO, 1997; Brandi *et al.*, 1999). Thus, AIDS and antibiotic-resistant infections especially in developing countries, such as South Africa necessitate an understanding of the relationship between opportunistic HPC bacteria and their environment (Barbeau *et al.*, 1998).

The detection of potentially virulent pathogens among the heterotrophic plate count bacteria in this study seems to fully justify the need for re-evaluation of the current microbiological water quality guidelines in South Africa and the need for more detailed studies on the potential health risk of heterotrophic bacteria in treated drinking water supplies. It is therefore, vital to maintain a quality of water that is acceptable to the consumer and that is in line with specifications and recommendations in the rest of the world .

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APPENDIX I

CULTURE MEDIA AND REAGENTS

I. ISOLATION AND PURIFICATION OF HETEROTROPHIC PLATE COUNT BACTERIA

Pour plate method (Tortora *et al.*, 1992; Atlas, 1997):

1. Water samples were mixed by shaking (bottle) or rotating (test tube).
2. Serial dilutions (10^0 , 10^{-1} , 10^{-2} , 10^{-3}) of the water samples were prepared.
3. Aliquots of 1.0 millilitre of each dilution were placed directly in the petri dishes, this was done in triplicate.
4. Sterilized agar medium was cooled to 50°C and 15-20 ml were poured aseptically in the petri dishes covering the inoculum.
5. After solidification of the agar, the plates were inverted and incubated at 37°C for 24 h.

Spread plate method (Tortora *et al.*, 1992; Atlas, 1997):

1. Water samples were mixed by shaking (bottle) or rotating (test tube).
2. Serial dilutions (10^0 , 10^{-1} , 10^{-2}) of the water samples were prepared.
3. Aliquots of 0.1 ml of the dilutions were placed directly on petri dishes containing pre-formed sterile agar layers, this was done in triplicate.
4. The inoculum was spread aseptically over the surface of the agar plates using a sterile spreader.
5. The agar plates were inverted and incubated at 37°C for 24 h.

Gram-staining method (Tortora *et al.*, 1992; Atlas, 1997):

1. A loopful of distilled water was placed on a clean microscopic slide.
2. A single colony of a 24 h bacterial culture was removed aseptically with a sterile inoculation loop and placed in a droplet of water on the microscopic slide.

3. The bacterial smear was air-dried and fixed.
4. The bacterial smear was covered with crystal violet for 60 sec.
5. After rinsing the smear with tap water, it was covered with Gram iodine for 60 sec.
6. The smear was de-colourized with acetone alcohol solution and rinsed with tap water.
7. Counter-staining using safranin for 60 sec was performed on the smear.
8. After rinsing the smear with tap water, the slide was dried and examined under the microscope.
9. Gram-positive bacteria appeared purple, whereas Gram-negative bacteria appeared pink.

II. ENZYMATIC ANALYSES

1. Chondroitinase test (Calculations):

- a) 4 mg.ml⁻¹ of chondroitin sulphate A

$$C_{\text{initial}} = 4000 \mu\text{g.ml}^{-1} \text{ of chondroitin sulphate A}$$

$$C_{\text{final}} = 400 \mu\text{g.ml}^{-1} \text{ of chondroitin sulphate A}$$

$$V_{\text{final}} = 100 \text{ ml}$$

$$C_{\text{initial}} \times V_{\text{initial}} = C_{\text{final}} \times V_{\text{final}}$$

$$(4000 \mu\text{g.ml}^{-1}) \times V_{\text{initial}} = (400 \mu\text{g.ml}^{-1}) \times 100 \text{ ml}$$

$$V_{\text{initial}} = (400 \mu\text{g.ml}^{-1}) \times 100 \text{ ml} / (4000 \mu\text{g.ml}^{-1})$$

$$V_{\text{initial}} = 10 \text{ ml}$$

- b) 5% bovine albumin fraction V

$$0.05 \text{ g.ml}^{-1}$$

$$50 \text{ mg.ml}^{-1}$$

$$50\,000 \mu\text{g.ml}^{-1}$$

$$C_{\text{initial}} = 50\,000 \mu\text{g.ml}^{-1} \text{ of bovine albumin fraction V}$$

$$C_{\text{final}} = 10\,000 \mu\text{g.ml}^{-1} \text{ of bovine albumin fraction V}$$

$$V_{\text{final}} = 100 \text{ ml}$$

$$C_{\text{initial}} \times V_{\text{initial}} = C_{\text{final}} \times V_{\text{final}}$$

$$(50\,000\ \mu\text{g}.\text{ml}^{-1}) \times V_{\text{initial}} = (10\,000\ \mu\text{g}.\text{ml}^{-1}) \times 100\ \text{ml}$$

$$V_{\text{initial}} = (10\,000\ \mu\text{g}.\text{ml}^{-1}) \times 100\ \text{ml} / (50\,000\ \mu\text{g}.\text{ml}^{-1})$$

$$V_{\text{initial}} = 20\ \text{ml}$$

Thus, 10 ml of chondroitin sulphate A and 20 ml of bovine albumin fraction V were added in the brain heart infusion medium to make up 100 ml.

- c) Preparation of 100 ml of 2 M CH_3COOH from a concentrated solution with a specific gravity of 1.05 and 99.7% (w/w) of CH_3COOH ($60.05\ \text{g}.\text{mol}^{-1}$).

$$C_{\text{CH}_3\text{COOH}} = (1.05 \times 10^3\ \text{g reagent}) / \text{L reagent} \times (99.7\ \text{g CH}_3\text{COOH}) / 100\ \text{g reagent} \times (1\ \text{mol CH}_3\text{COOH}) = 1046.85\ \text{g}.\text{L}^{-1}$$

$$C_{\text{CH}_3\text{COOH}} = 1046.85\ \text{g}.\text{L}^{-1} \times (1\ \text{mol CH}_3\text{COOH}) / (60.05\ \text{g CH}_3\text{COOH}) = 17.4\ \text{M}$$

$$C_{\text{conc}} \times V_{\text{conc}} = C_{\text{dil}} \times V_{\text{dil}}$$

$$(17.4\ \text{mol}.\text{L}^{-1}) \times V_{\text{conc}} = (2.0\ \text{mol}.\text{L}^{-1}) \times 100\ \text{ml}$$

$$V_{\text{conc}} = 12\ \text{ml}$$

Thus, 12 ml of the concentrated CH_3COOH were diluted with 88 ml distilled water.

2. DNase test (Calculations):

Preparation of 100 ml of 1 M HCl from a concentrated solution that has a specific gravity of 1.16 and 32% (w/w) of HCl ($36.5\ \text{g}.\text{mol}^{-1}$).

$$C_{\text{HCl}} = (1.16 \times 10^3\ \text{g reagent}) / \text{L reagent} \times (32\ \text{g HCl}) / 100\ \text{g reagent} \times (1\ \text{mol HCl}) = 371.2\ \text{g}.\text{L}^{-1}$$

$$C_{\text{HCl}} = 371.2\ \text{g}.\text{L}^{-1} \times (1\ \text{mol HCl}) / (36.5\ \text{g HCl}) = 10.2\ \text{M}$$

$$C_{\text{conc}} \times V_{\text{conc}} = C_{\text{dil}} \times V_{\text{dil}}$$

$$(10.2\ \text{mol}.\text{L}^{-1}) \times V_{\text{conc}} = (1.0\ \text{mol}.\text{L}^{-1}) \times 100\ \text{ml}$$

$$V_{\text{conc}} = 10\ \text{ml}$$

Thus, 10 ml of the concentrated HCl were diluted with 90 ml distilled water.

3. Hyaluronidase test (Calculations):

- a) $2\ \text{mg}.\text{ml}^{-1}$ of hyaluronic acid

$$C_{\text{initial}} = 2000\ \mu\text{g}.\text{ml}^{-1}\ \text{of hyaluronic acid}$$

$C_{\text{final}} = 400 \mu\text{g.ml}^{-1}$ of hyaluronic acid

$V_{\text{final}} = 100 \text{ ml}$

$C_{\text{initial}} \times V_{\text{initial}} = C_{\text{final}} \times V_{\text{final}}$

$(2000 \mu\text{g.ml}^{-1}) \times V_{\text{initial}} = (400 \mu\text{g.ml}^{-1}) \times 100 \text{ ml}$

$V_{\text{initial}} = (400 \mu\text{g.ml}^{-1}) \times 100 \text{ ml} / (2000 \mu\text{g.ml}^{-1})$

$V_{\text{initial}} = 20 \text{ ml}$

b) 5% bovine albumin fraction V

0.05 g.ml^{-1}

50 mg.ml^{-1}

$50\,000 \mu\text{g.ml}^{-1}$

$C_{\text{initial}} = 50\,000 \mu\text{g.ml}^{-1}$ of bovine albumin fraction V

$C_{\text{final}} = 10\,000 \mu\text{g.ml}^{-1}$ of bovine albumin fraction V

$V_{\text{final}} = 100 \text{ ml}$

$C_{\text{initial}} \times V_{\text{initial}} = C_{\text{final}} \times V_{\text{final}}$

$(50\,000 \mu\text{g.ml}^{-1}) \times V_{\text{initial}} = (10\,000 \mu\text{g.ml}^{-1}) \times 100 \text{ ml}$

$V_{\text{initial}} = (10\,000 \mu\text{g.ml}^{-1}) \times 100 \text{ ml} / (50\,000 \mu\text{g.ml}^{-1})$

$V_{\text{initial}} = 20 \text{ ml}$

Thus, 20 ml of hyaluronic acid and 20 ml of bovine albumin fraction V were added in the brain heart infusion medium to make up 100 ml.

III. CYTOTOXICITY ANALYSIS

a) **HEp-2 cell line cultivation (Whitaker, 1972):**

1. Five percent of Eagle's Minimum Essential Medium (MEM) (Highveld Biological, Lyndhurst, RSA) were used as the growth medium for the cultivation of HEp-2 cells.
2. The medium was replaced twice weekly. A yellow colour indicated the growth of cells and the production of metabolic substances due to acid excretion.
3. As soon as a monolayer of HEp-2 cells was formed on the base of the flask (200 cm^3), the

cells were passaged in a ratio of 1:2 to 1:6, depending on the thickness of the monolayer.

4. The medium in the flask was discarded and the walls of the flask were washed with 5 ml phosphate-buffered saline (PBS) (Sigma).
5. Three millilitre of trypsin EDTA (National Institute for Virology, RSA) were added to the flask and incubated at 37°C for 1 min.
6. The cells were detached from the bottom of the flask by lightly tapping the flask firmly against the hand.
7. An amount of 3 ml of a 10% MEM (Highveld Biological) was added to the flask and swirled to neutralize the effect of trypsin EDTA (National Institute for Virology, RSA).
8. The whole suspension was transferred from the flask to a centrifuge tube, which was centrifuged (BHG Roto-Uni II, Separation Scientific, RSA) at 500 x g for 2 min in order to pellet the cells.
9. The supernatant was discarded and the pellet of cells was re-dissolved in 10% MEM (Highveld Biological).
10. After thorough mixing, 1 ml of the cell suspension was transferred into each flask containing 14 ml of the 5% MEM (Highveld Biological) that has been preheated to 37°C. Flasks were incubated at 37°C in a 5% CO₂ incubator (Galaxy CO₂ Incubator- Biotech, Northants, England).

b) Preparation of 5% MEM (Whitaker, 1972):

1. Fetal calf serum (FCS) (Delta Bioproducts, Kempton Park, RSA) was decomplexed in a water bath (Gallenkamp, England) at 56°C for 30 min.
2. Thirty millilitre of serum-free medium were abstracted from a 500 ml MEM (Highveld Biological) bottle.
3. Twenty five millilitre of filter sterilised fetal calf serum and 5 ml of sterile Pen/Strep Fungizone Mix (BioWhittaker, Walkersville, MD) were added aseptically to the medium.

c) Preparation of 10% MEM (Whitaker, 1972):

1. Fetal calf serum (FCS) (Delta Bioproducts) was decomplexed in a water bath

(Gallenkamp, England) at 56°C for 30 min.

2. Fifty five millilitre of serum-free medium were abstracted from a 500 ml MEM (Highveld Biological) bottle.
3. Fifty millilitre of filter sterilised fetal calf serum and 5 ml of sterile Pen/Strep Fungizone Mix (Bio Whittaker) were added aseptically to the medium.

d) Determination of total HEp-2 and CaCo-2 cell counts (Bird *et al.*, 1981):

1. The total number of viable cells of a monolayer of HEp-2 cells (CaCo-2 HTB-37 cells) formed on the base of the flask (200 cm³) was determined.
2. The medium in the flask was discarded and the walls of the flask were washed with 5 ml phosphate-buffered saline (PBS) (Sigma).
3. Trypsin EDTA (National Institute for Virology, RSA) (3 ml) was added to the flask. The flask was incubated at 37°C for 1 min.
4. The cells were detached from the bottom of the flask by lightly tapping the flask firmly against the hand.
5. Three millilitre of a 10% MEM (Highveld Biological) were added to the flask and swirled to neutralize the effect of trypsin EDTA.
6. The suspension was transferred from the flask to a centrifuge tube, which was centrifuged (BHG Roto-Uni II, Separation Scientific, RSA) at 500 x *g* for 2 min to pellet the cells.
7. The supernatant was discarded and the pellet of cells was re-dissolved by mixing thoroughly in serum-free media, with a volume (generally, $V_{\text{initial}} = 10 \text{ ml}$) dependent on the thickness of the monolayer.
8. In a separate centrifuge tube, 500 µl of 0.4% Trypan Blue stain (Sigma) were added. This cell counting method was based on the fact that viable cells (with intact membranes) do not take up the trypan blue stain, whereas dead (non-viable) cells do.
9. An equal amount of the cell suspension (500 µl) (dilution factor of 2) was added to the trypan blue and mixed thoroughly to receive an even suspension without excessive clumping. The Trypan Blue-cell suspension was stained for 5 minutes.
10. A cover-slip was placed on one of the two counting chambers of a Neubauer hemacytometer

(Superior, Germany). This counting chamber has four identical ruled squares, each measuring 1 by 1 mm. The space between the cover-slip and the ruled squares (with surface areas of 1 mm²) was 0.1 mm. Therefore, the volume of one ruled square was 0.1 mm³, or 10⁻⁴ cm³. Using a Pasteur pipette, a small amount of the Trypan Blue-cell suspension (10 µl) was transferred to the counting chamber by carefully touching the edge of the cover-slip with the pipette tip and allowing the chamber to fill by capillary action. The chamber was not allowed to overfill or underfill.

11. The counting chamber was placed and examined under a light microscope. The low-power objective was focused on the ruled squares and all of the cells in the four 1 mm² corner squares were counted. Cells lying outside the borders of the squares were not counted.
12. The total number of viable cells in the 4 squares was divided by 4 to determine the mean count per square. This represented the number of cells per 0.1 mm³. This number is multiplied by 10 000 to determine the number of cells per cubic centimetre. Since 1 cm³ is equivalent to 1 ml, the cell number can be expressed per millilitre. The final number is adjusted by the appropriate dilution factor.

Determination of the total number of cells (Bird *et al.*, 1981):

Cells per ml = the average count per square x 10⁴

Total cells = cells per ml x the dilution factor x the original volume of fluid from which cell sample was removed.

Example:

$$C_{\text{initial}} = 24 \times 10^4 \text{ cells.ml}^{-1}$$

$$C_{\text{final}} = 2 \times 10^5 \text{ cells.ml}^{-1}$$

$$V_{\text{initial}} = 10 \text{ ml}$$

$$2 \times C_{\text{initial}} \times V_{\text{initial}} = C_{\text{final}} \times V_{\text{final}}$$

$$2 \times (24 \times 10^4 \text{ cells.ml}^{-1}) \times 10 \text{ ml} = (2 \times 10^5 \text{ cells.ml}^{-1}) \times V_{\text{final}}$$

$$V_{\text{final}} = (4.8 \times 10^6 \text{ cells.ml}^{-1}) / (2 \times 10^5 \text{ cells.ml}^{-1})$$

$$V_{\text{final}} = 24 \text{ ml}$$

13. The pellet of cells was dissolved in a serum-free media and centrifuged (BHG Roto-Uni II,

Separation Scientific, RSA) at 500 x g for 2 min to pellet the cells.

14. The supernatant was discarded and the pellet of cells was re-dissolved in the necessary volume (V_{final}) of 5% growth medium.

e) Freezing of HEp-2 and Caco-2 cell culture for storage:

Add the following to prepare the freeze medium:

1. Twenty five millilitre of filter sterilised double-strength Eagle's Minimum Essential Medium (Highveld Biological).
2. Twenty millilitre of filter sterilised fetal bovine serum (Delta Bioproducts).
3. Five millilitre of filter sterilised glycerol (Sigma).
4. Sterile Pen/Strep Fungizone Mix (0.5 ml) (Bio Whittaker).
5. Mix together.

Procedure:

1. Cells were trypsinised and neutralised with 10% MEM (Highveld Biological) medium.
2. The cell suspension was centrifuged (BHG Roto-Uni II, Separation Scientific, RSA) at 500 x g to pellet cells.
3. The supernatant was removed and cells were resuspended in 1 ml of freeze-medium.
4. The cell suspension was carried over to a sterile cryogenic vial (Corning Costar Corporation, Cambridge, MA, Canada) and frozen at 4°C for 2 hours.
5. After 2 h, the cell suspension was frozen at -20°C for 24 h and stored at -70°C.

f) Preparation of the crystal violet-formaldehyde stain:

1. Crystal violet (1.3 g) (Merck) was dissolved in 50 ml of isopropanol (Merck).
2. Three hundred millilitre of a 37% formalin were suspended in the solution, which was diluted to 1000 ml by the addition of sterile distilled water.
3. The solution was mixed thoroughly.

IV. ANALYSIS OF CELL ADHERENCE:

a) Caco-2 cell line cultivation (Whitaker, 1972):

1. Fifteen to Twenty percent of Eagle's Minimum Essential Medium (MEM) (Highveld Biological) were used as the growth medium.
2. As soon as a monolayer of Caco-2 HTB-37 cells was formed on the base of the flask (200 cm³), the cells were passaged in a ratio of 1:2 to 1:6, depending on the thickness of the monolayer.
3. The medium was discarded and the walls of the flask were washed with 5 ml phosphate-buffered saline (PBS).
4. Three millilitre of trypsin EDTA (National Institute for Virology, RSA) were added and the flask was incubated at 37°C for 1 min.
5. Cells were detached from the bottom of the flask by lightly tapping the flask firmly against the hand.
6. Three millilitre of a 15% MEM (Highveld Biological) were added and the flask was swirled to neutralize the effect of trypsin EDTA.
7. The suspension was transferred from the flask to a centrifuge tube and centrifuged (BHG Roto-Uni II, Separation Scientific, RSA) at 500 x g for 30 sec to pellet the cells.
8. The supernatant was discarded and the pellet was re-dissolved in 15% MEM (Highveld Biological).
9. After thorough mixing, 1 ml of the cell suspension was transferred into each flask containing 14 ml of the 20% MEM (Highveld Biological) that was preheated to 37°C. Flasks were incubated at 37°C in a 5% CO₂ incubator (Galaxy CO₂ Incubator- Biotech, Northants, England).

b) Preparation of 15% MEM (Whitaker, 1972):

1. Fetal calf serum (FCS) (Delta Bioproducts) was decomplexed in a water bath (Gallenkamp, England) at 56°C for 30 min.
2. Eighty millilitre of serum-free medium were abstracted from a 500 ml MEM (Highveld

Biological) bottle.

3. Seventy five millilitre of filter sterilised fetal calf serum and 5 ml of sterile Pen/Strep Fungizone Mix (Bio Whittaker) were added aseptically to the medium.

c) Preparation of 20% MEM (Whitaker, 1972):

1. Fetal calf serum (FCS) (Delta Bioproducts) was decomplexed in a water bath (Gallenkamp, England) at 56°C for 30 min.
2. Hundred and five millilitre of serum-free medium were abstracted from a 500 ml MEM (Highveld Biological) bottle.
3. Hundred millilitre of filter sterilised fetal calf serum and 5 ml of sterile Pen/Strep Fungizone Mix (Bio Whittaker) were added aseptically to the medium.

d) Preparation of 0.5 M acetate buffer (pH 3.5):

The molarity of sodium acetate (C_{CH_3COONa}) was 0.5 mol.L⁻¹:

$$C_{CH_3COONa} = n \text{ (no. of moles of solute)} \div V \text{ (no. of L of solution)}$$

$$0.5 \text{ mol.L}^{-1} = n \div 0.500 \text{ L}$$

$$n = 0.25 \text{ mol}$$

$$\text{Molar mass } (M_{CH_3COONa}) = \text{mass } (m_{CH_3COONa}) \div n_{CH_3COONa}$$

$$m_{CH_3COONa} = (82.03 \text{ g.mol}^{-1}) \times (0.25 \text{ mol})$$

$$m_{CH_3COONa} = 20.5 \text{ g}$$

Thus 20.5 g of sodium acetate were dissolved in 500 ml of distilled water.

The pH of the acetate buffer was adjusted to 3.5 by adding concentrated HCl acid or concentrated NaOH base.

V. ANALYSIS OF CELL INVASION:

Caco-2 and HEp-2 cells were cultivated using 5%, 10% and 20% MEM. Surviving bacteria were counted using the spread plate method. A volume of 0.1 ml inoculum from each of the 24-well tissue culture plates were spread on the agar plates and incubated for 24 h at 37°C. The number of cfu.ml⁻¹ was used to calculate the invasiveness index.

APPENDIX II

ARTICLES

Identification of potentially pathogenic heterotrophic plate count bacteria isolated from selected drinking water supplies in South Africa

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Abstract

The Heterotrophic plate count (HPC) is commonly used to assess the general microbiological quality of drinking water, therefore, it is included in water quality specifications world-wide. The South African Bureau of Standards specifies a HPC limit of 100 cfu.ml⁻¹ for drinking water. HPC bacteria are considered harmless, therefore, some countries allow HPCs as high as 500 cfu.ml⁻¹ in drinking water. However, a number of recent studies revealed evidence that these commonly used indicator bacteria may not be as harmless as generally accepted. It appears that immuno-compromised individuals are particularly at risk. The objective of this study was to determine the potential health risk of HPC bacteria isolated from selected drinking water supplies in South Africa. In this study, 339 bacterial colonies were isolated at random using heterotrophic plate count tests. In a first step to screen for potentially pathogenic properties, 188 (55.5%) of the isolates showed α - or β -haemolysis on human- or horse-blood agar media. Subsequent analysis of the haemolytic isolates for enzymatic properties associated with pathogenicity revealed the presence of chondroitinase in 5.3% of the isolates, coagulase in 16.0%, DNase in 60.6%, elastase in 33.0%, fibrinolysin in 53.7%, gelatinase in 62.2%, hyaluronidase in 21.3%, lecithinase in 47.9%, lipase in 54.8%, and proteinase in 64.4%. Fluorescein and pyocyanin were not produced by any of the isolates. Among the

haemolytic isolates, 77.7% were resistant to oxacillin (1 µg), 59.6% to penicillin G (2 units), 47.3% to penicillin G (10 units), 54.3% to ampicillin (10 µg) and 43.1% to ampicillin (25 µg). Cell culture studies revealed that 96% of haemolytic isolates were cytotoxic to HEp-2 cells and 98.9% of the 181 cytotoxic isolates adhered to HEp-2 or Caco-2 cells. The average index of adherence for Gram-negative bacteria was 20-30 bacteria per HEp-2 cell, compared to 3-7 for Gram-positive bacteria. HEp-2 cells were invaded by 43.6% and Caco-2 cells by 49.7% of the 181 cytotoxic isolates. The invasion index on HEp-2 cells was 1.9×10^{-1} to 8.9×10^{-6} , compared to 7.7×10^{-2} to 8.3×10^{-6} on Caco-2 cells. The most commonly isolated genera with these potentially pathogenic features were *Aeromonas*, *Acinetobacter*, *Aureobacterium*, *Bacillus*, *Chryseobacterium*, *Corynebacterium*, *Klebsiella*, *Moraxella*, *Pseudomonas*, *Staphylococcus*, *Tsukamurella* and *Vibrio*. Our results support earlier findings on potentially pathogenic features of bacteria detected by heterotrophic plate counts on drinking water. These findings seem to be in agreement with some epidemiological studies, which indicated an association between HPCs of drinking water and the incidence of gastroenteritis in consumers. However, the extent of the health risk concerned needs to be defined in detail for meaningful revision of quality guidelines for HPCs in drinking water.

Key words: Adherence, cytotoxicity, haemolysis, heterotrophic, invasiveness, virulence factors

Introduction

One way of evaluating the general microbiological quality of finally treated and disinfected drinking water is the use of heterotrophic plate counts (Grabow, 1996). The heterotrophic plate count method, referred to as standard or total plate count, determines bacterial quality changes in water during treatment, storage and distribution (Reasoner, 1990; Edberg *et al.*, 1996; Grabow, 1996). The test is approved by the U.S. Environmental Protection Agency (USEPA) for reporting heterotrophic plate counts when residual disinfectant levels are less than 0.2 mg.L⁻¹ (Jackson *et al.*, 2000). The organisms are detected by propagation on non-selective media rich in nutrients to support the multiplication of the widest possible range of bacteria, which may include other organisms such as yeasts (Grabow, 1996; WHO, 2001).

In view of the above features the HPC is included in water quality specifications world-wide. The South African Bureau of Standards specifies a HPC limit of 100 cfu.ml⁻¹ for drinking water (SABS, 2001). This limit is endorsed by the Departments of Health and Water Affairs (South Africa), and is in line with specifications and recommendations in the rest of the world (WHO, 2001). In the case of bottled water a maximum HPC of 50 cfu.ml⁻¹ is generally specified (WHO, 1997).

However, the application of HPC specifications is a contentious issue all over the world. Reasons are basically that under circumstances largely depending on the quality of raw water sources, intensive and expensive treatment is required to produce drinking water which meets HPC specifications. In addition, the general perception of the HPC is that it determines harmless organisms and reflects no meaningful health risk. This issue is growing in concern and importance particularly in the face of accumulating evidence that HPCs may actually detect organisms of meaningful health significance (Payment *et al.*, 1994; Edberg *et al.*, 1996; Hellard *et al.*, 1997; Rusin *et al.*, 1997). Factors which increase the urgency of questions concerning HPC specifications include the escalating component of highly vulnerable individuals among consumers of bulk drinking water supplies. These individuals include the very young and very old, as well as individuals with compromised immune systems due to diseases such as AIDS, and medical interventions with immune systems as part of procedures such as organ transplantation and cancer therapy (Grabow, 1996; Rusin *et al.*, 1997; Barbeau *et al.*, 1998).

Heterotrophic plate count bacteria are known to be responsible for gastroenteritis, meningitis, pneumonia, septicaemia, as well as skin, eye and urinary tract infections (Rusin *et al.*, 1997; Brandi *et al.*, 1999). The risk of infection by heterotrophic plate count bacteria in drinking water has been estimated at levels as low as 7.3 per billion for low exposures to *Aeromonas*, to 98 per 100 for patients on antibiotic treatment exposed to high levels of *Pseudomonas* (Rusin *et al.*, 1997). Therefore, opportunistic pathogens pose a threat to the immuno-compromised population and in South Africa with its high HIV positive cases their importance increases. Therefore, the purpose of this study was to determine the potential health risk of HPC bacteria isolated from selected drinking water supplies in South Africa, with special emphasis on bacteria with virulence factors such as

cytotoxicity, adherence and invasiveness.

Materials and methods

• *Isolation of HPC bacteria*

Traditionally, the pour plate procedure with the use of Plate Count Agar (PCA) medium has been applied in the enumeration of HPC bacteria found in drinking water (APHA, 1996). In this study heterotrophic plate counts were carried out on 153 pour plate samples (exceeding the 100 cfu.ml⁻¹ guideline set by the SABS) using PCA medium (Merck, Darmstadt, Germany) and incubation at 37°C for 24 h (SABS, 2001). The samples were obtained from selected drinking water supplies in three different areas of South Africa during the period of 2000-02-25 to 2000-06-15. The spread plate method was applied on an additional number of 20 river water samples. Representative numbers of HPC bacteria were randomly selected from the samples and purified by the streak plate technique, Gram-stained and stored in 50% glycerol (Sigma) at -20°C for future analysis.

The first step in screening the HPC isolates for potentially pathogenic features consisted of testing their ability to grow on human- and horse-blood agar media. Pure 24 h bacterial cultures were streaked aseptically on blood agar plates and incubated at 37°C for 24 h (Atlas, 1997). Bacterial isolates displaying α - or β -haemolysis were selected for further examination for the production of virulence factors.

• *Enzymatic analyses*

In order to cause an infection, microorganisms should possess virulence factors associated with pathogenesis (Edberg *et al.*, 1996). Therefore, isolated haemolytic HPC bacteria were examined for the production of the following 12 selected extracellular enzymes according to the methods indicated below:

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| 2.1 | <i>Chondroitinase</i> and <i>Hyaluronidase</i> | (Smith <i>et al.</i> , 1968; Edberg <i>et al.</i> , 1996) |
| 2.2 | <i>Coagulase</i> | (Pro-Lab Diagnostics; Edberg <i>et al.</i> , 1996) |
| 2.3 | <i>DNase</i> and <i>Fibrinolysin</i> | (Janda <i>et al.</i> , 1981; Edberg <i>et al.</i> , 1996) |
| 2.4 | <i>Elastase</i> | (Sbarra <i>et al.</i> , 1960; Edbarg <i>et al.</i> , 1996) |

- | | | |
|-----|---|--|
| 2.5 | <i>Gelatinase</i> | (Edberg <i>et al.</i> , 1976; Edberg <i>et al.</i> , 1996) |
| 2.6 | <i>Lecithinase</i> and <i>Lipase</i> | (Edberg <i>et al.</i> , 1996) |
| 2.7 | <i>Proteinase</i> | (Burke <i>et al.</i> , 1991; Edberg <i>et al.</i> , 1996) |
| 2.8 | <i>Pyocyanin</i> and <i>Fluorescein</i> | (The United States Pharmacopeia, 1995) |

- ***Antibiotic susceptibility testing of HPC bacteria***

The antibiotic susceptibility of the HPC isolates was tested using the Kirby-Bauer quality controlled disk diffusion method (Raphael *et al.*, 1983; Atlas, 1997). Haemolytic HPC isolates were tested to several natural and synthetic antibiotics (Mast Diagnostics, Mast group Ltd, Merseyside, U.K.). Plates, smeared with bacterial inoculum and containing antibiotic disks were incubated aerobically and the inhibition zone diameters were measured after 16 - 18 h (Raphael *et al.*, 1983; Atlas, 1997). The end point was taken as the complete inhibition of growth as it appeared to the unaided eye (Raphael *et al.*, 1983; Atlas, 1997). Organisms were reported as either resistant, intermediate or sensitive to each antimicrobial tested (Raphael *et al.*, 1983; Atlas, 1997).

- ***Identification of the isolated HPC bacteria***

In this study, the VITEK Automated Microbiology System (BioMerieux Vitek, Inc. USA) and Biolog MicroPlates (Biolog, Inc., Hayward, CA, USA) were used for determining the identity of haemolytic HPC isolates found in the selected drinking water samples. The VITEK 32 analyser was designed to provide an accurate miniaturised automated system for identifying a large variety of bacteria based on different biochemical reactions (VITEK Senior/Junior procedures manual, BioMerieux VITEK, Inc.). The VITEK 32 analyser could identify Gram-negative or Gram-positive aerobic and anaerobic bacteria, however in this study it was primarily used to identify the Gram-negative HPC isolates, because it is known that the system does not provide reliable results for Gram-positive bacteria.

Biolog GP2 and GN2 MicroPlates were employed in the identification of Gram-positive and Gram-negative HPC bacteria unidentifiable by the VITEK 32 analyser (Biolog, 1999). The GN2 and GP2 Microplate performance characteristics have been determined by establishing a database from a large collection of clinical and environmental stock microorganisms (Biolog, 1999). The database was

designed to give identifications of all species in the database, in accordance with current standards of classical identification methods and current taxonomic nomenclature (Biolog, 1999).

- ***Determination of cytotoxicity***

The cytotoxicity of haemolytic HPC isolates was determined using pure 24 h bacterial cultures. One millilitre of each of the bacterial suspensions (concentration of 10^8 cells per ml) was passed through 0.45 μ m Cellulose Nitrate Filters (Sartorius AG, Germany) (Payment *et al.*, 1994). To increase the expression of cytotoxins, each of the membrane filters containing the HPC isolates was placed on human blood agar plates for 24 h at 37°C prior to initiation of the analysis (Payment *et al.*, 1994). Overlay medium stock was prepared using filter sterilised double-strength Eagle's Minimum Essential Medium, consisting of 96% MEM (Highveld Biological, Lyndhurst, RSA) and 4% fetal bovine serum (Delta Bioproducts, Kempton Park, RSA) (Lye *et al.*, 1991; Payment *et al.*, 1994). A 2% Sea Kem ME Agarose solution (FMC Bioproducts, ME, USA) was prepared separately (Lye *et al.*, 1991; Payment *et al.*, 1994). Equal amounts of the double-strength MEM and the agar were mixed at 50°C to give a final concentration of 1% agar (Lye *et al.*, 1991; Payment *et al.*, 1994). The overlay medium was poured aseptically in each tissue culture plate with the pre-formed HEp-2 cell CCL-23 (American Type Culture Collection, VA, USA) monolayer at the bottom (a concentration of 2×10^5 cells per ml) (Lye *et al.*, 1991; Payment *et al.*, 1994). Each membrane, containing the HPC isolates, was placed on the agar overlay covering the HEp-2 cell monolayer (Lye *et al.*, 1991; Payment *et al.*, 1994). The presence of areas of plaques of cytolysis after removal of the agar overlays indicated a positive test (Lye *et al.*, 1991; Payment *et al.*, 1994).

- ***Determination of cell adherence and invasion***

Caco-2 HTB-37 (American Type Culture Collection, VA, USA) and HEp-2 cells were employed to evaluate the adherence and invasive potential of HPC bacteria (Darfeuille-Michaud *et al.*, 1990; Payment *et al.*, 1994). After a 10 day incubation period, Caco-2 (human adenocarcinoma cell line) cells have the characteristics of human enterocytes (Darfeuille-Michaud *et al.*, 1990; Payment *et al.*, 1994). Pure 24 h cytolytic HPC isolates (concentration of 10^8 cells per ml for a final bacterium-to-cell ratio of 100:1) were suspended in sterile test tubes containing 10 ml of sterile tryptic soy broth

In order to evaluate the cell adherence displayed by HPC isolates, each bacterial suspension was introduced into 16-well Lab-Tek chambers (Nalge Nunc, Denmark) containing the Caco-2 cell monolayers (concentration of 8×10^4 cells per ml) for 3 h at 37°C in a 5% CO_2 atmosphere incubator (Galaxy CO_2 Incubator- Biotech, Northants, England) (Payment *et al.*, 1994). The monolayers were washed three times with phosphate-buffered saline (Sigma) and fixed with 100% methanol (BDH Laboratory Supplies, UK) for 30 min (Payment *et al.*, 1994). After discarding the methanol, Caco-2 cell monolayers were stained with 0.01% acridine orange (Merck) in 0.5 M acetate buffer pH 3.5 (Sigma) for 15 min and examined under UV fluorescence by using a 100X oil immersion objective (Payment *et al.*, 1994). The index of adherence was defined as the average number of bacteria per cell and was determined by counting the number of bacteria adhering to 100 Caco-2 cells (Darfeuille-Michaud *et al.*, 1990; Payment *et al.*, 1994). Experiments were performed in triplicate. The same procedure was followed when studying the adherence of HPC bacteria using HEp-2 cells.

In evaluating cell invasion by cytotoxic HPC bacteria, 24-well tissue culture plates (Corning Costar Corporation, Cambridge, MA, Canada) containing Caco-2 monolayers were drained of their medium (Payment *et al.*, 1994). One millilitre of each bacterial suspension (concentration of 10^8 cells per ml) was added to the single wells (Payment *et al.*, 1994). After incubation for 3 h at 37°C in a 5% CO_2 incubator (Galaxy CO_2 Incubator- Biotech, Northants, England), the cells were washed three times with phosphate-buffered saline (PBS) (Sigma) and incubated for 1 h in Minimum Essential Medium (Highveld Biological) containing 100 μg of gentamicin (Boehringer Mannheim GmbH, Germany) per ml to kill non-invasive bacteria (Payment *et al.*, 1994). Each monolayer was washed with PBS and lysed with 1 ml of 1% Triton X -100 (Whittaker M.A. Bioproducts, Maryland, USA) for 20 min to extract bacteria that had penetrated the cells (Payment *et al.*, 1994). Surviving bacteria were counted by a plate dilution method using PCA medium (Merck) (Payment *et al.*, 1994). The invasion index was calculated as follows (Payment *et al.*, 1994): $\text{Invasion index} = (\text{number of colonies obtained} / \text{number of inoculated bacteria}) \times 100$. The same procedure was followed when studying the invasiveness of HPC bacteria using HEp-2 cells.

Results and discussion

A total of 339 HPC bacteria were randomly isolated, purified and gram-stained. After streaking the purified bacterial isolates on human and horse blood agar plates, a total of 188 (55.5%) HPC isolates were α - or β -haemolytic and consisted of 56.4% Gram-negative and 43.6% Gram-positive isolates. Alpha haemolysis was indicated by 26% (48 isolates) of the HPC isolates, of which 37 isolates were Gram-negative and 11 Gram-positive bacteria. Beta-haemolysis was observed for 74% (140 isolates) of the HPC isolates, of which 69 isolates were Gram-negative and 71 Gram-positive bacteria. Gram-negative haemolytic isolates, such as *Aeromonas veronii* biovar *sobria* showed the highest percentage occurrence in the drinking water samples (18.1%), followed by *Pseudomonas* species (4.8%) and *Aeromonas hydrophila* (3.7%). Dominant haemolytic Gram-positive isolates included: *Tsukamurella inchoensis* (13.3%), *Staphylococcus* species (6.9%) and *Corynebacterium* species (3.7%). Similar HPC bacteria (except *Tsukamurella inchoensis*) were isolated from drinking water by other researchers such as Payment *et al.* (1994), Ashbolt *et al.* (1995) and Edberg *et al.* (1996).

After analyzing the 188 haemolytic HPC isolates against a panel of 12 enzymes the following positive results were obtained: chondroitinase (5.3%), coagulase (16.0%), DNase (60.6%), elastase (33.0%), fibrinolysin (53.7%), gelatinase (62.2%), hyaluronidase (21.3%), lecithinase (47.9%), lipase (54.8%) and proteinase (64.4%). Although no single extracellular enzyme has been proven to be the sole factor responsible for bacterial virulence, there is no doubt that such enzymes do play a role in their pathogenic process. One of the mechanisms whereby extracellular enzymes are capable of enhancing the bacterial virulence involves the destruction of host-protective macromolecules such as mucus, lipoprotein membranes, hyaluronic acid and immunoglobulins (Edberg *et al.*, 1996). In this manner, enzymes may enable pathogens to invade body cells as well as tissues and to interfere with normal cell functions (Edberg *et al.*, 1996).

Some of the HPC isolates (in total 12 isolates), such as *Acinetobacter iwoffii*, *Actinobacillus ureae*, *Aureobacterium terregens*, *Deinococcus radiopugnans* (2 isolates), *Klebsiella* spp. (3 isolates), *Micrococcus luteus*, *Staphylococcus* spp. (2 isolates) and *Vibrio metschnikovii* did not produced any of the extracellular enzymes. None of the HPC isolates produced fluorescein and pyocyanin, which

are characteristic of *Pseudomonas aeruginosa*. Different *Pseudomonas* spp. were isolated in this study, however, no *Pseudomonas aeruginosa* was identified in the drinking water samples. DNases, gelatinases and proteinases were the most commonly produced enzymes. These enzymes are known to destroy cell components, such as nucleic acids and proteins. Isolates which produced more than two extracellular enzymes associated with pathogenesis consisted of: *Aeromonas* spp., *Acinetobacter* spp., *Actinobacillus ureae*, *Aureobacterium terregens*, *Bacillus* spp., *Brevibacterium mcbrellneri*, *Brochothrix campestris*, *Burkholderia cocovenenans*, *Cellulomonas cellasea*, *Chromobacterium violaceum*, *Chryseobacterium (Flavobacterium) spp.*, *Chryseomonas luteola*, *Clavibacter michiganense*, *Corynebacterium* spp., *Curtobacterium citreum*, *Dermabacter hominis*, *Deinococcus radiopugnans*, *Eikenella corrodens*, *Flavimonas oryzihabitans*, *Rathayibacter tritici*, *Rhodococcus equi*, *Pseudomonas* spp., *Serratia* spp., *Staphylococcus* spp., *Tsukamurella inchoensis* and *Vibrio* spp. These HPC isolates were the most virulent among all HPC bacteria isolated from the selected drinking water supplies in South Africa. However, approximately 24 HPC bacteria produced only one virulence factor.

Table 1 shows, that the highest incidence of antibiotic resistance among haemolytic HPC isolates was recorded against natural antibiotics such as penicillin G 2 units (59.6%), penicillin G 10 units (47.3%), ampicillin 10 µg (54.3%) and ampicillin 25 µg (43.1%) than to synthetic agents. Synthetic antibiotics are active against a broad range of bacteria, including heterotrophic plate count bacteria, because these antibiotics were developed from chemical modifications of the natural antibiotics (Edberg *et al.*, 1996). However, an exception was observed for the synthetic antibiotic oxacillin 1 µg, since 77.7% of the HPC isolates were resistant to this antibiotic. All isolates were susceptible to ciprofloxacin 5 µg and gentamicin 100 µg. In total, 51 (27.1%) of the 188 haemolytic HPC isolates were sensitive to all of the antibiotics or displayed resistance to only one antibiotic. The data on antibiotic resistance of HPC bacteria are in agreement with those of Edberg and colleagues (1996).

Some bacterial isolates were tested with both identification systems (VITEK 32 analyser and BiologMicroPlate) in order to check the accuracy of these systems. One of the isolate was identified as *Acinetobacter calcoaceticus baumannii* complex by both systems and the level of certainty was

higher with the VITEK 32 analyser (99%). Two isolates could not be identified with neither of the systems and were therefore defined as unidentifiable.

Cytotoxicity analysis was carried out on a HEp-2 cell line CCL-23 (human epithelial cells). The test revealed that a total of 181 (96%) of the 188 haemolytic HPC bacteria were cytotoxic to HEp-2 cells. As shown in Figure 1 (A), the positive control *Pseudomonas aeruginosa* completely destroyed the HEp-2 cell monolayer, whereas in Figure 1 (B) the cell layer remained intact.

Adherence analyses revealed that 179 (98.9%) of the 181 cytotoxic HPC isolates were adherent to the HEp-2 and Caco-2 cells. An index of adherence was defined as the average number of bacteria per cell. The average index of adherence for Gram-negative bacteria was between 20 - 30 bacteria per HEp-2 cell, compared to 3 - 7 Gram-positive bacteria per HEp-2 cell. Gram-negative isolates such as *Aeromonas*, *Acinetobacter* and *Pseudomonas* spp. adhered to the cells in larger numbers than Gram-positive bacteria, except for *Staphylococcus* and *Micrococcus* species for which the adherence index was as high as 30 - 40 bacteria per HEp-2 cell because of their grouping in pairs or tetrads of cocci. On the other hand, Gram-positive bacilli adhered in smaller numbers possibly due to their cell morphology and formation of chains of single, thick bacilli, compared to Gram-negative bacilli which were relatively small in size. Caco-2 cells appeared to be the more suitable cell line investigating the adhesive capacity of HPC bacteria, possibly due to their ability to obtain the characteristics of human enterocytes after a 10-day incubation period, when the cell monolayers were postconfluent (Darfeuille-Michaud *et al.*, 1990). The adherence index for Gram-positive bacteria on Caco-2 cells was higher than for HEp-2 cells, ranging between 5 - 12 bacteria per cell. A similar tendency was observed with the Gram-negative isolates.

Analysis of invasiveness revealed that 79 (43.6%) of the 181 cytotoxic HPC isolates invaded HEp-2 cells. Of these 79 invasive HPC isolates, 42 (53.2%) were Gram-positive, whereas 37 (46.8%) were Gram-negative. Some of the HPC isolates involved with potential invasiveness on HEp-2 cells included: 9.4% *Tsukamurella inchoensis*, 4.4% *Acinetobacter* spp., 3.9% *Aeromonas* spp., 3.3% *Staphylococcus* spp., 2.2% *Corynebacterium diphtheriae*, 2.2% *Vibrio* spp., 1.6% *Pseudomonas* spp.

and 1.6% *Rhodococcus equi*.

A higher number of cytotoxic HPC isolates were invasive on Caco-2 cells, possibly due to their ability to obtain the characteristics of human enterocytes after a 10-day incubation period. In total 90 (49.7%) of the 181 cytotoxic HPC isolates invaded the Caco-2 monolayer, of which 49 (54.4%) were Gram-positive isolates, compared to 41 (45.6%) were Gram-negative. Invasive HPC isolates on Caco-2 cells included: 8.8% *Aeromonas* spp., 8.3% *Tsukamurella inchoensis*, 5.0% *Staphylococcus* spp., 3.3% *Pseudomonas* spp., 2.8% *Acinetobacter* spp., 2.8% *Corynebacterium diphtheriae*, 2.2% *Chryseobacterium* spp., 1.7% *Bacillus* spp., 1.7% *Vibrio* spp. The invasion index on Hep-2 cells ranged from 1.9×10^{-1} to 8.9×10^{-6} , whereas the invasion index on Caco-2 cells varied between 7.7×10^{-2} to 8.3×10^{-6} .

Although, it is known that high concentrations of HPC bacteria can develop in favourable locations in a distribution system, there is paucity of data on human health effects resulting from exposure to these organisms following ingestion or inhalation (aerosols) (Reasoner, 1990; Edberg *et al.*, 1997). Therefore, three large epidemiological studies (Calderon *et al.*, 1988; Calderon *et al.*, 1991; Payment *et al.*, 1991; Payment *et al.*, 1994) have been conducted to examine the possible association of HPC bacteria in finished drinking water and gastroenteritis (Edberg *et al.*, 1996). These studies yielded mixed results. In two separate studies, Calderon and Mood (1988; 1991) found no association of HPC concentrations with point-of-entry devices and gastroenteritis (Edberg *et al.*, 1996). However, according to a study on filtered and non-filtered tap water conducted by Payment and colleagues (1991; 1994), an association was found between HPC concentrations and gastroenteritis.

Our findings are in agreement with earlier observations that heterotrophic bacteria detected by commonly used HPC tests may indeed include substantial numbers of bacteria which constitute a potential health risk in terms of hospital- and community-acquired infections (Payment *et al.*, 1994; Rusin *et al.*, 1997). Based on these results, it can be concluded that HPC bacteria present in drinking water may constitute a potential health risk to immuno-compromised individuals and particularly in South Africa with its high number of HIV positive cases the importance of HPC bacteria increases.

Conclusion

In this study, 53.4% of the HPC bacteria isolated randomly from selected drinking water supplies in South Africa displayed cytotoxicity compared to the 1.2% reported by Lye and colleagues (1991), which used nutrient-poor R₂A medium. Rusin and colleagues (1997) found some of the HPC bacteria such as *Pseudomonas*, *Acinetobacter*, *Xanthomonas*, *Aeromonas*, *Moraxella*, *Mycobacterium avium* and *Legionella* discussed in this study in less than 1% to as many as 50% of the samples tested. Payment and colleagues (1994) indicated that blood agar medium enhanced the production of extracellular enzymes and cytotoxins. According to their research, 25% of the HPC bacteria isolated on blood agar at 35°C were cytotoxic and had extracellular enzymes associated with pathogenesis. In our study as a first step to screen for potentially pathogenic properties, HPC isolates were grown on horse- and human-blood agar media. Thereafter, haemolytic HPC isolates were grown on blood agar media for 24 h before performing the cytotoxicity analysis. This would explain the high percentage of HPC bacteria isolated with potential virulence factors.

In conclusion, the most frequently isolated HPC bacteria in this study included *Aeromonas*, *Acinetobacter*, *Aureobacterium*, *Bacillus*, *Chryseobacterium*, *Corynebacterium*, *Klebsiella*, *Moraxella*, *Pseudomonas*, *Staphylococcus*, *Tsukamurella*, *Vibrio*. These isolates possessed various virulence factors, such as haemolysins, extracellular enzymes, cytotoxins, adherence and invasiveness associated with pathogenesis. *Aeromonas* spp., as the most isolated organisms, have been associated with various infections in humans, such as diarrhoea, peritonitis, endocarditis, meningitis, septicemia, urinary tract and wound infections (Pin *et al.*, 1997). The second most frequently isolated organism *Tsukamurella* have been responsible for chronic lung diseases, immuno-suppression, indwelling foreign bodies and postoperative infections (Larkin *et al.*, 1999). Infections caused by *Staphylococcus* species include deep and superficial abscesses, endocarditis, wound infections, mastitis, osteomyelitis, pneumonia, meningitis and sepsis (Atlas, 1997). *Pseudomonas* spp. cause a variety of clinical syndromes, such as endocarditis, respiratory infection, bacteremia, meningitis, and ear, bone, and joint, urinary tract, gastrointestinal, skin and soft tissue infections (folliculitis) (Hunter, 1997).

Evidence suggested, that it is vital to maintain a quality of water that is acceptable to the consumer and at the same time the costs associated with water quality surveillance and control must be carefully evaluated before developing national standards (WHO, 2001). “*HPC Bacteria in Drinking Water- Public Health Implications?*” marks the first international symposium on heterotrophic plate count (HPC) bacteria in drinking water and its public-health implications, which takes place in Geneva, Switzerland (2002). According to the NSF Center for Public Health Education, the timing for this symposium is of global importance and organizations around the world are currently focusing on drinking- water safety. Various organizations around the world such as the NSF, WHO and European Member States are aiming as well at achieving scientifically based consensus regarding the appropriate interpretations of HPC measurements in drinking water and at adopting new drinking water directives. Therefore, these data seem to fully justify the need for reevaluation of the current microbiological water quality guidelines in South Africa and the need for more detailed studies on the potential health significance of heterotrophic bacteria in treated drinking water supplies.

The *Mycobacterium avium* complex (MAC) group and many other heterotrophic organisms in water with potentially pathogenic properties, were not addressed in this study. However, it is strongly recommended that this group be thoroughly investigated, because the incidence of MAC infections in HIV positive patients has increased exponentially over the past years and is the second most common cause of death (Singh *et al.*, 1994). Therefore, future work on HPC bacteria should include determination of the incidence of MACs and other viable but non-culturable bacteria in selected drinking water distribution systems of South Africa.

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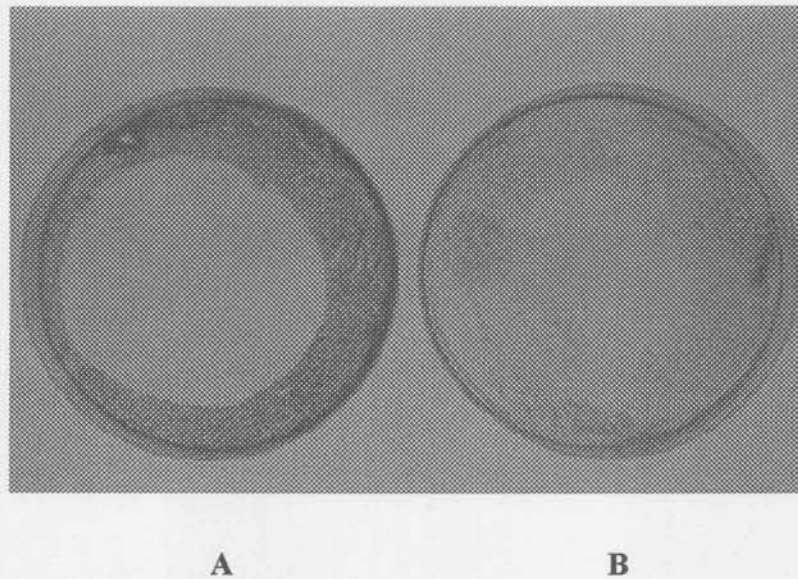


Figure 1: Cytotoxicity displayed by haemolytic HPC isolates. (A) Positive control (*Pseudomonas aeruginosa* ATCC 49189) completely destroying the HEp-2 cell monolayer. (B) Negative control (*Bacillus subtilis* ATCC 6683) HEp-2 cells remained unaffected.



Table 1: The resistance of HPC bacteria isolated from selected drinking water supplies to synthetic and natural antibiotics

| Natural antibiotics | Percentage of bacteria resistant | Synthetic antibiotics | Percentage of bacteria resistant |
|----------------------------|---|------------------------------|---|
| penicillin G 2 units | 59.6% | oxacillin 1 µg | 77.7% |
| ampicillin 10 µg | 54.3% | cefoxitin 30 µg | 17.0% |
| penicillin G 10 units | 47.3% | piperacillin 75 µg | 3.2% |
| ampicillin 25 µg | 43.1% | gentamicin 10 µg | 2.7% |
| erythromycin 10 µg | 13.8% | ciprofloxacin 1 µg | 2.1% |
| erythromycin 15 µg | 10.6% | ciprofloxacin 5 µg | 0% |
| streptomycin 10 µg | 10.6% | gentamicin 100 µg | 0% |
| kanamycin 30 µg | 6.9% | | |
| streptomycin 25 µg | 5.3% | | |

Determination of cytotoxicity and invasiveness of heterotrophic plate count bacteria isolated from drinking water

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Abstract

Evidence has been presented that some heterotrophic bacteria often detected in drinking water supplies possess features associated with pathogenicity. This suggests that even the low numbers of heterotrophic bacteria considered acceptable by drinking water specifications may constitute a health risk, particularly to immunocompromised consumers. In this study 339 bacteria were isolated at random from routine heterotrophic plate count (HPC) tests on selected drinking water supplies in South Africa. In a first screen for potentially pathogenic properties 188 of the isolates (55.5%) displayed α - or β -haemolysis on blood agar. Further analysis of the haemolytic isolates for enzymes associated with virulence revealed the presence of chondroitinase in 5.3% of the isolates, coagulase in 16.0%, DNase in 60.6%, elastase in 33.0%, fibrinolysin in 53.7%, gelatinase in 62.2%, hyaluronidase in 21.3%, lecithinase in 47.9%, lipase in 54.8%, and proteinase in 64.4%. No fluorescein or pyocyanin was detected in any of the isolates. Among the haemolytic isolates 68.6% were resistant to oxacillin (1 μ g), 59.6% to penicillin G (2 units), 47.3% to penicillin G (10 units), 53.7% to ampicillin (10 μ g) and 42.6% to ampicillin (25 μ g). Cytotoxicity, invasiveness and adherence properties of the haemolytic isolates was determined on HEp-2 and Caco₂ cell lines. Among the haemolytic isolates 96% were cytolytic on the HEp-2 cell line. All the haemolytic isolates adhered to HEp-2 and Caco₂ cells but gram-negative isolates tended to adhere in larger numbers than gram-positive isolates. HEp-2 cells were invaded by 42% of the haemolytic isolates. Heterotrophic bacteria, which most frequently revealed the above features associated with pathogenicity included species of the following genera: *Aeromonas*, *Acinetobacter*, *Aureobacterium*, *Bacillus*, *Klebsiella*,

Moraxella, *Pseudomonas*, *Staphylococcus*, *Tsukamurella* and *Vibrio*. The results obtained in this study support earlier indications that bacteria detected by routine heterotrophic plate counts on drinking water supplies may include bacteria associated with potentially pathogenic properties. The extent to which these bacteria in drinking water supplies may constitute a health risk remains to be investigated.

Key words: Heterotrophic plate count bacteria, health risk, drinking water, virulence factors, cytotoxicity, invasiveness, adherence

Introduction

The heterotrophic plate count (HPC), also known as the total or standard plate count, gives a valuable indication of the general microbiological quality of water (Reasoner, 1990; Grabow, 1996; WHO, 2001). The test is widely used to monitor the efficiency of treatment and disinfection processes, and to assess the quality of drinking water supplies. The test is also used to study the deterioration of the quality of water during storage and distribution.

Drinking water quality specifications used world-wide allow HPCs of 100 cfu.ml⁻¹ (WHO, 1996; WHO, 1997; SABS, 2001, WHO, 2001) and in some cases as high as 500 cfu.ml⁻¹ (LeChevalier *et al.*, 1980). HPC bacteria are generally regarded as harmless organisms, which constitute no meaningful health risk. Consequently HPCs exceeding specified limits tend not to be perceived as a serious violation of water quality specifications and are widely accepted. However, there is growing concern about accumulating evidence suggesting that HPCs may actually include organisms which are not as harmless as generally perceived (Ptak *et al.*, 1977; Payment *et al.*, 1994; Edberg *et al.*, 1996; Hellard *et al.*, 1997; Rusin *et al.*, 1997).

Some HPC bacteria isolated from water have been associated with opportunistic infections such as gastroenteritis, skin and mucous membrane infections (Rusin *et al.*, 1997). Evidence has also been presented that certain HPC bacteria isolated from drinking water are cytotoxic and can directly damage human cells in culture (Lye *et al.*, 1991). These observations suggest that HPC organisms in drinking water supplies which meet generally accepted quality specifications may constitute a meaningful health risk. This would particularly concern consumers with immune systems compromised by diseases such as AIDS, organ transplantation and chemotherapy. The very young

and very old would also be at elevated risk (WHO, 1996; Rusin *et al.*, 1997; WHO, 1997). In many communities world-wide the component of high risk consumers is increasing.

The purpose of this study was to determine the potential health risk of HPC bacteria isolated from selected drinking water supplies in South Africa.

Materials and methods

Isolation of heterotrophic plate count bacteria

Heterotrophic plate counts using Plate Count Agar (Merck) in pour plates and incubation at 37°C for 24 h (SABS, 2001), were carried out on 339 samples of selected drinking water supplies in three different areas of South Africa during the period 2000-02-25 to 2000-06-15. Representative numbers of HPC bacteria were randomly picked from the plates, purified and Gram-stained. Freeze cultures were prepared in 50% glycerol (Sigma) and stored at -20°C.

Growth of HPC bacteria on blood agar

Pure bacterial cultures were streaked onto human- and horse-blood agar plates and incubated at 37°C for 24 h. The observation of clear zones around the bacterial colonies indicated β -haemolysis, whereas green zones around the colonies suggested α -haemolysis and no haemolysis was referred to as γ -haemolysis.

Enzymatic analyses of HPC bacteria

The potential virulence of HPC isolates was determined by analysis for 12 selected enzymes (Table 1).

Antibiotic susceptibility testing of HPC bacteria

The Kirby-Bauer quality controlled disc diffusion method (Raphael *et al.*, 1983; Atlas, 1997) was used to determine susceptibility to the following antibiotics (Edberg *et al.*, 1996): (1) Natural and first generation antibiotics (Mast Diagnostics): Penicillin G 2 units, Penicillin G 10 units, Ampicillin 10 μ g, Ampicillin 25 μ g, Streptomycin 10 μ g, Streptomycin 25 μ g, Erythromycin 10 μ g, Erythromycin 15 μ g, Kanamycin 30 μ g. (2) Synthetic and later generation antibiotics (Mast

Diagnostics, Mast Group Ltd, Merseyside, U.K.): Ciprofloxacin 1 µg, Ciprofloxacin 5 µg, Piperacillin 75 µg, Gentamycin 10 µg, Gentamycin 100 µg, Cefoxitin 30 µg, Oxacillin 1 µg. Plates were incubated aerobically and the inhibition zone diameters were measured after 16-18 h (Raphael *et al.*, 1983; Atlas, 1997).

Table 1: Enzymatic analyses of HPC bacteria isolated from drinking water

| Enzymes | References |
|----------------------------------|---|
| Chondroitinase and Hyaluronidase | (Smith <i>et al.</i> , 1968; Edberg <i>et al.</i> , 1996) |
| Coagulase | (Pro-Lab Diagnostics; Edberg <i>et al.</i> , 1996) |
| DNase and Fibrinolysin | (Janda <i>et al.</i> , 1981; Edberg <i>et al.</i> , 1996) |
| Elastase | (Sbarra <i>et al.</i> , 1960; Edberg <i>et al.</i> , 1996) |
| Gelatinase | (Edberg <i>et al.</i> , 1976; Edberg <i>et al.</i> , 1996) |
| Lecithinase and Lipase | (Edberg <i>et al.</i> , 1996) |
| Proteinases | (Burke <i>et al.</i> , 1991; Edberg <i>et al.</i> , 1996) |
| Pyocyanin and Fluorescein | (The United States Pharmacopeia, 1995; Edberg <i>et al.</i> , 1996) |

Analyses of Cytotoxicity, Invasiveness and Adherence of HPC bacteria

The cell lines HEp-2 CCL-23 and Caco₂ HTB-37 (American Type Culture Collection, VA, USA) were used to determine adherence of bacteria (Payment *et al.*, 1994), and the HEp-2 CCL-23 cell line to determine cytotoxicity and invasiveness (Lye *et al.*, 1991; Payment *et al.*, 1994).

Identification of the HPC isolates

A VITEK 32 analyser (BioMerieux Vitek, Inc. USA) was used to identify the Gram-negative haemolytic HPC isolates. Biolog GP and GN plates (Biolog, Inc., Hayward, CA, USA) were employed in the identification of Gram-positive haemolytic HPC isolates and the Gram-negative bacteria unidentifiable by the VITEK 32 analyser.

Results and discussion

Haemolytic isolates

A total of 188 (55.5%) of the 339 heterotrophic bacteria isolated from drinking water supplies were haemolytic. Of these 26% were α - and 74% β -haemolytic. The haemolytic HPC isolates consisted of 56.4% Gram-negative and 43.6% Gram-positive bacteria (Table 2). Gram-negative haemolytic isolates, such as *Aeromonas veronii* biovar *sobria* and *Pseudomonas* species, showed the highest percentage occurrence in the drinking water samples (Table 2). *Tsukamurella inchoensis* and *Staphylococcus* species were the dominant haemolytic Gram-positive isolates (Table 2). Similar HPC bacteria (except *Tsukamurella inchoensis*) were isolated from drinking water by Payment *et al.* (1994), Ashbolt *et al.* (1995) and Edberg *et al.* (1996).

Enzymes related to pathogenicity

After analyzing the 188 haemolytic isolates against a panel of enzymes the following positive results were obtained: chondroitinase (5.3%), coagulase (16.0%), Dnase (60.6%), elastase (33.0%), fibrinolysin (53.7%), gelatinase (62.2%), hyaluronidase (21.3%), lecithinase (47.9%), lipase (54.8%), and proteinase (64.4%). No fluorescein or pyocyanin were detected in any of the isolates. DNases, gelatinases and proteinases were the most commonly produced enzymes. These enzymes are known to destroy cell components, such as nucleic acids and proteins. Isolates which produced three or more extracellular enzymes associated with pathogenesis consisted of: *Aeromonas* species, *Acinetobacter* species, *Bacillus cereus*, *Brevibacterium mcbrellneri*, *Chryseobacterium* (*Flavobacterium*) species, *Corynebacterium diphtheriae*, *Rhodococcus equi*, *Pseudomonas* species, *Serratia marcescens*, *Staphylococcus* species, *Tsukamurella inchoensis*, and *Vibrio* species. It is generally considered necessary to contain more than one extracellular enzyme in order for a microbe to be virulent (Edberg *et al.*, 1996). Some of the HPC isolates, such as *Klebsiella* species, *Morganella morganii*, *Pasteurella haemolytica*, *Pseudomonas mendocina*, *Rahnella aquaticus* and *Shewanella putrefaciens* contained only one or two enzymes associated with pathogenicity.

Antibiotic susceptibility

The highest incidence of resistance among haemolytic HPC isolates was against oxacillin 1 µg (68.6%), penicillin G 2 units (59.6%), penicillin G 10 units (47.3%), ampicillin 10 µg (53.7%) and ampicillin 25 µg (42.6%). The results suggested that a higher percentage of HPC bacteria were resistant to natural antibiotics than to synthetic agents (Table 3). Chemical modifications of the natural antibiotics led to the development of synthetic antibiotics that were active against a broad range of bacteria, including heterotrophic plate count bacteria (Edberg *et al.*, 1996). The data on resistance recorded here are in agreement with those of Edberg *et al.* (1996).

Table 3: The resistance of HPC bacteria isolated from drinking water to synthetic and natural antibiotics

| Natural antibiotics | Percentage of bacteria resistant | Synthetic antibiotics | Percentage of bacteria resistant |
|-----------------------|----------------------------------|-----------------------|----------------------------------|
| penicillin G 2 units | 59.6% | oxacillin 1 µg | 68.6% |
| ampicillin 10 µg | 53.7% | cefoxitin 30 µg | 17.0% |
| penicillin G 10 units | 47.3% | gentamicin 10 µg | 2.7% |
| ampicillin 25 µg | 42.6% | piperacillin 75 µg | 2.7% |
| erythromycin 10 µg | 13.2% | ciprofloxacin 1 µg | 2.1% |
| erythromycin 15 µg | 10.6% | ciprofloxacin 5 µg | 0% |
| streptomycin 10 µg | 10.6% | gentamicin 100 µg | 0% |
| kanamycin 30 µg | 7.4% | | |
| streptomycin 25 µg | 5.3% | | |

Table 2: Identification and percentage occurrence of 188 haemolytic HPC bacteria isolated from selected drinking water supplies in South Africa

| Gram-negative HPC isolates | Percentage occurrence in drinking water | Gram-positive HPC isolates | Percentage occurrence in drinking water |
|---|--|---|--|
| <i>Aeromonas veronii</i> biovar <i>sobria</i> | 18.6% | <i>Tsukamurella</i> <i>inchonensis</i> | 13.8% |
| <i>Pseudomonas</i> spp. | 4.9% | <i>Staphylococcus</i> spp. | 6.9% |
| <i>Chryseobacterium</i> spp. | 4.3% | <i>Corynebacterium</i> spp. | 4.8% |
| <i>Aeromonas</i> <i>hydrophila/caviae</i> | 3.7% | <i>Aureobacterium</i> <i>terregens</i> | 2.7% |
| <i>Acinetobacter</i> <i>calcoaceticus baumannii</i> complex | 3.7% | <i>Bacillus</i> spp. | 2.7% |
| <i>Vibrio</i> spp. | 3.2% | <i>Brevibacterium</i> <i>mcbrellneri</i> | 2.7% |
| <i>Klebsiella</i> spp. | 2.1% | <i>Deinococcus</i> <i>radiopugnans</i> | 2.7% |
| <i>Acinetobacter iwoffii</i> | 1.6% | <i>Micrococcus</i> spp. | 1.6% |
| <i>Actinobacillus ureae</i> | 1.1% | <i>Rhodococcus equi</i> | 1.6% |
| <i>Achromobacter</i> <i>cholinophagum</i> | 1.1% | <i>Dermabacter hominis</i> | 1.1% |
| <i>Francisella philomiragia</i> | 1.1% | <i>Brochothrix campestris</i> | 0.5% |
| <i>Xanthomonas campestris</i> | 1.1% | <i>Cellulomonas cellasea</i> | 0.5% |
| <i>Acidovorax temperans</i> | 1.1% | <i>Clavibacter</i> <i>michiganense</i> | 0.5% |
| <i>Moraxella catarrhalis</i> | 1.1% | <i>Curtobacterium citreum</i> | 0.5% |
| <i>Serratia marcescens</i> | 1.1% | <i>Exiguobacterium</i> <i>acetylicum</i> | 0.5% |
| <i>Chromobacterium</i> <i>violaceum</i> | 0.5% | <i>Rathayibacter tritici</i> | 0.5% |
| <i>Burkholderia</i> <i>cocovenenans</i> | 0.5% | <i>Listeria monocytogenes</i> | 0.5% |

Cytotoxicity

A total of 181 (96%) of the 188 haemolytic bacteria were found to be cytolytic to the Hep-2 cell line (human epithelial cells) (Figure 1). Cytotoxic characteristics were not displayed by the following isolates: *Francisella philomiragia* (1 out of 2 isolates), *Vibrio tubiashii* (1 out of 3 isolates), *Serratia fonticola* (2 isolates), *Achromobacter cholinophagum* (1 out of 2 isolates), *Xanthomonas campestris* (1 out of 2 isolates) and *Morganella morganii* (1 out of 1 isolate).

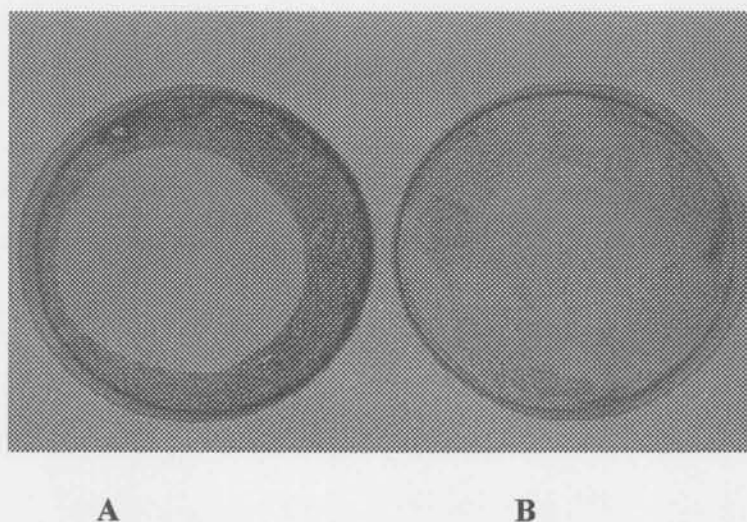


Figure 1: Cytotoxicity displayed by haemolytic heterotrophic bacteria. (A) Positive control (*Pseudomonas aeruginosa* ATCC 49189) completely destroying the HEp-2 cell monolayer. (B) Negative control (*Bacillus subtilis* ATCC6683) HEp-2 cells remained unaffected.

Adherence

All 181 haemolytic HPC isolates adhered to HEp-2 and Caco₂ (human colorectal adenocarcinoma) cells. The gram-negative isolates such as *Aeromonas*, *Acinetobacter* and *Pseudomonas* species adhered to the cells in larger numbers than gram-positive bacteria, except for *Staphylococcus* and *Micrococcus* species. An index of adherence was defined as the average number of bacteria per cell. The average index of adherence for Gram-negative bacteria was between 20-30 bacteria per HEp-2 cell, compared to 3-7 Gram-positive bacteria per HEp-2 cell. However, for some Gram-positive bacteria such as *Staphylococcus* species, the adherence index was as high as 30-40 bacteria per HEp-

2 cell. The adherence index for Gram-positive bacteria on Caco₂ cells was higher than for HEp-2 cells, ranging between 5-12 bacteria per cell. A similar tendency was observed with the Gram-negative isolates. Caco₂ cells appeared to be more suitable for the study of the adhesive capacity of HPC bacteria than the HEp-2 cells, possibly due to their ability to obtain the characteristics of human enterocytes after a 10-day incubation period (Darfeuille-Michaud *et al.*, 1990). A study conducted by Darfeuille-Michaud and colleagues (1990) showed that 15 day-old confluent cultures of Caco₂ cells were covered by typical brush border microvilli that projected out perpendicular to the cell surface. The surface of the Caco₂ monolayer was irregular and formed domes where bacteria preferentially adhered to (Darfeuille-Michaud *et al.*, 1990). Adherence of bacteria such as *Aeromonas* to the intestinal mucosa followed by invasion is essential for the development of gastrointestinal infections such as diarrhoea (Majeed *et al.*, 1994).

Invasiveness

A total of 76 (42%) of haemolytic HPC isolates invaded the HEp-2 cells. The invasive bacteria consisted of 44 Gram-positive isolates (57.8%) and 32 Gram-negative isolates (42.2%). The following invasion index was established: (number of colonies obtained/number of inoculated bacteria) x100. The invasion index for Gram-positive isolates ranged from 2.4×10^{-2} to 1.43×10^{-6} , compared to 1.9×10^{-1} to 5.0×10^{-6} for Gram-negative isolates. The highest invasion index was recorded for *Actinobacillus ureae* at 1.9×10^{-1} . HPC isolates involved with potential invasiveness included: 3.3% *Aeromonas* species, 3.9% *Acinetobacter* species, 1.1% *Aureobacterium terregens*, 1.6% *Bacillus* species, 1.1% *Brevibacterium mcbrellneri*, 2.8% *Chryseobacterium* species, 0.6% *Chromobacterium violaceum*, 2.1% *Corynebacterium diphtheriae*, 0.6% *Eikenella carrodensis*, 1.1% *Klebsiella* species, 0.6% *Moraxella catarrhalis*, 1.1% *Pseudomonas* species, 1.6% *Rhodococcus equi*, 3.2% *Staphylococcus* species, 9.4% *Tsukamurella inchoensis*, and 1.7% *Vibrio* species.

The percentage of heterotrophic bacteria with virulence factors (53.4%) isolated from drinking water in this study is higher than the 1.2% reported by Lye *et al.* (1991). Differences may be due to factors such as the nutrient-poor R2A medium used by the latter authors. Payment *et al.* (1994) found that 25% of bacteria isolated on blood agar at 35°C were cytolytic and had other virulence factors. According to Payment *et al.* (1994), blood agar at 35°C is useful to detect bacteria of health

significance. Horse- and human-blood agar media were, therefore, used in this study as a first screen for α - and β -haemolytic isolates. Moreover, the blood agar medium enhanced the production of extracellular enzymes and cytotoxins (Payment *et al.*, 1994).

Conclusions

Results obtained in this study are in agreement with earlier observations that heterotrophic bacteria detected by commonly used HPC tests may indeed include substantial numbers of bacteria which constitute a potential health risk in terms of hospital- and community-acquired infections (Payment *et al.*, 1994; Rusin *et al.*, 1997). The extent to which the presence of these organisms in drinking water constitutes a risk of infections remains to be investigated in more detail. The results of epidemiological studies on consumers of treated drinking water supplies suggested that regrowth of HPC bacteria such as *Aeromonas* and *Bacillus* species may have accounted for at least some of the cases of gastroenteritis recorded among consumers (Payment *et al.*, 1991, 1994).

Conventional HPC tests fail to detect many culturable micro-organisms in water. Among the bacteria, which fail to produce visible colonies under the conditions concerned is the large group of mycobacteria (Grabow, 1996; Covert *et al.*, 1999). This group includes organisms known as the *Mycobacterium avium* complex (MAC)(Grabow, 1996; Covert *et al.*, 1999). The MAC group is of increasing concern because it includes known pathogens exceptionally resistant to water treatment and disinfection processes, and these pathogens are able to proliferate in raw water sources and drinking water distribution systems (Grabow, 1996; Covert *et al.*, 1999). The MAC group and many other heterotrophic organisms in water with potentially pathogenic features, were not addressed in this study.

HPC bacteria isolated in this study possessed various virulence factors, such as haemolysins, cytotoxins, enterotoxins, adherence and invasiveness associated with pathogenesis. Therefore, these data seem to fully justify more detailed studies on the potential health significance of heterotrophic bacteria in treated drinking water supplies.

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