CYTOTOXICITY AND INVASIVENESS OF HPC BACTERIA

Report to the Water Research Commission
on the Project:
"DETERMINATION OF CYTOTOXICITY AND INVASIVENESS OF HETEROTROPHIC PLATE COUNT BACTERIA ISOLATED FROM DRINKING WATER"

by

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EXECUTIVE SUMMARY

1. Background and Motivation

Heterotrophic plate counts (HPCs) are commonly used to assess the general microbiological quality of drinking water. Drinking water quality specifications worldwide recommend HPC limits ranging from 50 cfu.ml^{-1} up to 500 cfu.ml^{-1} (USEPA, 1989; WHO, 1997). The South African Bureau of Standards specifies a HPC limit of 100 cfu.ml^{-1} for drinking water (SABS, 2001). HPCs exceeding specified limits tend not to be perceived as a serious violation of water quality specifications and are widely accepted and therefore guidelines are often disregarded due to difficulties in producing and delivering water, which conforms strictly to such limit.

Studies conducted in Canada and the USA revealed evidence that these commonly used indicator bacteria may not be as harmless as generally perceived (Payment *et al.,* 1991a; Payment *et al.,* 1991b). These studies indicated that some of the heterotrophic bacteria identified from drinking water samples are opportunistic pathogens. Worldwide these research findings have raised concerns on the health implications, which these bacteria might have for the consumer. Many of these potentially pathogenic bacteria are associated with primary and secondary infections in immuno-compromised individuals (Rusin *et al.,* 1997). This would include the very young and very old, patients with diseases such as AIDS, the malnourished as well as patients on therapy for purposes such as organ transplantation and cancer treatment. It would appear that immuno-compromised individuals, who represent an increasing component of many consumer populations, are particularly at risk. South Africa with its high incidence of AIDS patients is therefore no exception to this rule.

The purpose of this project was to conduct a study in South Africa to determine the presence of HPC bacteria showing potential pathogenic features in treated drinking water samples that exceeded the HPC water quality guideline of 100 cfu.ml^{-1}. According to these results the potential health implications for consumers could be determined and recommendations for the re-evaluation of existing water quality guidelines could be made.

2. Objectives

The objectives of this study were the following:

- To determine the identity and nature of HPC bacteria in cases where the guideline of 100 cfu.ml^{-1} is exceeded in the distribution network.
- To determine if HPC bacteria possess virulence components known to be associated with pathogenesis.
- To re-evaluate current microbiological water quality guidelines for HPC bacteria in South Africa based on these results.

3. Literature Review

A complete literature review has been carried out by Mr Dobromir Nikolov Pavlov as part of his MSc degree.

4. Results

In this study, 339 bacterial colonies were isolated at random from selected drinking water supplies in South Africa using routine heterotrophic plate count tests. In a first step to screen for potentially pathogenic properties, the bacterial isolates were streaked onto human- and horse- blood agar media. The purpose of this test was to determine whether these bacteria were able to destroy red blood cells. A total of 188 (55.5%) of the HPC isolates showed α- or ß-haemolysis.

Haemolytic analyses were followed by examining the production of extracellular enzymes associated with pathogenicity by the HPC isolates. These tests 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 fact that environmental isolates carry antibiotic resistance is important because of their ability to pass it on to opportunistic pathogens.Therefore, antibiotic analysis was conducted in order to determine whether HPC bacteria isolated from drinking water displayed resistance to various antibiotics commonly occurring in the environment. This test showed that 77.7% of the haemolytic isolates 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) using the Kirby-Bauer quality controlled disc diffusion method.

In order to determine the cytotoxicity, adherence and invasiveness of HPC bacteria, HEp-2 and Caco-2 cell lines were used (Payment *et al.,* 1994). These cancer epithelial cells were selected because of their continuous growth and similarity to the cells lining the human respiratory and intestinal tract. These cell culture studies revealed that 96% of the 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 ranged from $1.9x10^{-1}$ to $8.9x10^{-6}$, whereas the invasion index on Caco-2 cells varied between $7.7x10^{-2}$ and $8.3x10^{-6}$.

The most commonly isolated genera displaying potentially pathogenic features were *Aeromonas,Acinetobacter, Aureobacterium, Bacillus, Chryseobacterium, Corynebacterium, Klebsiella, Moraxella, Pseudomonas, Staphylococcus, Tsukamurella* and *Vibrio*. Many bacterial strains included in these genera are known opportunistic

pathogens and can therefore constitute a health risk for consumers specifically for those with compromised immune-systems. These findings would seem to be in agreement with results of epidemiological studies conducted in Canada and the USA (Payment *et al.,* 1991a; Payment *et al.,* 1991b).

A major benefit of this study was the expertise and technology which have been established in a very important area of water quality analysis that were not only of significance for public health but also for water utilities and institutions setting up water quality guidelines. An infrastructure is in place for future research on this topic. After all, future research is essential for the meaningful assessment of public health implications regarding HPC bacteria as well as other more fastidious bacteria in this group such as *Legionella* spp. and *Mycobacterium avium* complex. This information is essential in order to formulate and re-evaluate existing water quality guidelines for HPC bacteria.

5. Cost estimates

A survey of tariffs quoted by leading service providers in the South African market indicates that on average the tariffs for routine monitoring of heterotrophic plate counts vary between R 30 to R 60 per sample. Large scale testing reduces the cost per sample.

Costs for the specialised analyses (haemolysis, enzyme analysis, antibiotic susceptibility, cell culture work to determine cytotoxicity, adherence and invasiveness as well as bacterial identification) are not feasible and are therefore not suggested for routine monitoring by water utilities. The cost per HPC isolate will increase between R 2 500 to R 3 500 and a period of approximately 15 to 20 days is required (per isolate) for the accomplishment of the various tests. This implies that these specialised tests are expensive and time consuming. Furthermore, the tests are complicated, require special laboratory facilities and staff with high levels of training in advance technology as well as expertise. Therefore, these tests are not feasible for all laboratories engaged in routine water quality analysis. Special laboratories are needed to conduct research on aspects such as the prevalence of these potentially pathogenic HPC organisms and their epidemiology, as well as to improve techniques for the isolation and characterisation of HPC bacteria. Hence, these analyses are solely for research purposes but the information obtained is extremely important for water treatment facilities to make them aware of the possible health implications if water quality guidelines are not strictly complied with.

6. Capacity building

- Mr DN Pavlov obtained a MSc degree on the work conducted in this project.
- Technologists and research assistants in the Department of Medical Virology gained experience with the technology concerned.
- Three MSc and one PhD student from the University of Venda gained experience in the field as part of a training programme conducted at the Department of Medical Virology, UP.

7. Technology transfer

During the project term the technology developed during this project has been transferred to personnel of other institutions that visited the Department of Medical Virology, UP. Students and researchers of the following institutions were educated in the isolation techniques of HPC bacteria and in determining the production of potential virulence factors by these isolates: Free State Technikon, Rand Water, Umgeni Water and University of Venda.

Information obtained during this project is of extreme importance to small and large water treatment utilities including the bottled water industry. Technology transfer can be made available to researchers, operators as well as management of these facilities in the form of a workshop or as a pamphlet.

8. Conclusions

All objectives of the project have been met and the following conclusions were made:

1. New techniques for the isolation and characterisation of HPC bacteria found in drinking water have been established in the Department of Medical Virology, University of Pretoria. The most frequently isolated HPC bacteria from plates exceeding the 100 cfu.ml^{-1} guideline and those possessing various potentially pathogenic features included *Aeromonas, Acinetobacter, Aureobacterium, Bacillus, Chryseobacterium, Corynebacterium, Klebsiella, Moraxella, Pseudomonas, Staphylococcus, Tsukamurella, Vibrio* spp. Many of these genera contain species that may act as opportunistic pathogens.

2. A high number of bacteria isolated during this study possessed various virulence factors, such as α- and ß-haemolysins, extracellular enzymes, cytotoxins, adherence and invasiveness associated with bacterial pathogenesis.

3. The high number of opportunistic pathogens among the heterotrophic plate count bacteria detected in this study seem to fully justify the need for re-evaluation of the current HPC water quality guidelines in South Africa. According to the results, it is crucial that water utilities comply to HPC water quality guidelines since in case of exceeding these guidelines the potential risk of a consumer (especially immuno-compromised individuals) being infected by an opportunistic pathogen increases.

4. 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. A single HPC guideline will prove inadequate for all possible scenarios concerning exposures, types of heterotrophs, infectious doses, weakened host conditions and economic realities. At the very least, identification of the composition of the heterotrophic population is needed more than any single level of regulation based on CFU.ml^{-1}.

9. Future Research

The following areas of research need further attention:

- Assessing the risk of infection associated with HPC bacteria, such as *Aeromonas* and *Pseudomonas* spp.
- Determination of the seasonal fluctuations of potentially pathogenic HPC bacteria in drinking water.
- Detection of viable but non-culturable organisms in drinking water and assessment of the importance of these organisms to human health.
- Determination of the incidence of *Mycobacterium avium* complex (MAC) in selected drinking water supplies in South Africa. This is of extreme importance in South Africa with its high number of HIV positive individuals since MACs are the second most important cause of death in these patients.
- Assessment of the contributing factors for persistence of MACs in water distribution systems.
- Assessment of the increase in host susceptibility to opportunistic pathogens due to antibiotic use or immune-suppression.
- The costs involved in water treatment should be considered before re-evaluation and application of water quality guidelines.

10. Publications and Presentations emanating from the Project

10.1 Publications

- 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.. *Water Supply* **2:** 115-122.

- Potentially pathogenic features of heterotrophic plate count bacteria isolated from treated and untreated drinking water by Pavlov, D.N., de Wet, C.M.E., Grabow, W.O.K., Ehlers, M.M. by Pavlov, D.N., de Wet, C.M.E., Grabow, W.O.K., Ehlers, M.M.. Accepted for publication in the *International Journal of Food Microbiology.*

- 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.. Manuscript published in the conference preprint book 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).

- 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.. Published in a book form to be part of the WHO series on water quality.

10.2 Conference presentations

National:

- 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.

- 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.

International:

- 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. Oral presentation (B0309) at the IWA 2nd World Water Congress, 15-19 October 2001, Berlin.

- 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 first NSF International / WHO symposium *"HPC Bacteria in Drinking Water - Public Health Implications?"* Geneva, Switzerland (22-24 April 2002).

10.3 Report

- Report on the outcomes of the first NSF International / WHO symposium on heterotrophic plate count (HPC) bacteria in drinking water *"HPC Bacteria in Drinking Water-Public Health Implications?"* Geneva, Switzerland.

10.4 Thesis

- DN Pavlov (2002) Determination of cytotoxicity and invasiveness of heterotrophic plate count bacteria isolated from drinking water. MSc Thesis.

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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
GP:	Gram-positive
HEp-2:	Human epithelial carcinoma
HPCs:	Heterotrophic plate counts
ID_{50}:	Infectious dose
LRF:	Large restriction fragment
MAC:	*Mycobacterium avium* complex
MEM:	Minimum Essential Medium
MIC:	Minimum inhibitory concentration
MRSA:	Methicillin Resistant *Staphylococcus aureus*
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

1. INTRODUCTION

Heterotrophic plate count (HPC) 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 include other organisms such as yeasts (Grabow, 1996). Plates are generally incubated from 24 h to 48 h, at temperatures ranging from 20°C to 37°C (Grabow, 1996). The 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 test and its application in water quality assessment and monitoring have been described by researchers such as Grabow (1990, 1996), Reasoner (1990), States and Sykora (1995) and WHO (1996, 1997, 2001).

In view of the above features, the HPC test is included in water quality specifications and recommendations worldwide. The South African Bureau of Standards specifies a HPC limit of 100 cfu.ml^{-1} for drinking water (SABS, 1999; SABC, 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.

Evidence has been presented that heterotrophic plate counts may include bacteria, which are not 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 higher 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 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 (Rusin *et al.,* 1997). 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). Several epidemiological studies have been conducted to examine the potential health risk of HPC bacteria found in drinking water.
In two separate studies in USA, 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 in Canada 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 *et al.* (1981), Lye *et al.* (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 of the virulent HPC bacteria.

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 individuals 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 objective of this project was to determine the potential health risk of HPC bacteria isolated from selected drinking water supplies in South Africa and depending on the results suggest the possible re-evaluation of current drinking water quality guidelines for HPC bacteria. The intention was to focus on HPC bacteria with special emphasis on virulence factors such as cytotoxicity, adherence and invasiveness.

2. MATERIALS AND METHODS

2.1 Isolation of HPC bacteria

Heterotrophic plate counts, using the pour plate method, PCA medium (Merck, Darmstadt, Germany) 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 laboratories of the water suppliers, were obtained for microbiological analyses. The spread plate method, PCA medium and incubation at 37°C were applied on 20 river water samples (Atlas, 1997). Incubation at 37°C was carried out in order to select for possible human pathogens. Representative numbers of HPC bacteria (in total 339 bacterial colonies) were randomly selected from samples of the water distribution systems and purified by the streak plate technique, Gram-stained (in order to differentiate between Gram-positive and Gram-negative bacteria) and stored in 50% glycerol at -20°C for future analysis.

2.2 Growth of HPC isolates on blood agar media

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 (Department of Medical Microbiology, University of Pretoria, RSA). This test is commonly used when the production of three kinds of haemolysins (α, ß and γ) by the bacterial isolates is to be determined. When haemolysin-producing bacteria are grown on blood agar plates, zones of clearing may be seen around the 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 erythrocytes is referred to as α-haemolysis (Atlas, 1997). Therefore, pure 24 h bacterial cultures were streaked aseptically onto the blood agar plates and

incubated at 37°C for 24 h (Atlas, 1997).

2.3 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 *et al.,* 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, which include chondroitinase, coagulase, DNase, elastase, haemolysin, hyaluronidase, gelatinase, lecithinase, lipase and proteinase (Finlay *et al.,* 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 *et al.,*1999). Therefore, haemolytic HPC isolates were examined for the production of the following extracellular enzymes:

2.3.1 Chondroitinase

Chondroitinase is an enzyme that attacks chondroitin sulfate in human tissues (Smith *et al.,* 1968; Atlas, 1997). 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 *et al.,* 1968; Janda *et al.,* 1981; 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 sterilised separately using 0.20 μm Minisart filter units (Sartorius AG, Goettingen, Germany) (Smith *et al.,* 1968; Edberg *et al.,* 1996). The appearance of a clear zone around the bacterial colonies represented a positive test (Smith *et al.,* 1968; Edberg *et al.,* 1996).

2.3.2 Coagulase

Coagulase is capable of clotting plasma by converting fibrinogen to fibrin thus enhancing the bacterial virulence (invasiveness) (Atlas, 1997). 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). A positive coagulase test was represented by any degree of clotting (Pro-Lab Diagnostics) (Edberg *et al.,* 1996).

2.3.3 DNase

DNases are a class of extracellular endonucleases capable of hydrolysing polymerised DNA into smaller oligonucleotides (Edberg *et al.,* 1996). DNase agar (Difco Laboratories) supplemented with 0.01% toluidine blue O (Sigma) was employed as the substrate (Janda *et al.,* 1981; 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).

2.3.4 Elastase

Elastase is capable of degrading elastin (cellular glue) and is the substance responsible for haemorrhagic skin lesions and necrosis (Edberg *et al.,* 1996). Elastin powder from bovine neck ligament (Sigma) was prepared as a 1% suspension in nutrient agar (Difco Laboratories) (Sbarra *et al.,* 1960; Janda *et al.,* 1981; Edberg *et al.,* 1996). After incubation, inoculated 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).

2.3.5 Fibrinolysin

Fibrinolysin catalyzes the lysis of fibrin clots and in this way pathogens are free to spread to surrounding areas (Atlas, 1997). Fibrinolysis was determined using fibrinogen type III: from human plasma (Sigma) (Janda *et al.,* 1981; Edberg *et al.,* 1996). Nutrient agar medium (Difco Laboratories) was autoclaved separately at 121°C for 15 min (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).

2.3.6 Gelatinase

Gelatinase is an enzyme that enhances the invasiveness of HPC bacteria by breaking down proteins (gelatin) into smaller peptides and amino acids used for cellular transport as well as metabolism (Edberg *et al.,* 1996). 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 removal of the gelatin coating from the surface of the X-ray film, exposing the blue photographic film base, represented a positive test (Edberg *et al.,* 1976; Edberg *et al.,* 1996).

2.3.7 Hyaluronidase

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). One gram of Noble agar (Difco Laboratories) was added to 100 ml of Brain Heart Infusion Broth (Oxoid) (Smith *et al.,* 1968; 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 sterilised separately using 0.20 µm Minisart filter units (Sartorius AG, Goettingen, Germany)(Smith *et al.,* 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 *et al.,* 1968; Edberg *et al.,* 1996).

2.3.8 Lecithinase

Lecithinase, 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). 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). A white precipitate around or beneath the inoculum spot was taken as a positive test (Edberg *et al.,* 1996).

2.3.9 Lipase

Lipase is an enzyme that enhances the invasiveness of HPC bacteria by hydrolyzing long-chain fatty acid esters (Edberg *et al.,* 1996). Trypticase soy agar (Difco Laboratories) supplemented with 1% Tween 80 (polyoxyethylene sorbitan monooleate) (Duchefa, Netherlands) served as the substrate (Edberg *et al.,* 1996). The appearance of a turbid halo around the inoculum spot was taken as a positive test (Edberg *et al.,* 1996).

2.3.10 Proteinases

Proteinases are a class of enzymes that attack the peptide bonds of protein molecules, forming small peptides (Atlas, 1997). 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). A zone of clearing around the inoculum spot was regarded as a positive test (Burke *et al.,* 1991; Edberg *et al.,* 1996).

2.3.11 Pyocyanin

This enzyme is used for detecting and differentiating *P. aeruginosa* from other pseudomonads based on the appearance of pyocyanin (Janda *et al.,* 1981; Edberg *et al.,* 1996). 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). 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).

2.3.12 Fluorescein

This enzyme is used for detecting and differentiating *P. aeruginosa* from other pseudomonads based on the appearance of fluorescein (Janda *et al.,* 1981; Edberg *et al.,* 1996). 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). 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).

2.4 Antibiotic susceptibility tests on HPC bacteria

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.

The antibiotic susceptibility of the bacterial isolates was tested using the Kirby-Bauer quality controlled disk diffusion method (Raphael *et al.,* 1983; Atlas, 1997). Antibiotic disks (Mast Diagnostics, Mast group Ltd, Merseyside, UK) were placed on the inoculated plates with sterile forceps (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). Bacteria were reported as either resistant or sensitive to each anti-microbial tested (Raphael *et al.,* 1983; Atlas, 1997).

2.5 Analysis of cytotoxicity

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 of the host cell (cytolysis) and the detection of toxins or enzymes produced by these isolates are methods used in clinical microbiology (Payment *et al.,* 1994). The type of cell cultures used in this study (HEp-2 and Caco-2) are similar to those found in the intestinal and respiratory tract of humans, however they are cancerous cells growing continuously and thus making them suitable for research purposes. These cancer epithelial cells were selected because of their continuous growth and similarity to the cells lining the human respiratory and intestinal tract. After a 10 day of incubation period, Caco-2 cells have the characteristics of human enterocytes (Darfeuille-Michaud *et al.,* 1990; Payment *et al.,* 1994).

The cytotoxicity of haemolytic HPC isolates was determined using pure 24 h bacterial cultures. One millilitre of each of the bacterial suspensions (a concentration of 10^8 cells.ml^{-1}) 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 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 double-strength MEM and 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 cells CCL-23 (American Type Culture Collection, VA, USA) monolayer at the bottom (2 x 10^5 cells.ml^{-1}) (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 (destruction of the cell monolayer) after removal of the agar overlays indicated a positive test (Lye *et al.,* 1991; Payment *et al.,* 1994).

2.6 Analysis of cell adherence and invasiveness

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). During the process of invasiveness, microorganisms invade human cells and tissues to establish an infection within the body (Atlas, 1997). In this way, invasive microorganisms may destroy the tissues they infect (Atlas, 1997). Therefore, HPC bacteria were tested for their ability to adhere to and invade

human epithelial cells. Caco-2 HTB-37 (human adenocarcinoma cell line) (American Type Culture Collection, VA, USA) and HEp-2 cells were employed in the evaluation of the adherent and invasive potential of HPC bacteria (Darfeuille-Michaud *et al.,* 1990; Payment *et al.,* 1994). Pure 24 h cytolytic HPC isolates (a concentration of 10^8 cells.ml^{-1} for a final bacterium-to-cell ratio of 100:1) were suspended in sterile test tubes containing 10 ml of sterile tryptic soy broth (Difco Laboratories) (Payment *et al.,* 1994).

In order to evaluate the cell adherence displayed by HPC isolates, 16-well Lab-Tek chambers (Nalge Nunc, Denmark), containing pre-formed Caco-2 monolayers (a concentration of $8x10^4$ cells.ml^{-1}) were used (Payment *et al.,* 1994). Each bacterial suspension was introduced into these chambers 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 inoculated monolayers were washed three times with phosphate-buffered saline (pH 7) (Sigma) and 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.

In the evaluation of cell invasion by cytotoxic HPC bacteria, 24-well tissue culture plates (Corning Costar Corporation, Cambridge, MA, Canada) containing Caco-2 cell monolayers were drained of their medium before use (Payment *et al.,* 1994). One millilitre of each bacterial suspension (a concentration of 10^8 cells.ml^{-1}) 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 inoculated cells were washed three times with phosphate-buffered saline (PBS; pH 7) (Sigma) and incubated for 1 h in Minimum Essential Medium (Highveld Biological), which contained 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): Invasion index = (number of colonies obtained / number of inoculated bacteria) x 100. The same procedure was followed when studying the invasiveness of HPC bacteria using HEp-2 cells.

2.7 Identification of the HPC isolates

HPC bacteria can be identified by the substrates they utilise and the products of their metabolism (Atlas, 1997). The API 20-E system employs twenty miniature capsule reaction chambers in which twenty metabolic characteristics are determined (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 such as HPC bacteria (Atlas, 1997).

The VITEK Automated Microbiology System was designed to provide an accurate automated system for identifying a large variety of environmental as well as clinical bacterial isolates

(VITEK Senior/Junior procedures manual, BioMerieux VITEK, Inc.). The VITEK 32 analyzer can identify Gram-negative or Gram-positive aerobic and anaerobic bacteria; however in this study it was primarily used to identify Gram-negative HPC isolates, because it is known that the system does not provide reliable results with Gram-positive bacteria (VITEK Senior/Junior procedures manual, BioMerieux VITEK, Inc.). Biolog microplates provided 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). Biolog GP2 and GN2 MicroPlates were employed in the identification of Gram-positive and Gram-negative HPC bacteria unidentifiable by the VITEK 32 analyzer (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).

3. RESULTS AND DISCUSSION

3.1 Isolation of HPC bacteria

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 researchers such as Carter *et al.* (2000) and were important for interpreting changes in the microbiological quality of finished water (Geldreich *et al.,* 1990; Rusin *et al.,* 1997).

3.2 Growth of HPC isolates on blood agar media

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 19.7% (37 isolates) were Gram-negative and 5.9% (11 isolates) Gram-positive bacteria. 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. 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* spp. (4.8%) and *Aeromonas hydrophila* (3.7%). Dominant haemolytic Gram-positive isolates included: *Tsukamurella inchonensis* (13.3%), *Staphylococcus* spp. (6.9%) and *Corynebacterium* spp. (3.7%). Similar HPC bacteria (except *Tsukamurella inchonensis*) were isolated from drinking water by other researchers such as Payment *et al.* (1994), Ashbolt *et al.* (1995) and Edberg *et al.* (1996).

3.3 Extracellular enzymes related to pathogenicity

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%). 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). None of the HPC isolates produced fluorescein and pyocyanin, which are characteristic of *Pseudomonas aeruginosa*. Various *Pseudomonas* spp. were isolated in this study. However, no *Pseudomonas aeruginosa* was identified in the drinking water samples.

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). 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 inchonensis* and *Vibrio* spp.

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 extracellular enzyme. Some of the HPC isolates (in total 12 isolates): *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 produce any of the extracellular enzymes.

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 inchonensis* and *Vibrio* spp. were the most 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.

3.4 Antibiotic susceptibility of the HPC isolates

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 natural

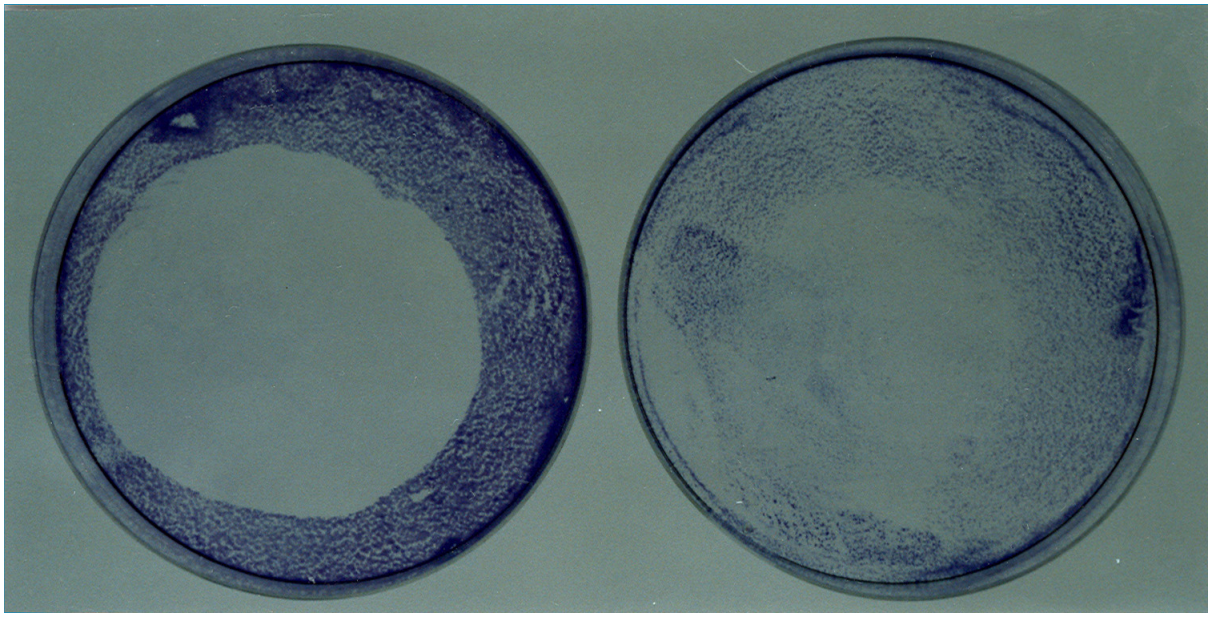
antibiotics (Edberg *et al.,* 1996). However, an exception was observed for the synthetic antibiotic oxacillin 1 µg, since 77.7% of the HPC isolates were resistant to this antibiotic. All isolates were susceptible to ciprofloxacin 5 µg and gentamicin 100 µg. In total of 51 (27.1%) of the 188 haemolytic 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).

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%		

3.5 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. The release of cytotoxic products from the bacterial colonies was recognised by zones of cellular lysis (Figure 1) (Lye *et al.,* 1991). Optimal detection of cytotoxic activity in the HPC bacteria was accomplished using blood agar medium (Lye *et al.,* 1991). The HEp-2 cell line was sensitive in detecting those bacterial colonies causing cytotonic effects (rounding up of cells) and was, therefore, recommended as the tissue culture cell line for detecting cytotoxin-producing bacteria (Lye *et al.,* 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).



A B

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 subtillis* ATCC 6683) HEp-2 cells remained unaffected.

3.6 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. 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.

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. 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 *et al*., 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 number 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.

3.7 Invasiveness displayed by HPC bacteria

Analyses of invasiveness revealed that 79 (43.6%) of the 181 cytotoxic HPC isolates invaded HEp-2 cells. Seventy nine 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 inchonensis*, 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 inchonensis*, 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 $1.9x10^{-1}$ to $8.9x10^{-6}$, whereas the invasion index on Caco-2 cells varied between $7.7x10^{-2}$ and $8.3x10^{-6}$. The highest invasion index was recorded for *Actinobacillus ureae* at $1.9x10^{-1}$ 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 have the potential of causing disease in humans. However, there is no clear-cut evidence that HPC bacteria as a whole pose a public health risk (Rusin *et al.,* 1997).

3.8 Identification of the haemolytic HPC isolates

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.* Two Gram-positive isolates (bacilli; catalase positive) could not be identified with neither of the systems (VITEK 32 and BiologMicroplates) and were therefore defined as unidentifiable. In agreement with reports by other researchers, all of the above genera contain species that can be possible opportunistic pathogens and which may cause various diseases such as gastroenteritis, meningitis, pneumonia and septicemia (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 consumers.

3.9 Drinking water quality guidelines

In the past decade, there has been growing concern about the safety of public water supplies (Payment *et al.,* 1991). Water disinfected at 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.,* 1991). It was assumed that current water quality standards were sufficient to protect the public against the risk of gastrointestinal disease (Payment *et al.,* 1991). However, there is non-trivial endemic level of unreported gastrointestinal diseases due to the consumption of tap water (Payment *et al.,* 1991). In a study, conducted by Payment and colleagues (1991), 35% of gastrointestinal illness was attributed to the consumption of drinking water meeting current 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 $cfu.ml^{-1}$. Drinking water quality specifications used worldwide 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).

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 (Payment *et al.,* 1994; Edberg *et al.,* 1996; Grabow, 1996; 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 developments imply that drinking water suppliers expose themselves to offences 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 simply not be in the position to afford it.

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). 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.

4. 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. According to these studies, some members of the HPC bacteria produce virulence factors and therefore, can 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 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 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 indicated that HPC isolates capable of producing more than two extracellular enzymes associated with bacterial pathogenesis were the following: *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).

A total of 181 HPC isolates (53.4% from the initial 339 isolates) displayed cytotoxic characteristics. However, Lye and colleagues (1991) used nutrient-poor R_2A 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 was *Tsukamurella* (13.30%). *Tsukamurella* spp. have been implicated in chronic lung diseases, immuno-suppression, indwelling foreign bodies and postoperative infections particularly in HIV positive patients (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).

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 prioritise 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).

The HPC method does not detect all culturable microorganisms 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, 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 in these individuals (Singh *et al.,* 1994).

Some HPC bacteria may form populations of viable but nonculturable cells that can not be cultured on standard microbiological media (Bogosian *et al.,* 2000). Therefore, the detection of viable but nonculturable organisms in drinking water and assessment of the importance of these bacteria to human health are areas of future investigation.

In conclusion the first international symposium (22-24 April 2002, Geneva) on HPC bacteria in drinking water was of particular benefit to practitioners, experts, manufacturers, and associations engaged in areas such as public water supply, public health, regulatory and policy officials. Through posters and discussions the most current scientific knowledge was shared on the implications of microbial regrowth as quantified by HPC indicator measurements in drinking water. However, final conclusions on these implications would be published by the WHO organization at a later stage.

5. FUTURE RESEARCH

Future research should also include the following: 1) Assessing the risk of infection associated with HPC bacteria, such as *Aeromonas* and *Pseudomonas* spp. 2) Determination of the incidence of *Mycobacterium avium* complex (MACs) in selected drinking water supplies in South Africa.

The high number of opportunistic pathogens among the heterotrophic plate count bacteria detected in this study seem to fully justify the need for re-evaluation of the current microbiological water quality guidelines in South Africa. There is also a 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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