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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 (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%) of the isolates. No fluorescein or pyocyanin was detected in any of the isolates. Among the haemolytic isolates 68.6% were resistant to oxacillin (1 μg), 59.6% to penicillin G (2 units), 47.3% to penicillin G (10 units), 53.7% to ampicillin (10 μg) and 42.6% to ampicillin (25 μg). Cytotoxicity, invasiveness and adherence properties of the haemolytic isolates was determined on HEp-2 and $Caco_2$ cell lines. Among the haemolytic isolates 96% were cytolytic on the HEp-2 cell line. All the haemolytic isolates adhered to HEp-2 and $Caco_2$ 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.

Keywords Adherence; cytotoxicity; drinking water; health risk; heterotrophic plate count bacteria; invasiveness; virulence factors

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 worldwide allow HPCs of 100 $cfu.ml^{-1}$ (WHO, 1996, 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 and Ginsburg, 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 and Dufour, 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 worldwide, 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 hours (SABS, 2001), were carried out on 339 samples of selected drinking water supplies in three different areas of South Africa during the period 25 February–15 June 2000. 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 hours. The observation of clear zones around the bacterial colonies indicated β-haemolysis, whereas green zones around the colonies suggested α-haemolysis. 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):

- *Natural and first generation antibiotics*: (Mast® Diagnostics): 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).
- *Synthetic and later generation antibiotics*: (Mast Diadnostics, Mast Group Ltd, Merseyside, UK): 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 hours (Raphael *et al*., 1983; Atlas, 1997).

Analyses of cytotoxicity, invasiveness and adherence of HPC bacteria

The cell lines HEp-2 CCL-23 and $Caco_2$ HTB-37 (American Type Culture Collection, VA, USA) were used to determine adherence of bacteria (Payment *et al*., 1994), and the HEp-2

Table 1 Enzymatic analyses of HPC bacteria isolated from drinking water

Enzymes	References
Chondroitinase and hyaluronidase	Smith and Willett, 1968; Edberg *et al*., 1996
Coagulase	Pro-Lab Diagnostics; Edberg *et al*., 1996
DNase and fibrinolysin	Janda and Bottone, 1981; Edberg *et al*., 1996
Elastase	Sbarra *et al*., 1960; Edbarg *et al*., 1996
Gelatinase	Edberg *et al*., 1976; Edberg *et al*., 1996
Lecithinase and lipase	Edberg *et al*., 1996
Proteinases	Burke *et al*., 1991; Edberg *et al*., 1996
Pyocyanin and fluorescein	The United States Pharmacopeia, 1995; Edberg *et al*., 1996

CCL-23 cell line to determine cytotoxicity and invasiveness (Lye and Dufour, 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% were 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 of occurrence in the drinking water samples (Table 2). *Tsukamurella inchonensis* and *Staphylococcus* species were the dominant haemolytic gram-positive isolates (Table 2). Similar HPC bacteria (except *Tsukamurella inchonensis*) were isolated from drinking water by Payment *et al*. (1994), Ashbolt *et al*. (1995) and Edberg *et al*. (1996).

Enzymes related to pathogenicity

After analysing 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 was 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. The enzymes pyocyanin and fluorescein were not detected in any of the isolates. 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 inchonensis*, 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.

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

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).

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).

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%		

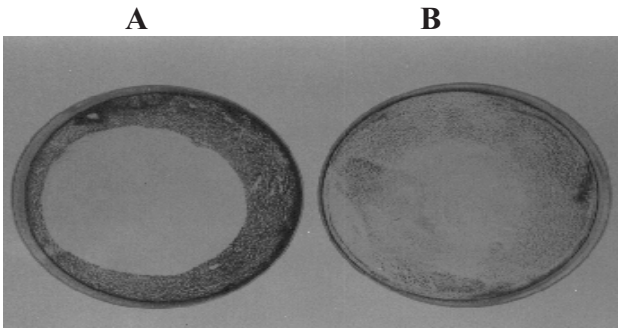

Figure 1 Cytotoxicity displayed by haemolytic heterotrophic bacteria: (A) positive control (*Pseudomonas aeruginosa* ATCC 49189) completely destroying the HEp-2 cell monolayer; (B) negative control (*Bacillus subtillis* ATCC6683) HEp-2 cells remained unaffected

Adherence

All 181 haemolytic HPC isolates adhered to HEp-2 and $Caco_2$ (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 $Caco_2$ 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 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 showed 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 (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 consisited 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) $\times$ 100. 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: *Aeromonas* species (3.3%), *Acinetobacter* species (3.9%), *Aureobacterium terregens* (1.1%), *Bacillus* species (1.6%), *Brevibacterium mcbrellneri* (1.1%), *Chryseobacterium* species (2.8%), *Chromobacterium violaceum* (0.6%), *Corynebacterium diphtheriae* (2.1%), *Eikenella carrodens* (0.6%), *Klebsiella* species (1.1%), *Moraxella catarrhalis* (0.6%), *Pseudomonas* species (1.1%), *Rhodococcus equi* (1.6%), *Staphylococcus* species (3.2%), *Tsukamurella inchonensis* (9.4%), and *Vibrio* species (1.7%).

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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