Pathogenic features of heterotrophic plate count bacteria from drinking-water boreholes

Suranie Horn, Rialet Pieters and Carlos Bezuidenhout

ABSTRACT

Evidence suggests that heterotrophic plate count (HPC) bacteria may be hazardous to humans with weakened health. We investigated the pathogenic potential of HPC bacteria from untreated borehole water, consumed by humans, for: their haemolytic properties, the production of extracellular enzymes such as DNase, proteinase, lipase, lecithinase, hyaluronidase and chondroitinase, the effect simulated gastric fluid has on their survival, as well as the bacteria's antibiotic-susceptible profile. HuTu-80 cells acted as model for the human intestine and were exposed to the HPC isolates to determine their effects on the viability of the cells. Several HPC isolates were α- or β-haemolytic, produced two or more extracellular enzymes, survived the SGF treatment, and showed resistance against selected antibiotics. The isolates were also harmful to the human intestinal cells to varying degrees. A novel pathogen score was calculated for each isolate. *Bacillus cereus* had the highest pathogen index: the pathogenicity of the other bacteria declined as follows: *Aeromonas taiwanensis* > *Aeromonas hydrophila* > *Bacillus thuringiensis* > *Alcaligenes faecalis* > *Pseudomonas* sp. > *Bacillus pumilus* > *Brevibacillus* sp. > *Bacillus subtilis* > *Bacillus* sp. These results demonstrated that the prevailing standards for HPCs in drinking water may expose humans with compromised immune systems to undue risk.

Key words | cytotoxicity, extracellular enzymes, HPC bacteria, human duodenal cells, pathogenicity, simulated gastric fluid

Suranie Horn (corresponding author)
Rialet Pieters
Carlos Bezuidenhout
Unit for Environmental Sciences and Management,
Potchefstroom Campus, North-West University,
Private Bag X6001,
Potchefstroom 2520,
South Africa
E-mail: *suraans@gmail.com*

INTRODUCTION

There is evidence that heterotrophic bacteria are dangerous to human health (Edberg & Allen 2004; Pavlov *et al.* 2004) and may contribute to what is generally referred to as acute gastrointestinal illness (AGI), resulting in fever, nausea and diarrhoea and/vomiting (Macler & Merkle 2000). Most of the AGI are acute, self-resolving and do not have major consequences to healthy individuals. This is however not the case for immuno-compromised individuals.

Heterotrophic bacteria use organic nutrients as their energy source and are present in water, air, soil and food (Edberg & Allen 2004). Heterotrophic plate count (HPC) bacteria are a subset of heterotrophic bacteria and can be isolated in the laboratory by using culture-based methods under a predetermined set of conditions (WHO 2002).

There is much controversy over the usefulness of HPC bacteria as indicators of microbial water quality (Stelma *et al.* 2004; Donskey 2006). Previous studies investigated the potentially pathogenic features of HPC isolates (Pavlov *et al.* 2004). The authors of this paper subscribe to the view of Casadevall & Pirofski (2001) that pathogenicity refers to the ability to cause disease mediated by specific virulence factors. A number of studies reported HPC bacteria to have virulent characteristics associated with potential pathogenicity such as haemolysis, secretion of extracellular enzymes (Pavlov *et al.* 2004), which cause them to be cytotoxic to cells (Lye & Dufour 1991), to adhere to cells (Pavlov *et al.* 2004), and to survive passing through the gastric fluids of the stomach (Janda & Bottone 1981; Yuk & Marshall 2004).

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According to the World Health Organization (WHO 2003), about 15% of the global population lives in areas with water stress and 1.1 billion people do not have access to good quality drinking water. In South Africa, 90% of the population had access to piped water in 2011, leaving an estimated 5.4 million people denied potable water in 2015 (Statistics South Africa 2011). Given the limited availability of treated drinking water, rural communities often use water directly from untreated sources, exposing them to waterborne diseases such as diarrhoea, shigellosis, cholera, salmonellosis and a variety of bacterial, viral, fungal and parasitic infections (Oparaocha *et al.* 2010). One such source of untreated drinking water is boreholes and there is a general misconception among many people that this type of water is safe for human consumption. A section of the population which is particularly susceptible to waterborne diseases includes those with underdeveloped, compromised or weakened immune systems, such as very young children, individuals living with HIV/AIDS, and the elderly (Pavlov *et al.* 2004).

In the study reported here the potential pathogenicity of HPC bacteria isolated from untreated drinking water from boreholes was investigated in a novel way. This was achieved by first using standard methods such as the haemolysin assay (Hoult & Tuxford 1991) and enzyme production analysis (Janda & Bottone 1981). Previous studies showed that HPC bacteria that are α- or β-haemolytic and produce two or more extracellular enzymes are potentially pathogenic (Pavlov *et al.* 2004). Second, the effect of HPC bacteria on human intestinal cells – the HuTu-80 cells acted as a model for the human small intestine – was evaluated. Third, the extent to which HPC isolates with virulence characteristics can withstand the effect of gastric juices was investigated. Mimicking gastric fluids experimentally allows for a closer simulation of conditions within the human body, because these fluids act as an important first line of defence against ingested pathogens, especially when an individual lacks a fully functioning immune system. The South African National Standards (SANS) 241 drinking water specifications stipulate that good quality drinking water should not exceed an HPC bacterial count of 1,000 colony-forming units (CFU)/mL (SANS 2011). However, the bacterial load corresponding to a count of 1,000 CFU/mL from one water source might be more virulent than those with the same count from a different source. This might lead to the incorrect assumption that if a water sample meets the criterion of 1,000 CFU/mL the water is deemed safe for human consumption.

METHODS

Sampling

Water samples were collected from untreated ground-water sources via boreholes on farms surrounding the town of Potchefstroom in the North West province, South Africa. The samples were stored at 4 °C until analysed, but not longer than 24 hours. All the experiments were conducted under aseptic conditions to prevent contamination.

Isolation of HPC bacteria

In order to isolate specifically the HPC bacteria in the water, the low nutrient containing R2A agar (Merck, Germany), that is the conventional growth medium for HPCs, was used. The samples were serially diluted (10^{-1} to 10^{-5} mL) and 100 µL of each diluent was spread plated on the agar (Zhou *et al.* 2010). The plates were incubated for 48 h at 37 °C. Colonies with varying morphologies were selected as HPC representatives and purified for further investigation (no replicates of the same organism were isolated). The aim of this step was solely to harvest only HPC bacteria, and not to imitate intestinal conditions where a variety of bacterial species might thrive.

Haemolysin and extracellular enzyme production assays

Media that contained substrates specific for particular enzymes were used for the assessment of different extracellular enzymes (Israil *et al.* 2011).

Haemolysin

Isolates were subjected to this test first as the ability to secrete haemolysin has proved to be a characteristic of virulent bacteria (Payment *et al.* 1994) that are associated with infections (Giridha Upadhyaya *et al.* 2010). HPC isolates

were streaked on 5% sheep blood agar (BioMérieux, RSA) and incubated at 37 °C for 24 h (Xiao *et al.* 2009). A distinct greenish-black ring, or colourless zone, surrounding the inoculums indicated α- or β-haemolytic activity, respectively (Xiao *et al.* 2009). Isolates that tested positive for α- or β-haemolysin, or both, underwent further testing for production of other extracellular enzymes.

Lipase

Tryptone soy agar (prepared according to manufacturer's instructions) (Merck, Germany) was supplemented with 1% Tween-80 (Sigma, Germany) and served as the substrate to determine lipase production by bacteria (Israil *et al.* 2011). A positive reaction for lipase was the appearance of a turbid halo around the inoculation spot after 72 h at 37 °C (Janda & Bottone 1981; Pavlov *et al.* 2004).

Proteinase

Isolates were screened for proteinase on skimmed milk agar plates (Janda & Bottone 1981). The plates contained equal volumes of 3% (w/v) skimmed milk (Oxoid, UK) and brain heart infusion broth (BHIB) (Merck, Germany) with the addition of 3 g agar (Merck, Germany) per 100 mL (Pavlov *et al.* 2004). Isolates were inoculated and the plates incubated at 37 °C for 48 h. The development of transparent zones around the colonies indicated proteolytic activity (Boominadhan *et al.* 2009).

Lecithinase

Lecithinase production was determined by using McClung–Toabe egg yolk agar (Difco, France) (prepared according to manufacturer's instructions) (Steffen & Hentges 1981). A 1:9 mixture of 50% egg yolk mix (Merck, RSA) and agar were used to prepare the plates. A distinct zone of opacity around or beneath the inoculums after 72 h of incubation at 37 °C indicated the production of lecithinase (Jula *et al.* 2011).

DNase

DNase agar (Merck, RSA) was prepared according to the manufacturer's instructions. The indicator toluidine blue (Sigma, Germany) forms a complex with the DNA in the agar. As soon as this DNA is hydrolysed by the DNase of the test organism, colourless zones appear around the colonies. These zones are formed after 48 h incubation at 37 °C and become visible when the plates are flooded with 1 mol HCl (Merck, USA) (Gündoğan *et al.* 2006).

Hyaluronidase

A medium containing 1 g Noble agar (Difco, France) for every 100 mL of BHIB (Merck, Germany) was prepared as well as a second aqueous substrate containing 2 mg/mL hyaluronic acid (Merck, Germany) and 5% bovine albumin fraction V (final concentration) (Roche, Germany). A mixture 1:1 of the two substrates was poured into plates (De Assis *et al.* 2003). Isolates were inoculated and the plates incubated at 37 °C for 48 h. Inoculums that secreted hyaluronidase created a clear zone around their colonies (Pavlov *et al.* 2004).

Chondroitinase

Chondroitin sulphatase activity was evaluated by the incorporation of a chondroitin sulphatase aqueous solution into 1 g Noble agar for every 100 mL of BHIB (Pavlov *et al.* 2004). The plates contained 4 mg/mL chondroitin sulphate A from bovine trachea (Roth, Germany) and 5% bovine albumin fraction V (final concentration) (Roche, Germany) (Steffen & Hentges 1981; De Assis *et al.* 2003). After incubation for 48 h at 37 °C, a clear zone surrounding the inoculation spot confirmed chondroitinase activity (Xiao *et al.* 2009).

Identification of HPC isolates using molecular methods

The identity of the HPC isolates that were positive for haemolysin as well as two or more other enzymes was determined using molecular methods. Extraction of DNA, polymerase chain reaction, electrophoresis and sequencing were conducted according to Carstens *et al.* (2014). The 16S rRNA gene sequences were submitted to GenBank and given National Center for Biotechnology Information accession numbers (KU253259 to KU253268; see Table 1 in Results and discussion).

Cell maintenance

HuTu-80 cells (HTB-40™), obtained from the American Type Culture Collection (Manassas, VA, USA), are adherent cells and had been isolated from the human intestine. They were selected for this study as the small intestine is one of the areas of the body that is exposed directly to imbibed water and where infections could be initiated. They were cultured in Dulbecco's modified Eagle's medium (Sigma, Germany) at 37 °C in a humidified atmosphere and 5% CO_2 as described by Prinsloo *et al.* (2013).

Cell viability due to isolate exposure

HPC isolates that were already proven to have virulent characteristics by testing positive for haemolysin production and two or more extracellular enzymes were regarded as potentially pathogenic (Pavlov *et al.* 2004). The extent of their capacity to be harmful to human health was further investigated by determining any cytotoxic effect of the microbes on the human intestinal cells. The effect of each HPC isolate on cell viability was measured using the xCELLigence real time cell analyser system. The analyser measures electrical impedance across tiny gold electrodes that occur at the bottom of a 96-well micro-titre plate, where the cells attach. This system provides information on the cell numbers, morphology and viability (Atienza *et al.* 2006), which is expressed as the cell index (CI) (Kloetzel *et al.* 2013). Percentage viability was calculated using the equation (Wu *et al.* 2011):

$$\%viability = \frac{CI\ of\ exposed\ cells}{CI\ of\ unexposed\ cells} \times 100$$

Cytotoxic effects were evaluated based on the time period in which cell viability was significantly decreased (Mann–Whitney, $P \leq 0.05$). The quicker the isolate caused a significant decrease in cell viability, the more pathogenic it was considered to be as the total effect of the isolate was determined by this method. The HuTu-80 cells were seeded at 80,000 cells/mL and allowed to adhere for 13.5 hr before exposure (Handfield *et al.* 1996). After reaching approximately 90% confluency, the HuTu-80 cells were exposed to 10 µL of nutrient broth containing the different HPC isolates, all of the same density. The cells were exposed in triplicate and incubated for 24 h. The real-time cell analysis was performed under cell culture conditions described in the section on cell maintenance (Atienza *et al.* 2006).

The effect of simulated gastric fluid on HPC bacterial viability

Human gastric fluid can kill or inactivate ingested pathogens (Yuk & Schneider 2006). Isolates capable of surviving gastric juices are considered more dangerous to human health than those that die. The effect of gastric fluid on HPC isolate survival was determined by exposing them to a mixture of simulated gastric fluid (SGF) containing 8.3 g/L proteose–peptone (Conda Pronadisa, Spain), 3.5 g/L D-glucose (Merck, RSA), 0.05 g/L bile salts (Difco, France), 0.1 g/L lysozyme from chicken egg white (Merck, Germany), 13.3 mg/L pepsin (Merck, Germany), 2.05 g/L NaCl (Merck, RSA), 0.6 g/L KH_2PO_4 (Merck, RSA), 0.11 g/L $CaCl_2$ (Merck, RSA) and 0.37 g/L KCl (Merck, RSA) in distilled water. The final pH of the SGF was adjusted to 2.5 with sterile 5.0 mol HCl (Yuk & Schneider 2006). The solution was sterilized by filtering through 0.22 µm bottle-top filters (Corning, NY, USA). HPC isolates were added to different dilutions of SGF at 37 °C and exposed for 20 min, as this is the average time for liquids to travel from the stomach to the duodenum (Chavanpatil *et al.* 2006). The dilutions were 50:50, 70:30, and 90:10 (HPC bacterial isolate:SGF) and represented actual volumes when water is consumed and was based on the following: (1) a fasting stomach has 25 mL of gastric fluid and when a glass of water (250 mL) is consumed the gastric fluids are diluted 90:10; (2) when humans are about to eat or drink, the stomach is stimulated to produce 250 mL of gastric fluid, which is diluted 50:50 when a glass of water is imbibed; and (3) the 70:30 dilution was chosen as an intermediate to investigate possible effects that could have been overlooked between the highest (50:50) and lowest (90:10) concentrations of SGF. Sterile broth exposed to SGF acted as a control containing no viable bacteria. After exposure, 100 µL of the mixture (SGF and HPC bacteria) was transferred to a 96-well plate for a viability assay.

Dehydrogenase activity of living cells reduces the yellow tetrazolium salt (MTT) to a blue-purple formazan product (Mosmann 1983). Live bacteria also do the same (Prinsloo

et al. 2013). Each well received 100 μL of 0.5 mg/mL MTT (Sigma, Germany) and after 30 min 200 μL dimethyl sulphoxide (Merck, RSA) was added to dissolve the formazan crystals. Optical density (OD) was measured at 540 nm (Térouanne et al. 2000) in a micro-plate reader (Berthold TriStar LB 941, Germany). The OD of the solubilized formazan is directly proportional to the number of viable cells per well (Madsen et al. 2008). OD values obtained for each exposed isolate were expressed in terms of the OD values of the sterile broth for the same dilution to give fold viability (FV) values, i.e. the number of times the OD of the bacteria-containing mixture was greater than that of the broth control. A value of FV >1 implies surviving and viable bacteria; FV ≤ 1 signifies no surviving bacteria. Statistically significant differences were calculated with the Mann–Whitney test ($P \leq 0.05$) by comparing the OD value of the exposed HPC cells with the corresponding values of the control that contained sterile broth.

Antibiotic resistance

The HPC bacterial strains isolated in this study that had already been shown to have virulent characteristics by the enzyme tests and survival of SGF were tested for antibiotic resistance. Although all ecosystems will have a certain percentage of bacteria resistant to antibiotics, it would be more detrimental to human health if that percentage also exhibits a number of virulent characteristics. This strengthens the argument for improved microbial tests regarding drinking water quality. The Kirby–Bauer disk diffusion method (Pavlov et al. 2004) was used to determine antibiotic resistance of HPC isolates. Antibiotics from four different classes, based on their mechanisms of action, were used (Kohanski et al. 2010). Cell wall synthesis inhibitors included: ampicillin 10 μg (AP10), amoxillin 10 μg (A10), vancomycin 30 μg (VA30) and cephalothin 30 μg (KF30). Protein synthesis inhibitors included: neomycin 30 μg (NE30), tetracycline 30 μg (T30), oxytetracycline 30 μg (OT30) and streptomycin 25 μg (S25) for the 30S ribosomal subunit and chloramphenicol 30 μg (C30) as a 50S ribosomal subunit inhibitor. Trimethoprim (2.5 μg, TM2.5) was chosen as a folic acid synthesis inhibitor. Antibiotic disks (diameter 6 mm) (Mast Diagnostics, UK) were incubated on Mueller-Hinton spread plates for 24 h at 37 °C. After incubation, inhibition zones were measured and compared to an interpretative chart to classify the isolates as resistant, intermediate or susceptible to the antibiotics (Jeena et al. 2006).

Statistical analysis

Basic statistics were performed using SPSS version 20. Sample size dictated that non-parametric tests had to be performed. The non-parametric tests included Mann–Whitney and Spearman's test and differences in viability were deemed statistically significant for the cytotoxic tests and SGF exposures when $P < 0.05$.

RESULTS AND DISCUSSION

Haemolysin and enzyme production

Ten isolates passed all the tests for virulence characteristics of which four were α-haemolytic and six others were β-haemolytic (Table 1). When the isolates were investigated for enzyme secretions four isolates were found to produce two enzymes, and six secreted three types of enzymes. The identities of those isolates that produced haemolysin and two or more enzymes are summarised in Table 1.

Aeromonas spp.

Although these two isolates originated from the same morphological group, they produced two different sets of extracellular enzymes: *Aeromonas taiwanensis* produced lecithinase and lipase whereas *Aeromonas hydrophila* secreted DNase, chondroitinase and hyaluronidase (Table 1). These results contradicted Cumberbatch et al. (1979), Mateos et al. (1993) and Handfield et al. (1996), who reported that *A. hydrophila* strains produce proteinases. According to Mateos et al. (1993), haemolytic and cytotoxic effects are more severe at 37 °C than at environmental temperatures (−5–25 °C), which underlines the significance of these extracellular enzymes in the pathogenic process. *Aeromonas hydrophila* is widely distributed in aquatic habitats and can easily adapt to them. The presence of these strains in drinking water is a major health concern. Some cases of gastroenteritis and wound infections

Table 1 | Identification and virulence characteristics of each isolate

		[a]Enzyme production							SGF survival			
HPC isolate ^Accession number	Haemolysis	C	D	H	Le	Li	P	Time (hr) for cytotoxicity	50%	30%	10%	#Antibiotic resistance
Aeromonas hydrophila ^KU253261	β	x	x	x				1.0		✓	✓	1
Aeromonas taiwanensis ^KU253267	α				x	x		0.3		✓	✓	1
Alcaligenes faecalis ^KU253259	α		x	x			x	None			✓	4
Bacillus cereus ^KU253262	β		x		x	x		1.0	✓	✓	✓	3
Bacillus sp. ^KU253265	β		x			x	x	18.7				0
Bacillus pumilus ^KU253266	β		x		x			19.5			✓	2
Bacillus subtilis ^KU253263	β		x			x	x	19.7				0
Bacillus thuringiensis ^KU253260	α		x	x	x			5.3			✓	3
Brevibacillus sp. ^KU253268	α				x		x	6.8				2
Pseudomonas sp. ^KU253264	β				x		x	6.0		✓	✓	2

[a]C = Chondroitinase; D = DNase; H = Hyaluronidase; Le = Lecithinase; Li = Lipase; P = Proteinase.
#The number of antibiotic classes the isolate was resistant to, a maximum of 4 classes were tested.
^Accession numbers.

during diver training were associated with high numbers of *Aeromonas* reported in the Anacostia River in Washington, DC (Seidler *et al.* 1980).

Alcaligenes faecalis sp.

Thangam & Rajkumar (2000) confirmed production of proteinase by *Alcaligenes faecalis*, which was also true in this study. However, we report here evidence of the products of two additional enzymes: DNase and hyaluronidase (Table 1). *Alcaligenes faecalis* is present in a wide variety of niches such as water, soil and various clinical samples, such as faeces, blood and other body fluids (Kahveci *et al.* 2011). It is not known to cause infections in humans, but is considered to be pathogenic in patients with peritonitis (Kahveci *et al.* 2011).

Bacillus spp.

According to previous studies, most *Bacillus* spp. are known for their production of lecithinase (Molva *et al.* 2009; Cadot *et al.* 2010; Chaves *et al.* 2011). The results from our study concurred with this as four of the six *Bacillus* isolates produced lecithinase (Table 1). *Bacillus cereus* isolates in the present study also produced DNase and lipase (Table 1). This finding is similar to that of Molva *et al.* (2009). Both Cadot *et al.* (2010) and Chaves *et al.* (2011) also found *B. cereus* to be β-haemolytic, as did we (Table 1). *Bacillus cereus* is known to cause diarrhoea and emesis, as well as some non-gastrointestinal infections in humans (Kotiranta *et al.* 2000). The latter is caused by secretion of toxins such as haemolysins, emesis-inducing compounds, phospholipases, non-haemolytic enterotoxins and cytotoxin K (Bottone 2010). This organism's natural environment includes fresh and marine waters, decaying organic matter, soil, vegetables and the intestinal tract of invertebrates. *Bacillus thuringiensis* was responsible for the production of DNase, lecithinase and hyaluronidase (Table 1). Both *B. subtilis* and the *Bacillus* sp. produced proteinase, DNase and lipase whereas *B. pumilus* produced DNase and lecithinase (Table 1). Hoult & Tuxford (1991) found similar results for strain M38 of *B. pumilus*, which secreted lecithinase, but strain M11 was negative for the same enzyme.

Brevibacillus sp.

Brevibacillus spp. isolated from medical waste have been shown to be opportunistic pathogens (Park *et al.* 2009). The *Brevibacillus* sp. isolated in our study also showed potential pathogenic activity. These were haemolytic activity and production of two extracellular enzymes, proteinase and lecithinase (Table 1). This corroborates the findings of Huang *et al.* (2005), who found that *B. laterosporus* G4 produces proteinase.

Pseudomonas sp.

Pseudomonas aeruginosa is also an opportunistic pathogen and known to cause infections in humans. Kida *et al.* (2011) ascribed its pathogenic potential to proteinase and haemolysin production. The *Pseudomonas* sp. of our study produced haemolysin, proteinase and lecithinase (Table 1), indicating the pathogenic potential of this isolate. Sasikala & Sundararaj (2012) found that all the strains of *Pseudomonas* they investigated produced haemolysin, as did our strain. They also reported that 81% of the *Pseudomonas* they isolated, produced proteinase, 77% lipase and 13% lecithinase. The *Pseudomonas* isolate in our study did not secrete lipase (Table 1).

The results thus far reported are of the standard microbial tests which confirmed virulence characteristics of the HPC bacterial isolates. The next section elaborates on the results of the additional tests with which the degree of the detrimental effects of these isolated strains were explored.

Intestinal cell viability after exposure to individual isolates

Prinsloo *et al.* (2013) previously demonstrated that human intestinal cells are a good model to determine the cytotoxic effects of bacteria in water. The measure of virulence of the isolates was judged by how quickly each isolate caused a significant decrease in cell viability, regardless of the specific characteristic, or combination of characteristics (e.g. DNAse and proteinase or growth rate). All the strains, except for *A. faecalis*, were cytotoxic to the human duodenal cells (Table 1). *Aeromonas taiwanensis* was responsible for the quickest significant decrease in cell viability, followed by *A. hydrophila* and *B. cereus* (Table 1). The remaining isolates could significantly decrease viability only during longer exposure times in the following order: *Bacillus thuringiensis, Pseudomonas* sp., *Brevibacillus* sp., *Bacillus* sp., *B. pumilus* and *B. subtilis.* It is clear from these results that the detrimental effect of the various bacterial strains became evident over varying exposure periods. It is, however, important to keep in mind that the intestinal cells, grown in tissue culture dishes, are more susceptible to bacteria than what they would be in the living body with its many defences. The *in vitro* study is more representative of individuals with a compromised immune response.

In a study by Handfield *et al.* (1996), *Aeromonas* isolated from food and water was cytotoxic to human intestinal cells (HT-29). Both the *Aeromonas* spp. isolated in our study caused a significant decrease in viability of the duodenal cells, which is consistent with the results of Handfield *et al.* (1996). Pang *et al.* (2010) also demonstrated the cytotoxicity of *B. thuringiensis* isolates to a Chinese hamster ovary cell line. These authors concluded that the enterotoxins released by these microorganisms would induce diarrhoea, vomiting and abdominal pain in humans.

Bacterial survival after exposure to SGF

Gastric fluids act as a natural defence against ingested pathogens (Yuk & Schneider 2006) and were therefore included in the assessment of bacterial pathogenicity. At the lowest concentration of SGF, i.e. 90:10 (HPC bacterial isolate:SGF), seven isolates survived ($P \leq 0.05$) (Table 1). After exposure to the 70:30 (HPC bacterial isolate:SGF) mixture, *A. hydrophila*, *B. cereus, Pseudomonas* sp. and *A. taiwanensis* (Table 1) withstood the inactivation effects of SGF ($P \leq 0.05$). *Bacillus cereus* survived exposure to all three concentrations. It was therefore clear that some of these isolates tolerated acidic conditions similar to those found in the human gastric environment. This is possible because bacteria can evolve and adapt relatively easily to unfavourable conditions and environments (Valentine 2007). Gastric fluids as a first line of defence may not be effective against HPCs as the HPCs of our study demonstrated varying degrees of resistance.

Antibiotic resistance profile

When the natural defence mechanisms of the human body are failing, a common course of action is to treat infections with antibiotics. In this part of our study, we investigated whether the HPC isolates identified were resistant or susceptible to antibiotics. The HPC isolates in this study were obtained from aquatic environments where they could have been exposed to unknown substances, possibly contributing to their antibiotic resistance profiles.

The different antibiotic classes against which the isolates were resistant are indicated in Table 1. Table 2 presents the results of specific antibiotic resistance. Although the various antibiotics belonging to the same class have the same mechanism of action, the isolates responded inconsistently: ampicillin, amoxillin, cephalothin and vancomycin are all inhibitors of cell wall synthesis; *B. thuringiensis* was resistant to ampicillin, amoxillin and cephalothin, but susceptible to vancomycin (Table 2). *Bacillus thuringiensis* and *B. cereus* had the same antibiotic resistance profile (Table 2). Our evidence of *B. cereus*'s resistance to tetracyclin corroborates the findings of Kiyomizu *et al.* (2008) and of Savini *et al.* (2009); its multiple resistances support the results of Bottone (2010). The last concluded that *B. cereus* has always been resistant to different generations of β-lactam antibiotics such as penicillin, cephalosporin and ampicillin as well as trimethoprim, but frequently susceptible to erythromycin, clindamycin, vancomycin, chloramphenicol, the aminoglycosides, and tetracycline. *Alcaligenes faecalis* was the only organism resistant to streptomycin. Two isolates were susceptible (with some intermediate resistance) to all the antibiotics and one was resistant to only trimethoprim (Table 2). In contrast to the findings of Sasikala & Sundararaj (2012), *Pseudomonas* sp. isolated in our study was not resistant to multiple antibiotics.

Pathogen score based on virulence characteristics of each isolate

The HPC bacterial isolates were virulent in various ways that contributed to their pathogenicity (Table 1). We transformed these results into a pathogen index by allocating a weighted score for the individual virulent factors, depending on their contribution to a pathogenic profile. This index was used to compare and evaluate the degree to which these organisms have the potential to cause disease in humans.

The scoring was done according to the following scheme: Isolates responsible for α- and β-haemolysis were awarded 0.1 and 0.2, respectively. β-Haemolysis causes complete lysis of red blood cells (Pakshir *et al.* 2013) and is therefore regarded as having a more severe effect on the host than α-haemolysis, which causes only partial haemolysis of red blood cells (Miyake *et al.* 2010). For every enzyme

Table 2 | Resistance, intermediate resistance and susceptibility of the HPC isolates to antibiotics

	Antibiotic	*Aeromonas hydrophila*	*Aeromonas taiwanensis*	*Alcaligenes faecalis*	*Bacillus cereus*	*Bacillus* sp.	*Bacillus pumilus*	*Bacillus subtilis*	*Bacillus thuringiensis*	*Brevibacillus* sp.	*Pseudomonas* sp.
	Gram reaction	−	−	−	+	+	+	+	+	+	−
Cell wall synthesis inhibitors	Ampicillin 10 µg (AP10)	R	R	R	R	S	S	S	R	S	S
	Amoxillin 10 µg (A10)	R	R	R	R	IR	R	S	R	S	IR
	Cephalothin 30 µg (KF30)	R	R	IR	R	S	S	S	R	S	R
	Vancomycin 30 µg (VA30)	R	R	R	S	S	S	S	S	S	R
Protein synthesis inhibitors 30S ribosomal subunit	Tetracycline 30 µg (T30)	IR	IR	R	R	S	IR	S	R	R	S
	Oxytetracycline 30 µg (OT30)	IR	IR	R	R	IR	R	IR	R	R	IR
	Streptomycin 25 µg (S25)	S	S	R	S	IR	S	IR	S	IR	IR
	Neomycin 30 µg (NE30)	S	S	S	S	S	S	S	S	S	S
Protein synthesis inhibitors 50S ribosomal unit	Chloramphenicol 30 µg (C30)	S	S	R	S	S	R	S	S	S	S
Folic acid inhibitor	Trimethoprim 2.5 µg (TM2.5)	IR	IR	R	R	S	S	S	R	R	R

R = resistant; IR = intermediate resistance; S = susceptible.

produced, 0.1 was added to the pathogen score. These scores for cytotoxicity ranged from 1.2 (at 0 hr) to 0 (after 24 hr). The score was decreased by increments of 0.1 for every increase by 2 hr of the exposure period. For example, if cytotoxicity was evident within the first 2 hr of exposure, an isolate was awarded 1.2. Evidence of cytotoxicity was deemed valid when it was statistically significant. Isolates that survived exposure to the most concentrated SGF (50:50) received a score of 0.5, the second highest (70:30) a value of 0.3, and survival at the lowest concentration (90:10) was given a value of 0.1. A score of 0.1 was also added to the tally for resistance against antibiotics. Such resistance earned a score of 0.1 for every antibiotic class to which a strain was resistant, with a maximum of 0.4 (because only four antibiotic classes were evaluated). The highest pathogen score indicated the most pathogenic HPC bacteria investigated. These results in decreasing order of the pathogenic score were: *B. cereus* > *A. taiwanensis* > *A. hydrophila* > *B. thuringiensis* > *A. faecalis* > *Pseudomonas* sp. > *B. pumilus* > *Brevibacillus* sp. > *B. subtilis* > *Bacillus* sp.

The results obtained in this study only reflect the effects of culturable HPC bacteria and it does not account for the number of bacteria present in water sources that are viable, but not culturable (VBNC). The VBNC bacteria might be pathogenic; however their effects cannot be detected and are overlooked. New technologies such as environmental DNA (Bohmann *et al.* 2014) may be enlisted to identify unculturable bacteria, but this method cannot predict enzyme secretions, ability to survive gastric juices or effect on viability of intestinal cells.

CONCLUSION

Currently, there is not a single test available to predict the effect HPC bacteria have on human health. However, in this study a series of tests provided answers regarding their potential pathogenicity. A novel pathogen index summarised the virulence degree of each isolate according to its virulent characteristics and indicated the extent to which these organisms have the potential to cause disease in humans.

Although the borehole water in this study is not subjected to water quality control, because they are privately owned and people incorrectly regard water from a borehole as pristine after percolating through layers of soil, sand and rock to be free from any contaminants. When the only water quality guideline that gives information on the HPC condition of the water was applied (1,000 CFUs/mL), only eight of the 16 boreholes exceeded this level (data not shown). However, pathogenic HPCs were isolated from three of the eight boreholes not exceeding the standard guidelines (and a number of boreholes that did exceed the guideline did not have HPCs with virulence characteristics). Therefore, merely applying a quantity measure of HPCs does not guarantee protection from pathogenic HPCs should humans consume the water.

In a country such as South Africa where 11.2% are living with AIDS (Wakefield 2015) it would be wise to address current drinking water quality guidelines related to HPCs. The authors suggest that the following two measures be applied by water utilities: (i) lowering the acceptable CFUs to, for example, 500 CFU/mL and (ii) introduce additional tests for bacterial virulence. A third measure would be for South Africa to continue its infrastructure improvement and supply piped water to the lacking 10% of the population that depend on other water sources.

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