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Full Length Research Paper

Effect of oxidative stress on viability and virulence of environmental *Acinetobacter haemolyticus* isolates

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The effect of oxidative stress exerted by 0.03% crystal violet, 0.3% bile salt, 4.0% NaCl and 8% ethanol on the survival and production of virulence factors among *Acinetobacter haemolyticus* isolates obtained from wastewater and river water samples was investigated. Though generally there was insignificant lethal effect against all the isolates, crystal violet exerted the highest lethal effect followed by ethanol and bile salt, NaCl exerted the least effects as compared to crystal violet and ethanol. Isolates from wastewater demonstrated the highest rate of resistance compared to isolates from river water. Presence of resistant verotoxic *A. haemolyticus* in the environments investigated is a cause for concern. Resistance exhibited by the bacteria means that the efficacy of these salts, chemicals and temperature conditions frequently employed as control/preservation agents in hospitals and food industries may be undermined.

Key words: Cell viability, hydrophobicity, contamination, food-borne pathogens, oxidative stress, virulence.

INTRODUCTION

The emergence of multidrug resistant nosocomial or community-acquired infections of *Acinetobacter* spp. is a result of high adaptability to adverse environmental conditions, ability to persist in harsh environments (for example, hospital environment), increased use of broad spectrum antibiotics, vulnerability of individuals or patients, and rapid transformation. Contamination of food and water sources with these bacterial agents results in outbreak of various forms of infections. *Acinetobacter* spp. has been recovered from vegetables and fruits and has also been implicated in the spoilage of bacon, chicken, meat, fish, eggs and hospital foods. Food safety and public health, has preoccupied many governments, including those of developing countries (FDA, 2001; WHO, 2005). *Acinetobacter haemolyticus* and other *Acinebacter* spp. have been causative agents of resistant nosocomial infections and costs associated with controlling such infections are staggering (Kurcik-Trajkovska, 2009), forcing some institutions to close entire units as a control measure. The major problem with *Acinetobacter* spp. is their resistance to antibiotics. Recently, the organisms were associated with high rate of mortality among hospital patients investigated (Lee et al., 2011). It has been reported that the organisms are most commonly resistant to ampicillin, cephalothin, carbenicillin, gentamicin, amikacin, chloramphenicol, tetracycline, co-trimoxazole, ciprofloxacin and cefoperazone. Resistance to these antibiotics has hindered therapeutic management, causing growing concern the world over (Manchanda et al., 2010; Doughari et al., 2011b). Verotoxins also present treatment challenges. Abong' o and Momba (2009) reported complications arising from antibiotic treatment of verotoxic bacteria. The toxins are released into the medium as the bacterial cells are lysed by the antibiotics causing further health complications such as the bloody diarrhea (Abong' o and Momba, 2009) and sometimes kidney complications. This informed the need to

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Table 1. Cell Kill Index (CKI) (%) of *A. haemolyticus* from wastewater and river water samples.

#*Acinetobacter* isolates	Cell Kill Index (CKI) (%)			
	Crystal violet (0.03%)	Bile salt (0.3%)	NaCl (4%)	Ethanol (8%)
Treatment plant wastewater				
A. haemolyticus RWW1v	5.77±0.023	9.80±0.000	2.44±0.000	19.05±0.012
A. haemolyticus PSW2i	2.02±0.000	1.33±0.000	15.00±0.000	9.80±0.121
A. haemolyticus PSW2ii	3.85±0.031	11.1±0.017	8.37±0.000	12.7±0.301
A. haemolyticus FEW2iv	4.56±0.011	7.96±0.044	4.76±0.000	42.86±0.032*
Abattoir wastewater				
A. haemolyticus FSE1iv	53.85±0.001*	7.88±0.037	16.67±0.000	8.00±0.000
A. haemolyticus FSE1v	33.02±0.036	7.14±0.033	6.90±0.000	2.40±0.000
A. haemolyticus PST1i	3.23±0.001	3.70±0.000	23.33±0.034	8.62±0.130
A. haemolyticus PST2i	7.00±0.000	7.00±0.000	2.44±0.027	6.76±0.068
A. haemolyticus PST2ii	31.67±0.021	6.90±0.000	8.89±0.026	10.00±0.032
River Berg water				
*A. haemolyticus*RBD1i	56.06±0.052*	9.76±0.071	6.67±0.0022	5.33±0.111
*A. haemolyticus*RBD1ii	30.60±0.000	10.83±0.034	8.50±0.000	6.31±0.023
*A. haemolyticus*RBD1iii	10.64±0.010	12.50±0.000	6.00±0.000	15.91±0.047
*A. haemolyticus*RBI1i	62.86±0.005*	15.32±0.005	3.23±0.038	11.04±0.000
A. haemolyticus RBI2i	20.00±0.000	17.86±0.016	27.27±0.021	42.25±0.000*
Control				
A. haemolyticus 19002	34.75±0.022*	57.69±0.009*	43.13±0.041*	34.88±0.000*

#Isolates were non motile, negative for sulphide production, oxidase reaction and arginine fermentation but positive for indole production, and fermented triglyceride, glucose, citrate, urea, tryptophane, as well as oxidation of sodium nitrate; Gram-negative coccobacilli and tiny, blue, mucoid colonies on Eosin Methylene Blue (EMB); Pink colonies diffused into the medium on Leeds Acinetobacter Medium (LAM) and all were haemolytic on Sheep blood agar.*significant at (P>0.05)

investigate the efficacy of various salts, chemicals and temperature conditions frequently employed as control agents in hospitals and food industries with a view to developing more effective control measures. Thus the study reports the effect of oxidative stress exerted by low temperature stress, crystal violet, bile salt, and sodium chloride on some virulence factors of environmental isolates of *A. haemolyticus.*

MATERIALS AND METHODS

Source of bacterial strains and blood sample

From Athlone wastewater Treatment Plant): *A. haemolyticus* RWW1v, *A. haemolyticus* PSW2i, *A. haemolyticus* PSW2ii and *A. haemolyticus* FEW2iv. From Winelands Pork abattoir wastewater: *A. haemolyticus* FSE1iv, *A. haemolyticus* FSE1v, *A. haemolyticus* PST1i, *A. haemolyticus* PST2i and *A. haemolyticus* PST2ii and from River Berg: *A. haemolyticus* RBD1i, *A. haemolyticus* RBD1ii, *A. haemolyticus* RBD1iii, *A. haemolyticus* RBI1i and *A. haemolyticus* RBI2i (from River Berg) were used for this study. All sample sites were located in Cape Town South Africa.

Confirmation of isolates

Before use, the bacteria were reconfirmed by culture on Eosin methylene blue (EMB, Oxoid SA) and Leeds Acinetobacter medium (LAM, Hardy Diagnostics USA) and characterized using biochemical reagents and Rapid NF plus identification kits (Merck, SA) (Table 1) and confirmed (Doughari et al., 2011a) as positive for verotoxins, cell surface hydrophobicity, serum resistance and haemolysin production using standard methods (Chou and Cheng, 2000; Hedge et al., 2009). The cultures were maintained on trypton soy agar slants (TSAS, Oxoid, SA) in the Microbiology Laboratory, Department of Biotechnology, Faculty of Applied Sciences Cape Peninsula University of Technology, Cape Town South Africa. To screen for virulence factors, bacteria were subcultured on chemically defined medium (CDM) as described by Hedge et al. (2009). The non pathogenic strain *A. haemolyticus* 19002 (static culture) was used as control. For each experiment, bacteria were sub-cultured onto trypton soy agar (TSA, Oxoid, and SA) and incubated at 37°C for 18 h before use. Sheep blood erythrocyte was purchased fresh from the National Research Council (NRC, Cape Town, South Africa). The blood was used without further treatment as a source of serum complement.

Antimicrobial susceptibility testing

The bacteria were tested for susceptibility against ampicillin (10 µg), cefuroxime (30 µg), cephalexin (30 µg), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), aztreonam (30 µg), nalidixic acid (30 µg), amikacin (30 µg), tetracycline (30 µg), gentamicin (10 µg), ofloxacin (5 µg), ciprofloxacin (5 µg) (Oxoid UK) (WHO 2002) using the disc diffusion method (Aksoy et al., 2007).

All antibiotics were obtained from Quantum Biotechnologies, South Africa.

Effect of stress on bacterial viability and virulence

Effect of oxidative stress on surface hydrophobicity of bacterial cells

Bacterial strains from TSA were sub-cultured twice in shake flasks with CDM (Hedge et al., 2009) and each time incubated at 37°C for 24 h. After the final subculture, the cells were washed thrice in sterile physiological saline (0.85% w/v NaCl) and finally suspended in saline to get a solution of OD_{600} 0.1 (0.5 McFarland turbidity standard). CDM (10 ml) containing various concentrations (0.1, 0.2 and 0.3 M) of H_2O_2 were dispensed aseptically in three sets of 100 ml Erlenmeyer flasks. The bacterial suspensions were inoculated into each of the flasks to obtain cell concentration of approximately 10^6 cells/ml. A flask containing *A. haemolyticus* ATCC 19002 suspended in CDM without H_2O_2 was used as control. The flasks were then incubated at 37°C in rotary water bath at 160 rpm for 24 h, centrifuged at 3000 rpm/10 min and retained packed cells were washed with sterile phosphate buffered saline (PBS, 10 ml) then suspended in PBS to get a density of 0.3 at OD_{600} (OD Initial). To this bacterial suspension (3 ml), *p*-xylene 0.3 ml, was added and vortexed for 1 min, left for 30 min at ambient conditions and final OD_{600} (OD Final) determined. Degree of hydrophobicity retention was calculated as percent hydrophobicity index (HI) (Equation 1):

$$HI = \frac{OD\,I - OD\,F}{OD\,I} x100$$

Where, HI = hydrophobicity index, ODI = initial optical density, ODF = final optical density.

Effect of oxidative stress on bacterial haemolysin production

This was carried out using the quantitative α-haemolysin assay. The H_2O_2 treated (0.3 M) bacteria suspension earlier grown in the Erlenmeyer flasks (10 ml), was transferred into sets of tubes and centrifuged at 3000 rpm for 10 min. The supernatant was collected and diluted in 0.8% calcium chloride solution (10 ml), 1 ml withdrawn and mixed with 1% (v/v) sheep erythrocyte suspension and incubated at 37°C for 1 h. After the incubation, 2 ml of 0.8% NaCl saline was added to each tube exhibiting partial haemolysis and the bacterial suspensions centrifuged at 1500 rpm for 10 min to pellet the unlysed erythrocytes. The supernatant fluid was separated and the OD_{540} determined. Fifty percent (50%) haemolysis standard prepared by mixing 1 ml of 1% (v/v) sheep erythrocyte suspension and 3 ml of diluents was used as control. Inverse of the dilution which caused 50% lysis was recorded as HU 50 (50% haemolysis units) (Hedge et al., 2009). A tube containing non-H_2O_2 treated bacterial suspension (*A. haemolyticus* ATCC 19002) suspended in CDM was used as control (Equation 2):

$$HU\,50 = \frac{ODs - ODt}{ODs} x100$$

Where, OD_s = 50% haemolysis standard, OD_{ex} = final haemolysis measured, HU 50 = 50% haemeolysis.

Effect of oxidative stress on bacterial serum resistance

The effect of oxidative stress on bacterial serum resistance was determined using the quantitative serum bactericidal assay (Hughes et al., 1982). H_2O_2 treated (0.3 M) bacterial suspension (0.5 ml) was mixed with 1.5 ml of fresh undiluted serum (from blood) and incubated at 37°C. Cell viability was determined turbidiometrically at 600 nm after 3, 6 and 18 h. Serum resistance index (SRI) of bacteria was calculated (Equation 3):

$$SRI\ (\%) = \frac{ODI - ODF}{ODI} x100$$

Where, SRI = serum resistance index, ODI = initial turbidiometric reading, ODF = Final turbidiometric reading. A tube containing non-H_2O_2 treated bacterial suspension (*A. haemolyticus* ATCC 19002) suspended in CDM was used as control.

Effect of low temperature stress

A 1 ml aliquot of bacterial suspension (initial density 10^8 CFU/ml) grown at 37°C was inoculated into 9 ml trypton soy broth (TSB) in two different sets of test tubes, thoroughly mixed and then stored at -5, -18 and -28°C for 21 days. The tubes were removed from the freezers after every 5 days, and thawed under running tab water for 5 min then returned to the freezer. After 21 days, the cultures were removed and 2 to 3 loopfuls inoculated into TSB, incubated for 18 h at 37°C and the viable cell index (VCI) determined by taking the OD_{600} values first at 0 min (OD Initial) then at 10 min interval for 1 h (Chou and Cheng, 2000) and VCI on the scale of 100% calculated as follows (Equation 4):

$$VCI = \frac{OD\,I - OD\,F}{OD\,I} x100$$

To confirm that viable bacteria were in a culturable state, viable counts were made by making serial dilutions of 2-3 loopfuls of bacterial culture in 10 ml TSB and surface spread by inoculating onto NA plates, incubating at 37°C and determining percentage survival after 18 h. Bacterial suspension containing non-H_2O_2 treated and non-freeze-thawed bacterial (*A. haemolyticus* ATCC 19002) suspension in CDM was used as control.

Effect of ionic salt concentrations and other chemicals on cell viability and verotoxin production

Low-temperature stressed culture suspension (0.2 ml) was inoculated into solution (10 ml) containing either 0.03% crystal violet, 0.3% bile salt, 4% NaCl, or 8% ethanol and incubated at ambient conditions for 1 h. Bacterial culture (*A. haemolyticus* ATCC 19002) not subjected to H_2O_2 stress, low temperature storage, or alcohol and salts was used as control. After incubation, each of the samples was serially diluted in Butterfield's phosphate diluents (USFDA, 2001) (appendix iv) and 0.1 ml surface plated on TSA and incubated at 37°C for 10 (initial) and 30 min (final) after which, the percent cell kill index (CKI, %) was determined (Chou and Cheng, 2000) (Equation 5):

$$CKI\,\% = \frac{Initial\ Population - Final\ Population}{Initial\ Population} x100$$

For effect of H_2O_2 (0.3 M) stress and temperature freeze thawing on verotoxin production, the Duoperth kit (Merck, SA) antibody-based rapid slide agglutination assay was employed according to the manufacturer's instructions. The H_2O_2 stressed or freeze thawed bacterial isolates from three different sets of TSA (-5, -18, and

-28°C isolates) were first cultured in 1 ml casaminoacid yeast extract (CAYE) broth and incubated at 37°C at 100 rpm for 24 h. After incubation, 10 µl of the broth culture (approximately 1 x 10^7 cells/ml) was inoculated into fresh CAYE broth and further incubated for 16 h at 100 rpm at 37°C, then centrifuged at 5000 rpm/5 min, cell pellets retained, washed thrice with phosphate buffered saline (PBS, 5 ml), then suspended in 0. 25 ml 0.01 M Tris-HCl (pH 7.5). To the pellets, 0.5 ml distilled water containing 50 µg/ml polymyxin B was added and the suspension incubated at 37°C for 30 min. Culture suspension (200 µl) was then transferred onto the test device using a sterile Pasteur pipette and result read after 10 min. The appearance of red bands on the vtx1 or vtx2 bands denoted the presence of either one of or both verotoxins.

Statistical analysis

Triplicates of values obtained were recorded as ± SEM using the SIGMAPLOT 8.0 statistical software at $p \leq 0.05$.

RESULTS

Confirmation of isolates

The isolates were Gram-negative coccobacilli, tiny, blue, mucoid colonies on EMB, pink colonies diffused into the medium on LAM and all were haemolytic on sheep blood agar. All fermented triglyceride (EST), glucose (Gluc), citrate (Cit), arginine (ADH), urea (URE), and tryptophane (IND) and produced nitrates (NO_3). They were oxidase (O) negative, sulphide negative and indole positive.

Effect of ionic salt concentrations and other chemicals on bacterial viability and production of virulence factors

Results showed that majority of the isolates did not show any significant (p>0.05) CKI values except for one isolate from treatment plant waste water; *A. haemolyticus* FEW2iv (CKI 42.86 ± 0.032% against 8% ethanol) one isolate from abattoir wastewater; *A. haemolyticus* FSE1iv (CKI 53.85 ± 0.001% against 0.03% crystal violet), and two isolates from river Berg; *A. haemolyticus* RBD1i, and *A. haemolyticus* RBI1i with respective CKI values of 56.06 ± 0.052, and 62.86 ± 0.005 against 0.03% crystal violet. *A. haemolyticus* RBI2i also showed a significant (p > 0.05) CKI value (42.25 ± 0.000) against 8% ethanol All the control isolates (*A. haemolyticus* 19002) showed significant (p > 0.05) CKI values of 34.75 ± 0.022 (against 0.03% crystal violet), 34.88 ± 0.000 against 8% ethanol, 43.13 ± 0.041 (against 4% NaCl) and 57.69±0.009% (against 0.3% bile salt) (Table 1).

Antimicrobial susceptibility and effect of low temperature stress on verotoxin production and bacterial viability

Results showed that most of the isolates from waste water sources were resistant to between 3 to 5 antibiotics including tetracycline (TE), gentamicin (CN), cefuroxime (CXM), ampicillin (AMP), nalidixic acid (NA), and ofloxacin (OFX) (Table 2).

Low temperature treatments (-5, -18 and -28°C) did not have any significant (P > 0.05) effect on either verotoxin production. Results also showed that the low temperature freeze-thaw stress conditions applied had no effect on the viability of the isolates as there was no significant differences in the initial cell population (ICP) and the final cell population (FCP) with the waste water samples consistently maintaining significantly (P > 0.05) higher population of viable cells as compared to the river water samples (Table 2).

Effect of oxidative stress on surface hydrophobicity, haemolysin production and serum resistance of bacterial isolates

Results (Table 3) showed that at 0.3 M H_2O_2 the least HI value (0.009 ± 21,) was exhibited by *A. haemolyticus* PST2ii (from abattoir wastewater) and the highest value (0.789 ± 31) was exhibited by *A. haemolyticus* PSW2ii (from Athlone Treatment Plant wastewater). At 0.1M and 0.3 M H_2O_2, the respective HI values of 0.651±12 and 0.526 ± 25 for *A. haemolyticus* RWW1v (from Athlone wastewater) was recorded compared to 0.172 ± 00 (0.1M H_2O_2) and 0.023 ± 37% (0.3 M H_2O_2) of *A. haemolyticus* RBI2i (from river water). The highest SRI values of 85.23 ± 23, 76.42 ± 67 and 73.36 ± 27% were recorded for *A. haemolyticus* RWW1v, *A. haemolyticus* PSW2i and *A. haemolyticus* FSE1iv from treatment plant and abattoir waste waters compared to 67.60 ± 01% for *A. haemolyticus* RBD1i from river Berg. Similarly for HU 50%, *A. haemolyticus* PSW2i and *A. haemolyticus* PST2i from waste water and abattoir water recorded the highest values of 83.21±13 and 78.45 ± 31% respectively compared to the highest value of 58.12 ± 01% for *A. haemolyticus* RBD1iii from river Berg. The control isolate *A. haemolyticus* 19002 consistently showed the least values for HI, SRI, HU and CVI (Table 3). Results showed that the wastewater isolates were less affected by H_2O_2 and sheep blood erythrocytes/serum as compared to river water isolates.

DISCUSSION

Cell kill index (CKI) values give the degree of lethality of chemical agents and salts against the bacteria tested in this study; high CKI values indicate susceptibility, low CKI values indicates resistance to the chemicals tested. Though generally there was insignificant lethal effect against all the isolates, crystal violet exerted the highest lethal effect followed by ethanol and bile salt, NaCl exerted the least effects compared to crystal violet and ethanol. Also, wastewater isolates demonstrated significantly the highest rate of resistance (low CKI

Table 2. Effect of low temperature stress on verotoxin production among environmental *A. haemolyticus* isolates.

Acinetobacter isolates	Antibiotic resistance pattern	Temperature (°C) stress treatment/ verotoxin status and viability											
		37 (non treated)			5			-18			-28		
		Vtx1	Vtx2	ICP/ml	Vtx1	Vtx2	FCP/ml	Vtx1	Vtx2	FCP/ml	Vtx1	Vtx2	FCP/ml
Treatment plant wastewater													
A. haemolyticus RWW1v	TE, CN, CXM, AMP	-	-	5200	-	-	5200	-	-	5200	-	-	5100
A. haemolyticus PSW2i	AK, CXM,	+	-	6300	+	-	6300	+	-	6200	+	-	6200
A. haemolyticus PSW2ii	OFX, NA, AMP,TE	-	-	5200	-	-	5200	-	-	5000	-	-	5000
A. haemolyticus FEW2iv	AML	-	-	4820	-	-	4820	-	-	4700	-	-	4700
Abattoir wastewater													
A. haemolyticus FSE1iv	-	+	+	2600	+	+	600	+	+	2400	+	+	2600
A. haemolyticus FSE1v	AML, NA	-	+	5300	-	+	5300	-	+	5100	-	+	5300
A. haemolyticus PST1i	AMP, CN, OFX,NA,CN	+	-	6200	+	-	6200	+	-	6120	+	-	6000
A. haemolyticus PST2i	TE, CL	-	-	4000	-	-	4000	-	-	3820	-	-	4600
A. haemolyticus PST2ii	-	-	-	6000	-	-	6000	-	-	6000	-	-	5820
River Berg water	-												
A. haemolyticusRBD1i	-	+	-	3300	+	-	3300	+	-	3300	+	-	3100
A. haemolyticusRBD1ii	-	-	-	6340	-	-	6340	-	-	6240	-	-	6200
A. haemolyticusRBD1iii	OFX, NA	-	-	4700	-	-	4700	-	-	4500	-	-	4400
A. haemolyticusRBI1i	-	-	-	7000	-	-	7000	-	-	6700	-	-	7800
A. haemolyticus RBI2i		-	-	2000	-	-	2000	-	-	1800	-	-	2820
Control	TE												
A. haemolyticus 19002		-	-	4000	-	-	4000	-	-	3860	-	-	3800

SXT-sulphomethaxazole/trimethoprim; AMP-ampicillin; ATM-aztreonam; TE-tetracycline; AK-amikacin; OFX-ofloxacin; CAZ-ceftazidim; CL-cephalexin; CRO-ceftriazone; CXM-cefuroxime; AML-amoxycillin; CN-gentamicin; CFM-cefixime; CIP-ciprofloxacin; NA-nalidixic acid) +(positive); - (negative); ICP-Inicial cell population; FCP-final cell population.

values, P>0.05) compared to river water isolates. The demonstration of less sensitivity by *A. haemolyticus* to various stress conditions indicates potential to survive or rapidly adapt to harsh environmental conditions and chemical agents. This is the first report of presence of and resistance (to antibiotics and oxidative stress) among environmental isolates of *A. haemolyticus* in South Africa. Yuk and Marshall, (2003) reported that stress due to change in salts or chemical concentrations and freeze-thawing can compromise the integrity of bacterial cell walls and membranes resulting in the alteration of the protoplasmic content, conformational changes and structural damages. However, absence of any significant change in the cell population and physiological functions of bacteria in this study indicates minimal or absence of damage on their cell walls. Sodium chloride is one of the most important food adjuncts used for food preservation.

Table 3. Haemolysin unit (HU), Serum resistance index (SRI), and effect of hydrogen peroxide cell surface hydrophobicity (HI) and low temperature stress on *A. haemolyticus* viability (CVI).

A. haemolyticus isolates	Hydrophobicity index (HI, %) /molar concentrations (M) of H_2O_2			SRI (%)	HU 50%
	0.1M	0.2M	0.3M		
Treatment plant wastewater					
A. haemolyticus RWW1v	0.651±12	0.633±10	0.526±25	85.23±23	67.23±43
A. haemolyticus PSW2i	0.833±00	0.796±02	0.788±00	76.42±67	83.21±13
A. haemolyticus PSW2ii	0.906±04	0.183±00	0.789±31	43.76±23	65.21±00
A. haemolyticus FEW2iv	0.753.00	0.602.23	0.585.00	19.96±00	23.44±00
Abattoir wastewater					
A. haemolyticus FSE1iv	0.418±11	0.306±00	0.204±00	73.36±27	57.68±06
A. haemolyticus FSE1v	0.511±00	0.504±31	0.489±23	58.48±11	64.51±04
A. haemolyticus PST1i	0.669±23	0.632±01	0.602±43	68.66±34	45.87±11
A. haemolyticus PST2i	0.774±41	0.731±00	0.625±01	34.74±00	78.45±31
A. haemolyticus PST2ii	0.136±03	0.003±00	0.009±21	48.78±00	22.67±22
River berg water					
*A. haemolyticus*RBD1i	0.462±32	0.372±12	0.152±00	67.60±01	24.66±00
*A. haemolyticus*RBD1ii	0.227±25	0.172±14	0.031±00	48.46±34	38.33±00
*A. haemolyticus*RBD1iii	0.127±23	0.087±01	0.067±23	24.98±18	58.12±01
*A. haemolyticus*RBI1i	0.439±22	0.282±00	0.131±21	33.47±00	26.87±21
A. haemolyticus RBI2i	0.172±00	0.08±15	0.023±37	48.36±00	12.56±34
Control					
A. haemolyticus 19002	0.472.±00	0.183±13	0.114±00	22.56±09	10.83±22

Resistance to antibiotics among bacterial pathogens especially multidrug resistant nosocomial infections among *Acinetobacter* spp. is of current global concern (Lee et al., 2007). Savov et al. (2002) reported resistance among *A. baumanni, A. lwoffii, A. junii* and *A. johnsonii* to various antibiotics including ampicillin, cephalothin, carbenicillin, gentamicin, amikacin, chloramphenicol, tetracycline, co-trimoxazole, ciprofloxacin and cefoperazone. In this study, *A. haemolyticus* demonstrated the potential to be multidrug resistant.

Verotoxin production among *A. haemolyticus* isolates was first reported by Grotiuz et al. (2006). This is the first report on verotoxic *A. haemolyticus* isolates in South Africa and to our knowledge there is no report on effect of stress on these bacteria. In this study, low temperature stress had no effect on both bacterial population and verotoxin production. There was no significant difference between the initial viability index of isolates at -5°C compared to the final viability at -28°C, an indication of little or no effect of freeze-thaw temperatures on the bacterial cell population. During the handling of food and food ingredients from the farm to table, food-borne bacteria are exposed to different suboptimal physical and chemical environments including low temperature storage. Resistance to oxidative stress, antibiotics or suboptimal temperatures as demonstrated in this study might lead chemotherapeutic challenges in situations where the bacteria become sources of outbreaks (Ray and Bhunia, 2008). Resistance demonstrated by verotoxin producing *A. haemolyticus* in this study is worrisome because of the potential dangers associated with verotoxic resistant bacteria especially in environments with poor sanitation and inadequate potable water such as obtainable in developing countries. Outbreak of infections associated with this class of bacteria will have very serious health implications.

While surface hydrophobicity is one of the virulence factors which contribute to the adherence of microorganisms to host tissue (Wojnicz and Jankowski, 2007; Hedge et al., 2009), previous studies have shown correlation between this factor and bacterial adherence to epithelia (Jahnn et al. 1981; Hedge et al., 2009). The retention of surface hydrophobicity - demonstrated by low hydrophobicity index (HI%) values - by most isolates from this study means that the isolates are likely to adhere more to epithelial cells even if challenged with similar concentrations of salts or chemicals. Serum resistance on the other hand confers Gram-negative bacteria with the ability to resist the lytic effects of serum and to invade and survive in the human bloodstream (La Regione and Woodward, 2002). Surface hydrophobicity and serum

resistance is mediated by cell surface polysaccharides and proteins respectively whose specific roles remains poorly understood (Cross et al., 1986). In this study, isolates demonstrated high SRI and HU 50% values, an indication of the potential to resist host defense mechanisms.

The control cultures showed low HU 50% and SRI values indicating that they were more susceptible to stress than the environmental isolates. This might be due to the fact that the control isolates were not exposed to similar physiochemical conditions as the isolates from the wastewater or river waters investigated. Control isolates however also demonstrated low HI values and indication that they also have surface adherence potentials.

Conclusion

A. haemolyticus in this study exhibited resistance to oxidative stress conditions and some antibiotics as well as, freeze-thawing. Contamination of food and drinking water with these strains might mean the proliferation of more virulent and resistant nosocomial infections including severe bloody diarrhea. Diarrhea is of particular concern to developing countries with over 5 million cases and over 3 million deaths annually. More effective environmental monitoring and risk assessment studies of environmental and hospital wastes and survey of resistant strains of these bacteria should be considered.

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