Detection of *vanA* and *vanB* genes in vancomycin-resistant enterococci (VRE) from groundwater using multiplex PCR analysis

Collins Njie Ateba, Kgothatso Pontsho Lekoma and David Tonderai Kawadza

ABSTRACT

A total of 22 groundwater samples were randomly collected from three rural communities in the Mafikeng area. Bile esculin agar was used for selective isolation of enterococci. Standard preliminary tests (Gram staining, oxidase test, catalase test) and confirmatory tests (Prolex™ Streptococcal Grouping Rapid Latex Agglutination test kit) were used to determine the identities of presumptive enterococci. The antibiotic sensitivity test was performed on all positively identified enterococci; percentage resistance and multiple antibiotic resistance (MAR) phenotypes were generated. Multiplex polymerase chain reaction (PCR) was performed to detect *vanA* and *vanB* genes vancomycin-resistant enterococci (VRE). A total of 179 enterococci were positively identified and the proportion of isolates from Dibate (62.5%) was higher compared to those from Majemantsho and Motlhabeng (22.3 and 15.0, respectively). A large proportion (81.5 to 100%) of the isolates from Dibate, Motlhabeng and Majemantsho were resistant to ampicillin, vancomycin and penicillin G. Two main MAR phenotypes, PG-VA-Ap-A-OX and PG-VA-Ap-OX, were identified. Multiplex PCR analysis of 50 VRE indicated that 17 (34%) were positive for *vanA* and *vanB* genes. This highlights the need to determine the cause of vancomycin resistance in enterococci in the sampled sites and suggests that sequence analysis be used to confirm the identities of these amplicons.

Key words | antibiotic resistance, enterococci, multiplex PCR analysis, *vanA*, *vanB*, vancomycin-resistant enterococci (VRE)

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INTRODUCTION

Life depends on water, therefore the supply of safe drinking water to communities is very important (Meays *et al*. 2004). Access to safe drinking water is a fundamental human need and therefore a basic right of every individual (WHO 2003). About 3.3 million South African citizens had been identified to be living without access to potable water and 15.3 million did not have access to adequate sanitation (Council for Scientific and Industrial Research 2008). These largely include residents from rural communities who rely on water from alternative sources, such as rivers, dams and boreholes for drinking, and household and recreational activities (Chamaille-Jammes *et al*. 2007).

In most rural communities in South Africa, groundwater sources are usually constructed very close to pit toilets, as observed during collection. This setting facilitates constant contamination of these water bodies with microbes of faecal origin, such as *Escherichia coli*, *Enterococcus* species and *Salmonella* species, and this renders the water unsafe for drinking (Momba *et al*. 2003, 2005, 2006). In communities such as Motlhabeng, Dibate and Majemantsho in the North West Province of South Africa, the situation is not different since residents with low incomes obtain water from boreholes. The consumption of contaminated groundwater could serve as a potential source for the transfer and

doi: 10.2166/wh.2013.037

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spread of water-borne infections and antibiotic-resistant bacterial strains to humans (Sorum & L'Abeer-Lund 2002).

Enterococci are indicators that can be used to trace faecal contamination in water (Sapkota *et al.* 2007). In the past, vancomycin was used as the last line of treatment for enterococcal infections (Gambaratto *et al.* 2000; Reeves & Grant 2004). The emergence of vancomycin-resistant enterococci (VRE) and the problems caused by VRE in both human and veterinary medicine has been felt in South Africa and even in countries with more advanced health care facilities (Derby *et al.* 1998; von Gottberg *et al.* 2000; Rice 2001; Kühn *et al.* 2005). The presence of multiple antibiotic-resistant *Enterococcus* species in untreated groundwater sources in rural areas may therefore pose health risks to individuals in these communities (Murray 1990; Leclercq 1997; Takeuchi *et al.* 2005). Moreover, *Enterococcus* species have the potential to acquire and transfer resistant determinants to other bacterial species (Gambaratto *et al.* 2000). Vancomycin is not used in the area but a previous study that was conducted revealed the presence of VRE based on phenotypic antibiotic tests (Ateba & Maribeng 2011). However, it has been reported that polymerase chain reaction (PCR)-based techniques are efficient and reliable for the detection of vancomycin-resistant determinants in VRE (Patel *et al.* 1997; Fluit *et al.* 2001; Kiem *et al.* 2003). This study was designed to isolate and screen the VRE species from groundwater obtained from villages around Mafikeng in the North West Province of South Africa for the presence of *vanA* and *vanB* resistance gene determinants.

METHODS

Sampling site

Water samples were collected from boreholes in three villages around Mafikeng. Houses in Motlhabeng, Dibate and Majemantsho were randomly selected and groundwater samples were collected at different times.

Sample collection

Twenty-two water samples were collected during the winter season of 2011 into sterile 1 litre Duran Schout bottles from boreholes in Motlhabeng, Dibate and Majemantsho villages using sterile techniques. After each collection, the water samples were properly labelled and immediately transported on ice to the microbiology research laboratory in the Department of Biological Sciences for analysis. Tap water was used as a control during analysis.

Analysis of water samples using conventional microbiological techniques

Isolation of *Enterococcus* species

Water samples were analysed immediately upon arrival in the laboratory. Analysis was performed according to *Standard Methods* (APHA 1995). An aliquot of 50 mL from each sample was filtered using 0.45 μm grid filter units (Watman Glass Microfiber GS Filter paper) on a vacuum water pump machine (model Sartorius 16824). These grid filter units were placed on the surfaces of solidified bile esculin agar using a sterile forceps and plates were incubated aerobically at 37 °C for 24 h. Typical black colonies were sub-cultured on bile esculin agar plates and plates were incubated aerobically at 37 °C for 24 h. Presumptive *Enterococcus* isolates from each sample were randomly selected, subcultured on nutrient agar for long-term storage at room temperature and their identities confirmed using primary and secondary identification tests.

Enterococcus control strains

Enterococcus faecalis (ATCC 29212) and *Enterococcus faecium* (ATCC 6569) were used as positive controls during all experiments.

Bacterial identification

Presumptive isolates were identified using the following criteria:

- Cellular morphology
- Gram staining.

Presumptive isolates were Gram stained using standard methods (Cruikshank *et al.* 1975). Isolates that were

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Gram-positive cocci were subjected to both preliminary and confirmatory identification tests for *Enteroccocus* species.

Preliminary biochemical identification tests for *Enterococcus* species

Oxidase test

The oxidase test reagent (Pro-Lab Diagnostics, UK) was used in performing the test. In carrying out this procedure, a sterile wire loop was used to transfer a colony onto a filter paper (Whatman International Ltd, UK) and a drop of oxidase reagent was added to it. A sterile wire loop was used to mix the contents on the filter paper and results were read within 30 s. The formation of purple colour indicated a positive test while no colour change was regarded as a negative reaction. Enterococci are oxidase negative, thus all isolates that were oxidase negative were retained for confirmatory biochemical tests.

Catalase test

The catalase test is designed to detect the presence of the catalase enzymes in most aerobic and facultative anaerobic bacteria that contain the cytochrome system. Enterococci and streptococci are exceptions and do not possess the enzyme. This enzyme is responsible for protecting bacteria from hydrogen peroxide (H_2O_2) accumulation, which can occur during aerobic metabolism. Catalase enzymes decompose hydrogen peroxide to water and oxygen. The catalase test was performed by adding a drop of 2% hydrogen peroxide on viable presumptive *Enterococcus* cells that were placed on a clean microscope slide. *Enterococcus* species are catalase negative hence all isolates that satisfied this preliminary identification criterion were subjected to further confirmatory tests.

Confirmatory biochemical test for enterococci

Serotyping

A Prolex™ Streptococcal Grouping Latex test kit obtained from PRO-LAB Diagnostics, UK, was used for serological identification of *Enteroccocus* spp. into Lancefield groups D and G. The test was performed by reacting a cell wall specific carbohydrate extract of isolates with nitrous acid reagents.

Screening to determine the antibiotic-resistant profiles

Antibiotic susceptibility test

Antibiotic susceptibility tests were performed on all *Enteroccocus* species to determine their antibiotic-resistant profiles using the Kirby–Bauer disc diffusion technique (Kirby *et al.* 1966). Before antibiotic sensitivity testing, the isolates were revived by subculturing onto bile esculin plates, then plates were incubated aerobically at 37 °C for 24 h. Bacterial suspensions were prepared using pure isolates and aliquots of 100 μL from this suspension were spread plated on Mueller Hinton agar (Biolab, Merck, South Africa). The susceptibilities of the isolates were determined against a panel of eight different antimicrobial agents using discs obtained from Mast Diagnostics, UK. The antibiotic discs were gently pressed onto Mueller Hinton agar plates to ensure intimate contact with the surface and plates were incubated aerobically at 37 °C for 24 h (NCCLS 1999). The antibiotic inhibition zone diameters were measured and results obtained were used to classify isolates as being resistant, intermediate resistant or susceptible to a particular antibiotic based on standard reference values (NCCLS 1999). Table 1 indicates the different antibiotics used, their concentrations and standard reference values.

Table 1 | Details of antibiotics used in the study

Group	Antibiotics	Disc conc.	*R*	*I*	*S*
Aminoglycosides	K	30 μg[c]	≤13	14–17	≥18
Beta-lactams	Ap	10 μg[a]	≤11	12–14	≥15
	A	10 μg[a]	≤11	12–14	≥15
	GM	10 μg[a]	≤12	–	≥13
	PG	10 μg[a]	≤11	12–14	≥15
Glycopeptides	VA	30 μg[c]	≤9	10–11	≥12
Phenols	C	30 μg[c]	≤12	13–17	≥18
Macrolides	E	15 μg[b]	≤13	14–22	≥23

Ap, ampicillin; VA, vancomycin; C, chloramphenicol; K, kanamycin; GM, gentamicin; PG, penicillin; A, amoxicillin; E, erythromycin.
Superscripts [a] to [c] indicate the generally accepted concentrations of the discs according to the standard method stipulated by the manufacturer, Mast Diagnostics, Merseyside, UK.

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Multiple antibiotic resistance (MAR) phenotypes

MAR phenotypes were generated for isolates that were resistant to three and more antibiotics (Rota *et al.* 1996). Phenotypes were generated using the abbreviations that appear on antibiotic discs.

Molecular characterisation

Extraction of genomic DNA

DNA was extracted from all isolates that were resistant to vancomycin and control strains using Seeplex® VRE ACE Detection extraction kit (Seegene, Rockville, USA). Nutrient broth cultures were prepared for all isolates that were resistant to vancomycin. In preparing the broth, 6 μg/mL of vancomycin was added into the broth as indicated by the manufacturer (Seegene). The broths were incubated at 37 °C for 24 h while shaking. DNA was extracted following the manufacturer's protocol and 3 μL of the DNA extract from supernatant was used for VRE ACE specific multiplex PCR analysis.

PCR analysis

All isolates that were resistant to vancomycin were subjected to a multiplex PCR analysis for the detection of the presence of vancomycin-resistant genes (*vanA* and *vanB*) using the Seeplex® VRE ACE multiplex detection system (Seegene). PCR reactions were prepared in 20 μL volumes including 1X VRE PM solution, 1X multiplex master mix, 3 μL of template DNA and 3 μL of 8-Mop solution. During PCR reactions a no-template tube was used as a negative control while 3 μL of VRE PC provided by the manufacturer was used as a positive control. Amplifications were performed on a DNA thermal cycler (model-PTC-220 DYAD™ DNA engine) following the manufacturer's instructions. PCR cycling was performed with an initial denaturation at 94 °C for 15 min; 35 cycles of 94 °C for 30 s, 60 °C for 60 s and 72 °C for 60 s. A final elongation step was performed at 72 °C for 10 min. The PCR products were kept at 4 °C until electrophoresis.

Electrophoresis

The DNA extracted was resolved by electrophoresis on 1% (w/v) while PCR fragments were separated on a 2% (w/v) agarose gel, respectively, using a horizontal system. The use of a 2% (w/v) agarose gel was based on instructions from the manufacturer of the kit that was used for PCR amplifications (Seegene). Electrophoresis was performed at 60 V for 5 h using 1X TAE buffer (40 Mm Tris, 1 Mm EDTA and 20 Mm glacial acetic acid, pH 8.0). Each gel contained a 100 bp DNA molecular weight marker (Fermentas, USA). The gels were stained in ethidium bromide (0.001 μg/mL) for 15 min and amplicons were visualised under UV light at 420 nm wavelength (Sambrook *et al.* 1989). A Gene Bio Imaging System (Syngene, Synoptics, UK) was used to capture images using Gene Snap (version 6.00.22) software. Gene Tools (version 3.07.01) software (Syngene) was used to analyse the images in order to determine the relative sizes of the amplicons.

RESULTS AND DISCUSSION

Prevalence of *Enterococcus* spp. in groundwater samples

Twenty-two groundwater samples comprising 11, six and four samples from Dibate, Motlhabeng and Majemantsho villages, respectively, were collected directly from boreholes. These samples were analysed for the presence of *Enterococcus* species. The proportion of isolates that satisfied both the preliminary and confirmatory tests for *Enterococcus* species are shown in Table 2. The results in Table 2 indicate that the

Table 2 | The percentages of *Enterococcus* spp. isolated from three sampling sites. The percentages were obtained by dividing the number of a particular site by the total number of isolates obtained

Sample area	No. isolated	% of *Enteroccus* spp. isolated
Dibate	112	62.5
Motlhabeng	27	15.0
Majemantsho	40	22.3
Total	179	100

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Table 3 | Percentage of antibiotic resistance of *Enterococcus* spp. isolated from different sampling sites

Sampling site	Ap	VA	C	K	GM	PG	A	E
Dibate								
(NT = 112)	101	105	5	11	0	112	110	42
% Rest	90.2	93.8	4.5	9.8	0	100	98.2	37.5
Motlhabeng								
(NT = 27)	22	24.0	4.0	0	3	25	21	20
% Rest	81.5	88.9	14.8	0	11.1	92.6	77.8	62.9
Majemantsho								
(NT = 40)	35	37	2	1	0	37	24	24
% Rest	87.5	92.5	5.0	2.5	0	92.5	60.0	60.0

Ap, ampicillin; VA, vancomycin; C, chloramphenicol; K, kanamycin; GM, gentamicin; PG, penicillin; A, amoxicillin; E, erythromycin.

proportion of *Enterococcus* spp. isolated from Dibate (62.5%) was higher compared to those from Majemantsho and Motlhabeng (22.3 and 15.0, respectively). During analysis, all the isolates that grew on the selective culture medium were selected and from these results it is evident that the contamination with enterococci was higher in Dibate.

Percentage antibiotic resistance of *Enterococcus* spp. isolated from groundwater

The number of enterococci that were resistant to the different antibiotics from the various sampling sites was determined. Values obtained were expressed as percentages and results were as shown in Table 3. A large proportion (81.5 to 100%) of the isolates from Dibate, Motlhabeng and Majemantsho were resistant to ampicillin, vancomycin and penicillin G when compared to those that were resistant to chloramphenicol and kanamycin (4.5 to 9.8%). However, none of the isolates from Dibate and Majemantsho were resistant to gentamycin. The identification of VRE was a cause for concern.

MAR phenotypes *Enterococcus* spp. isolated

The antibiotic-resistant phenotypes for *Enterococcus* spp. isolated from groundwater obtained from different sampling sites are shown in Table 4. Generally, the two main phenotypes PG-VA-Ap-A-OX and PG-VA-Ap-OX were identified for isolates obtained from Dibate, Motlhabeng and Majemantsho. A large proportion of the isolates (85.2 to 98.2%) from these sampling sites exhibited the phenotype PG-VA-Ap-A-OX.

Table 4 | The multiple antibiotic-resistant (MAR) phenotypes of *Enterococcus* spp. isolated from the different sampling sites. MAR phenotypes were expressed using the abbreviation letters as they appear on the antibiotic paper discs

Sampling site	Phenotype	No. observed	Percentage observed (%)
Dibate (NT = 112)	PG-VA-Ap-A-OX	110	98.2
	PG-VA-Ap-OX	2	1.8
Motlhabeng (NT = 27)	PG-VA-Ap-A-OX	23	85.2
	PG-VA-Ap-OX	4	14.8
Majemantsho (NT = 40)	PG-VA-Ap-A-OX	36	90
	PG-VA-Ap-OX	4	10

Ap, ampicillin; VA, vancomycin; C, chloramphenicol; K, kanamycin; GM, gentamicin; PG, penicillin; A, amoxicillin; E, erythromycin.

Molecular characterisation

DNA extraction

Chromosomal DNA was extracted from 50 *Enterococcus* species that were resistant to vancomycin and a 1% (w/v) agarose gel is shown in Figure 1. The isolates consisted of 30 VRE from Dibate, and 10 from Majemantsho and Motlhabeng, respectively. The DNA was of good quality and with no fragmentation.

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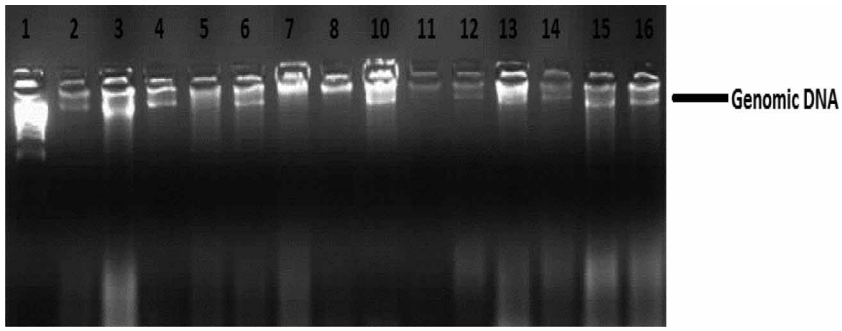

Figure 1 | Lane 1 = 100 bp DNA molecular marker; lanes =2–16 DNA extracted from VRE isolated from groundwater samples from the different sampling areas.

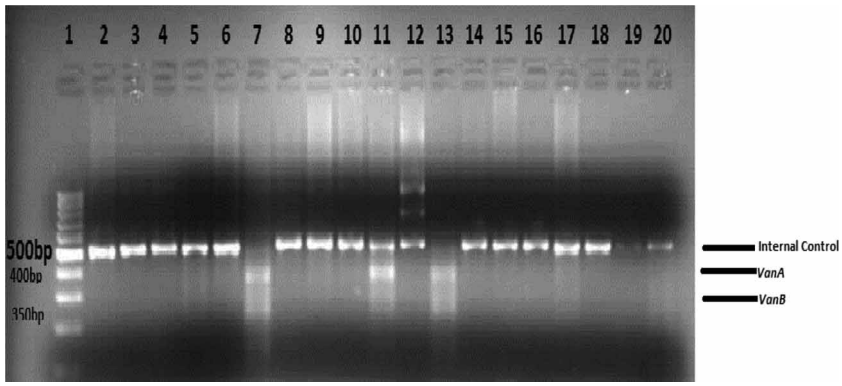

Figure 2 | Multiplex PCR analysis of the *vanA* and *vanB* genes from enterococci. Lane 1 = 100 bp DNA molecular weight marker; lanes 2–20= isolates screened for *vanA* and *vanB*; lanes 7, 11, 12 and 13: positive for *vanA* and *vanB*.

Multiplex PCR analysis

Multiplex PCR analysis for the presence of *vanA* and *vanB* using chromosomal DNA from the 50 vancomycin-resistant isolates indicated that 17 (34%) were positive for these genes. Despite the fact that not all the isolates that were phenotypically resistant to vancomycin possessed the genes screened, the presence of the *vanA* and *vanB* in some isolates was a cause for concern. Vancomycin is not used in human and veterinary medicine in the area and this highlights the need to determine the cause of vancomycin resistance in enterococci in the sampled sites. These isolates could potentially facilitate the transfer of resistant genes in the area. Figure 2 indicates a 2% (w/v) agarose gel of the vancomycin-resistant genes that were amplified. As shown in Figure 2, the isolates on lanes 7, 11, 13 and 17 had both the *vanA* and *vanB* gene fragments. However, from the isolate on lane 12 the *vanA* and *vanB* gene fragments were on the opposite side of the internal control.

CONCLUSIONS

All water samples collected from the different villages tested positive for *Enterococcus* species and this indicates the need to supply potable water to individuals who live in these villages, especially Dibate, so as to reduce the health risk for consumers. The identification of multiple antibiotic-resistant enterococci was a cause for concern. Moreover, the detection of both the *vanA* and *vanB* gene fragments indicated that these isolates could pose a severe challenge to the medical and veterinary professions (Fisher & Phillips 2009). It is therefore suggested that sequence analysis be used to confirm the identities of these amplicons.

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ACKNOWLEDGEMENTS

This study was supported by funding provided by the North West University – Mafikeng Campus. The contribution received from colleagues of the Faculty of Agriculture Science and Technology and the Department of Biological Sciences is greatly appreciated.

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First received 21 February 2013; accepted in revised form 18 July 2013. Available online 27 August 2013

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