Isolation of Group B *Streptococcus* in groundwater in the North West Province, South Africa

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Abstract: The normal hosts of *Streptococcus agalactiae* are humans and animals and are highly predominant as normal flora in the female genital tract, male urethra, pharynx as well as in gastrointestinal tract. However, *Streptococcus agalactiae* has been associated with a number of complications in humans ranging septicaemia, pneumonia, meningitis to still birth. The aim of study was to isolate and characterize antibiotic resistant *Streptococcus agalactiae* from groundwater in selected areas within the Northwest Province. A total of 25 groundwater samples were randomly collected from selected areas around the Northwest Province. Brain Heart Infusion (BHI) agar was used to isolate *Streptococcus* species. Standard preliminary tests (Gram staining and catalase test) and confirmatory tests (Prolex™ Streptococcal Grouping Rapid Latex Agglutination test kit) were used to determine the identities of *Streptococcus agalactiae*. The antibiotic susceptibility profiles of the isolates were determined on all positively identified *Streptococcus agalactiae.* A large proportion (52.5% to 87.5%) of the isolates from Dibate, Ramosadi, Vryburg and Motlhabeng and only 35% of those from Taung were Gram positive cocci. All the isolates from the different sampling stations were catalase negative. Similarly, all the isolates from Taung, Vryburg, Motlhabeng, Stella and Setlagole were oxidase negative. Large proportions (50% to 90%) of the isolates tested were positively identified using the latex agglutination test. A large proportion (71 -100%) of isolates from Taung, Setlagole, Stella and Motlhabeng were resistant to ampicillin and penicillin. Moreover, all the isolates from Stella and Taung were resistant to vancomycin and tetracycline. Despite the fact that a large proportion (75%) of the isolates from Taung were resistant to chloramphenicol, only a small proportion (23% to 37.5%) of the isolates from Stella, Setlagole and Dibate were resistant to this antibiotic. However, none of the isolates from Stella and Setlagole were resistant to clarithromycin. A large proportion (84.6% to 100%) of the isolates from Dibate, Motlabeng and Ramosadi were resistant to erythromycin and clarithromycin.

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1. Introduction

Group B *Streptococcus* are Gram positive coccus whose cells occur in chains and they are beta-hemolytic (Lachenauer *et al*., 2002). They are sensitive to bile and are able to hydrolyze sodium hippurate (Lachenauer *et al.,* 2002). These organisms are characterized by the presence of Group B Lancefield antigen. Streptococci occur as normal flora in the gastrointestinal tract of humans and animals (Bergy *et al.,* 1994). However, Group B *Streptococcus* is known to cause pneumonia and meningitis in neonates and their mothers (WHO, 2012). GBS can also colonize the intestine and the female reproductive tract and increasing the risk for premature rupture of membranes that facilitates the transmission of these organisms to the infant (Schuchat, 1998). They also cause septicemia in the elderly and some patients whose immune system are compromised (Tyrrell *et al.,* 2000; Schuchat *et al*., 2002). Based on the aforementioned, GBS infections remain a serious cause of disease and death in newborn babies (CDC, 2010).

Resistance to commonly used antibiotics is an emerging problem among *Streptococcus* species (Amyes, 2007). GBS have been found to be resistant to macrolides such as erythromycin and clindamycin that are considered the drugs of choice for the treatment of infections (Amyes, 2007). Resistance to these antibiotics may result either through the presence of macrolide ribosomal methylases that are encoded by *erm* genes or 14/15- membered macrolide-specific efflux pump coded for by a *mef* gene (Dipersio *et al*., 2011). The *erm* enzyme is known to methylate the bacterial ribosome, thereby reducing its ability to bind to macrolide antibiotics and thus resulting in resistance phenotype (Dipersio *et al*., 2011). Currently the proportion of GBS isolates resistant to clindamycin and erythromycin is rapidly increasing (Manning *et al*., 2003).

The natural hosts of Group B *Streptococcus* are humans and animals and the presence of these organisms in the environment is may be indicative of the hygienic conditions prevailing. Multiple antibiotic resistant strains in particular may present severe health complications in hospitalized and pregnant women.

Therefore, knowledge of the antibiotic resistance profiles of circulating strains would positively indicate the options that are available for treatment. The aim of study was to isolate and characterize antibiotic resistant *Streptococcus agalactiae* from groundwater in selected areas within the Northwest Province.

2. Material and Methods

2.1 Area of study, Sample collection and Laboratory analysis

A total of 25 water samples were collected from some areas around the North West using aseptic techniques. The sample sites and the number of samples obtained are shown in Table 1. The samples were properly labeled and transported to the laboratory for analysis. Aliquots of 100µl was passed through the membrane filter paper which was placed on brain heart infusion agar. The plants were incubated aerobically at 37°C for 24 hours. Isolates were sub-cultured on brain heart infusion agar and plates were incubated aerobically at 37°C for 24. Pure isolates were used for bacteria identification tests.

Table 1: Areas in which water samples was collected

Sample area	Number of samples
Dibate	5
Motlhabeng	5
Ramosadi	5
Taung	4
Stella	1
Setlagole	2
Vryburg	3

2.2 Bacterial identification

Presumptive isolates were identified using the following criteria:

2.2.1 Preliminary test

2.2.1.1 Gram staining test

Isolates were gram stained using standard method or technique (Cruinshank *et al*., 1975). A bacteria smear was prepared by mixing a pure colony with water on a microscope slide. The cells were then fixed by passing slide through flame. The slide was then dipped into crystal violet for 30 seconds and rinsed in tap water. Then it was also dipped into iodine for 30 seconds and rinsed with tap water. The slide was decolorized with 70% alcohol, then immediately flooded with carbul fuchsin for 30 seconds, rinsed with tap water and air dry. The slide was observed under the light microscope under oil immersion. (Cruinshank *et al*., 1975)

2.2.1.2 Catalase test

Using a sterile inoculating loop a small amount of colony was collected from a plate and smeared onto a microscope slide. A drop of 3% H_2O_2 was placed onto the colony and the slide was observed for the formation of bubble.

2.2.1.3 Oxidase test

This test was performed using the TestOxidase™ reagent (PL.390) Mast Diagnostics (Neston, Wirral, U.K.) in accordance with the manufacturer's protocol. With the help of a wire loop, a colony from each compartment in the plate was picked and rubbed onto a filter paper then one drop of oxidase reagent was placed on the organism smear. As observation ensued, positive results showed a colour change which was dark purple smear within 60 to 90 seconds and negative results showed a colourless smear (Neston, Wirral, UK).

2.2.1.4 Latex agglutination test for the detection of *Streptococcus agalactiae*

A Prolex™ Streptococcal Grouping Latex test kit obtained from PRO-LAB Diagnostics – U.K was used for serological identification of *Streptococcus agalactiae* based on specific agglutination reactions with the Lancefield group B antiserum. The test was performed by reacting a cellwall specific carbohydrate extract of isolates with a nitrous acid reagent (positive B antisera). Positive reactions were recorded when extracts agglutinated with specific reagents. However, the A antiserum that was provided in the kit was used as a negative control for the test isolates.

2.3 Antibiotic susceptibility test

Antibiotic susceptibility test was performed on all streptococci to determine their antibiotic resistant profiles using the Kirby-Bauer disc diffusion technique (Kirby *et al*., 1966). Prior to antibiotic sensitivity testing, isolates were revived by sub-culturing onto BHI agar plates. The plates were incubated aerobically at 37°C for 24 hours. Bacterial suspensions were prepared using pure cultures and aliquots of 100µl from these suspensionswere spread on Mueller Hinton agar (Biolab, Merch, South Africa). The susceptibilities of the isolates against a panel of seven different antibiotics obtained from Mast Diagnostics –United Kingdom were determined. The antibiotic discs were gently pressed onto the inoculated Mueller Hinton agar to ensure intimate contact with the surface and the plates were incubated aerobically at 37°C for 24 hours (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 2 indicates the details of antibiotics that were used in the study.

3. Results

3.1 Occurrence of Streptococci species in ground water samples

Twenty five ground water samples were collected from borehole pumps in the North West Province. These samples were analyzed for the

presence of *Streptococcus* species using a series of identification tests. Table 3 indicates the proportions of isolates that satisfied the preliminary and confirmatory identification tests.

Table 2: Details of antibiotics that were used in the study

Group	Antibiotic	Disc conc.	R	I	S
Beta-Lactams	Ap	10µg[a]	≤11	12-14	≥15
	PG	10µg[a]	≤13	14-16	≥29
Glycopeptides	VA	30µg[a]	≤19	10-11	≥12
Tetracyclines	T	30µg[a]	≤14	15-18	≥19
Phenols	C	30µg[a]	≤12	13-17	≥18
Macrolides	E	15µg[a]	≤13	14-22	≥23
	CLA	15µg[a]	≤13	14-22	≥3

The superscript [a] to [d] indicate the generally accepted concentrations of the discs according to the standard method stipulated by the manufacturer, Mast Diagnostic, Merseyside, United Kingdom.

Table 3: The percentages of results obtained from isolates from different sample sites.

Sample station	Gram straining (%) (+)	Catalase Test (%) (-)	Oxidase Test (%) (-)	Serotyping (%) (+)
Motlhabeng	87.5	0	100	50
Dibate	52.5	0	77.5	84
Ramosadi	62.5	0	90	75
Vryburg	62.5	0	100	77.5
Taung	35	0	100	80
Setlagole	100	0	100	90
Stella	100	0	100	50

As shown in Table 3, a large proportion (52.5% to 87.5%) of the isolates from Dibate, Ramosadi, Vryburg and Motlhabeng were Gram positive cocci. Despite the fact that only 35% of the isolates from Taung were Gram positive cocci, none of those isolated from Stella and Setlagole satisfied this criteria. All the isolates from the different sampling stations were negative for the catalase test. Similarly, all the isolates from Taung, Vryburg, Motlhabeng, Stella and Setlagole were oxidase negative. A relatively large proportion (77.5% to 90%) of the isolates from Dibate and Ramosadi were also oxidase negative. A large proportion (50% to 90%) of the isolates were positively identified using the latex agglutination test.

3.2 Antibiotic resistance profiles of isolates

All the isolates that were positively identified using the latex serological test were subjected the antibiotic to determine their antibiotic susceptibility profiles to a panel of seven different antimicrobial agents. Results obtained are shown on Table 3. A large proportion (71 -100%) of isolates from Taung, Setlagole, Stella and Motlhabeng were resistant to ampicillin and penicillin. Moreover, all the isolates from Stella and Taung were resistant to vancomycin and tetracycline. Despite the fact that a large proportion (75%) of the isolates from Taung were resistant to chloramphenicol, only a small proportion (23% to 37.5%) of the isolates from Stella, Setlagole and Dibate were resistant to this antibiotic. However, none of the isolates from Stella and Setlagole were resistant to clarithromycin. A large proportion (84.6% to 100%) of the isolates from Dibate, Motlabeng and Ramosadi were resistant to erythromycin and clarithromycin.

Table 3: Percentage results of Antibiotic resistance of samples from the selected areas.

Sampling site	AP (10)	PG (10)	VA (30)	T (30)	C (30)	E (15)	CLA (15)
Motlhabeng	100	100	77.5	70	42.5	100	92.5
Dibate	67.5	92.5	70	30.	23	84.6	92.5
Ramosadi	70	100	100	30.	0	100	100
Vryburg	25	71	37.5	46	8	29	20
Taung	100	100	100	100	75	62.5	50
Setlagole	100	100	31.3	100	31.3	68.8	0
Stella	100	100	100	100	37.5	0	0

Vancomycin (VA), Ampicillin (AP), Peniciliin (PG), Tetracycline (T), Erytromycin (E), Chloramphenicol (C), Clarithromycin (CLA)

4. Discussions

Water is a very important resource for life and access to safe drinking water is a fundamental human need and therefore a basic right of every individual (WHO, 2003). Faecal pollution of water that is intended for consumption can lead to public health problems due to the presence of infectious microorganisms (WHO, 2003; Blanch *et al.,* 2004). These microorganisms may contaminate water as a result of contact with faeces from humans, animals and untreated sewage (Blanch *et al.,* 2004). Water that is contaminated either by pathogenic microorganisms jeopardizes both physical and social health to consumers (WHO, 2003).

Little is documented about the occurrence of *Streptococcus agalactiae* in groundwater when compared to reports published indicating its presence in humans, fish, cattle and milk (Pereira *et al.,* 2010). Moreover, *Streptococcus agalactiae* is currently an important pathogen frequently associated with complications such as meningoencephalitis in fish, mastitis in cows, and neonatal meningitis in humans (Evans *et al.,* 2002; Mitchell, 2003; Oliveira *et al.,* 2006; Mian *et al.,* 2009). In aquaculture *S. agalactiae* is an emerging pathogen that has been associated with considerable morbidity and mortality in fish farms worldwide (Mian *et al.,* 2009). The ability of *S. agalactiae* to cross the interspecies barrier and therefore making it possible for human-derived strains to infect animals, or vice versa, is properly understood. It is therefore hypothesized that contaminated water and food such as fish may serve as potential sources for the transmission of these pathogens to humans.

The aim of study was to isolate and characterize antibiotic resistant *Streptococcus agalactiae* from groundwater in selected areas within the Northwest Province. This was motivated from the fact that no information on the occurrence of *Streptococcus agalactiae* has been documented in the area. Based on the serological agglutination test all groundwater samples were positive for streptococci. Despite the fact PCR methods have proven to be very reliable in the detection of *Streptococcus agalactiae* from different sources (Meiri-Bendek *et al.,* 2002; Chiang *et al.,* 2008), conventional methods that include serological latex agglutination tests have also been employed in routine identification of these species (Yildirim *et al.,* 2002). In the present study, preliminary large proportions (52.5% to 87.5%) of the isolates were Gram positive cocci. All the isolates from the different sampling stations were catalase negative and similar observations have been reported by Winn *et al*., (2006). Similarly, all the isolates from Taung, Vryburg, Motlhabeng, Stella and Setlagole were oxidase negative. Large proportions (50% to 90%) of the isolates tested were positively identified using the latex agglutination test. Yildrim *et al.,* (2002) reported that the group B-specific antiserum was very reliable in the identification of *Streptococcus agalactiae* when compared to 16S rRNA and the *cfb* gene PCR results performed simultaneous on the test isolates. In present study, *S. agalactiae* was isolated from groundwater that is consumed by individuals in rural areas. Despite the fact that the virulence gene profiles of the isolates were not determined it is suggested that these strains may have severe implications on consumers especially if their immune system is compromised.

Another objective of the study was to determine the antibiotic resistance profiles of the isolates. Emphasis was directed towards the identification of strains that are resistant to multiple antibiotics. A large proportion (71 -100%) of isolates from Taung, Setlagole, Stella and Motlhabeng were resistant to ampicillin and penicillin. Moreover, all the isolates from Stella and Taung were resistant to vancomycin and tetracycline. Despite the fact that a large proportion (75%) of the isolates from Taung were resistant to chloramphenicol, only a small proportion (23% to 37.5%) of the isolates from Stella, Setlagole and Dibate were resistant to this antibiotic. However, none of the isolates from Stella and Setlagole were resistant to clarithromycin. A large proportion (84.6% to 100%) of the isolates from Dibate, Motlabeng and Ramosadi were resistant to erythromycin and clarithromycin.

S. agalactiae isolates obtained in the present study had a high frequency of phenotypic resistance to tetracycline, penicillin, erythromycin and ampicillin. These results are similar to previous studies (Erskine *et al.,* 2002; Duarte *et al.,* 2005). It is therefore suggested that these antibiotics should be used cautiously in the treatment of animal infections in the area unless antimicrobial sensitivity testing is performed. A reasonable proportion of the isolates were resistant to macrolide antibiotics; erythromycin and clarithromycin. The detection of vancomycin resistant strain was a cause for concern.

Isolates that portray positive resistant phenotypes may however be negative for the resistance determinants based on genotypic PCR assays. This may result from the fact that some resistance phenotypes are caused by point mutations rather than gene acquisitions (Anzueto and Norris, 2004). On the contrary, some strains may be phenotypically sensitive to antibiotics but carry resistance genes (Carattoli, 2001). These may be due to the fact that resistance genes may not be expressed if they are distant from a promoter or associated with a weak promoter; resistant genes may not be expressed due to point mutations; and gene cassettes might lack

a promoter in front of the coding sequence (Carattoli, 2001). It is therefore important to perform routine surveillance of antibiotic resistance among bacteria isolates and also ensure that molecular tools are developed which can provide correlations between phenotypic and genotypic resistance data.

In conclusion, most *S. agalactiae* isolates exhibited resistance to at least one antibiotic. It is therefore suggested that molecular methods be used in determining the antibiotic resistance profiles of the isolates.

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