THE ANTIMICROBIAL SUSCEPTIBILITY AND GENE-BASED RESISTANCE OF *STREPTOCOCCUS AGALACTIAE* (GROUP B *STREPTOCOCCUS*) IN PREGNANT WOMEN IN WINDHOEK (KHOMAS REGION), NAMIBIA.

by

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DECLARATION

I, Fredrika Engelbrecht, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

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ABSTRACT

BACKGROUND AND OBJECTIVES: Group B *Streptococci* (GBS) can asymptomatically colonise the vagina and rectum of women. Studies have shown that this bacterium is the leading cause of septicemia, meningitis and pneumonia in neonates. In Namibia no known studies have investigated GBS colonisation and the antibiotic resistance profile of GBS isolates in pregnant women. This study accessed the GBS colonisation rate amongst the pregnant women who attended the Windhoek Central Hospital Antenatal Clinic (Khomas region), in Namibia for a period of 13 months. Furthermore, using the VITEK 2 system, the GBS isolates were tested against the following antimicrobial substances; benzylpenicillin, ampicillin, clindamycin, erythromycin, tetracycline, vancomycin, cefotaxime, ceftriaxone, linezolid and trimethoprim/sulfamethoxazole. Penicillin G is the drug of choice in the majority of studies, and seems to be the most effective drug for intrapartum antibiotic prophylaxis (IAP). All the GBS isolates found in this study were also analysed for the presence of selected genes known to be associated with resistance to key antibiotics using specific primers within a polymerase chain reaction (PCR).

STUDY DESIGN: Participants signed a consent form on recruitment, and were asked to complete a questionnaire. A lower vaginal swab and a rectal swab were collected from each willing participant from 35 weeks gestation onwards.

RESEARCH METHODOLOGY: All collected swabs were cultured on combinations of selective media for GBS isolation. Group B *Streptococci* isolates were then tested against selected antimicrobial substances, using the VITEK 2 system. All GBS strains were also screened for the presence of selected resistance genes [*tet*(M) and *tet*(O), *erm*(A/TR), *mef*(A/E), *aph*A-3 and *aad*-6] by means of the PCR.

RESULTS: The prevalence of GBS colonisation amongst the pregnant women who were screened at the Windhoek Central Hospital Antenatal Clinic in Windhoek during the time period was 13.6%. The median age of the GBS colonised women in this study was 28. Approximately 46.25% of the swabs tested positive for both the vagina and rectum, 36.25% tested positive for the vagina only and 17.5% tested positive for the rectum only. All swabs that were directly inoculated onto Columbia sheep blood agar containing 8 µg/ml of gentamycin powder and 15 µg/ml of nalidixic acid (BCNA) that were positive, were also positive when first enriched in Todd-Hewitt broth followed by BCNA plating (TH-BCNA). Thirty seven of the colonised participants presented positive on the direct inoculated BCNA cultures as well as on the TH-BCNA cultures. Thirty nine of the colonised participants presented with a negative direct inoculated BCNA culture and positive TH-BCNA cultures. The remaining four colonised participant were detected on the Granada agar only. The swabs from the first 419 participant were culture on BCNA and TH-BCNA plates, while the swab from the last 169 participant were culture on pre-prepared BCNA (pBCNA) produced

by bioMérieux, TH-BCNA and Granada media. When comparing the different media used in the thesis, the odds of testing positive are five times higher under the pBCNA, TH-pBCNA and Granada testing media as compared to the BCNA and TH-BCNA and is statistically significant with a p-value of 0.000.

Prevalence rates of 17.8%, 9.5% and 0% were seen amongst single, married and divorced women respectively. The results however, were not statistically significant with a P value of 0.315. When analysing the various levels of education, colonisation rates of 5.96%, 5.6% and 2.2% for women with below matric, matric and tertiary education, respectively were found. These results were also not statistically significant with a P value of 0.729. Employed, unemployed and self-employed women presented with 14.7%, 12.15% and 13.3% prevalence rates respectively, with no statistical significance (P = 0.125). Professionals, skilled and semi-skilled women showed colonisation rates of 19.8%, 11% and 13% respectively, with no statistical significance (P = 0.138). While colonisation rates of 15%, 15.6% and 10.7% were seen amongst women that rent their own house, are rent sharing or own their own house respectively (P = 0.407), with no statistical significance.

No antimicrobial resistance against benzylpenicillin, ampicillin, ceftriaxone, linezolid and vancomycin were seen. Levofloxacin showed 0.9% intermediate resistance, trimethoprim/sulfamethoxazole showed 6% resistance, tetracycline showed 94.1% resistance, erythromycin showed 11.1% resistance; clindamycin showed 8.5% full resistance and 18.8% intermediate resistance against clindamycin was detected. Inducible clindamycin resistance (ICR) was detected in 5.1% of the isolates.

One hundred and fourteen of the 117 samples presented with the gene *tet*(M). The gene *tet*(M) is associated with tetracycline resistance. Three isolates that presented with the *mef*(A/E) gene, also presented with erythromycin resistance. The gene *erm*(A/TR) was seen in one isolate that also presented with inducible clindamycin resistance. Furthermore, the six isolates that tested sensitivity against tetracycline did also present with *tet*(M) genes.

CONCLUSION: Group B *Streptococus* prevalence amongst the screened women in the current study is 13.6%, which is lower than that of our neighbouring countries. None of the investigated risk factors showed any statistical significance. A high resistance to tetracycline was reported which was not surprising, since studies have shown that GBS isolates have a high tetracycline resistance rate.

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DEDICATION

TO: My loving husband, without whom I would not have been able to accomplish this and who believed and supported me through everything, and whom I love and adore with all my heart

DEWALD ENGELBRECHT

AND

Our 3 sons that I love and adore

Hanru, Luan and Caleb Engelbrecht

AND

My loving parents for their example and unfailing love and support

Frikkie and Hester Henn

AND LAST BUT NOT LEAST

My parents in law for all your support and love

Koning and Elmarie Engelbrecht

GLOSSARY

Symbol	Description
°C	Degrees Celsius
+	Positive
-	Negative
(-)	weak negative reaction
(+)	weak positive reaction
%	Percentage
α	Alpha

Abbreviation	Description
AST	Antibiotic sensitivity testing
ATCC	American type culture collection
BCNA	Columbia sheep blood agar containing 8 µg of gentamycin and 15 µg of nalidixic acid/ml of agar.
bp	Base pairs
CDC	Centres for Disease Control and Prevention
CLSI	Clinical Laboratory Standards Institute
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
EOGBS	Early onset Group B *Streptococcus*
erm	Ribosomal methylase
g	Gram
x g	Gravity
GBS	Group B *Streptococcus*
GP	Gram positive
HLR-KM	High level resistance for kanamycin
I	Intermediate resistance
IAP	Intrapartum antibiotic prophylaxis
l	litre
LVS	Lower vaginal swab
$MgCl_2$	Magnesium Chloride
MIC	Minimum Inhibitory Concentration
ml	Milli litre
MLS	Macrolide, Lincosamide and Streptogramin
iMLS	Inducible Macrolide, Lincosamide and Streptogramin resistance
i/cMLS	Inducible or constitutive Macrolide, Lincosamide and Streptomycin resistance
mM	Millimolar
MOPS	Disodium Phosphate Anhidrous

Mwm	Molecular Weight Marker
NCBI	National Centre for Biotechnology Information
NUST	Namibia University of Science and Technology
pBCNA	Pre-prepared Columbia sheep blood agar containing 8 µg of gentamycin and 15 µg of nalidixic acid/ml of agar produced by bioMérieux
PCR	Polymerase chain reaction
QC	Quality Control
R	Resistance
rRNA	Ribosomal ribonucleic acid
RS	Rectal swab
Rpm	Revolutions per minute
S	Sensitive
SBA	Sheep blood agar
SPSS	Statistical Package for the Social Sciences
Srr	Serine-rich repeat
Taq	Polymerase produced by *Thermophilus aquaticus*
TH	Todd Hewitt broth containing 8 µg of gentamycin and 15 µg of nalidixic acid per broth/ml of broth.
µg	Micro gram
µl	Micro litre
µM	Micromolar
UNISA	University of South Africa
UTI	Urinary tract infection

TABLE OF CONTENTS

DECLARATION ii
ABSTRACT iii
ACKNOWLEDGEMENTS v
DEDICATION vi
GLOSSORY vii- ix
1. LITERATURE REVIEW
1.1 Introduction 1
1.2 Group B Streptococcus diseases 3
1.2.1 Group B *Streptococcus* (GBS) bacteria 3
1.2.2 Pathogenesis 4
1.2.3 Immune response to GBS infection 6
1.2.4 Symptoms of infection 6
1.2.5 Early onset and late onset Group B *Streptococcus* Infections 6
1.2.5.1 Early-onset disease 7
1.2.5.2 Late-onset disease 8
1.2.6 Prevalence 9
1.2.7 Prophylaxis treatment 10
1.2.8 Isolation and identification of GBS 11
1.2.9 Antimicrobial agents 12
1.2.9.1 Mechanisms of antimicrobial resistance 12
1.2.9.2 Antibiotic susceptibility 13
1.2.9.3 VITEK 2 sensitivities 21
1.3 Objectives and Aim of study 22
2. MATERIALS AND METHODS
2.1 Sample collection 23
2.2 Culture media 24
2.2.1 Columbia sheep blood agar 24
2.2.2 Prevalence of Columbia sheep blood agar with antibiotics 24
2.2.3 Preparation of Todd Hewitt broth 25
2.2.4 Glycerol broth 26
2.2.5 Granada agar 26
2.3 Sample processing 27
2.4 Isolation and identification 27
2.4.1 Catalase test 28
2.4.2 Identification of Group B beta-haemolytic *Streptococcus* 29
2.4.3 Preservation of Group B *Streptococcus* in glycerol broth 30
2.5 VITEK 30
2.5.1 VITEK identification 30
2.5.1.1 DENSICHECK PLUS 31
2.5.2 VITEK sensitivities 32
2.5.2.1 Preparation of the bacterial suspension for VITEK sensitivities 32
2.6 Molecular Biology
2.6.1 Genomic DNA Extraction 32
2.6.1.1 Boiling method 32
2.6.1.2 DNA purification method 33
2.6.2 Multiplex Polymerase Chain Reaction 34
2.6.3 Cleaning of the amplified PCR products 37
2.6.4 Sequencing 37
2.7 Statistical analysis 38
3. RESULTS

3.1 Bacteriology 39
3.1.1 Group B *Streptococcus* prevalence seen in this study 41
3.1.2 Culture media used to detect GBS 42
3.1.3 Group B *Streptococcus* identification 47
3.1.3.1 Catalase 47
3.1.3.2 Agglutination 47
3.1.3.3 VITEK identifications 47
3.2 Antimicrobial susceptibility results 47
3.3 Polymerase chain reaction 49
3.3.1 Optimisation of DNA extraction from GBS isolates 49
3.3.2 Optimisation of PCR protocol 50
3.3.3 Identification of specific resistance genes in the GBS isolates by Multiplex PCR 50
3.3.4 Sequencing 58
3.4 Questionnaire evaluation 58
3.4.1 Marital status 59
3.4.2 Educational levels 59
3.4.3 Employment 60
3.4.4 Occupation 60
3.4.5 Accommodation 61
3.4.6 History of Pain and Fever 61
3.4.7 Trauma during the pregnancy 62
4. DISCUSSION 63
4.1 Group B Streptococcus prevalence in the Khomas region 63
4.2 Culture media 64
4.3 Identification 65
4.4 Risk factors 65
4.5 Antimicrobial susceptibility 66
4.6 Antimicrobial resistance genes 69
5. CONCLUSION 71
6. LIMITATIONS AND RECOMMENDATIONS 73
7. REFERENCES 74
8. APPENDICES 83

LIST OF FIGURES

Figure 1.1	**Route of GBS infection.**	**5**
Figure 1.2	**Pathogenesis of GBS.**	**5**
Figure 1.3	**The procedure in preventing early-onset group B *Streptococcal* (GBS) disease in infants, through the evaluation of risk factors.**	**11**
Figure 2.1A	**Columbia sheep blood agars with 8µg of gentamycin/ml (BCNA) and 15 µg of nalidixic acid/ml with the growth of sample 440 as the beta-haemolytic *Streptococcus*.**	**28**
Figure 2.1B	**The Granada agar with the growth of sample 440 as orange carotenoid colonies.**	**28**
Figure 2.2	**On the left slide, a photo of a positive catalase test. On the right slide, a photo of a negative catalase test.**	**28**
Figure 2.3	**In the top, left circle, a negative agglutination test is seen. In the top centre circle, a positive agglutination test is seen.**	**30**
Figure 3.1	**A positive GBS culture presenting with clear beta-haemolysis seen on a BCNA agar plate.**	**40**
Figure 3.2	**A positive GBS culture presenting with slight beta-haemolysis seen on a BCNA agar plate.**	**40**
Figure 3.3	**Top half of plate: The Granada agar presenting with orange carotenoid colonies considered a positive GBS growth.**	**41**
Figure 3.4	**Group B *Streptococcus* prevalence rate from the current study.**	**42**
Figure 3.5	**On the left: Columbia sheep blood agars containing 8 µg of gentamycin/ml (BCNA) and 15 µg of nalidixic acid/ml presenting the growth of a GBS positive sample as the beta-haemolytic colonies. On the top right: The Granada agar with growth of a GBS positive sample presenting as orange carotenoid colonies.**	**43**
Figure 3.6	**Sample 422, showing no visible beta-haemolysis on the Columbia sheep blood agars with 8 µg of gentamycin/ml and 15 µg of nalidixic acid/ml (BCNA) the, but clear orange carotenoid colonies on the Granada agar.**	**44**
Figure 3.7	**The percentage of participants that tested positive on vaginal swab only, rectal swab only and both vaginal and rectal swabs.**	**46**
Figure 3.8	**Representative agarose gel electrophoresis showing uncut genomic DNA extracted from GBS isolates using the DNeasy Blood & Tissue Kit from Qiagen for extraction.**	**50**
Figure 3.9	**Agarose gel electrophoresis showing amplicons of DNA extracted from GBS strains for resistant genes using 9 specific primers pairs.**	**51**
Figure 3.10	**Agarose gel electrophoresis showing amplicons of DNA extracted from GBS strains for resistant genes using 9 specific primers pairs. .**	**51**

LIST OF TABLES

Table 1.1: Susceptibility profile of GBS isolates for Erythromycin, Tetracycline, Clindamycin and Penicillin 16
Table 1.2: Susceptibility profile of GBS isolates for Ampicillin, Vancomycin, Ceftriaxone and Trimethoprim-Sulfametoxazole. 17
Table 1.3: Micro dilution susceptibility analyses of GBS isolates determined to intermediate or resistant by disc diffusion method 17
Table 1.4: Relationship of phenotype to genotype in phenotypically erythromycin-resistant (n = 67) and/or clindamycin-resistant (n = 61) GBS isolates. 18
Table 1.5: Concordance agreement and discrepancies between MLS resistance with the VITEK 2 and disc diffusion method according to the MLS phenotype and genotype. 20
Table 1.6: Essential agreement (EA) and categorical agreement (CA) of VITEK compared to the reference method. 21
Table 1.7: Performance Characteristics for *Streptococcus* Species antimicrobial Susceptibility testing with the AST-ST01 card. 22
Table 2.1: Acceptable ranges for the calibration standards on the DENSICHEK PLUS. 31
Table 2.2: Oligonucleotide primers used in this study 34
Table 2.3: PCR master mix preparation before optimization as previously described by Zeng et al. 35
Table 2.4: PCR master mix and predicted sizes of Polymerase chain Reaction amplicons. 36
Table 2.5: Multiplex PCR cycle conditions. 37
Table 3.1: Summary of the number of GBS positive samples detected on different media used for the first 419 samples. 43
Table 3.2: Odds ratio and p value calculation for using the different sets of media for GBS isolation. 45
Table 3.3: Summary of the number of GBS positive samples detected on different media used for the last 169 samples. 46
Table 3.4: Antimicrobial susceptibility results for the Group B Streptococcus isolates from pregnant women in the Khomas region at 35 weeks gestation onwards (n = 117). 48
Table 3.5: Summary of antibiotic resistance genes and VITEK susceptibility profiles that was detected in GBS isolates for this study. 53
Table 3.6: Influence of marital status on the rate of GBS colonisation (n = 588). 59
Table 3.7: Chi-Square tests for the association of level of education with GBS colonisation. 59
Table 3.8: Influence of education on the rate of GBS colonisation (n = 587). 60
Table 3.9: Influence of employment on the rate of GBS colonisation (n= 588). 60
Table 3.10: Influence of occupation on the rate of GBS colonisation (n= 586). 61
Table 3.11: Rate of GBS colonisation versus different accommodation conditions (n = 586). 61
Table 3.12 Rate of GBS colonisation versus a history of pain and fever during the pregnancy (n = 588). 62
Table 3.13 Rate of GBS colonisation versus a trauma during the pregnancy (n = 583). 62

APPENDICES

Appendix A: Group B Streptococcus participant questionnaire 83

Appendix B: Consent form 86

Appendix C: Culture media on which GBS have grown and a summary of these findings. 87

Appendix D: Summary of the MIC results, antibiotic interpretation (VITEK), gel lanes and genes identified for individual isolates. 90

CHAPTER ONE: LITERATURE REVIEW

1.1 Introduction

Group B *Streptococcus* (GBS), is the leading cause of neonatal infection in new born babies (Hyde et al. 2002), classified as early or late onset. Early-onset infection is an infection that presents within 7 days of an infant's life and also poses the risk of long term disability or death. Group B *Streptococcus* infections in pregnant women are also associated with babies being born prematurely, abortions or still births (Moyo et al. 1995; 1996). Pregnant women that are colonised with GBS therefore have the possibility that the organism might infect their infant.

In addition to GBS detection, it is important to determine the antimicrobial susceptibility as well as resistance patterns for GBS among pregnant women. Penicillin is the drug of choice for intrapartum antibiotic prophylaxis (IAP), but due to some patients being allergic to penicillin, alternative drugs need to be used (Hyde et al. 2002). A reduction in early-onset GBS disease has been seen as a result of implementation of intrapartum antibiotic prophylaxis (IAP). Group B *Streptococcus* resistance to alternative drugs has been reported, and therefore it is advisable to do antimicrobial sensitivity testing on GBS isolates in order to ensure the correct profile. Knowledge regarding antimicrobial susceptibility and resistance patterns of GBS isolates in Windhoek, Namibia will aid clinicians and health officials in the management and treatment of GBS. This will provide guidance on the prevalence of antibiotic resistance amongst the GBS strains.

To our knowledge, GBS has not yet been studied in Namibia; there is no literature on studies done in Namibia. Screening for GBS colonisation amongst pregnant women and the availability of IAP could thus prevent stillbirths, infant deaths and infection that could have a long term effect on the child.

Since the 1930's, it has been shown that GBS have been associated with neonatal meningitis, though neonatal and perinatal infections due to GBS only became evident during the 1960s (Hood et al. 1961). In the early 1970s, 2-3 cases per 1 000 live births (from a study done in Egypt at a clinic at AlFayom University Hospital) were infected with Group B *Streptococcus* with case-fatality ratios of 50% being reported and 80% severe invasive infections presenting in the first week of life (early-onset neonatal disease), being caused by GBS (Elbaradie et al. 2009). These infections were due to the spread of the GBS through both intact and ruptured membranes into the amniotic fluid (Elbaradie et al. 2009). Early-onset GBS infections in the new born babies have been associated with a number of obstetric factors which include, the colonisation of the mother's vagina and/or rectum with GBS, premature delivery of the baby, prolonged rupture of membranes, young maternal age,

intra-amniotic infection (Verani et al. 2010), intrapartum fever, having a previously GBS infected baby (Carstensen et al. 1988; Faxelius et al. 1988; Christensen et al. 1981), women with heavy colonisation (Verani et al. 2010; Pass et al. 1979; Wood et al. 1981; Moller et al. 1984; Liston et al. 1979; Persson et al. 1985) associated with GBS bacteriuria =/< 104 colony forming units or a low GBS antibody level, as stated by the CDC.

The absence of GBS maternal antibodies is recognised as increasing an infant's risk of developing GBS disease (Washington et al. 2006). Colonisation of women by group B *Streptococcus* is usually occurring in the vagina and rectum. Colonisation rates of 10 – 35% have been found in pregnant women, and intermittent colonisation is seen in up to 60% of the women (Washington et al. 2006). Arisoy et al found a GBS colonisation rate of 10.6% in the Izmir, Turkey study done in 2000 and 66.7% of the GBS colonised women were positive for both the vaginal and rectal cultures (Arisoy et al. 2003). It is a possibility that vaginal colonisation occur due to rectum contamination of the vagina. GBS is primarily harboured in the gastrointestinal tract. Women colonised with Group B *Streptococcus* mostly do not present with any symptoms. It is now generally acceptable that colonisation in women constitutes colonisation of the vagina, rectum or both. South Africa (Bolukaoto et al. 2015; Gaurav et al. 2014), Zimbabwe (Mavenyengwa et al. 2010; Moyo et al. 2000), Malawi, Tanzania and Mozambique (de Steenwinkel et al. 2008) have reported significant maternal prevalence of GBS and therefore it could be an indication that such a study is needed in Namibia, to assess Namibia's GBS prevalence in pregnant women.

The infant's risk of a GBS infection is directly related to the GBS colonisation status of the mother. More or less half of the infants born to mothers that are colonised by GBS also become colonised either on the mucosal surfaces or the skin through vertical transmission from the mother (see **Figure 1.1**). This could occur during the birth process or while in utero. Infections caused by GBS may present in 1 to 4 new born babies per 1 000 live births, amongst the colonised infants (Washington et al. 2006).

Group B *Streptococcus* colonisation seems to be variable in pregnant women. Women being colonised by GBS early during their pregnancy are not necessarily colonised by GBS at the delivery of the baby (Regan et. al, 1996). Group B *Streptococcus* colonisation during the final trimester on the other hand is representative of the intrapartum colonisation (Boyer et al. 1983.). Therefore in order to get a representative result of the patient's GBS colonisation rate at delivery. Centres for Disease Control and Prevention (CDC) suggest the collection of vaginal and rectal swabs at 35 – 37 weeks gestation.

A reduction in early-onset GBS disease has been reported since intrapartum antibiotic prophylaxis (IAP) has been implemented. In the time frame of 1993 to 1999 a reduction of 70% in early-onset GBS disease was reported in San Francisco and Atlanta due to the use of IAP (Hyde et al. 2002). Before routine GBS screening and the use of intrapartum antibiotic prophylaxis in GBS colonised pregnant women, early onset sepsis due to GBS in neonates were seen in 1.7 per 1 000 live births. Since IAP was used, early onset GBS infections were reduced by 83% (Turrentine 2013).

Implementation of GBS screening was followed by a decrease in early-onset sepsis caused by GBS during the period 1992 – 2010. Universal screening for GBS at the gestational age of 35 – 37 weeks in pregnant women was recommended by the World Health Organisation (WHO) (Colleen et al. 2013).

1.2 Group B Streptococcus diseases

1.2.1 Group B Streptococcus (GBS) bacteria

Group B *Streptococcus* is a facultative anaerobic, gram positive, catalase negative coccus, which grows on different bacteriological media. On sheep blood agar GBS presents with greyish white, flat and mucoid colonies, 1-3 mm in diameter, showing a small zone of beta-haemolysis (Brown. 1937). A very small percentage, approximately 2%, of GBS isolates have been described as being non-haemolytic or alpha-haemolytic. The group B beta haemolytic *Streptococcus* (*Streptococcus agalactiae*) belong to the Group B of Lancefield-grouping system typed based on cell surface polysaccharide and protein antigens (Washington et al. 2006). The presence of this antigen also assists in some of the identification tests for GBS. Rebecca Lancefield, described the differentiation of haemolytic streptococci by means of serological tests (Lancefield. 1933).

Due to the presence of polysaccharide antigens in GBS, that are type-specific, the following serotypes of GBS have been identified: Ia, Ib, II, - IX (Slotved et al. 2007). In a study done by Mavenyengwa et al, 32 serovariants were identified in Zimbabwe of which the capsular polysaccharides revealed the following; capsular types Ia, Ib, II, III, V and NT were identified in 15.7%, 11.6%, 8.3%, 38.8% 24.0% and 1.7% of the isolates, respectively. These capsular types were identified together with 17.7% of the isolates presented with the Ci protein, 19.8% presented with the Cα protein, 22.3% presented with the Alp1 protein, 5.0% presented with the Alp3 protein, 46.3% presented with the R4/Rib protein, 27.3% presented with the R3 protein, 2.3% presented with the Z protein and 28.9% presented with the SAR5 protein. These proteins are known as the strain-variable proteins and encodes for the protein R5. One GBS isolate is able to possess or express up to four of these protein genes. Within this same study, it was noted that the type V GBS strains presented with a low Alp3 prevalence, which was different from the discoveries of the non-African GBS capsular type V strains

(Mavenyengwa et al. 2010). More or less one third of the GBS strains responsible for colonisation of neonates that do not present with any symptoms, are type III strains. The type III GBS strain accounts for more or less 1/3rd of early onset Group B Streptococcus (EOGBS) disease, and 2/3rds of late-onset infections, whereas serotype V is responsible for 13% of EOBGS and 5% of late-onset infections (Di John et al. 1990; Kogan et, al. 1996; Shet et, al. 2004).

1.2.2 Pathogenesis

Group B *Streptococcus* colonise the vaginal and gastrointestinal tracts in healthy women, with carriage rates ranging from 15% - 60% (Woods et al. 2014; Mavenyengwa et al. 2010). Group B *Streptococcus* colonisation of the vaginal tract is subsidiary to the GBS in the gastrointestinal tract, which seems to be the principal area of GBS reservoir in a women's body. Group B *Streptococcus* prevalence has been reported to be most frequently occurring in the rectum, followed by the vagina and lastly the cervix. It is therefore motivated that GBS screening during pregnancy should include both the vagina and rectum. Factors influencing GBS colonisation rate include age, ethnicity and also geographical location. Sexual transmission may also occur, since GBS are isolated from urethral swabs of 45% to 63% of men (Gardner et al. 1979; Franciosi et al. 1973).

Group B *Streptococcus* is able to attach itself to a number of different human cells. The cells to which GBS can attach itself include vaginal epithelial cells, placental membrane cells, respiratory epithelial cells as well as blood-brain barrier endothelium cells. In an acidic environment, GBS show maximal ability to adhere to host cells. The vaginal mucosal cells present with such an acidic pH, where GBS then adhere, exposing the unborn infant to the risk of vertical transmission of GBS. Group B *Streptococcus* have the ability to attach to immobilised fibronectin, through which colonisation of the mucosal cells are enabled. On the other hand GBS do not bind to soluble fibronectin, which could be an opsonin in the recognition of the GBS for phagocytosis by the host (Tamura et al. 1994). It also has been shown that GBS can enter into chorionic epithelial cells but it cannot invade amniotic cells. Group B *Streptococcus* strains that cause bacteraemia in infants are able to invade epithelial cells, whereas stains causing women to present with asymptomatic colonisation are not able to enter epithelial cells as easily (Kelly et al. 2004). An illustration on the pathogenesis of GBS can be seen in **Figure 1.2**.

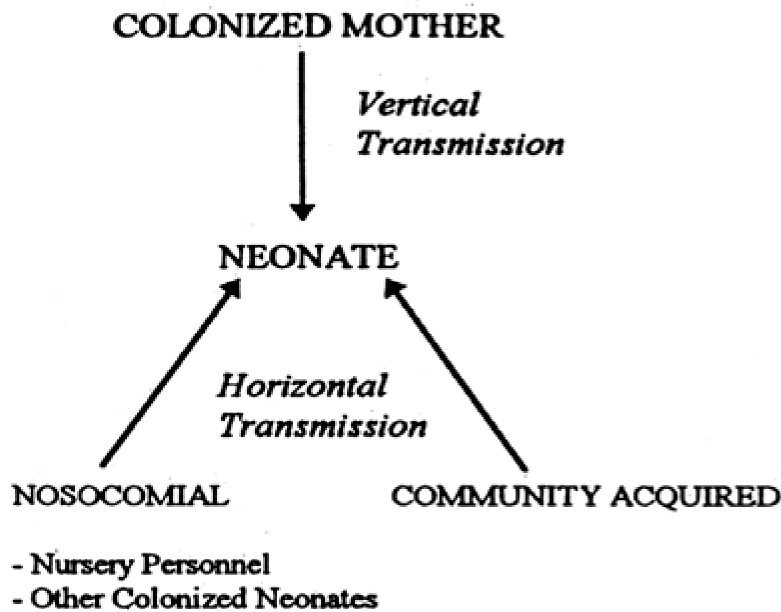

Figure 1.1: Route of GBS infection (Boyer et al. 1983; Hoggkamp-Korstanje et al. 1982; Ancona, 1980).

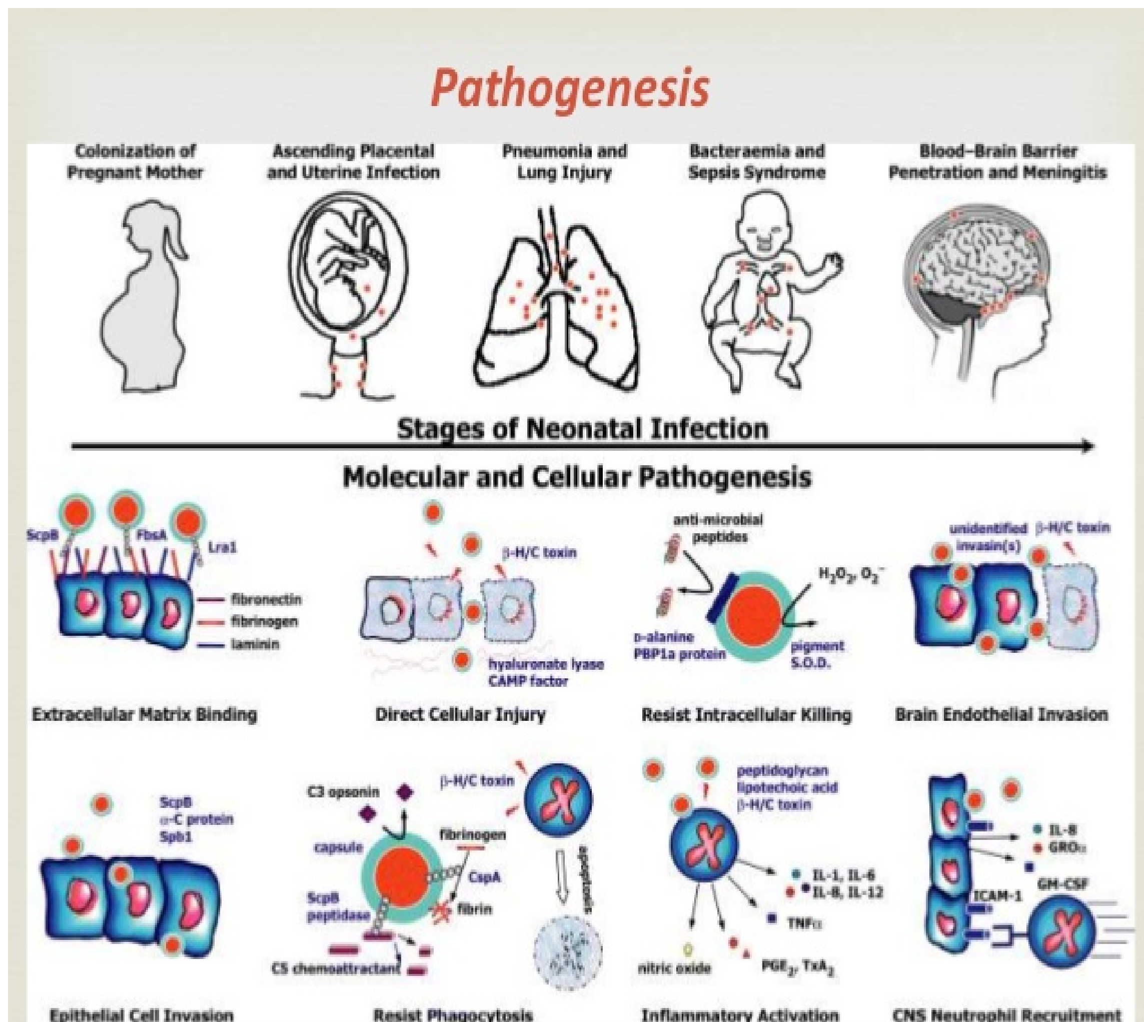

Figure 2.2: Pathogenesis of GBS (Doran and Nizet. 2004)

1.2.3 Immune response to GBS infection

Invasive GBS infections could potentially be haltered by the presence of antibodies, produced by the mother, against the capsular polysaccharide GBS antigens. Group B *Streptococcus* immunity is acquired through phagocytosis induced by the antibodies bound to the antigens. The mothers of infants that present with type III GBS sepsis at birth show lower type-specific antibodies in their serum compared to those delivering GBS colonised babies that are asymptomatic. The IgG antibody against type III GBS presents with reactivity against all GBS strains, and it also easily passes across the placenta. Maternal and cord blood antibodies show a very good correlation to each other (Baker et al. 1976, 1981; Gibbs et al. 1982). Studies have shown a significant decline in the occurrence of EOGBS disease in infants born to mothers with increased serum concentrations of maternal IgG specific to GBS type Ia and III (Baker et al. 2014).

1.2.4 Symptoms of infection

Symptoms presenting itself during early infant septicaemia are very similar to the symptoms seen in an animal model due to the extracellular toxin of GBS. Symptoms in the animal experimental model, when sheep were injected with an extracellular toxin that was produced by the virulent type III GBS strain, included a biphasic response that was identified by initial increase in pulmonary artery pressure, a fall in arterial partial pressure of oxygen (PaO_2) and also with a rise in temperature, granulocytopenia as well as an elevated pulmonary vascular permeability were seen at a later stage (Hellerqvist et al. 1981). Virulence of the GBS strain is also an influencing factor in GBS infection. Virulence factors of GBS are not very well defined, but an enzyme C_5a-ase that is known to rapidly inactivate chemoattractant C_5a of polymorphonuclear cells, is one of the contributing virulence factors in GBS infections. Other known virulence factors is the pili and serine-rich repeat (Srr) proteins that assist the bacterial cell in attaching itself to the host cell (Sheen et al. 2011). Neurological sequelae are seen in up to half of the survivors of GBS related neonatal meningitis. The presentation of the sequelae include a list of symptoms such as mental retardation, blindness, deafness, uncontrolled seizures, hydrocephalus, hearing loss, speech and language delays (de Louvois et al. 2005; Fluegge et al, 2006).

1.2.5 Early onset and late onset Group B Streptococcus infections

Early onset infection can be defined as an infection that presents within the first seven days of an infant's life, whereas late onset infection can be defined as an infection that occurs between the 7th and 90th days of an infant's life. Babies that are born prematurely and present with a low birth weight show an increased risk for invasive GBS infections. Group B *Streptococcus* has also been linked to preterm rupturing of membranes as well as preterm birth of the infant (<32 weeks gestation). Amongst the mothers that had early rupturing of the

membranes, 24.6% tested positive for cervical GBS colonisation and 38% of the early onset neonates were born to mothers that were colonised with GBS (Regan et al. 1981). There is a relation between mothers presenting with GBS in their urine and a high GBS concentration in the vagina or cervix, and therefore the GBS in the urine of a mother is also associated with early rupturing of membranes during pregnancy and infants being born prematurely (Regan. et al. 1981).

1.2.5.1 Early-onset disease

Respiratory distress, apnoea or other signs of sepsis within the first 24 – 48 hours of an infant's life, is generally representative of early onset GBS (EOGBS) disease. The most common clinical manifestation of EOGBS disease are sepsis and pneumonia; less frequently meningitis. About 70% of blood cultures from EOGBS infections are positive at birth (Boyer, et al. 1983). During the last decade, European countries presented with less than one baby per 1000 live births with EOGBS disease, and 0.1 up to two babies per 1000 live births from industrialized, non- European countries. Invasive infant GBS disease presents with case fatality rates of 4% up to 7.5% (Bergseng et al. 2007; CDC, 2007; Fluegge, et al. 2006; Heath et al. 2005; Schrag et al. 2004). In the group of babies born to GBS colonised mothers, a colonisation rate of between 40% and 70%, where the babies usually test positive for the same GBS serotypes that were isolated from the mother. Babies born by means of spontaneous vaginal delivery presented with a 10.1% GBS colonisation rate as compared to the 4.9% in caesarean section babies (Joachim et al. 2009). Group B *Streptococcus* infections do not only occur in neonates but it makes up between 60% and 70% of the total of GBS infections. The majority of EOGBS comprise of the following serotypes: Ia, II, III and V (le Doare et al. 2013). Early onset Group B *Streptococcus* only occur in an infant when the organism has been transmitted to the infant via a colonised mother, and this transmission usually takes place during labour or just prior to labour. Developed countries have more or less a 20 – 30% prevalence rate of GBS amongst pregnant women, half of their babies are colonised with GBS, and 1% of these babies present with early onset GBS disease (le Doare et al. 2013). Early onset Group B *Streptococcus* infections presenting with symptoms of pneumonia and sepsis are seen in > 90% of the reported cases within the first 12 hours of their life (le Doare et al. 2013). Mortality is higher in preterm infants compared to that of full term infants. Early onset Group B Streptococcus is acquired vertically through exposure to a mothers vagina colonised with GBS. GBS can invade through intact membranes, but primarily neonatal infections occur when GBS ascends from the vagina to the amniotic fluid after onset of labour or rupture of membranes (Narava et al. 2014). Group B *Streptococcus* infection of the amniotic fluid has shown an increased risk of the following factors, colonisation with the organism, ruptured membranes for more than 6 hours, more than

twelve hours of internal monitoring of the fetus and more than six vaginal examinations during the pregnancy (Yancey et al. 1994).

Group B *Streptococcus* has been isolated from both amniotic fluid and infants at birth and this suggest that the infection occur prior to birth. More or less 70% of neonates with EOGBS already have a bacteraemia at birth. This suggests that the bacteraemia already developed in the uterus due to the infant aspirating GBS infected amniotic fluid or due to the placenta being infected with GBS. The administration of antibiotics to preterm infants as well as to infants presenting with symptoms after birth is an important preventative measure for EOGBS infection (Boyer, 1986). Despite the fact that there is a high prevalence rate of GBS colonisation both in mother and new-borns, the rate of serious infections due to GBS is low.

The frequency of EOGBS infection occurs more often in the following three risk groups, low birth weight (<2500g), ruptured membranes for more than 18 hours, and mothers with an elevated body temperature of > 37.5°C. Group B *Streptococcus* colonisation of the infant, was significantly influenced by prolonged labour (Joachim et al. 2009). Early onset Group B *Streptococcus* sepsis cases per 1 000 live births that occur in the group presented with one of the three risk categories mentioned above, showed a 13 times higher chance of acquiring the infection than the group not falling in any of the three risk categories. Boyer et al found that about 70% of GBS infected neonates fell in at least one of the three risk categories. Additional risk factors for infant sepsis due to GBS include mothers presenting with a urinary tract infection due to GBS and mothers that have given birth to a baby with GBS sepsis before. As mentioned earlier, some studies show a correlation between GBS infections and a maternal age of < 20 years, black ethnicity and diabetes (Franciosi et al. 1973; Gardner et al. 1979).

Early onset GBS infection characteristics have been classified as follows: the onset occurs in less than seven days after the birth of the infant; the onset of the infections presents itself at a mean age of 20 hours; it is usually associated with obstetric complications; GBS is usually transmitted vertically, approximately 40% of the EOGBS infected new-borns present with pneumonia, approximately 30% presents with meningitis and septicaemia also occur amongst these new-borns; there is no predominant serotypes seen in EOGBS infected new-borns; the disease incidence is present in 1.8 per 1 000 live births and the mortality rate seen is 12 – 15% (Baker 1979).

1.2.5.2 Late-onset disease

Late onset GBS disease occurs between the 7th and 90th days of an infant’s life and it is occurs less common than EOGBS diseases. Late onset GBS disease is generally not

associated with obstetric complications in the mother. Transmission of GBS can occur from the mother to the infant due to close vicinity between mother and new born baby and this is known as vertical transmission. On the other hand, horizontal transmission of GBS can occur when an infant becomes infected either through contact with other GBS infected infants, healthcare or community workers (Narava et al. 2014). Group B *Streptococcus* serotype III is responsible for the majority of late onset GBS infections (le Doare, et al. 2013). Group B *Streptococcus* infections in new born babies present as meningitis in more than 80% of late onset GBS infections of which there is approximately 20% mortality rate. Dangor et al found that neonates that were affected by GBS compared to a control group, had a 21.48 elevated chance of developing neurological sequelae (Dangor et al. 2015).

Early onset GBS infection characteristics have been classified as follows: the onset occurs in less than seven days after the birth of the infant; the onset of the infections presents itself at a mean age of 20 hours; it is usually associated with obstetric complications; GBS is usually transmitted vertically, approximately 40% of the EOGBS infected newborns present with pneumonia, approximately 30% presents with meningitis and septicemia also occur amongst these newborns; there is no predominant serotypes seen in EOGBS infected newborns; the disease incidence is present in 1.8 per 1 000 live births and the mortality rate seen is 12 – 15% (Baker 1979).
Whereas late onset GBS infections characteristics have been classified as follows: the onset of the infection presents itself 7 days or more after the birth of the infant; the onset of the infections presents itself at a mean age of 24 days; it is usually not associated with obstetric complications; GBS is usually acquired through horizontal nosocomial transmission, approximately 80% of the late onset GBS infected newborns present with meningitis; serotypes III is commonly seen in late onset GBS infected newborns; the disease incidence is present in 0.4 per 1 000 live births and the mortality rate is 20% (Baker 1979).

1.2.6 Prevalence

Studies have shown that GBS prevalence seen in African countries differ greatly. According to Madhi et al. (2003) invasive GBS disease in South Africa is higher than in developed countries. In Zimbabwe, 60.3% (470/780) of the women screened for GBS tested positive, with colonisation rates at 47% at 20 weeks gestation, 24.2% at 26 weeks gestation and 21% at delivery (Mavenyengwa et al. 2010). The Zimbabwean study by Mavenyengwa et al was done in distinct rural areas, while Moyo et al reported a GBS prevalence of 31.6% in 2000 from the Chinhoyi General Hospital in Zimbabwe (Mavenyengwa et al. 2010; Moyo et al. 2000). Prevalence in Mozambique for GBS colonisation indicated 1.8% in one particular study (only 8mg/l gentamycin and human blood were incorporated in the media used for this study). Inhibitory substances present in human blood could have inhibited GBS growth and

resulted in possibly false low GBS prevalence shown by this study (de Steenwinkel, et al. 2008). GBS prevalence in Gambia was reported at 22% (Suara et al. 1994).

Group B *Streptococcus* carriers vary in relation to the geographical area and/or a number of demographic factors. The prevalence rate of GBS colonisation in late pregnancy was 18% in the survey done by Busetti in a mother-and-child hospital of Trieste (North-Eastern Italy).
Group B *Streptococcus* was isolated from 23% pregnant women and from 8.9% neonates in a study done by Agricola et al between October 2009 and March 2009 in Dar es Salaam, Tanzania (Agricola et al. 2009). A vaginal colonisation rate of 9.66% was found in a study done by Narava in India (Narava et al. 2014).

In the study done by Feng-Ying et al, 24.5% prenatal cultures were positive for GBS. Pregnant women in Oakland presented a GBS prevalence rate of 30.2% and 18.5% were seen in Houston ($P<0.001$) (Feng-Ying et al. 2011). Of women screened for GBS during labour, 1031 (18.8%) were positive (19.0% in Houston and 18.4% in Oakland). Different carriage rates were seen in this study amongst different race groups: 27.9% were seen in women of black ethnicity, women of white ethnicity presented with 17.7%, women of Hispanic ethnicity showed 17.5% and women of Asian ethnicity 13.6% (Feng-Ying et al. 2011).

Most of the GBS diseases in Africa, Eastern Mediterranean, America, Europe and Western Pacific are due to Ia, Ib, II, III and V serotypes (Le Doare et al. 2013).

In 2000 a study was done on pregnant women in Zimbabwe which showed the predominant GBS serotype to be III and V (Moyo et al. 2000). In 2010 another study done in Zimbabwe showed serotypes Ia, Ib, II, III and V to be the most common amongst the GBS strains (Mavenyengwa et al. 2010). Group B *Streptococcus* serotypes identified in South Africa were mostly III, Ia and II and also some V, Ib and IV serotypes were isolated (Madzivhandila et al., 2011).

1.2.7 Prophylaxis treatment

Intrapartum antibiotic prophylaxis (IAP) for GBS colonised mothers is described as the administration of the relevant antimicrobial substances (drug of choice is Penicillin) to the colonised mother during delivery, but preferably at least 4 or more hours before the baby is born. The described IAP seems to be the most effective in the prevention of early-onset GBS infections (Turrentine, 2014). Antibiotic protocol for IAP is presented in **Figure 1.3**.

In the Feng-Ying study 38.3% of mothers received antibiotics during labour (45.7% in Oakland and 30.4% in Houston, $P<0.001$). In the prenatally GBS-positive women, 93.3%

received IAP. In 83.7% of those colonised women, GBS colonisation was prevented in their infants (Feng-Ying et al. 2011).

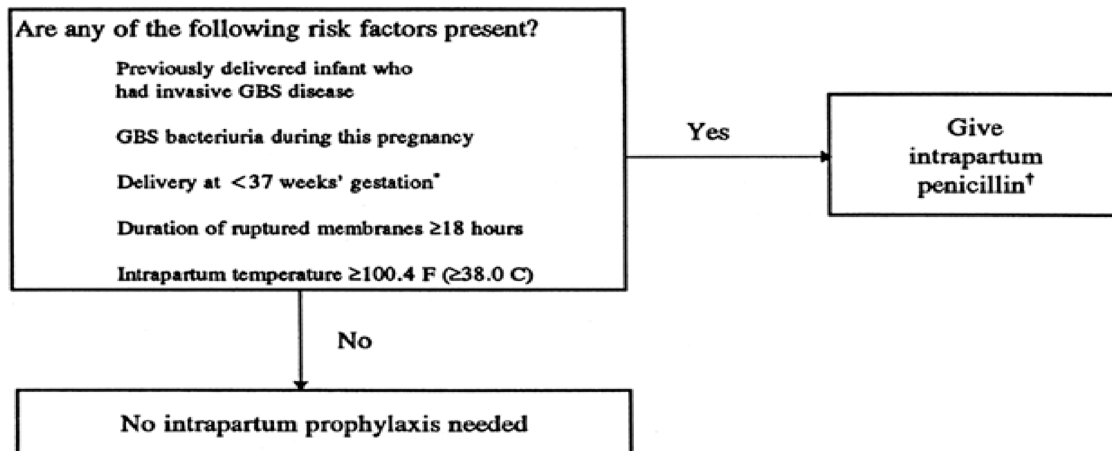

Figure 1.3: The procedure in preventing early-onset group B Streptococcal (GBS) disease in infants, through the evaluation of risk factors. *If a woman has not yet reached 37 weeks gestation and rupturing of occur without going into labour, specimens for the culturing of GBS culture should be obtained and either (1) antibiotics should be administered until culture results are negative, or (2) antibiotics could be administered only once a positive GBS cultures is obtained (Schuchat et al.1996).

The CDC provides guidelines in preventing EOGBS infections (Eschenbach, 2004). Pregnant women presenting with a urinary tract infection (UTI) due to GBS are normally heavily colonised and should be treated for this infection when it is diagnosed, and also during the birth of the baby, without any additional GBS cultures being performed.

Pregnant mothers that present with a positive GBS culture or that haven't been screened for GBS and presents with risk factors (as discussed in 1.5.2.1 and seen in **Figure 1.3**) should be given prophylaxis (Eschenbach, 2004). The Centres for Disease Control and Prevention (CDC) suggests that pregnant mothers that present with GBS positive cultures during 35 to 37 weeks of the pregnancy, should be given prophylaxis, whether they have other risk factors or not.

1.2.8 Isolation and identification of GBS

GBS can successfully be isolated on non-selective blood agar plates from a swab collected from the infected or colonised area. Currently common practise for GBS isolation from vaginal and rectal swabs is done by the use of broth mediums that is selective for the organism. Examples of such broth medium are Todd-Hewitt or Lim broth, which contains antibiotics that prevent other bacteria from growing. The antibiotics used to prevent the growth of the other organisms include nalidixic acid, gentamycin and or colistin (Schuchat et al. 1996). After the swabs are incubated in the selective broth medium for 18 -24 hrs, it then needs to be cultured onto sheep blood agar plates. The majority of GBS colonies present on blood agar plates as 1 to 2 mm in diameter, grey-white in colour and beta-haemolytic. Some

strains might be alpha or gamma haemolytic (Eschenbach, 2004). Disadvantages associated with the conventional culturing of GBS, includes the time required for both the growth and identification of the organism.

Specific cell wall antigens found in all GBS are used for identification of the organism. In a study by Cieslewicz et al, it is evident that of the 10 known serotypes of GBS, 8 seem to be structurally and genetically closely related. As for serotypes VI and VIII, a more distant relatedness is noted (Cieslewicz et al. 2004). A variety of methods, for more rapid identification of GBS have been employed over the years for the identification of these group specific antigens which includes methods like, latex agglutination, enzyme-linked immunosorbent assay, immunoelectrophoresis and indirect immunofluorescence. Due to the availability and simplicity of the latex agglutination test it is widely used to identify streptococci. The majority of GBS isolates produce CAMP factor. The CAMP factor is a known heat stable extracellular protein that is diffusible and presents with enhanced haemolysis in the presence of the beta-lysin produced by *Staphylococcus aureus* when grown together on a sheep blood agar (Hordes et al. 1995).

VITEK identification for GBS is done by the use of the gram positive (GP) card. The VITEK (an automated identification and susceptibility testing system for bacteria, produced by bioMérieux) uses advanced colorimetric reading to determine the identification of micro-organisms. Sixteen readings are taken in each well on the VITEK card, read at approximately 15-minute intervals. Colorimetric identification cards are based on established biochemical methods, and newly developed substrates, measuring carbon source utilisation, enzymatic activity and resistance to growth in certain media. Each reaction is read at wavelengths as determined in development for that reaction. There are 2 modes for identification interpretation that may be used. For the kinetic identification the second level Algorithm uses the kinetic identification algorithm. All the tests are not interpreted at the same time. This allows the system to report an identification call before getting a final reaction evaluation for all the tests, provided that the information brought by the tests already interpreted is sufficient to make a decision, and low risk that the call would change later due to the interpretation of additional tests (Joyanes et al. 2001).

1.2.9 Antimicrobial agents

1.2.9.1 Mechanisms of antimicrobial resistance

Tetracycline resistance amongst GBS isolates are often identified. Tetracycline resistance is frequently seen due to one of two mechanisms; ribosomal protection or sometimes an efflux pump. The genes *tet*(M) or *tet*(O) encodes for the ribosomal protection, whereas the genes *tet*(K) or *tet*(L) encodes for the efflux pump. The Tn*916* conjugative transposon commonly carries the *tet*(M) gene (Zeng et al. 2006).

Transposons also carry genes encoding for resistance against erythromycin (a macrolide). These genes are known as *erm*(B, A/TR, or C) (Zeng et al. 2006) and encode for either methylation of the 23S ribosomal ribonucleic acid (rRNA) by erythromycin ribosomal methylase (erm) enzymes or an efflux pump which is the cause of erythromycin resistance amongst GBS. Methylation of 23S rRNA by *erm* enzymes confers resistance to other antibiotics through the blocking of Macrolide, Lincosamide and Streptogramin (MLS) binding to the 50S ribosomal subunit (Arisoy et al. 2003).

Bacterial isolates present with constitutive or inducible resistance against clindamycin, which is most frequently caused by a mechanism resulting in the modification of a target site facilitated by *erm* genes (Prabhu et al. 2011). Inducible clindamycin resistance (ICR) is a challenge to detect in the routine laboratory since it present as erythromycin sensitive, and what seems to be clindamycin sensitive (in vitro). If the erythromycin and clindamycin discs is placed next to each other in close proximity (15 mm apart), it is known as the D-test, and an ICR strain can be identified by the flattening(D-shaped) of the zone of inhibition around the clindamycin disc it presents on the plate (Prabhu et al. 2011). Cross-resistance between erythromycin and clindamycin is caused by a resistance mechanism to MLS, which is shared amongst GBS (Capanna et al. 2013).

Kanamycin phosphorylase encoded for by the gene *aphA*-3 as well as streptomycin adenylylase encoded for by the gene *aad*-6 is the mechanisms responsible for aminoglycoside resistance (Zeng et al. 2006). In the GBS isolates investigated by Zeng et al, from three different countries, the *aphA*-3 gene was identified in 8 isolates from Hong Kong, 10 from South Koria and 2 from New Zealand (Zeng et al. 2006)

Fourteen- membered as well as 15-membered macrolides (which includes erythromycin) resistance is seen in the presence of the efflux pump encoded for by the genes *mef*(A) and *mef*(E) (Zeng et al. 2006). The *mef* (A/E) gene has been identified amongst GBS isolates at a percentage of 16% in Hong Kong, 4% in South Korea , 1% in Australia and 1% in New Zealand (Zeng et al. 2006).

1.2.9.2 Antibiotic susceptibility

Penicillin G is the prophylaxis agent of choice (Hyde et al. 2002) and is recommended rather than ampicillin due to the level of resistance of other bacteria existing against ampicillin (Eschenbach, 2004). According to numerous studies both penicillin and ampicillin still seem to have uniform efficacy against GBS (Dutra et al. 2014; Eskandarian et al. 2014; Tazi et al. 2007; Arisoy et al. 2003). Studies published in 2014 show that there is no resistance

amongst GBS against beta-lactam antibiotics so far (Eskandarian et al. 2014). It is clear that from 1994 an increase in penicillin minimum inhibitory concentrations (MIC's) have been reported and it is therefore of utmost importance to monitor these MIC values through continual studies (Dutra et al. 2014).

A minimum of 10% of patients classify themselves as being allergic to penicillin. Before 2002, clindamycin and erythromycin were the preferable antibiotics for prophylaxis in pregnant women that are allergic to penicillin (Kimberly et al. 2013). Unfortunately erythromycin treatment for GBS positive pregnant mothers does not decrease preterm delivery (Regan et al. 1996). Resulting from the more frequent use of clindamycin (lincosamide) and erythromycin (macrolide) it has been reported that there is an elevation in resistance to these drugs amongst GBS isolates (Eskandarian et al. 2013 and Dutra et al. 2014). The emergence of resistance to erythromycin and clindamycin amongst Group B *Streptococcus* has initiated the review of Centres for Disease Control and Prevention (CDC) guidelines for preventing perinatal GBS prevention guideline of the. It is recommended in the guidelines to assess the history of penicillin allergic mothers to determine their risk of a fatal allergic reaction against penicillin.

Many patients mistakenly report to be allergic to penicillin, while actually experiencing antibiotic side effects (Kimberly et al. 2013). Pregnant women that have had reactions to penicillin not classified as type 1 hypersensitivity reactions or have endured alternative beta-lactam agents previously, presents with a low anaphylaxis risk to cephalosporins (Kimberly et al. 2013). Such patients could therefore use a cephalosporin for GBS prophylaxis (Kimberly et al. 2013). Thus, GBS colonised women, not presenting with a high risk for a fatal allergic reaction against penicillin, should receive the cephalosporin, cefazolin, as intrapartum antibiotic prophylaxis.

It is recommended that all GBS-positive women that are truly allergic to penicillin should have the GBS strain tested for its susceptibility to clindamycin. A study in the United States showed clindamycin resistance amongst GBS as high as 38.4% and erythromycin resistance as high as 50.7% (Wang et al. 2014). In a study between 1991 till 2008 in Taiwan, erythromycin resistance amongst GBS isolates progressed from 30% to 44% (Janapatla et al. 2008). Dutra et al detected 4.1% and 3% resistance against erythromycin and clindamycin respectively in Brazil (Dutra et al. 2014). Capanna et al detected 30% resistance to erythromycin and 28% resistance to clindamycin amongst the 124 GBS isolates from their study done at a University Hospital in Geneva, Switzerland (Capanna et al. 2013). In 2000, Arisoy et al detected 21.2% resistance against erythromycin and 9.1% resistance against clindamycin in Izmir, Turkey (Arisoy et al. 2003). Frouhesh-Tehrani et al detected 21.2%

GBS isolates to be resistant, 23% of the isolates to be intermediate resistant and 55.8% of the isolates to be sensitive to erythromycin amongst the 104 GBS isolates in their study done in Tehran. Of the total GBS isolates from the Tehran study, 9.5% tested positive for inducible clindamycin resistance (Frouhesh-Tehrani et al. 2015). An Australian study detected 6.4% erythromycin resistance, 4.2% clindamycin resistance and 3.4% cross resistance against clindamycin (Garland et al. 2011). Due to the expression of this inducible resistance, Back et al queried the fact that clindamycin is an adequate drug for IAP (Back et al. 2011). On the other hand the importance of inducible resistance for IAP is debatable since the clinical significance of inducible resistance when the drug is only used for limited time in not known.

Sulfamethoxazole/trimethoprim resistance of 17.95% were detected amongst the GBS isolates in Rio de Janeiro, Brazil (Soares et al. 2013).

A study performed in Detroit detected a surprisingly high resistance rate of GBS isolates against ceftriaxone of 30%. Resulting in a statement by Simoes et al that 2nd and 3rd generation cephalosporins do not seem to present itself as a good alternative treatment to penicillin (Simoes et al. 2004). Although the CLSI guidelines do not contain criteria for the reporting of ceftriaxone on GBS, it was stated that the staphylococcal criteria were used in such cases as recommended by CLSI (Simoes et al. 2004). Very limited studies report on linezolid susceptibility, but 100% sensitivity was detected amongst GBS isolates in Geneva, Switzerland (Capanna et al. 2013) as well as in Misiones (Quiroga et al. 2008).

No resistance amongst GBS isolates were reported for vancomycin and levofloxacin in the following studies, Brazil (March 2005 till December 2009), Malaysia (June 2010 till October 2011), Paris (1998 till 2006) and Turkey (March till December 2000) as well as South Africa (February 2012 till December 2012) (Dutra et al. 2014; Eskandarian et al. 2014; Tazi et al. 2007; Arisoy et al. 2003; Bolukaoto et al. 2015). Vancomycin should be reserved for women that are colonised with GBS isolates presenting with resistance against erythromycin or clindamycin or if the sensitivity of the GBS is unknown (Kimberly et al. 2013; Allardice et al. 1982).

Although it was recommended in both the 2002 and 2010 guidelines from the CDC that GBS colonisation management for penicillin-allergic obstetric patient should be done by performing susceptibility testing on the isolates, adherence to these guidelines has been reported to be suboptimal and therefore potentially exposing many neonates to GBS (Kimberly et al. 2013). A penicillin allergic patient with a detailed clinical history can provide very important information in the decision and effectiveness of GBS prophylaxis. Clindamycin

susceptibility of GBS isolates found at Magee-Women's Hospital, Pittsburgh, USA were 48% (Kimberly et al. 2013).

Selected antibiotic sensitivity results determined by disk diffusion and micro dilution by Simoes et al in the Woman's Hospital of Texas in 1999 are shown in **Table 1.1**, **Table 1.2** and **Table 1.3** (Simoes et al. 2004). Antibiotics included in **Table 1.1** and **Table 1.2** is antibiotics that were also under investigation in this study.

Table 1.1: Susceptibility profile of GBS isolates for Erythromycin, Tetracycline, Clindamycin and Penicillin.

Location	Number of isolates	Erythromycin			Tetracycline			Clindamycin			Penicillin			Author
		S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	
Difco Lab, Detroit.	52	65.4	9.6	25	Not reported			80.8	0	19.2	84.6	15.4	0	Simoes et al. 2004
Misiones, Argentina	62	90.2	0	9.8	71	0	29	96.7	0	3.3	100	0	0	Quirago et al. 2007

S = sensitive; I = intermediate; R = resistance; Lab = Laboratory.

Note that for the Simoes et al. study, Kirby Bauer disc diffusion as well as micro dilution techniques, using CLSI guidelines. Where no references were available in the CLSI guidelines for *Streptococc*i, *Staphylococcus* values recommended by the CLSI were used.
Note that for the Quirago et al study, Clinical and Laboratory Standards Institute (CLSI) breakpoint for *Streptococcus* spp. other than Streptococcus pneumonia were used.

Table 1.2: Susceptibility profile of GBS isolates for Ampicillin, Vancomycin, Ceftriaxone and Trimethoprim-Sulfametoxazole.

Location	Number of isolates	Ampicillin			Vancomycin			Cefriaxone			Trimethoprim-Sulfametoxazole			Author
		S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	S (%)	I (%)	R (%)	
Difco Lab, Detroit.	52	62.7	17.3	0	100	0	0	50	0	15.4	Not reported			Simoes et al. 2004
Misiones, Argentina	62	[a]100	0	0	[a]100	0	0	Not reported			[b]46.8	0	[b]53.2	Quirago et al. 2007

S = sensitive; I = intermediate; R = resistance; Lab = Laboratory.

Note that for the Simoes et al study, Kirby Bauer disc diffusion as well as micro dilution techniques, using CLSI guidelines. Where no references were available in the CLSI guidelines for *Streptococc*i, *Staphylococcus* values recommended by the CLSI were used.
Note that for the Quirago et al study, [a]Clinical and Laboratory Standards Institute (CLSI) breakpoint for *Streptococcus* spp. Other than *Streptococcus pneumonia* or [b]CLSI breakpoint for *S. pneumonia* were used.

Table 1.3: Micro dilution susceptibility analyses of GBS isolates determined to intermediate or resistant by disc diffusion method

	Micro dilution*		
Disc diffusion	Susceptible	Intermediate	Resistant
Penicillin G intermediate-susceptible	2/10 (20.0%)	8/10 (80.0%)	-
Ampicillin intermediate-susceptible	10/19 (52.6%)	9/19 (47.4%)	-
Clindamycin resistant	-	-	10/10 (100%)

According to NCCLS (Simoes et al. 2004)

Zeng et al conducted a study where 512 isolates were tested. Amongst these isolates, 88% showed phenotypic resistance to tetracycline, and 92% of these presented with the *tet*(M) gene, 5% presented with the *tet*(O) gene, 1% presented with both, and 2% with neither. Five of these isolates that have the *tet*(M) gene, were tested with an Etest and showed intermediate resistance to tetracycline with minimum inhibitory concentrations (MIC) of 3 – 4 µg/ml and four isolates showed tetracycline susceptibility with MIC's of 0.125 – 0.5 µg/ml (Zeng et al. 2006). Thirteen percent of the 512 isolates presented with phenotypic resistance to erythromycin, while 12% showed phenotypic resistance against clindamycin. **Table 1.4** show phenotype and genotype distributions of the erythromycin and/or clindamycin-resistant isolates in the Zeng et al study (Zeng et al. 2006).

Table 1.4: Relationship of phenotype to genotype in phenotypically erythromycin-resistant (n = 67) and/or clindamycin-resistant (n = 61) GBS isolates (Zeng et al. 2006).

Phenotype[a]	No. of strains with genotype					Total
	erm(B)	*erm*(A/T)	*mef*(A/E)	*erm*(B) + *mef*(A/E)	No E resistance gene[b] detected.	
$cMLS_B$[c] (E-R; CD-R)	31	5		3		39
$iMLS_B$[c]		7			3	10
M[c] (E-R; CD-S)			18			18
E-S; CD-S	1		1		421	423
E-S/I; CD-R	2				20[d]	22
Total	34	12	19	3	444	512

[a] E-S=erythromycin susceptible; -R=resistant; –I=intermediate, or, CD-S=clindamycin susceptible; –R=resistant.
[b] Inducible *erm*(C).
[c] M=resistant to erythromycin only; $cMLS_B$=constitutively resistant to erythromycin and clindamycin; $iMLS_B$=resistant to erythromycin and inducible resistant to clindamycin (all which appeared susceptible to clindamycin on single-disk testing).
[d] Two of these isolates had intermediate resistance to erythromycin.
E=erythromycin.

Three isolates from the study done by Zeng et al presented with resistance to erythromycin, and of which the $iMLS_B$ phenotype was confirmed through retesting and Etests (MIC, 32 µg /ml). These isolates were not encoded by any of the four erythromycin resistance genes. From the same study three GBS isolates containing the *erm*(B) gene and one containing the *mef*(A/E) showed erythromycin susceptibility through Etests (MIC = 0.19 to 0.25 µg /ml) and disk diffusion tests. Twenty of the 61 GBS isolates that presented with clindamycin resistance in the Zeng et al study showed erythromycin susceptibility (MIC's = 0.38 µg /ml and 0.75 µg /ml) and 4 clindamycin resistant isolates presented with intermediate resistance to erythromycin. One or more erythromycin resistance genes were detected in 13%, which includes 3 isolates showing the presence of both *erm*(B) and *mef*(A/E) genes. Forty seven percent of these isolates contained one (*aphaA*-3, 4; *add*-6, 1), or both aminoglycoside resistance genes. Out of 512 isolates, 37 presented with the *erm*(B) gene, 21 of these presented with *int*-TN and *tet*(M) genes, and of these, 16 also presented with 1 or both aminoglycoside resistance genes. They detected 13 isolates containing both *erm*(B) and

tet(O) genes of which 12 presented additionally with *aphaA*-3 and *aad*-6 genes (Zeng et al. 2006).

Dutra et al detected genes *tet*(M) in 99.3% and *tet*(O) in 1.8% of the total GBS isolates in their study done in Brazil (Dutra et al. 2014). Amongst the erythromycin resistant isolates from the Brazil study, constitutive macrolide-lincosamide streptogramin B (cMLSB) phenotypes were detected in 13 of the 18 isolates. Three of the 18 erythromycin resistant isolates from the Brazil study presented with the M phenotype, while 2 of the 18 GBS isolates presented with the inducible MLSB phenotype (Dutra et al. 2014).

Selected antimicrobial sensitivity patterns of GBS isolated from pregnant women in Misiones, Argentina during the period January 2004 to December 2006 are shown in **Table 1.1** and **Table 1.2**.

GBS strains isolated from two general university hospitals in Paris indicated the following susceptibility results: all 110 GBS strains showed 100% susceptibility to teicoplanin, benzylpenicillin, vancomycin and ampicillin. These antibiotics, showed a 100% agreement between the susceptibility results of the disc diffusion method and the VITEK 2 system (Tazi, et al. 2007). The MIC results of the VITEK 2 were as follows: teicoplanin was </= 0.5 mg/L, benzylpenicillin; </= 0.12 mg/L, vancomycin; </= 1 mg/L and ampicillin, </= 0.25 mg/L in the Tazi et al study. Twenty-five (22.7%) isolates that were encoded by the *aphA*-3 gene showed high-level kanamycin resistance (MIC_{90} > 1024 mg/L). High-level resistance to kanamycin was always associated with tetracycline resistance. No high resistance to gentamycin was seen amongst these isolates (Tazi, et al. 2007). In *streptococci* and *enterococci*, two resistance mechanisms have been reported for minocycline and tetracycline namely: ribosome protection [(*tet*(M), *tet*(O), *tet*(S) and *tet*(T)] and the efflux by proton antiporters (*tet*(L) and *tet*(K). The *tet*(M) resistance gene was detected in 88%, *tet*(O) in 10.5% and in association with *tet*(M) in < 1% according to the study done by Tazi et al. In the Tazi et al study, *tet*(L), *tet*(K) and *tet*(S) were not detected. A category agreement of 98% (94/95) was found for tetracycline between VITEK 2 and the disc diffusion method. One strain that contained the *tet*(M) and *tet*(O) resistance determinants showed susceptibility through disc diffusion methods, presented with intermediate resistance by VITEK 2 (MIC = 2 mg/L) (Tazi et al. 2007). Erythromycin resistance was seen in 73.6% through the disc diffusion method. **Table 1.5** show different resistance phenotypes were observed (Tazi et al. 2007).

Table 1.5: Concordance agreement and discrepancies between MLS resistance with the VITEK 2 and disc diffusion method according to the MLS phenotype and genotype (Tazi et al 2007).

Phenotype	Genotype	No. of strains (%)				
		CA		**mE**	**ME**	**VME**
M (ERY^{R} CLI^{s}) *n = 6*	*mefA*		ERY CLI	6(100) 6(100)	0 0	0 0
MLS_{B-C} ($ERY^{R}CLI^{R}$) *n = 50* *n = 47*	*erm(B)*	ERY	35 (74.5)	0	0	12 (25.5)
		CLI	33 (70.2)	0	0	14 (29.8)
n = 3	*erm(A)*	ERY	3 (100)	0	0	0
		CLI	3 (100)	0	0	0
MLS_{B-i} ($ERY^{R}CLI^{S}$) *n = 25* *n = 1*	*erm(B)*	ERY	0		0	1 (100)
		CLI	1 (100)	0	0	0
n = 24	*erm (A)*	ERY	9 (37.5)	0	0	15 (62.5)
		CLI	24 (100)	0	0	0

ERY=erythromycin; CLI=clindamycin; R=resistant; S=susceptible; CA=category agreement; mE=minor error; ME=major error; VME=very major error; MLS=Macrolide-lincosamide-streptogramin antibiotics.

Macrolide-lincosamide-streptogramin (MLS) antibiotic resistance is due to two major mechanisms in *streptococci*. The first major mechanism is encoded for by the *erm* (erythromycin resistance methylase) gene, presenting with the methylation of the 23S rRNA by a methyltrasferase as well as resulting in cross-resistance to all MLS_B antibiotics (Tazi et al. 2007). The second major mechanism is encoded for by the *mef* (macrolide efflux) gene and result in proton-dependent active drug efflux system encoded which is responsible for 14- and 15-membered macrolide resistance (Tazi et al. 2007). According to Tazi et al. the presence of the genes *erm*(B) or *erm*(A) resulted in MLS_B resistant phenotypes. These genes is seen and dispersed otherwise in strains expresses constitutive [47/50 (94%) and 3/50 (6%), respectively] or inducible [1/25 (4%) and 24/25 (96%), respectively] resistance, respectively (Table 3) (Tazi et al. 2007). All strains in the Tazi et al study exhibiting an M phenotype harboured the *mef*(A) gene (**Table 1.5**). No detection of the determinant *erm*(C) was found. The *erm*(B) and to a lesser extend the *erm*(A) genes are widely distributed among GBS strains (Tazi et al. 2007). In a study done by Poyart et al, high level resistance to kanamycin (HLR-KM) were detected in 11 of the tested GBS strains and Ounissi et al have found that HLR-KM is associated with the presence of the *aphA*-3 gene (Ounissi et al.

1990). Eight of these 11 strains have also shown streptomycin high level resistance and in 7 of them the *aad*-6 gene were present (Poyart et al. 2003).

1.2.9.3 VITEK 2 sensitivities

Antimicrobial sensitivity testing on the VITEK is done by the use of an antimicrobial susceptibility test (AST) card. The company bioMérieux has a range of different AST cards available containing different panels of antimicrobial substances.

In a study by Lo-Ten-Foe et al, VITEK sensitivities compared to that of the standard broth dilution methods showed good correlation (Lo-Ten-Foe et al. 2007). Another study by Tazi et al in 2007 where VITEK 2 was compared with that of reference methods and disk diffusion testing showed a 100% category agreement for the Macrolide-lincosamide-streptogramin (MLS) susceptible strains. In this study the VITEK 2 was unable to detect GBS strain that were resistant to both erythromycin and clindamycin. Amongst the erythromycin resistant strains the VITEK 2 were able to detect 76% of the resistance and 72% of the clindamycin resistance amongst these strains were detected by the VITEK 2. The VITEK 2 showed an accuracy of 36% for the inducible resistant strains amongst the investigated GBS strains (Tazi et al. 2007). In the same study by Tazi et al (2007), the VITEK 2 did not show reliable results for macrolide resistance amongst the tested GBS strains, the VITEK AST card used for this study were the AST-P532.

The VITEK card AST-ST01 was launched by bioMérieux for better *Streptococcus* susceptibility results in 2013. A study done by Longtin et al on the AST-ST01 card showed a 98.1% overall essential agreement between the E-test method, broth micro dilution method and the VITEK. There were no major errors (ME) detected in this study (Longtin et al. 2013). **Table 1.6** shows a summary of the Longtin et al study results. For individual drugs, essential agreement (EA) between the reference methods and the VITEK ranged from 95.9% to 100% (**Table 1.7**).

Table 1.6: Essential agreement (EA) and categorical agreement (CA) of VITEK compared to the reference method (Longtin et al. 2013).

	EA VITEK	ME VITEK	miE VITEK	CA VITEK
Penicillin IV	96	0	4	96
Penicillin CSF	96	0	0	100
Penicillin oral	96	0	11	89
Ceftriaxone IV	99	1	1	97
Ceftriaxone CSF	99	0	1	99
Levofloxacin	100	0	4	96
Overall	98	0	3	97

Results presented by bioMérieux themselves regarding the performance of the AST-ST01 cards can be seen in **Table 1.7** (Longtin et al. 2013).

Table 1.7: Performance Characteristics for *Streptococcus* Specie's antimicrobial Susceptibility testing with the AST-ST01 card (bioMérieux, Marcy l'Etoile, France; http://www.biomerieux-diagnostics.com/).

Anti-microbial	Bp	Essential Agreement				Category Agreement				Repro-ducibility
			% Error				% Error			
		% EA	VME	ME	mE	% CA	VME	ME	mE	
AM	CLSI	99.0	0.0	0.2	0.2	96.5	0.0	0.2	3.3	100
CRO	CLSI	98.9	0.0	0.2	0.1	97.7	0.0	0.2	2.1	100
CD	CLSI	N/A	N/A	N/A	N/A	97.2	1.1	1.1	1.7	100
E	CLSI, FDA	97.9	0.8	0.2	0.5	98.7	0.8	0.2	0.9	96.7
ICR	CLSI	N/A	N/A	N/A	N/A	99.2	0.0	0.2	N/A	100
TE	CLSI	96.8	1.9	0.2	0.3	96.4	1.9	0.2	2.7	100
SXT	CLSI	-	-	-	-	96.1	0.0	0.0	3.9	100
VA	CLSI, FDA	95.8	0.0	0.0	0.0	100	0.0	0.0	0.0	100

Abbreviations: Bp=breakpoint; EA=essential agreement; CA=category agreement; VME=Very Major Error (Susceptible results with resistant reference result); ME=Major Error (resistant result with susceptible reference result); mE=minor Error (susceptible or resistant result with an intermediate reference results, or an intermediate result with a susceptible or resistant reference result). FDA breakpoints are used in the CLSI Interpretation Standard (breakpoint committee) in the VITEK 2 System Software.
AM=Ampicillin; CRO=Cefriaxone; CD=Clindamycin; E=Erythromycin; ICR=Inducible Clindamycin resistance; TE = Tetracycline; SXT=Trimethoprim/Sulfamethoxazole; VA=Vancomycin.

1.3 Objectives and Aim of study:

The aim of this study was to assess the GBS prevalence amongst pregnant women in the Windhoek (Khomas) region, Namibia. The GBS isolates identified during this study have been tested for antimicrobial susceptibility and gene based resistance. Lower vaginal and rectal swabs were collected from informed, willing participants mainly from the Windhoek Central antenatal clinic over a period of 13 months.

The objectives of the study were:

1. To culture all lower vaginal and rectal swabs obtained from participants in and on supporting media to obtain the prevalence rate of GBS amongst pregnant women in the Windhoek (Khomas) region, Namibia.
2. To obtain the antimicrobial susceptibility patterns of GBS isolates from this study through minimum inhibitory concentration results against specific antimicrobial substances.
3. To optimise the polymerase chain reaction (PCR) to detect specific resistance genes present in the GBS isolates from this study.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Sample collection

A total of 1176 samples were included in this study, which were collected from 588 random participants (two swabs collected from each participant, one lower vaginal and one rectal swab) in Windhoek, Namibia. All participants included in this study, had a gestational age of 35 weeks or later. The random participants for this study were obtained by means of a convenience sample, since any willing participant, with the appropriate gestational age visiting the clinic during the time the nurse was available for collection, were included. No clear bias was created by the collection of samples. Of the 588 participants, 5 were random from a private practice (Eros Family Private Practice in Windhoek) while the rest of the participants were state patients visiting the Antenatal clinic at Windhoek Central Hospital. The Windhoek Central Hospital Antenatal clinic is a state facility open to any pregnant women that wants to be assessed during her pregnancy. The majority of the patients visiting this clinic are state patients that do not belong to a private medical aid. The sample collection period was 13 months, from August 2013 up until and including August 2014. The sample size was calculated with the assistance of a statistician. Using the sample size calculation offered by creative research systems survey software (Creative research system, 1982) with a confidence interval of 5 and a confidence level of 99% used, a sample size of 575 was calculated from Windhoek Central Hospital. The sample size was based on the number of deliveries at Windhoek Central Hospital for 2012, which gave an indication of how many pregnancies occur in the specific area per annum. Although this sample size was calculated, it was decided to rather use a collection period than a sample size. It was agreed that a collection period of approximately 13 months will be used, which is comparable to the collection periods used in most previous studies (Bolukaoto et al. 2015; Zeng-Ying et al. 2011; Simoes et al. 2004; Heelan et al. 2004). Since 588 participants partook in this study within a period of 13 months, this number exceeded the suggested number of 575. Participants completed a questionnaire (**Appendix A**) which included questions pertaining to socio-economic status, previous and current obstetric history, parity, and current health status. A similar questionnaire was used in a study done previously in Zimbabwe (Mavenyengwa et al. 2010). Due to the ethical consideration, all participants needed to give written consent to participate in the study (**Appendix B**). Since the Centers for Disease Control and Prevention morbidity and mortality weekly report of May 1996 stated that the best screening procedure for GBS colonisation was to collect a rectovaginal swab from pregnant women between the 35th and the 37th week of pregnancy (CDC, 1996). In this study, we also collected swabs during this period. In this study, two swabs were collected by a registered nurse from each of the 588 participants: a lower vaginal swabs (a sterile swab was inserted about 2 centimeter (cm) into the vagina and the peri-urethra area and the

medial aspects of the labia were swabbed) and 1 rectal swab (collected by inserting a sterile cotton wool swab 1cm into the anus, rubbing the walls of the anal canal). The guidelines on collection of vaginal and rectal swabs for Group B *Streptococcus* (GBS) screening was provided by the Centers for Disease Control and Prevention (CDC) in 2010. Sterile cotton wool swabs were used for collection and were transported in Amies transport media to the microbiology laboratory of the Namibia University of Science and Technology within four hours after collection for further processing.

2.2 Culture media

2.2.1 Columbia sheep blood agar

One day before the agar was prepared; sheep blood received from the local veterinary laboratory was cultured on an agar plate and incubated at 37°C for 18 to 24 hours to determine if the received blood was sterile. A blood agar that presented with no growth on the culture plate after incubation indicated that the blood could be used in the next step of preparing the sheep blood agar (SBA) plates.

Molten agar was prepared, autoclaved and then cooled to a temperature of 50°C. Fifty millilitre of sterile sheep blood was then added to the Columbia blood agar base, and mixed gently and thoroughly by swirling the bottle. The media was then poured into the sterile labelled petri dishes.

Once solidified, a quality control and sterility check was done on a representative 10% of each prepared batch. The quality control was done by culturing *Streptococcus agalactiae* (ATCC 12403) on prepared SBA plates and incubating at 37°C for 18 to 24 hours. The sterility check was done by incubating the un-inoculated prepared SBA plates from the same batch at 37°C for 18 to 24 hours. The rest of the SBA plates that were prepared was then stored in the fridge at 4°C. After incubation of the quality control plates, plates were acceptable to use when plates containing the *Streptococcus agalactiae* American type culture collection (ATCC) 12403, presented with beta-haemolytic colonies on the SBA plate and the un-inoculated plate presented with no growth. If this was the case, that specific batch of SBA plates was ready to be used for specimen analysis. The expiry date was 6 weeks after production.

2.2.2 Preparation of Columbia sheep blood agar with antibiotics

In preparation of the Columbia sheep blood agar containing 8 µg of gentamycin and 15 µg of nalidixic acid per millilitre of (BCNA) media, sheep blood was required (Joachim et al. 2009). As mentioned previously, one day before the agar was prepared; sheep blood received from the local veterinary laboratory was cultured on an agar plate and incubated at 37°C for 18 to

24 hours to determine if the received blood was sterile. If the blood agar presented with no growth on the culture plate after incubation, the blood could be used in the next step of preparing the BCNA agar.

Molten agar was prepared, autoclaved and then cooled to a temperature of 50°C. Fifty millilitre of sterile sheep blood was then added to the Columbia blood agar base, and mixed gently and thoroughly by swirling the bottle. When 5 l of BCNA was prepared, 40 milligram (mg) of gentamycin powder and 75 mg of nalidixic acid were dissolved in 20 ml of sterile distilled water. Dissolved antibiotics were then added to the rest of the prepared agar (4980 ml) at the cooled temperature and the agar was once again mixed gently and thoroughly after the addition of the antibiotics. The media was then poured into the sterile labelled petri dishes. The incorporation of 15 µg nalidixic acid per millilitre of agar and 8 µg of gentamycin per millilitre of agar is to inhibit the growth of normal flora. The sheep blood is incorporated in the agar plates to demonstrate the beta-haemolysis that one expects to see for the majority of the beta-haemolytic Group B *Streptococcus* organisms.

Quality control and sterility checks on the prepared BCNA was performed as described in 2.2.1 If this was the case, that specific batch of BCNA plates was ready to be used for specimen analysis. The expiry date was 14 days after production, due to the addition of the antibiotics.

The prepared BCNA plates were used for samples received from participants 1 to 419. Thereafter, pre-prepared BCNA plates (pBCNA) were sourced from bioMérieux and used for swabs received from participants 420 to 588.

2.2.3 Preparation of Todd Hewitt broth

Todd Hewitt broth was incorporated since it is highly nutritious due to its content of peptones, dextrose and salts. The dextrose stimulates haemolysin production. Sodium phosphate and sodium carbonate provide buffering action to counteract the acidity produced during fermentation of dextrose; thereby protecting the haemolysin from inactivation by the acid. Selectivity for Group B *Streptococci* is obtained by the inclusion of gentamicin and nalidixic acid in the medium. Selective enrichment broths include the advantages of both enrichment and selection by providing conditions conducive to the growth of Group B *Streptococci* while inhibiting the growth of contaminants.

In preparation of the Todd Hewitt broths, Todd Hewitt Broth powder was prepared in 980 ml of sterile distilled water. After the autoclave cycle was done, the media was left to cool to room temperature after which the antibiotics (8 mg of gentamycin powder and 15 mg of

nalidixic acid were dissolved in 20 ml of sterile distilled water) were added to the Todd Hewitt broth. The broth was gently and thoroughly mixed and was dispensed (3 ml of the Todd Hewitt broth) into sterile labelled individual tubes. Quality control and sterility checks were done on a representative 10% of each prepared batch. Broths from each batch was inoculated with *Streptococcus agalactiae* [American type control collection (ATCC 12403)] and incubated at 37°C for 18 to 24 hours as a positive quality control (QC). Un-inoculated media tubes from the same batch were also incubated at 37°C for 18 to 24 hours as a sterility check. The rest of the batch was stored in the fridge at 4°C. For the quality control was acceptable when the inoculated broth showed turbidity after incubation and the un-inoculated broth was clear after incubation. If the quality control results were satisfactory, the batch was ready to be used for specimen analysis. The expiry date was 14 days after production, due to the addition of the antibiotics.

2.2.4 Glycerol broth

The glycerol broth was prepared to preserve frozen GBS in the laboratory. Firstly a nutrient broth was prepared by adding 13 grams (g) of broth powder to 1 liter (L) of distilled water and soaked for 15 minutes at room temperature. After the broth powder had soaked for the 15 minutes, 160 ml of glycerol was added to the broth and mixed well. Individual bottles were labelled as glycerol broth with the production date. The broth mixture was dispensed into 5 ml aliquots in small bottles. Bottles were autoclaved for 15 minutes at 121°C. After it was autoclaved, a representative 10% of the bottles from the batch were incubated at 37°C for 18 to 24 hours, as the quality control on the sterility of the broth. The rest of the batch was stored at 4°C. The glycerol broths were ready to be used when the quality control broth remained clear after incubation.

2.2.5 Granada agar

All Granada agar plates were ordered directly from the production company bioMérieux. Granada agar is a chromogenic agar produced and distributed by bioMérieux and yields orange carotenoid pigmented colonies when GBS is present on the plate. The Granada agar base is a selective and differential medium for the rapid detection of beta-haemolytic *Streptococcus agalactiae* [Lancefield group B *Streptococcus* (GBS)] from clinical samples. An easy method for detecting and identifying beta-haemolytic GBS is pigment detection. The production of an orange carotenoid pigment is a unique characteristic of beta-haemolytic GBS isolated from humans and serves as the basis of several media for the detection and identification of GBS from clinical specimens. Granada medium contains proteose peptone N°3, soluble starch, colloid protector, dextrose, magnesium sulphate, hemisodium salt, disodium phosphate anhydrous, crystal violet, methotrexate, colistin sulphate and metronidazole. Proteose Peptone Nº 3 provides nitrogen, vitamins, minerals and amino acids

essential for growth. Soluble starch in the medium acts as a growth factor, functioning like a colloid protector and neutralizes toxic products that form during the growth of the organisms. It also enhances the pigment formation. Dextrose is the fermentable carbohydrate providing carbon and energy. Magnesium sulphate is a magnesium ion required in a big variation of enzymatic reactions, including DNA replication. Hemisodium salt and Disodium Phosphate Anhidrous (MOPS) acts as a buffer system. Crystal violet inhibits gram-positive bacteria and bacteriological agar is the solidifying agent. Methotrexate, added to the mixture acts as a pigment enhancer and colistin sulphate and metronidazole inhibits undesired flora.

2.3 Sample processing

All 1176 swabs were inoculated onto BCNA and streaked out for single colonies. Within the results section, this will be referred to as direct BCNA. After direct inoculation of the collected swabs onto the agar plate, the swabs were also inoculated into Todd Hewitt broth containing 8 µg of gentamycin/ml and 15 µg of nalidixic acid/ml. After 18 – 24 hours incubation, the Todd Hewitt broths were sub-cultured onto BCNA and streaked out for single colonies and incubated in ambient air at 35°C – 37°C for 18 – 24 hours. Within the results section, this is referred to as TH-BCNA. From sample 420 up to sample 588 (336 swabs), the direct BCNA, TH-BCNA was used together with Granada media. Pre-prepared BCNA (pBCNA) was now used as explained in Section 2.2.2. Granada media was inoculated in a similar way as direct BCNA, with the collected swab streaked out onto the media for single colonies. The plates and the broths were then incubated for 18 – 24 hours at 35°C – 37°C in ambient temperature. After incubation, the BCNA plates that were sub cultured from the Todd Hewitt broth (TH-BCNA) were inspected for presumptive GBS colonies.

2.4 Isolation and identification

After 18 – 24 hours of incubation at 35 – 37°C in ambient air, all plates were inspected for presumptive *Streptococcus agalactiae* (GBS). Beta- haemolytic colonies on the BCNA (as demonstrated in **Figure 2.1A**) and orange carotenoid pigmented colonies on the Granada agar plates (as demonstrated in **Figure 2.1B**), were regarded as presumptively positive. One hundred and seventeen samples were identified in this manner.

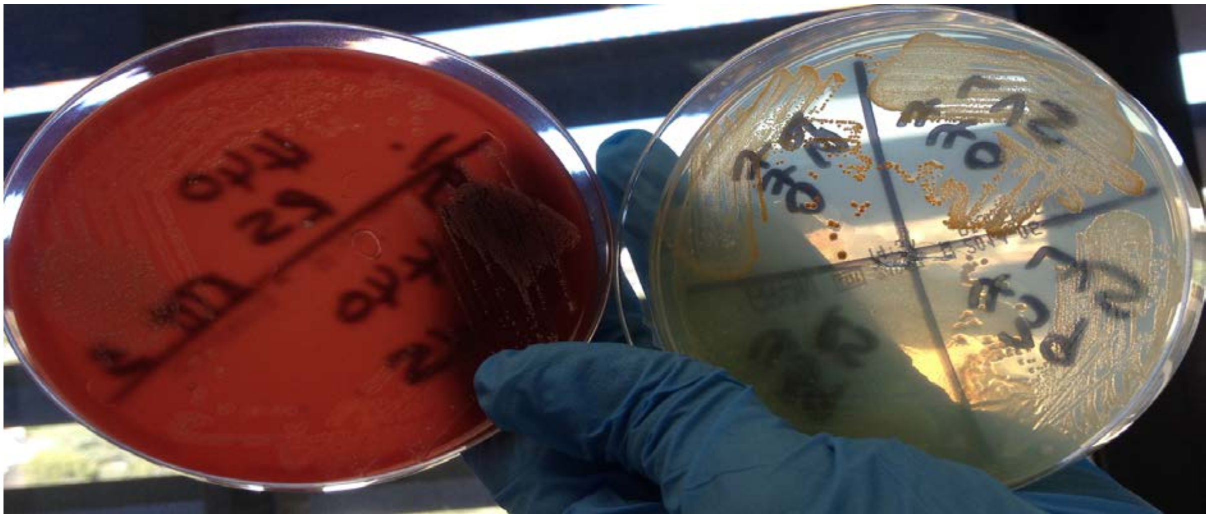

Figure 2.1A: On the left: Columbia sheep blood agars with 8µg of gentamycin/ml (BCNA) and 15 µg of nalidixic acid/ml with the growth of sample 440 as the beta-haemolytic streptococcus Group B. Figure 2.1B: On the right: The Granada agar with the growth of sample 440 as orange carotenoid colonies.

2.4.1 Catalase test

The catalase test was performed on all 117 presumptive positive samples that yielded beta-haemolytic colonies from the selective plates. Colonies were picked with a wooden applicator stick and deposited onto a clean glass slide. Picking the colonies from a blood agar plate was done with great care so as not to touch the agar itself, as the erythrocytes in the blood agar will give a peroxidase reaction, showing delayed and weak bubble production, and this might be difficult to distinguish from the actual catalase reaction of the organism being tested. One drop of 3% hydrogen peroxide was then deposited directly onto the colony. If an organism contains catalase enzymes, the enzyme will act as a catalyst and break down the hydrogen peroxide into water and oxygen producing visible bubbles. Therefore, if bubbles were produced it meant that the colony was catalase positive, and if no bubbles were produced, the colony was catalase negative (as demonstrated in **Figure 2.2**).

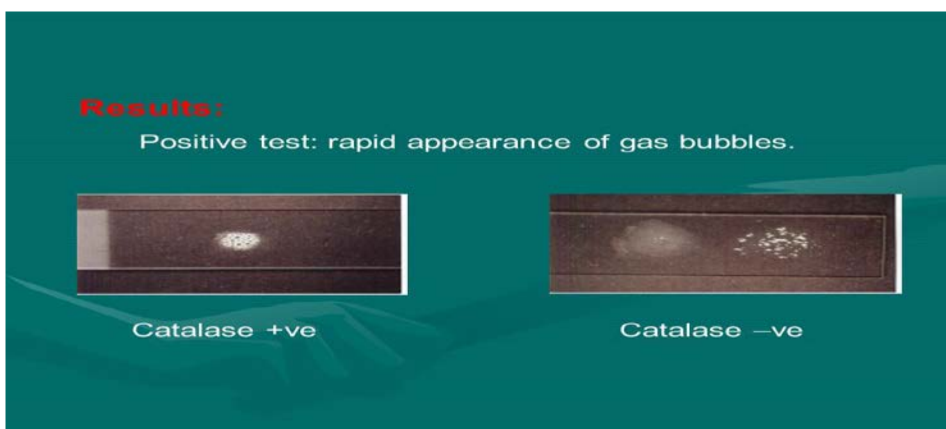

Figure 2.2: On the left slide, a photo of a positive catalase test. On the right slide, a photo of a negative catalase test.

For the catalase test, a known *Staphylococcus aureus* (ATCC 25923) was used as a positive control and known *Streptococcus* species (ATCC 12403) was used as a negative control. *Streptococcus agalactiae* does not contain catalase enzymes and is thus expected to give a negative catalase result. On all catalase negative, beta-haemolytic colonies, an immunological agglutination test was furthermore performed using the Oxoid Streptococcal Grouping kit.

2.4.2 Identification of Group B beta-haemolytic *Streptococcus*

Beta-haemolytic streptococci can be differentiated into Lancefield groups based on specific carbohydrate antigens. Differentiation is necessary for clinical treatment. For extraction of the group specific antigen prior to grouping, a variety of methods have been used including hot acid, hot formamide and enzyme extraction methods. The Oxoid Streptococcal Grouping Kit (DR585) utilises an enzyme extraction technique. This requires a 10 minute incubation period and efficiently extracts streptococci antigens of Lancefield Groups A, B, C, D, F and G6. Extracted antigens are then identified by agglutination.

In this study, three to five colonies of each of the 117 presumptive positive samples were inoculated in the extraction enzyme and incubated for 10 minutes. After 5 minutes of incubation, the samples were mixed thoroughly before the last 5 minute incubation period. After the 10 minutes incubation, the extraction-enzyme colony mixture was tested with Group B latex test reagent for the presence of *Streptococcus agalactiae*. One drop of the extraction-enzyme –colony mixture was mixed with one drop of Group B latex test reagent for 1 minute. A positive reaction would present with blue agglutination (**Figure 2.3**). A positive and negative control provided with the Oxoid kit was included in each batch of agglutination to ensure the performance of the kit is accurate.

All 117 presumptively GBS samples that tested negative for the catalase presented with a positive Oxoid Streptococcal Grouping test through the presentation of agglutination. Some of the specimens were tested with the Oxoid Streptococcal Grouping kit more than once to confirm colonies growing on different agar plates for the same specimen, as being either negative or positive.

Figure 2.3: In the top, left circle, a negative agglutination test is seen. In the top center circle, a positive agglutination test is seen.

2.4.3 Preservation of Group B *Streptococcus* in glycerol broth

All colonies that gave a positive reaction were sub-cultured on sheep blood agar and incubated for 18 – 24 hours at 35°C – 37°C in ambient air to obtain a pure growth. Following the incubation of the sheep blood agar plates containing the cultures that agglutinated with the Oxoid Streptococcal Grouping Kit, a pure growth of beta-haemolytic *Streptococcus* group B was harvested and stored in a 16% glycerol nutrient broth at -80°C. The addition of glycerol stabilizes the frozen bacteria, preventing damage to the cell membranes and keeping the cells alive. A glycerol stock of bacteria can be stored stably at -80°C for many years (Preserving bacterial cultures, 2015).

All *Streptococcus agalactiae* isolates that were isolated from the participants were stored at -80°C, labelled and batched until all collections were done.

2.5 VITEK

VITEK sensitivities were performed on all 117 GBS positive isolates, together with random VITEK identifications on some of the isolates as spot checks (to confirm the accuracy of the immunological identification test that was used). The organisms stored in the 16% glycerol nutrient broth, were thawed from the -80°C freezer, sub cultured onto sheep blood agar plates, streaked out for single colonies and incubated at 35°C – 37°C, for 18 – 24 hours in ambient air.

2.5.1 VITEK Identification

The gram positive (GP) identification card is based on the use of established biochemical methods and newly developed substrates, that is included. There are 43 biochemical tests measuring carbon source utilization, enzymatic activities and resistance. The reagent cards used on the VITEK 2 Compact machine have 64 reaction wells per card. Each well contains an individual test substrate. The substrates present in the well of the VITEK identification cards measure a variety of metabolic activities including acidification, alkalization, enzyme hydrolysis, and growth in the presence of inhibitory substances. A clear plastic (polystyrene)

test tube (12 x 75mm) was filled with 3.0 ml Sterile VITEK saline provided by bioMérieux (0.45 – 0.50% NaCl aqueous suspension with a pH of 4.5 – 7.0).

A sterile swab or applicator stick was then used to transfer the required number of colonies of a pure culture and to suspend the microorganism in the tube containing the 3.0 ml sterile VITEK saline. The turbidity of all the suspensions for the Group B *Streptococcus* identification was adjusted to give a McFarland value of between 0.5 and 0.62. A turbidity meter called the DENSICHECK PLUS was used to measure the McFarland values of all prepared bacterial suspensions, before the VITEK identification card was added to the suspension and loaded into the VITEK machine. Raw data obtained from the reading of the cards were used to perform calculations. These calculations were compared to thresholds and used to determine reactions for each test on the identification cards.

On the VITEK 2 Compact, test reaction results appeared as "+"," –", "(–)" or "(+)". Reactions that appeared in parentheses were indicative of weak reactions that were too close to the test threshold.

2.5.1.1 DENSICHEK PLUS

This DENSICHECK PLUS is designed to measure the optical density of the bacterial suspension in a tube containing liquid medium. This specific machine is designed to take McFarland readings using VITEK tubes. Before using of this instrument, it needed to be verified on its measurement performance by the use of provided standards. The turbidity of each of these standards should have produced a McFarland standard that was within the acceptable range (**Table 2.1**), indicated on the package insert of the calibration standards.

Table 2.1: Acceptable ranges for the calibration standards on the DENSICHEK PLUS.

Standard	Acceptable range	
0.5 McFarland	0.44	0.56
2.0 McFarland	1.85	2.15
3.0 McFarland	2.79	3.21

When all calibration standards were within acceptable limits, then specimen suspension readings were taken to ensure that a bacterial suspension of 0.5 – 0.62 McFarland were prepared from the organism that needs to be identified using the VITEK.

2.5.2 VITEK sensitivities

2.5.2.1 Preparation of the bacterial suspension for VITEK sensitivities

The colonies of all 117 GBS positive isolates that were cultured from the thawed 16% glycerol broths were used to prepare the bacterial suspensions for the VITEK sensitivities.
VITEK tubes, containing 3 ml of sterile VITEK Saline (0.45 – 0.5% NaCl) were inoculated with the GBS colonies, to match a broth equivalent to a McFarland standard of 0.5 – 0.62. McFarland standard broths were confirmed by the use of a DENSICHEK PLUS meter to measure and verify the McFarland of the prepared broths (See section 3.5.1.1 on the DENSICHEK PLUS). From the GBS prepared broth containing a 0.5 – 0.62 McFarland, 280 µl was transferred to a new VITEK tube (in the VITEK cassette) that contained 3 ml of sterile VITEK saline. A drop of this mixture was inoculated on sheep blood agar, streaked out for single colonies and incubated at 35°C – 37°C for 18 – 24 hours in ambient air to check the purity of the tested inoculum. A specific VITEK sensitivity card (AST-ST01) was added to the prepared tube in the VITEK cassette. The AST-ST01 VITEK card is the sensitivity card specifically to test for *Streptococcus agalactiae* sensitivities. After the sensitivity card was sealed, it was moved to the carousel and a reading of each well in the VITEK sensitivity card (containing different antibiotics) was taken every 15 minutes. Through the reading of each tested antibiotic at 15 minute intervals, the system allows for the kinetic analysis of antibiotic test results. The VITEK machine has a multichannel fluorimeter and a photometer that makes up the optical system, these combined reading were used to record fluorescence, turbidity and colourimetric signals. The results obtained through the optical system were then used to determine the minimum inhibitory concentration (MIC) value for each antibiotic on the cards. The VITEK sensitivity results for this study, using the AST-ST01 card, were completed between nine and ten hours after incubation started.

2.6 Molecular Biology

2.6.1 Genomic DNA Extraction

DNA was extracted from all 117 GBS isolates obtained in this study. The control that was included in this study was an ATCC strain known as ATCC 12403, which was obtained from University of South Africa (UNISA).

2.6.1.1 Boiling method

Initially, the boiling method as described by De Azavedo et al.1999 and Gygax et al. 2007 was used. Briefly, 30 µl of sterile distilled water was added to an Eppendorf tube and a single colony grown overnight on blood agar was picked using a sterile swab emulsified in the water. The suspensions were then boiled at 100ºC for 10 minutes, cooled down on ice for 5 min and centrifuged for 10 minutes at 8000 repetitions per minute (rpm). The supernatant

was transferred to a sterile Eppendorf tube and ready to be use PCR analysis was performed.

2.6.1.2 DNA purification method

A sweep of an 18 – 24 hour bacterial colonies from a 5% sheep blood agar was suspended in 180 µl of enzymatic lysis buffer. The enzymatic lysis buffer was prepared by using 20 mM Tris-Cl, pH 8.0. 2 mM sodium EDTA and 1.2% Triton X-100. Immediately before the lysis buffer was used, 20 mg/ml of lysozyme were added. The bacterial suspensions were then incubated for 30 minutes at 37°C in ambient air. After this pretreatment; 18.75 µl proteinase K and 150 µl Buffer AL (without ethanol) from the DNeasy Blood & Tissue Kit from Qiagen were added and mixed by vortexing. Bacterial suspensions were then incubated at 56°C for 30 minutes, followed by incubation at 95°C for 5 minutes. One hundred and fifty microliter ethanol (96-100%) was added to the samples and mixed thoroughly by vortexing. The complete mixture was then pipetted into the DNeasy Mini spin column that was placed in a 2 ml collection tube. The samples were centrifuged at 6000 x g for 1 minute. Flow-through and collection tubes were discarded. The DNeasy Mini spin column was then placed in a new 2 ml collection tube and 500 µl Buffer AW1 was added. This was then centrifuged for 1 minute at 6000 x g. Flow-through and the collection tube were then discarded. The DNeasy Mini spin column was then placed in a new 2 ml collection tube, and 500 µl Buffer AW2 was added and centrifuged for 3 minutes at 20 000 x g. Flow-through and the collection tubes were then discarded. The collection tubes were then emptied and reused in another centrifugation for 1 minute at 20 000 x g (dry spin). The DNeasy Mini spin column was then placed in a sterile 1.5 ml micro centrifuge tube and 100 µl Buffer AE was pipetted directly onto the DNeasy membrane. This was then incubated at room temperature for 1 minute followed by the centrifugation step of 1 minute at 6000 x g to elute the DNA from the filter within the column. The DNeasy Mini spin column was then placed into the same 1.5 ml micro centrifuge tube and a further 100 µl Buffer AE was pipetted directly onto the DNeasy membrane. After a 1 minute incubation period at room temperature, the tubes were centrifuged for 1 minute at 6000 x g.

DNA products from the above mentioned extraction method was checked by running the products on a 1.5% agarose gel (4 µl ethidium bromide was added to the gel during the preparation of 1.5% agarose gel) for 1 hour at 90 volts.

2.6.2 Multiplex Polymerase Chain Reaction

Table 2.2 lists the primers that were used in this study (Zeng et al. 2006).

Table 2.2 Oligonucleotide primers used in this study (Zeng et al. 2006).

Primers	Target	Sequence (5' – 3')	Size (bp)
tetmS-F	tet(M)	GTCTTGCATATATACGCCTTTATAGTGGAGTACTACATTTACGAG	
tetmA-R	tet(M)	CCACGTAATATCGTAGAAGCGGATCACTATCTGAG	347
tetoS-F	tet(O)	CGTATATATAGCGGAACATTGCATTTGAGGG	
tetoA-R	tet(O)	CGGCTCTATGGACAACCCGACAGAAG	548
ermatrS-F	erm(A/TR)	AAAATAATAGAAATTAAATCAGGAAAAGGACATTTTACC	
ermatrA-R	erm(A/TR)	CCCATTTATAAACGAAAAATCTATACTTTTTGTAGTCCTTCTT	498
mefS-F	mef(A/E)	GCAGGGCAAGCAGTATCATTAATCACTAGTGC	
mefA-R	Mef(A/E)	TGCGATAATTAAATCRGCACCAATCATTATCTTCTTC	209
apha3S-F	aphA-3	AGCTGCCTGTTCCAAAGGTCCTGA	
apha3A-R	aphA-3	CAGCTCGCGCGATCTTTAAATGG	304
aad6S-F	aad6S	TGAACGTATTCGAATTGTGACCCTTGAGG	
aad3A-R	aad3A	CATACTCCCTTGCGCTTCCGTTTCTTAC	397

These selected six-primer-pairs were included in this study, since these primers were described in previous studies to be associated with resistance genes in streptococci (Poyart et al. 2003).

The six-primer-pair multiplex polymerase chain reaction (PCR) mixture as previously described by Zeng et al (**Table 2.3**) was prepared as indicated in **Table 2.3.** The reactions components for the master mix, as indicated in **Table 2.3**, were set up in a nuclease free microfuge tube.

Table 2.3: PCR master mix preparation before optimization as previously described by Zeng et al.

Component	PCR Multiplex (µl)	[Final]	Size (bp)
Sterile water	2.25		
dNTP mix (10 mM)	2.5	0.2 mM	
TetmS-F (10 µM)	1.25	1 µM	347
TetmA-R (10 µM)	1.25	1 µM	
TetoS-F (10 µM)	1.25	1 µM	548
TetoA-R (10 µM)	1.25	1 µM	
ErmatrS-F (10 µM)	1.25	1 µM	498
ErmatrA-R (10 µM)	1.25	1 µM	
MefS-F (10 µM)	1.25	1 µM	209
MefA-R (10 µM)	1.25	1 µM	
Apha3S-F (10 µM)	1.25	1 µM	304
Apha3A-R (10 µM)	1.25	1 µM	
Aad6S-F (10 µM)	1.25	1 µM	397
Aad6A-R (10 µM)	1.25	1 µM	
Template nucleic acid	2.5		
$MgCl_2$ (25 mM)	1.25	3.5 mM	
5 x PCR buffer	1.25	1 x	
Taq Polymerase Enzyme (5u/µl)	0.25	0.1 unit	
TOTAL VOLUME	25 µl		

After optimization of the PCR ingredients, the master mix indicated in **Table 2.4** was used. The reactions components for the master mix were set up in a nuclease free microfuge tube. Briefly, the dNTP concentration was increased from 0.2 mM to 0.4 mM; 5 µl of template DNA was used; the $MgCl_2$ concentration was increased from 3.5 mM to 4.5 mM and 5 units of *Taq* was used.

Table 2.4: PCR master mix and predicted sizes of Polymerase Chain Reaction amplicons.

Component	PCR Multiplex (µl)	[Final]	Size (bp)
Sterile water	1.75		
dNTP mix (10 mM)	6.25	0.4 mM	
TetmS-F (10 µM)	0.25	0.1 µM	347
TetmA-R (10 µM)	0.25	0.1 µM	
TetoS-F (10 µM)	0.25	0.1 µM	548
TetoA-R (10 µM)	0.25	0.1 µM	
ErmatrS-F (10 µM)	0.25	0.1 µM	498
ErmatrA-R (10 µM)	0.25	0.1 µM	
MefS-F (10 µM)	0.25	0.1 µM	209
MefA-R (10 µM)	0.25	0.1 µM	
Apha3S-F (10 µM)	0.25	0.1 µM	304
Apha3A-R (10 µM)	0.25	0.1 µM	
Aad6S-F (10 µM)	0.25	0.1 µM	397
Aad6A-R (10 µM)	0.25	0.1 µM	
Template nucleic acid	5		
$MgCl_2$ (25 mM)	3	4.5 mM	
5 x PCR buffer	5	1 x	
Taq Polymerase Enzyme (5u/µl)	1	5 unit	
TOTAL VOLUME	25 µl		

All samples together with a blank (water) control as well as the ATCC 12403 control organism was then placed in a thermocycler (Biorad T100 termocycler) and the conditions as shown in **Table 2.5** was applied.

The PCR amplification conditions originally used by Zeng et al were: 95°C for 15 min(one cycle), 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, (repeated for 35 cycles), 72°C for 10 min (1 cycle), and 22°C hold. Some minor changes were made to these conditions in order to optimise the PCR cycle conditions (as seen in **Table 2.5**) for our study.

Table 2.5: Multiplex PCR cycle conditions.

Cycles	Temperature and Time		
1 x	Initial denaturation	95°C	15 minutes
30 x	Denaturation	94°C	30 seconds
	Annealing	60°C	30 seconds
	Elongation	72°C	1 minute
1 x	Use a prolonged elongation time 72°C up to 10 minutes. 14°C hold		

After the PCR cycles were done, polymerase chain reaction amplicons were confirmed by electrophoresis on a 1.5% agarose gel. One times concentration TAE electrophoresis buffer was used and the gel was run for 1 hour at 90 volts.

2.6.3 Cleaning of the amplified PCR products

Cleaning of the amplified PCR products were done using the Promega Wizard® SV Gel and PCR Clean-Up System. After electrophoresis of the PCR products, representative bands of the different amplicon sizes were excised from the gel. Firstly the gel slices were dissolved as follows: gel slices were placed in individual 1.5 ml labelled micro centrifuge tubes and 10 µl membrane binding solution were added for every 10 mg of gel slice. The mixture was then mixed by vortexing and incubated at a temperature of 50 – 65°C until the agarose gel was completely dissolved,. The DNA mixture was then transferred to a SV minicolumn attached to a collection tube. After 1 minute incubation at room temperature the mixture was centrifuged at 16 000 xg for 1 minute and the flow through was discarded and the minicolumn reinserted into the collection tube. The minicolumns containing the DNA was washed as follows: 700 µl of membrane wash solution was added to each tube and again centrifuged at 16000 xg for 1 minute after which the flow through was once again discarded and the minicolumn reinserted into the collection tube; 500 µl of membrane wash solution was then added to each tube and then centrifuged at 16000 xg for 5 minutes. Collections tubes were then emptied and the columns re-centrifuged for 1 minute with an open lid to allow residual ethanol evaporation. Lastly the DNA was eluted as follows: each minicolumn were transferred to a clean 1.5 ml microcentrifuge tube, 50 µl of nuclease free water were added to the minicolumn and incubated for 1 minute at room temperature after which it was centrifuged at 16000 xg for another 1 minute. The minicolumns were discarded and the eluted DNA stored at -20°C.

2.6.4 Sequencing

All sequencing was completed at the University of Stellenbosch's Central Sequencing Facility, with the same forward primers used for each respective PCR. Nucleotide sequences

were analysed using BLAST analysis at National Centre for Biotechnology Information (NCBI) (Altschul et al. 1990).

2.7 Statistical analysis

All data obtained from the completed GBS study questionnaire, laboratory identification and sensitivity results from the collected swabs were imported into the Statistical Package for Social Sciences (SPSS) version 22, 2013, software system for data analysis. Statistical Package for Social Sciences is the statistical analysis program available at and licensed to be used at my current institution, and therefore the statistical tool of choice. Furthermore, with the assistance of a statistician and using the SPSS software system, the odds ratio was calculated for the different sets of media that were used in the isolation of GBS in this study.

CHAPTER THREE: RESULTS

3.1 BACTERIOLOGY

Reporting of GBS isolates when detected on the culture media were done as follows:
When a participant had GBS growth on the direct inoculated BCNA agar, it was reported as positive. When the same participant also had GBS growth on the BCNA plate after sub-culturing from the Todd Hewitt enrichment broth (TH-BCNA) it was not reported as positive for a second time. **Figure 3.1** illustrates a positive GBS culture seen on BCNA agar. Some GBS isolates showed clear beta-haemolysis as seen in **Figure 3.1** while other GBS isolates showed only slight beta-haemolysis (could even be considered as alpha-haemolysis by some scientists) as seen in **Figure 3.2**.
Example: BCNA positive; TH-BCNA positive, but considered as one colonisation.

Whenever a participant had GBS growth for the first time on the BCNA plate after sub-culturing from the Todd Hewitt enrichment broth, it was reported as positive from the TH-BCNA.
Example: BCNA negative; TH-BCNA reported as positive

Whenever a participant tested positive on BCNA (direct inoculation) or TH-BCNA and Granada agar it was reported as a positive on the initial media and the Granada agar in order to compare the pre-prepared BCNA (pBCNA) purchased from bioMérieux and Granada detection rate for GBS. When a participant tested positive on both, the participant were still only counted once as a positive participant (being GBS colonised).
Example: pBCNA positive; TH-pBCNA positive (first direct inoculation reported); Granada positive (**Figure 3.3**).
Example: pBCNA negative; TH-pBCNA positive (first positive growth); Granada positive

Whenever a participant had GBS growth only on the Granada agar, it was reported as such.
Example: pBCNA negative; TH-pBCNA negative; Granada positive

Whenever a participant had GBS growth on any of the culture media, the participant is considered colonised with GBS. Some colonised participants presented with more than one GBS isolate.
Example: The participant had GBS growth on both the lower vaginal and rectal swabs. This participant will then be considered GBS colonised, presenting with two GBS isolates (one colonisation, but two isolates). Capsular typing would have to be done on GBS isolates in order to distinguish the isolates from each other. Since, capsular typing was originally included within the objectives of this study, it was not done. Studying the susceptibility profiles (**Appendix D**), it was shown that some of the isolates from the two different

anatomical sites of one participant presented with different MIC results on the VITEK (27LVS & 27RS; 426LVS & 426RS; 466LVS & 466RS; 469LVS & 469RS; 478LVS & 478RS; 539LVS & 539RS; 665LVS & 665RS) while some of the isolates from the two different anatomical sites presented with different PCR results (49LVS & 49RS; 450LVS & 450RS; 539LVS & 539RS; 575LVS & 575RS; 580LVS & 580RS). For this reason, for the purpose of this study all isolated GBS strains were presumed to be different isolates.

Note that when a single swab from a participant presented with growth on more than one of the culture media used, it was reported as such but only considered as one colonisation and one isolate.

Figure 3.1: A positive GBS culture presenting with clear beta-haemolysis seen on a BCNA agar plate.

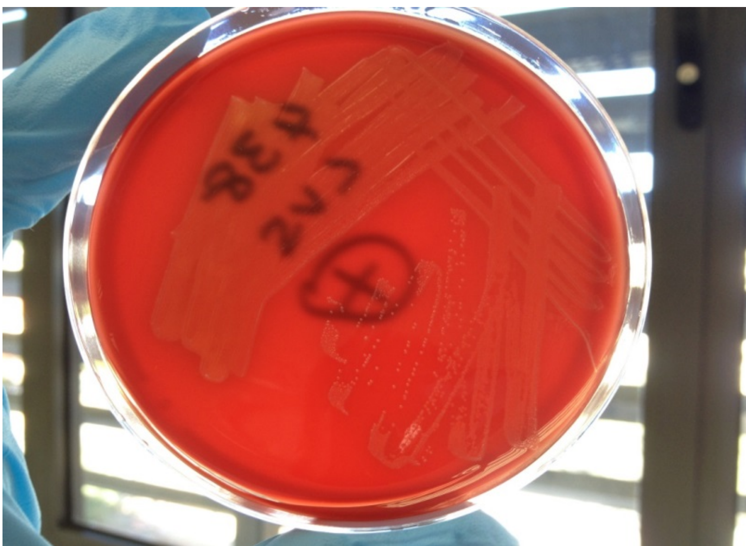

Figure 3.2: A positive GBS culture presenting with slight beta-haemolysis seen on a BCNA agar plate.

Figure 3.3: Top half of plate: The Granada agar presenting with orange carotenoid colonies considered a positive GBS growth.

3.1.1 Group B *Streptococcus* prevalence seen in this study

A total number of 588 willing participants took part in this study. From each participant both a lower vaginal and rectal swab was collected resulting in a total sample number of 1176 samples. Participants were mainly from the antenatal clinic at Windhoek Central Hospital. Sample collection took place over a 13 month period, from August 2013 up till and including August 2014. Initially, the total number of participant swabs that were cultured directly on prepared BCNA followed by enrichment in Todd Hewitt broth (containing 8 µg of gentamycin/ml and 15 µg of nalidixic acid/ml) with subsequent BCNA plating, was 419 participants. The last 169 participant samples were inoculated directly on both pBCNA agar and Granada agar (bioMérieux) followed by enrichment in Todd Hewitt broth with subsequent pBCNA plating. As mentioned previously, at the time of the sampling cycle when the Granada agars were included, ready prepared BCNA plates (pBCNA), produced by bioMérieux were used instead of the self-prepared BCNA (Section 2.2.2) since the blood source of the self-prepared BCNA plates were not well controlled, and haemolysis on the self-prepared BCNA not as convincing. Of the 588 participants, 80 participants tested positive for GBS, with a median age of 28 amongst the GBS colonised women from this study. The reporting of GBS positives is explained within section 3.1. This gives a GBS colonisation prevalence rate of 13.6% (**Figure 3.4**). Although 80 participants tested positive for GBS, 117 GBS isolates were found amongst these 80 participants. Some participants tested positive for both lower vaginal and rectal swabs and in such cases, both isolates were investigated further [(therefore in some cases there were two GBS isolates (referred to as one GBS colonisation) obtained from one participant]).

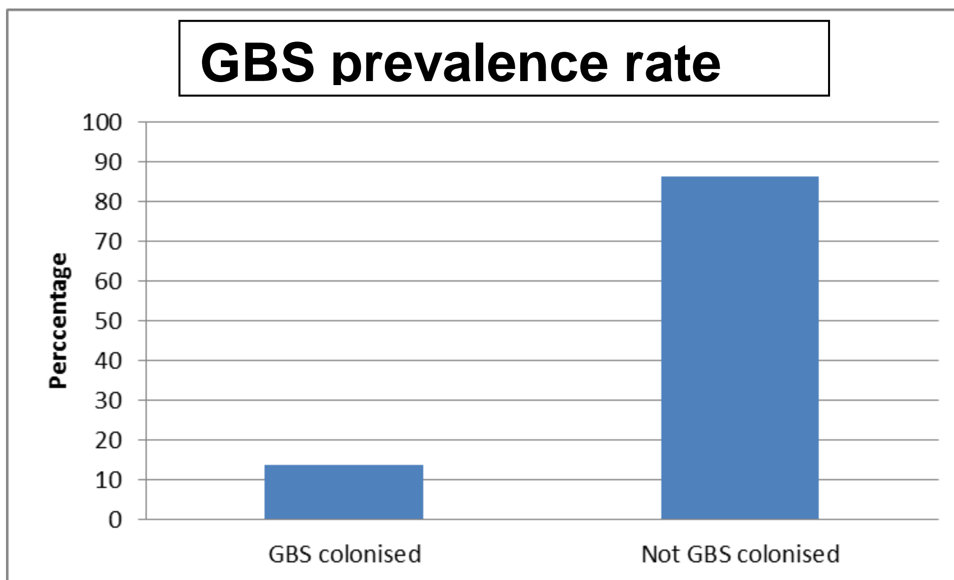

Figure 3.4: **Group B *Streptococcus* prevalence rate from the current study.**

3.1.2 Culture media used to detect GBS

As mentioned above, initially, the total number of participant swabs that were cultured directly on BCNA followed by enrichment in Todd Hewitt broth was cultured on BCNA and enriched in Todd Hewitt broth (containing 8 µg of gentamycin/ml and 15 µg of nalidixic acid/ml) with subsequent BCNA plating, was 419 participants. Of these participants, 31 participants tested positive for GBS. A prevalence of 7.4% was found amongst these participants.

For the initial 419 participants (838 samples – both lower vaginal and rectal swab from each participant) whose swabs were not inoculated simultaneously on Granada agar plates the following were seen:

- five of the GBS positive participants showed positive growth on the direct inoculated BCNA agar for both their lower vaginal and rectal swabs; six of the GBS positive participants showed positive growth on the direct inoculated BCNA agar for their lower vaginal swabs only, while seven participants showed GBS on the directly inoculated BCNA agar from their rectal swabs only (**Appendix C**).
- one GBS positive participants showed positive growth from the sub-cultured Todd Hewitt broth onto BCNA (TH-BCNA) for both her lower vaginal and rectal swabs; 11 of the GBS positive participants showed positive growth on the TH-BCNA for their lower vaginal swabs only while only one GBS positive participant showed positive growth on the TH-BCNA for her rectal swab only (**Table 3.1**).

The total number of GBS colonised participants for the first 419 participants was thus 31 while 37 GBS isolates were identified. This included participants that were positive for the directly inoculated BCNA cultures and those positive for the TH-BCNA cultures.

Table 3.1: Summary of the number of GBS positive samples detected on different media used for the first 419 samples.

Culture media used	No. of participant positive for GBS on both lower vaginal & rectal swabs	No. of participant positive for GBS on lower vaginal swab only	No. of participant positive for GBS on rectal swab only
Direct inoculated BCNA	5	6	7
TH-BCNA	1	11	1

No.=number; BCNA=Columbia sheep blood agar with 8 µg of gentamycin and 15 µg of nalidixic acid/ml; TH=Todd Hewitt broth with 8 µg of gentamycin/ml and 15 µg of nalidixic acid/ml; GBS=Group B *Streptococcus*.

The last 169 participant samples were inoculated directly on both pre-prepared pBCNA agar and Granada agar (bioMérieux) followed by enrichment in Todd Hewitt broth with subsequent pBCNA plating. An example of such positive cultured plates can be seen in **Figure 3.5** and **3.6**. Since there was both a rectal swab (RS) and lower vaginal swab (LVS) collected from each participant, the total number of samples processed on pBCNA, Granada and enriched in Todd Hewitt broths was 338 (two samples for each of the 169 participants).

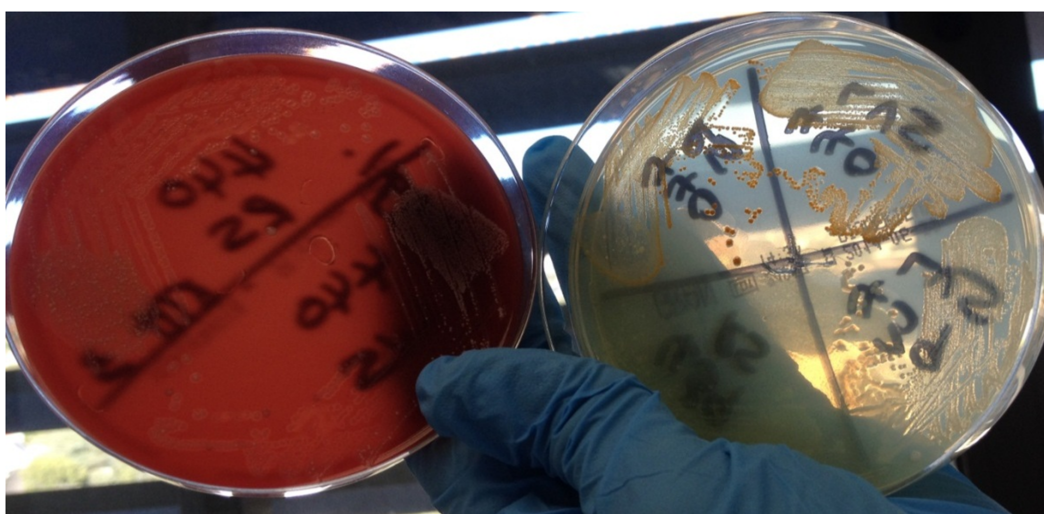

Figure 3.5: On the left: Columbia sheep blood agars containing 8 µg of gentamycin/ml (BCNA) and 15 µg of nalidixic acid/ml presenting the growth of a GBS positive sample as the beta-haemolytic colonies. On the top right: The Granada agar with growth of a GBS positive sample presenting as orange carotenoid colonies.

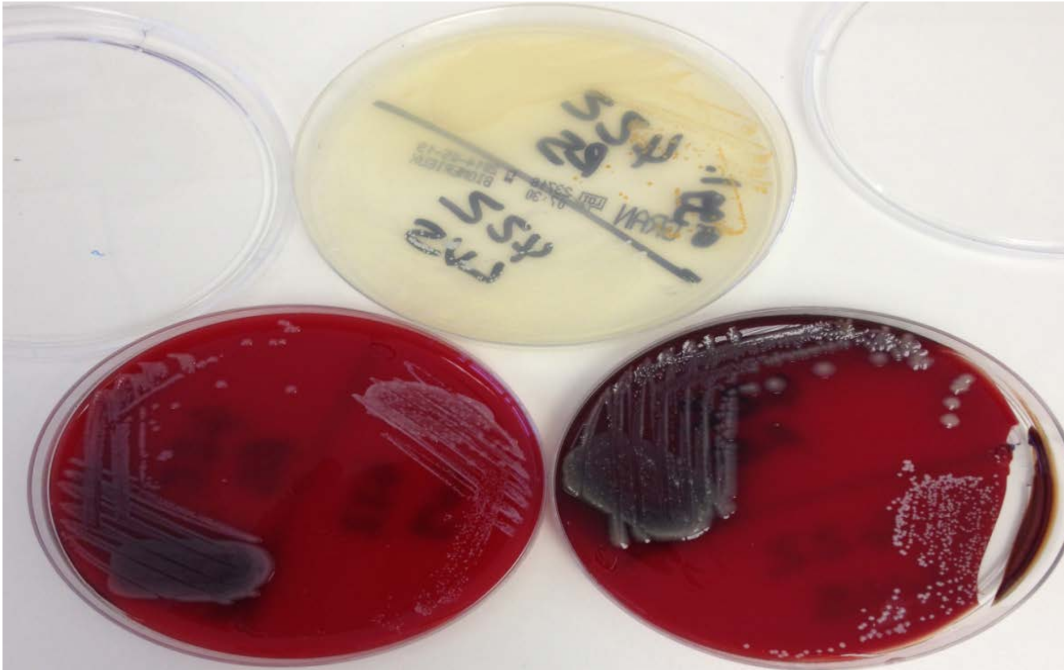

Figure 3.6: Sample 422, showing no visible beta-haemolysis on the Columbia sheep blood agars with 8 µg of gentamycin/ml and 15 µg of nalidixic acid/ml (BCNA) the, but clear orange carotenoid colonies on the Granada agar.

For samples 420 to 588, both the Granada agar and pBCNA from bioMérieux were used together with the Todd Hewitt enrichment broths. The total number of participants for whom the samples were processed in this way added up to 169. Since there was both a rectal and lower vaginal swab collected from each participant, the total number of samples processed in this way was 338.

After the incorporation of the Granada agar and the pBCNA plates from bioMérieux, the following were seen:

- Seventeen of the GBS colonised participants showed positive growth on the direct inoculated pBCNA agar as well as the Granada agar for both their lower vaginal and rectal swabs; two of the GBS colonised participants showed positive growth on the direct inoculated pBCNA agar and the Granada agar for their lower vaginal swabs only; and none of the participants presented with only a positive rectal swab on the direct inoculated pBCNA agar and Granada agar.
- Fourteen GBS colonised participants showed positive growth on the TH-pBCNA and the Granada agar for both their lower vaginal and rectal swabs; nine GBS colonised participants showed positive growth on the TH-pBCNA and the Granada agar for their lower vaginal swabs only, while three GBS colonised participants showed positive growth on the TH-pBCNA and the Granada agar for their rectal swabs only.

- There were three GBS colonised participants that were present only on the Granada agar from rectal swabs and one GBS colonised participant that were present only on the Granada agar from her lower vaginal swab (**Table 3.2)**.

The total amount of GBS colonised participants for the last 169 participants that were GBS positive was thus 49 while 80 GBS isolates were identified. This included participants that were positive for the directly inoculated pBCNA plates and Granada plates; those pBCNA plates that were only positive after the Todd Hewitt enrichment as well as on the Granada plates and those only positive on the Granada plates. Of the 169 participant whose samples had a Granada agar added to the rest of the culture media, 49 tested positive for GBS colonisation and therefore presented a prevalence rate of 28.99% (49/169).

The odds of testing positive are five times higher under the pBCNA, TH-pBCNA and Granada testing media as compared to the BCNA and TH-BCNA and are statistically significant with a p-value of < 0.000 (**Table 3.2**).

Table 3.2: Odds ratio and p value calculation for using the different sets of media for GBS isolation.

Media used	**Test results**		**Nr of cases**
	Positive	**Negative**	**Total**
pBCNA, TH-pBCNA and Granada	49	120	169
BCNA and TH-BCNA	31	388	419
Total	80	508	588
	Point Estimate		**(95% Confidence interval)**
Odds ratio	5.1108		(3.1179-8.3774)
p-value	<0.0000		

Appendix C provides more detail as to which media the positive GBS isolates presented itself on.

For the total 588 participants, 80 were thus positive for GBS colonisation while 117 GBS isolates were identified. Of the 80 GBS colonised participants, 14 were positive from the rectal swabs only, 29 from the lower vaginal swabs only and 37 from both lower vaginal and rectal swabs. Therefore 46.25% tested positive on both lower vaginal and rectal swabs, 36.25% tested positive on the lower vaginal swab only and 17.5% tested positive on rectal swab only (**Figure 3.7)**.

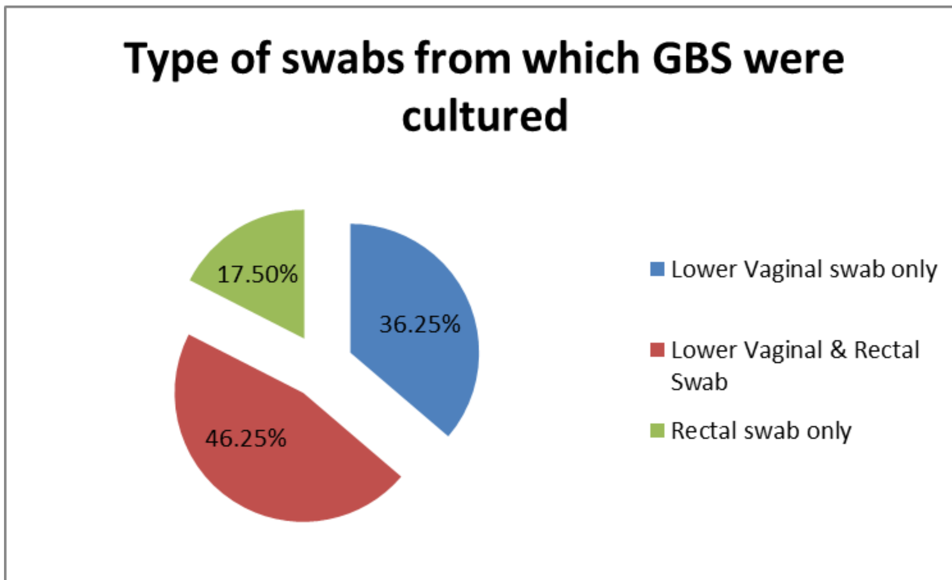

Figure 3.7: The percentage of participants that tested positive on vaginal swab only, rectal swab only and both vaginal and rectal swabs.

A summary of the number of GBS positive samples on different culture media for the last 169 samples can be seen in **Table 3.3**.

Table 3.3: Summary of the number of GBS positive samples detected on different media used for the last 169 samples.

Culture media used	No. of participant positive for GBS on both lower vaginal & rectal swabs	No. of participant positive for GBS on lower vaginal swab only	No. of participant positive for GBS on rectal swab only
Direct pBCNA & Granada	17	2	0
Granada & TH	14	9	3
Granada only	0	1	3

No.=number; pBCNA=Ready to be used bought Columbia sheep blood agar with 8 µg of gentamycin and 15 µg of nalidixic acid/ml produced by bioMérieux; TH=Todd Hewitt broth with 8 µg of gentamycin/ml and 15 µg of nalidixic acid/ml produced by bioMérieux; Granada=Granada agar; GBS =Group B *Streptococcus*.

All the direct inoculated pBCNA positive cultures also presented with positive TH-pBCNA cultures. Thirty seven of the colonised participants presented positive on the direct inoculated pBCNA culture as well as on the TH-pBCNA cultures. Thirty nine of the colonised participants presented with a negative direct inoculated pBCNA cultures and positive TH-pBCNA cultures. The remaining four colonised participants were detected on the Granada agar only.

3.1.3 Group B *Streptococcus* identification

3.1.3.1 Catalase

The catalase test was performed in triplicate for the GBS isolates (117 positive swabs). All colonies tested negative.

3.1.3.2 Agglutination

The identification of all 117 GBS isolates was done by the use of the Oxoid Streptococcal Grouping Kit (DR585), which is a latex agglutination test. Agglutination tests for GBS identifications were done on every presumptive GBS positive isolate (see section 2.4.1 for which details when an isolate is considered presumptively GBS positive) from the current study. Analysis of the 117 isolates from the current study showed that all 117 tested positive for *Streptococcus agalactiae*.

3.1.3.3 VITEK identifications

VITEK identifications on random GBS isolates from this study were done to confirm identification using bacteriology. All isolates presented with a GBS identification of 96% - 99%, which is an excellent identification confidence level.

3.2 ANTIMICROBIAL SUSCEPTIBILTIY RESULTS

Antimicrobial susceptibility testing was done by obtaining the minimum inhibitory concentrations (MIC) of each tested antimicrobial substance from the VITEK revealed the following results. Benzylpenicillin, ampicillin, ceftriaxone, linezolid and vancomycin showed 100% sensitivity, indicating that all the tested GBS isolates were sensitive to these five antimicrobial substances. These antimicrobial substances are said to be sensitive because the minimum inhibitory concentration (MIC) for all five these drugs is within the sensitive zone interpreted according to the Clinical Laboratory Standards Institute (CLSI) guidelines. A MIC interpreted as sensitive, indicates that therapeutically safe concentrations of the drugs in the human body will effectively kill the tested GBS isolates. Levofloxacin presented intermediate resistance in one of the isolates, although the MIC tested sensitive, the Advance Expert System of the VITEK 2 system, changed the result to intermediate resistance.

Antimicrobial substances said to be resistant has a MIC detected for that specific drug falling in the resistant zone as prescribed by the CLSI guidelines. This indicates that there is no therapeutically safe concentration (for that specific antimicrobial substance) in the human body that would effectively kill the tested organism. Antimicrobial substances said to be intermediate resistant indicates that the MIC detected is between the sensitive and resistant zone prescribed by the CLSI guidelines.

Resistance rates seen amongst the screened GBS isolates included: trimethoprim/sulfamethoxazole with 6% (7/117) resistance, tetracycline with 94.9% (111/117) resistance, erythromycin with 11.1% (13/117) resistance and clindamycin with 8.5% (10/117) resistance. Amongst the screened GBS isolates, 5.1% (6/117) showed inducible resistance against clindamycin. In the case of inducible clindamycin resistance (ICR), the clindamycin test sensitive on its own (in vitro), but when placed next to an erythromycin disc (15 mm apart) ICR is presented through the flattening of the inhibition zone (see section 1.2.9 for more detail on ICR). Complete resistance against clindamycin were seen in 8.5% (1/117) of the screened GBS isolates. Ceftriaxone presented with 100% (117/117) susceptibility for all tested GBS isolates.

The susceptibility profiles of the GBS isolates from this study for the antimicrobial substances investigated are presented in **Table 3.4**.

Table 3.4 Antimicrobial susceptibility results for the Group B *Streptococcus* isolates from pregnant women in the Khomas region at 35 weeks gestation onwards (n = 117).

Antibiotic (tested with VITEK)	**Number (%) of the isolates with S/I/R to the tested antibiotics**		
	Susceptible	**Intermediate**	**Resistant**
Benzylpenicillin	(n=117) 100 %	0 %	0 %
Ampicillin	(n=117) 100 %	0 %	0 %
Cefotaxime	*(n=116) 100 %	0 %	0 %
Cefriaxone	(n=117) 100 %	0 %	0 %
Levofloxacin	(n=116) 99.1 %	(n=1) 0.9 %	0 %
Erythromycin	(n=104) 88.9%	0 %	(n=13) 11.1 %
Clindamycin	(n=85) 72.6 %	(n=22) 18.8%	(n=10) 8.5 %
ICR	Positive in (n=6) 5.1% of all the GBS isolates		
Linezolid	(n=117)100 %	0 %	0 %
Vancomycin	(n=117)100 %	0 %	0 %
Tetracycline	(n=6) 5.1 %	0 %	(n=111) 94.9 %
Trimethoprim/ Sulfamethoxazole	(n=100) 94 %	0 %	(n=7) 6 %

Abbreviations: S=Susceptible; I=Intermediate; R=Resistant; ICR=Inducible Clindamycin Resistance; n=the actual number of isolates. *Discussed under cefotaxime in paragraph below.

The highest MIC (minimum inhibitory concentration) for penicillin that were detected in a total of seven isolates from this study were 0.12 (still being sensitive), and the remaining 110 isolates presented with a MIC of </= 0.06, indicating sensitivity. Amoxicillin showed a uniform MIC of </= 0.25 (sensitive) for all GBS isolates from this study. Cefotaxime presented with a uniform MIC value of </= 0.12 (sensitive), *except for one sample that had a modified MIC of one (sensitive) according to the VITEK report, but it is unclear why the MIC were modified by the Advanced Expert System (AES) on the VITEK report. Ceftriaxone also presented with a constant MIC reading of </= 0.12 (sensitive) for all isolates.

For levofloxacin, 21 of the isolates presented with a MIC reading of 0.5 (sensitive), one sample presented with a MIC reading of two (sensitive, but were changed by the Advanced Expert System on the VITEK to intermediate resistant) and the remaining 95 all gave an MIC reading of one (sensitive). Erythromycin had 11 isolates that presented with a MIC of two (resistant), four presented with a MIC of >/= 8 (resistant), and the remaining 102 showed a MIC value of </= 0.12 (sensitive). As for clindamycin, 22 isolates showed a MIC of 0.5 (intermediate), 12 a MIC of >/= 1 (resistant), and the rest of the isolates (83/117) presented with a MIC of </= 0.25 (sensitive). Vancomycin presented with a constant MIC of </= 0.25 (sensitive) for all GBS isolates from this study. For tetracycline five isolates presented with MIC's of two (sensitive), while the rest of the isolates (112/117) presented >/=16 (resistant) as MIC value. Of the total 117 GBS isolates, three presented with a MIC of >/= 320 (resistant), four presented with MIC values of >/=160 (resistant) and the remaining 110 presented MIC's of </=10 (sensitive) for trimethoprim/sulfamethoxazole. Five GBS isolates tested positive for ICR, whereas the rest (112) of all the tested GBS isolates were negative for ICR. In some cases the advanced expert system programmed on the VITEK changed sensitive interpretations to either intermediate resistance or fully resistant although the MIC might have been sensitive according to CLSI guidelines. An example of such a case would be when a sample is positive for ICR, although the MIC of the clindamycin might seem to be sensitive, the interpretation of the result were changed to resistant, since treatment with clindamycin in such a case would be inappropriate.

Appendix D is a summary of all the isolates with the MIC and sensitivity interpretation results.

3.3 POLYMERASE CHAIN REACTION

3.3.1 Optimisation of DNA extraction from GBS isolates

The initial method for the extraction of the bacterial deoxyribonucleic acid (DNA) from the GBS isolates in this study was done by using the boiling method (see section 2.6.1.1) as previously outlined by De Avaredo et al and Gygax et al. (De Avaredo et al. 1999; Gygax et al. 2007). In order to ensure that the DNA was successfully extracted during the boiling method, products were analysed on a 1.5% agarose gel, but did not yield any DNA.

The DNA extraction method described in the DNeasy Blood & Tissue Kit from Qiagen (see section 2.6.1.2) was used for a second attempt to extract DNA, which showed a significant yield of DNA from all 117 GBS isolates after analysis on a 1.5% agarose gel as illustrated in **Figure 3.8.**

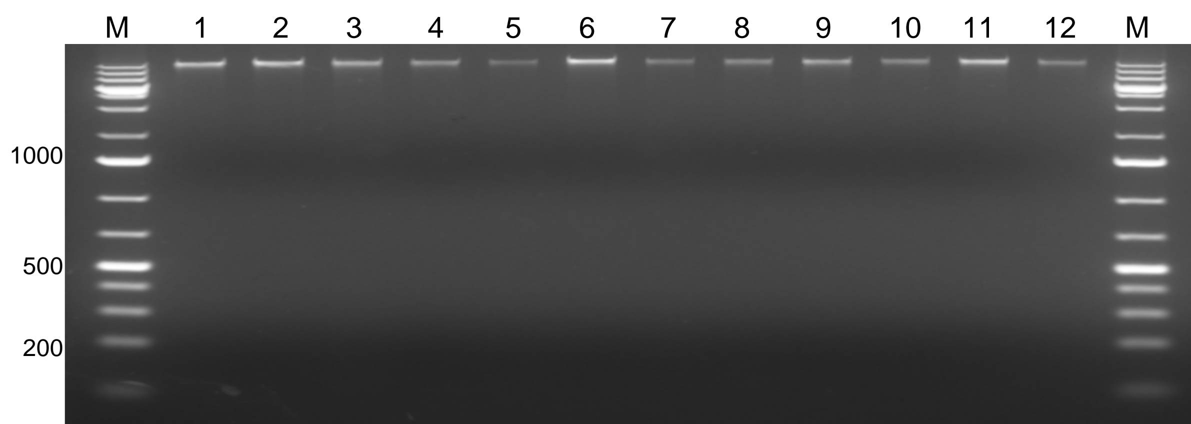

Figure 3.8 Representative agarose gel electrophoresis showing uncut genomic DNA extracted from GBS isolates using the DNeasy Blood & Tissue Kit from Qiagen for extraction. Lanes M: Molecular weight marker (GenerulerTM 1 kb plus DNA ladder, Fermentas); Lane 1 – 12 presenting GBS samples 27 RS, 27 LVS, 31 RS, 40 LVS, 42 LVS, 42 RS, 44 LVS, 45 LVS, 49 RS, 49 LVS and 59 LVS.

3.3.2 Optimisation of PCR protocol

Previously published specific primers (Zeng et al. 2006) were used to amplify potential resistance genes in the GBS isolates obtained from this study. Genes tested for in this study included the *tet*(M) and *tet*(O), *erm*(A/TR), *mef*(A/E), *aph*A-3 and *aad*-6 genes. Group B *Streptococcus* is often resistant to tetracycline, and this is usually because of the presence of *tet*(M) or *tet*(O) genes. Inducible cross-resistance or constitutive resistance to macrolides streptogramin B antibiotics and lincosamine are encoded for by the *erm*(A/TR) gene. The *mef*(A or E) gene is usually responsible for antimicrobial resistance to erythromycin, due to the encoding of the efflux pump in the bacteria (Zeng et al. 2006). Resistance to aminoglycosides are encoded for by both the *aph*A-3 and *aad*-6 genes.

The PCR results that presented on the 1.5% agarose gel after electrophoresis from the master mix described in **Table 2.3** yielded much clearer and more distinct bands than that of the master mix previously described by Zeng et al (Zeng et al. 2006).

3.3.3 Identification of specific resistance genes in the GBS isolates by Multiplex PCR

The optimised PCR protocol (**Table 2.3**) was used when performing the multiplex PCR. The GBS samples from this study were amplified by means of PCR cycles described in **Table 2.4** along with the ATCC 12403 control strain.

The results of the multiplex PCR after electrophoresis was performed on a 1.5% agarose gel for all samples. All 117 isolates were amplified in the same run and therefore only one control was included in the run. Examples of the gels presenting with amplified products is presented in **Figures 3.9** and **3.10.** Faintly visible bands were seen for amplicon size 639 bp (which was considered an artefact) on the sample gel seen in **Figure 3.9** and **3.10**.

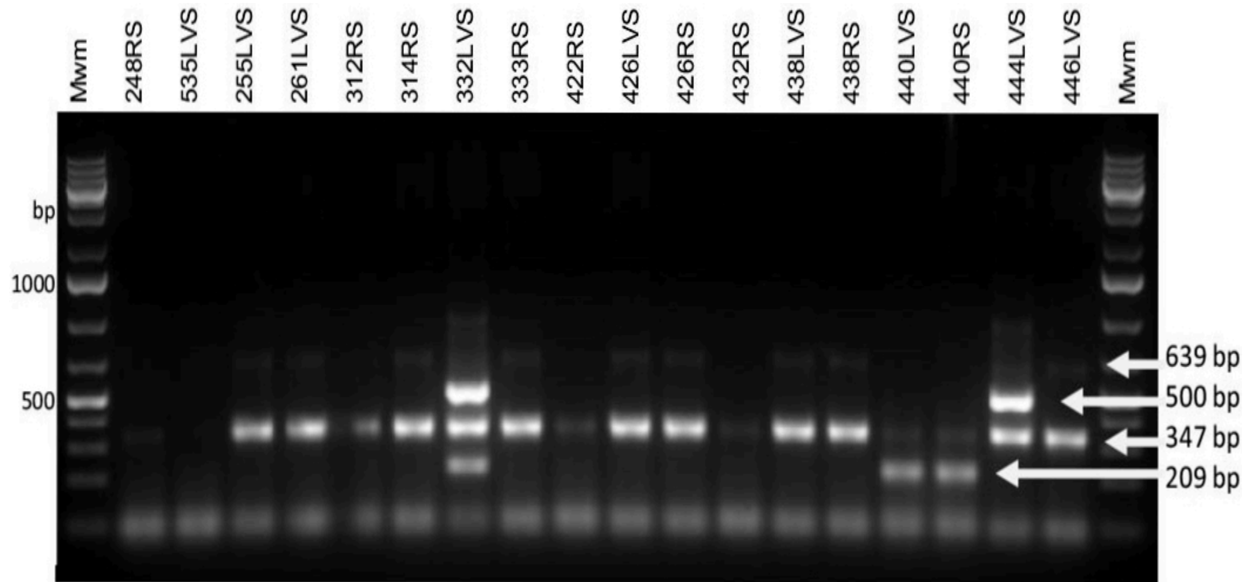

Figure 3.9: Agarose gel electrophoresis showing amplicons of DNA extracted from GBS strains for resistant genes using 9 specific primers pairs. Lane Mwm: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas), followed by samples; 248 RS, 535 LVS, 255 LVS, 261 LVS, 312 RS, 314 RS, 332 LVS, 333 RS, 422 RS, 426 LVS, 426 RS, 432 RS, 438 LVS, 438 RS, 440 LVS, 440 RS, 444 LVS, 446 LVS.

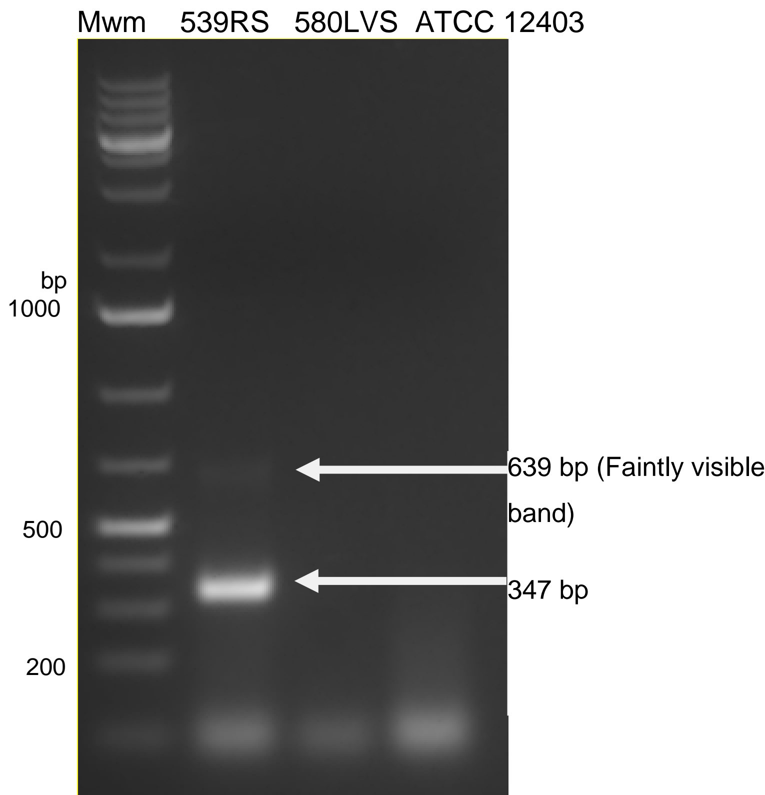

Figure 3.10: Agarose gel electrophoresis showing amplicons of DNA extracted from GBS strains for resistant genes using 9 specific primers pairs. Lane Mwm: Molecular Weight Marker (Generuler^{TM} 1 kb plus DNA ladder, Fermentas), followed by isolates 539 RS, 580 LVS, ATCC 12403.

Results obtained, for all GBS isolates, from the PCR after electrophoresis on the 1.5% agarose gels, is summarised in **Table 3.5**.

Table 3.5: Summary of antibiotic resistance genes and VITEK susceptibility profiles that was detected in GBS isolates for this study.

Isolate	Amplification by Multiplex PCR						Antibiotic resistance (VITEK profile)				
	Tetracycline		i/cMLS	Erythromycin	Aminoglycosides		Tetracycline	Macrolides (Erythromycin)	Lincosamide (Clindamycin)	Penicillin	ICR
	tet(M) (347 bp)	*tet*(O) (548 bp)	*erm*(A/TR) (498 bp)	*mef*(A/E) (209 bp)	*aphA* (304 bp)	*aad*-6 (397 bp)					
27LVS	+	-	-	-	-	-	R	R	R	S	-
27RS	+	-	-	-	-	-	R	S	I	S	-
31LVS	+	-	-	-	-	-	R	S	S	S	-
40LVS	+	-	-	-	-	-	R	S	S	S	-
42RS	+	-	-	-	-	-	R	S	I	S	-
42LVS	+	-	-	-	-	-	R	S	I	S	-
43LVS	+	-	-	-	-	-	R	S	S	S	-
44LVS	+	-	-	-	-	-	R	S	S	S	-
45LVS	+	-	-	-	-	-	R	S	I	S	-
49LVS	+	+	-	-	-	-	R	S	I	S	-
49RS	+	-	-	-	-	-	R	S	I	S	-
59LVS	+	-	-	-	-	-	R	S	S	S	-
65LVS	+	-	-	-	-	-	R	S	S	S	-
68RS	+	-	-	-	-	-	R	S	I	S	-
70RS	+	-	-	-	-	-	R	S	S	S	-
71LVS	+	-	-	-	-	-	S	S	I	S	-
73LVS	+	-	-	-	-	-	R	S	I	S	-
74LVS	+	-	-	-	-	-	R	S	S	S	-
79LVS	+	-	-	-	-	-	R	S	S	S	-
102LVS	+	-	-	-	-	-	R	S	S	S	-
212LVS	+	-	-	-	-	-	R	S	S	S	-
218LVS	+	-	-	-	-	-	R	S	S	S	-
224LVS	+	-	-	-	-	-	R	S	S	S	-
234RS	+	-	-	-	-	-	R	S	S	S	-
234LVS	+	-	-	-	-	-	R	S	I	S	-
238RS	+	-	-	-	-	-	R	S	S	S	-
238LVS	+	-	-	-	-	-	R	S	S	S	-
240LVS	+	-	+	-	-	-	R	S	S	S	+
240RS	+	-	+	-	-	-	R	R	R	S	+
244LVS	+	-	-	-	-	-	R	S	S	S	-
244RS	+	-	-	-	-	-	R	S	S	S	-
248LVS	+	-	-	-	-	-	R	S	I	S	-
248RS	+	-	-	-	-	-	R	S	I	S	-
255LVS	+	-	-	-	-	-	R	S	S	S	-

Isolate	Amplification by Multiplex PCR						Antibiotic resistance (VITEK profile)				
	Tetracycline		i/cMLS	Erythromycin	Aminoglycosides		Tetracycline	Macrolides (Erythromycin)	Lincosamide (Clindamycin)	Penicillin	Inducible Clindamycin Resistance (ICR)
	tet(M) (347 bp)	*tet*(O) (548 bp)	*erm*(A/TR) (498 bp)	*mef*(A/E) (209 bp)	*aphA* (304 bp)	*aad*-6 (397bp)					
261LVS	+	-	-	-	-	-	R	S	I	S	-
312RS	+	-	-	-	-	-	R	S	I	S	-
314RS	+	-	-	-	-	-	R	S	S	S	-
332LVS	+	-	+	+	-	-	R	R	R	S	+
333RS	+	-	-	-	-	-	R	S	S	S	-
422RS	+	-	-	-	-	-	R	S	S	S	-
426LVS	+	-	-	-	-	-	R	S	I	S	-
426RS	+	-	-	-	-	-	R	S	S	S	-
432LVS	+	-	-	-	-	-	R	S	S	S	-
432RS	+	-	-	-	-	-	R	S	I	S	-
438LVS	+	-	-	-	-	-	R	S	S	S	-
438RS	+	-	-	-	-	-	R	S	S	S	-
440LVS	+	-	-	+	-	-	R	R	S	S	-
440RS	+	-	-	+	-	-	R	R	S	S	-
444LVS	+	-	+	-	-	-	R	R	R	S	+
446LVS	+	-	-	-	-	-	R	S	S	S	-
446RS	+	-	-	-	-	-	R	S	S	S	-
447LVS	+	-	-	-	-	-	R	S	S	S	-
447RS	+	-	-	-	-	-	R	S	S	S	-
448RS	+	-	-	-	-	-	R	S	S	S	-
450LVS	+	-	-	-	-	-	R	S	S	S	-
450RS	-	-	-	-	-	-	R	S	S	S	-
466LVS	+	-	-	-	-	-	R	S	S	S	-
466RS	+	-	-	-	-	-	R	S	I	S	-
469LVS	+	-	-	-	-	-	R	S	I	S	-
469RS	+	-	-	-	-	-	R	S	S	S	-
470LVS	+	-	-	-	-	-	R	S	S	S	-
471LVS	+	-	-	-	-	-	R	S	S	S	-
471RS	+	-	-	-	-	-	R	S	S	S	-
473LVS	+	-	-	-	-	-	R	S	S	S	-
473RS	+	-	-	-	-	-	R	S	S	S	-
478LVS	+	-	-	-	-	-	R	S	I	S	-
478RS	+	-	-	-	-	-	R	S	S	S	-
482LVS	+	-	-	-	-	-	S	S	S	S	-
482RS	+	-	-	-	-	-	S	S	S	S	-

Isolate	Amplification by Multiplex PCR						Antibiotic resistance (VITEK profile)				
	Tetracycline		i/cMLS	Erythromycin	Aminoglycosides		Tetracycline	Macrolides (Erythromycin)	Lincosamide (Clindamycin)	Penicillin	Inducible Clindamycin Resistance (ICR)
	tet(M) (347 bp)	*tet*(O) (548 bp)	*erm*(A/TR) (498 bp)	*mef*(A/E) (209 bp)	*aphA* (304 bp)	*aad*-6 (397bp)					
483LVS	+	-	-	-	-	-	S	S	S	S	-
483RS	+	-	-	-	-	-	S	S	S	S	-
490LVS	+	-	-	-	-	-	R	S	S	S	-
490RS	+	-	-	-	-	-	R	S	S	S	-
491LVS	+	-	-	-	-	-	R	S	S	S	-
508LVS	+	-	-	-	-	-	R	S	S	S	-
508RS	+	-	-	-	-	-	R	S	S	S	-
525LVS	+	-	-	-	-	-	R	S	I	S	-
525RS	+	-	-	-	-	-	R	S	I	S	-
530LVS	+	-	-	-	-	-	R	S	S	S	-
530RS	+	-	-	-	-	-	R	S	S	S	-
535LVS	+	-	-	-	-	-	R	S	S	S	-
535RS	+	-	-	-	-	-	R	S	S	S	-
538LVS	+	-	-	-	-	-	R	S	S	S	-
538RS	+	-	-	-	-	-	R	S	S	S	-
539LVS	+	-	-	-	-	-	R	R	R	S	-
539RS	+	+	-	-	-	-	S	S	S	S	-
548LVS	+	-	-	-	-	-	R	S	S	S	-
549LVS	+	-	-	-	-	-	R	S	S	S	-
552LVS	+	-	-	-	-	-	R	S	S	S	-
575LVS	+	-	-	-	-	-	R	R	R	S	+
575RS	-	-	-	-	-	-	R	R	R	S	+
580LVS	-	-	-	-	-	-	R	S	S	S	-
580RS	+	-	-	-	-	-	R	S	S	S	-
588RS	+	-	-	-	-	-	R	S	S	S	-
588LVS	+	-	-	-	-	-	R	S	S	S	-
590LVS	+	-	-	-	-	-	R	S	S	S	-
590RS	+	-	-	-	-	-	R	S	S	S	-
595LVS	+	-	-	-	-	-	R	S	S	S	-
596LVS	+	-	-	-	-	-	R	S	S	S	-
603LVS	+	-	-	-	-	-	R	R	R	S	-
613LVS	+	-	-	-	-	-	R	R	S	S	-
625RS	+	-	-	-	-	-	R	S	S	S	-
626RS	+	-	-	-	-	-	R	S	S	S	-
626LVS	+	-	-	-	-	-	R	S	S	S	-
628LVS	+	-	-	-	-	-	R	S	S	S	-

Isolate	Amplification by Multiplex PCR						Antibiotic resistance (Vitek profile)				
	Tetracycline		i/cMLS	Erythromycin	Aminoglycosides		Tetracycline	Macrolides (Erythromycin)	Lincosamide (Clindamycin)	Penicillin	Inducible Clindamycin Resistance (ICR)
	tet(M) (347 bp)	*tet*(O) (548 bp)	*erm*(A/TR) (498 bp)	*mef*(A/E) (209 bp)	*aphA* (304 bp)	*aad*-6 (397 bp)					
628RS	+	-	-	-	-	-	R	S	S	S	-
641LVS	+	-	-	-	-	-	R	S	S	S	-
641RS	+	-	-	-	-	-	R	S	S	S	-
644RS	+	-	-	-	-	-	R	S	S	S	-
665LVS	+	-	-	-	-	-	R	S	I	S	-
665RS	+	-	-	-	-	-	R	S	S	S	-
670LVS	+	-	-	-	-	-	R	S	S	S	-
670RS	+	-	-	-	-	-	R	S	S	S	-
682LVS	+	-	-	-	-	-	R	R	R	S	-
682RS	+	-	-	-	-	-	R	R	R	S	-
691LVS	+	-	-	-	-	-	R	S	S	S	-
692RS	+	-	-	-	-	-	R	S	S	S	-
Total nr	114	2	3	3	0	0					

i/cMLS= inducible or constitutive Macrolide, Lincosamide and Streptomycin resistance; nr= number.

Isolates 27LVS, 27RS, 31LVS, 40LVS, 42RS, 42LVS, 43LVS, 44LVS, 45LVS, 49RS, 59LVS, 65LVS, 68RS, 70RS, 71LVS, 73LVS, 74LVS, 79LVS, 102LVS, 212LVS, 218LVS, 224LVS, 234RS, 234LVS, 238RS, 238LVS, 244LVS, 244RS, 248LVS, 248RS, 255LVS, 261LVS, 312RS, 314RS, 333RS, 422RS, 426LVS, 426RS, 432LVS, 432RS, 438LVS, 438RS, 446LVS, 446RS, 447LVS, 447RS, 448RS, 450LVS, 466LVS, 466RS, 469LVS, 469RS, 470LVS, 471LVS, 478LVS, 478RS, 482LVS, 482RS, 483RS, 490LVS, 490RS, 596LVS, 641LVS, 641RS, 644RS, 665LVS, and 682RS all had the same profile with a positive result for a 347 bp fragment. These results show possible resistance to tetracycline, since the product 347 bp is associated with the *tet*(M) gene encoding for tetracycline resistance. With the exception of isolates 71LVS, 482LVS, 482RS, 483RS and 665LVS that tested sensitive against tetracycline, all the other above mentioned isolates presented with tetracycline resistance (VITEK analysis).

Isolates 471RS, 473LVS, 473RS, 483LVS, 491LVS, 508LVS, 508RS, 525LVS, 525RS, 530LVS, 530RS, 535LVS, 535RS, 538LVS, 538RS, 539LVS, 548LVS, 549LVS, 552LVS, 575LVS, 580RS, 588RS, 588LVS, 590LVS, 590RS, 595LVS, 603LVS, 613LVS, 625RS, 626RS, 626LVS, 628LVS, 628RS, 665RS, 670LVS, 670RS, 682LVS, 691LVS and 692RS on the other hand showed positive reactions for both a 347 bp and 639 bp fragment. These results also shows possible resistance to tetracycline, because of the product 347 bp that is associated with the *tet*(M) gene encoding for tetracycline resistance, while the product size 639 bp is not associated with any of the genes investigated in this study. From these isolates with positive results for the 347 bp fragment, it is only isolate 483LVS that tested sensitive for tetracycline, the rest of these isolates presented with tetracycline resistance (VITEK analysis).

Isolate 49LVS showed positive results for a 347 bp and 548bp fragment, while isolates 240RS, 240LVS and 444LVS showed positive results for a 347bp and 498 bp fragment. From these results it shows possible resistance to tetracycline [both the 347 bp fragment (*tet(*M)) and the 548 bp fragment (*tet*(O))] for isolate 49LVS, while based on the PCR results, isolates 240RS and 444LVS have the presence of the *tet*(M) gene (347 bp fragment) as well as the *erm*(A/TR) gene (498 bp fragment). The VITEK analysis of sample 49LVS did show tetracycline resistance, while isolates 240RS, 240LVS and 444LVS presented with resistance to tetracycline, erythromycin, clindamycin as well and being positive for ICR. Based on the VITEK report for isolate 240RS and 240LVS the erythromycin and clindamycin showed sensitive MIC results, but were reported as being resistant due to the ICR being positive, while isolate 444LVS presented with a sensitive MIC for clindamycin but were also reported as resistant due to the positive ICR result (VITEK analysis).

Fragments of 347 bp, 209 bp and 498 bp were seen for isolate 332LVS, while 347 bp and 209 bp were seen for isolates 440LVS and 440RS. Isolate 539RS showed a positive result for 347 bp, 498 bp and 639 bp, and lastly no amplified products were seen for isolates 450RS, 575RS and 580LVS. Possible resistance were expected against tetracycline, erythromycin as well as the presence of ICR based on the PCR results that identified the presence of genes *tet*(M), *mef*(A/E) and *erm*(A/TR) for isolate 332LVS. VITEK analysis of isolate 332LVS confirmed the resistance of this isolate against tetracycline, erythromycin and presenting with ICR. The clindamycin MIC for isolate 332LVS tested sensitive on the VITEK but were reported as being resistance due the positive result for ICR. Although erythromycin as well as tetracycline resistance is expected to be seen in isolates 440LVS and 440RS based on the PCR results that identified the presence of genes *tet*(M) and *mef*(A/E), these isolates only presented with tetracycline resistance (VITEK analysis). Isolate 539RS showed no resistance against any antimicrobial substance although it presented with tet(M) and tet(O) base on the PCR results. Isolates 450RS and 580LVS showed resistance to tetracycline (VITEK analysis) although no resistance genes were identified by PCR testing on these isolates. Lastly isolate 575RS showed resistance to tetracycline, erythromycin and were positive for ICR despite the fact that no resistance genes were identified by PCR testing. Clindamycin for isolate 575RS were also reported as resistant due to the identification of the ICR although the clindamycin MIC result showed intermediate resistance.

3.3.4 Sequencing

Sequencing was done on cleaned, representative bands of the amplified products to ensure that the amplified products were representative of the investigated genes. Blast results confirmed that the amplified products belonged to the genus *Streptococcus* with a similarity hit 94% or more.

3.4 QUESTIONNAIRE EVALUATION

After statistical analysis using the SPSS software system the following conclusions were obtained from the answers of participants on the questionnaires. Analysis was done by using a confidence level of 95%. The Chi-square (p value) of < 0.05 after analysis is considered statistically significant. It seems that there was a slightly higher percentage of single women, compared to married women that were colonised with GBS in this study, but presented with a P value of 0.315, and therefore do not show any statistical significance.

From all participants, only 2.4% of the participants indicated that they did experience trauma during the pregnancy, therefore no significant influence of this on GBS colonisation could have been evaluated. It is also confirmed with the P value of 0.360, indicating no statistical significance.

3.4.1 Marital status

The study revealed the highest prevalence in this category being single women (17.8%), when compared to those who are married (9.5%) and those who are divorced (0%) (**Table 3.6**). These findings is not statistical significant because the p-value is 0.315.

Table 3.6: Influence of marital status on the rate of GBS colonisation (n = 588).

Marital status	No. of cases	Rate of GBS colonisation (%)		
		No. of positive cases	No. of negative cases	Percentage (%) positive
Married	105	10	95	9.5%
Single	481	71	410	17.8%
Divorced	2	0	2	0%

3.4.2. Education levels

The study revealed a low prevalence of GBS colonisation in women from all educational categories investigated in this study. This includes a GBS prevalence of 5.96% for women with an educational level of below matric, a 5.6% prevalence of women with an educational level of matric and 2.2% prevalence of women with tertiary education as seen in **Table 3.7**. The association between GBS colonisation and educational levels is not statistical significant because the p-value 0.729 is more than 0.05. (**Table 3.8**).

Table 3.7 Chi-Square tests for the association of level of education with GBS colonisation.

	Value	Df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.633[a]	2	0.729
Likelihood Ratio	.605	2	0.739
Linear-by-Linear Association	.429	1	0.513
N of Valid Cases	587		

a. 0 cells (0.0%) have expected count less than 5. The minimum expected coun 10.76.

Table 3.8: Influence of education on the rate of GBS colonisation (n = 587).

Educational levels	No. of cases	Rate of GBS colonisation (%)		
		No. of positive cases	No. of negative cases	Percentage (%) positive
Below matric	265	35	230	5.96%
Matric	244	33	211	5.6%
Tertiary	78	13	65	2.2%

As seen in **Table 3.8**, the level of education in this study have shown a slight decrease in GBS colonisation rates as the level of education increase although no statistical significance were seen (P value of 0.729).

3.4.3. Employment

Employed women that were colonised by GBS was 37 out of 215 (14.7%) and amongst the unemployed women recruited in the study 37 out of 269 (12.15%) were colonised by GBS. The self-employed women yielded a colonisation rate of 13.3% (2/13).The association between employment and GBS colonisation can be seen in **Table 3.9**. Fifteen of the participant indicated not applicable on the questionnaire. None of these findings was statistical significant, with a p-value of 0.125.

Table 3.9: Influence of employment on the rate of GBS colonisation (n = 588).

Employment	No. of cases	GBS colonisation (%)		
		Positive	Negative	%(Positive)
Employed	252	37	215	14.7%
Unemployed	306	37	269	12.1%
Self-employed	15	2	13	13.3%
Others	15	5	10	33.3%

3.4.4 Occupation

GBS colonisation rate for professional, skilled, semi-skilled and not applicable were 19.8%, 11%, 13% and 13.2% respectively (**Table 3.10**). No statistical significance was seen here (P = 0.318).

Table 3.10: Influence of occupation on the rate of GBS colonisation (n = 586).

Occupation	No. of cases	GBS colonisation (%)		
		Positive	Negative	%(Positive)
Professional	91	18	73	19.8%
Skilled	100	11	89	11%
Semi-skilled	69	9	60	13%
Not applicable	326	43	283	13.2%

3.4.5 Accommodation

One hundred and ninety seven participants indicated that they had their own accommodation of which 21 (10.7%) were GBS colonised. Forty women indicated that they rent their own place (rent-whole) of which 6 (15.0%) were colonised by GBS and 347 were rent-sharing of which 54 (15.6%) were GBS colonised (**Table 3.11**). No statistical significance was seen with regards to accommodation and GBS colonisation with a p-value of 0.407.

Table 3.11: Rate of GBS colonisation versus different accommodation conditions (n = 586).

Accommodation	No. of cases	GBS colonisation (%)		
		Positive	Negative	%(Positive)
Own	197	21	176	10.7%
Rent-whole	40	6	34	15%
Rent-sharing	347	54	347	15.6%

Participants that were rent-sharing had a higher GBS colonisation rate of 66.7%, compared to those that own their own house, showing a GBS colonisation rate of 25.9% and those that rent their own house, showed 7.4% GBS colonization. Once again it is of no statistical significance (P-value = 0.407).

3.4.6 History of Pain and Fever

Only eight participants from the 588, indicated that they had a history of pain and fever during the current pregnancy. None of these participants were shown to be colonised with GBS (**Table 3.12)**.There was thus no statistical significance found between a history of pain and fever and that of GBS colonisation in this study (p-value of 0.607).

Table 3.12: Rate of GBS colonisation versus a history of pain and fever during the pregnancy (n = 588).

History of Pain and Fever	No. of cases	GBS colonisation (%)		
		Positive	Negative	%(Positive)
Yes	8	0	8	0%
No	579	80	499	14%

3.4.7 Trauma during the pregnance

Eight participants indicated that they experienced trauma during their current pregnancy. Two of them were shown to be colonised by GBS (**Table 3.13)**, which does not indicated any statistical significance between trauma during pregnancy and GBS colonisation in this study, with a p-value of 0.308.

Table 3.13: Rate of GBS colonisation versus a trauma during the pregnancy (n = 583).

History of Pain and Fever	No. of cases	GBS colonisation (%)		
		Positive	Negative	%(Positive)
Yes	8	2	6	25%
No	575	79	496	13.7%

CHAPTER FOUR: DISCUSSION

4.1 Group B *Streptococcus* prevalence in the Khomas region.

Amongst the participants that were investigated in the Khomas region (the majority was from the Windhoek Central Hospital's antenatal clinic); a GBS colonisation prevalence rate of 13.6% (80/588), with a median age of 28 were detected amongst the GBS colonised women that were screened in this study. This prevalence falls within the range that's been reported previously from southern African countries. Prevalence of GBS colonisation amongst pregnant women has been reported to vary between 1.8%, as reported in Mozambique (de Steenwinkel et al. 2008), up to 32% which was seen in Zimbabwe (Moyo et al. 2000). In 2010, 21% GBS colonisation was reported in Zimbabwe for women screened at delivery (Mavenyengwa et al. 2010). A study done in Soweto, Johannesburg, South Africa reported GBS colonisation rates of 28.7% and 28.4% seen at 31-35 weeks gestation and 37 weeks gestation and onwards respectively (Gaurav et al. 2014).

In Pretoria, South Africa Bolukaoto et al reported GBS colonisation rate of 30.9% (Bolukaoto et al. 2015). The prevalence rates of GBS colonisation in pregnant women seen at 35 weeks gestation and onwards in Zimbabwe and South Africa, who are Namibia's neighbours, seem to have more than double the prevalence rate seen in our study. The prevalence of GBS colonisation is lower in Namibia; however this is looking at Windhoek only.

In our study 46.25% of the GBS isolates were detected from both lower vagina- and rectum, 36.25% from lower vagina only and 17.5% were detected from rectum only. In Turkey a 10.6% GBS colonisation rate was found, of which 66.7% was positive for both the rectum and vagina (Arisoy et al. 2003). The high percentage of women that presented with both vaginal and rectal colonisation could indicate that personal hygiene could be a contributing factor, since the gut is known to be the reservoir of GBS, and the vagina is usually contaminated from the gut.

A study done on 400 000 infants indicated early-onset GBS sepsis in 160 of the infants. The study also indicated that amongst the infants that were born full term only 63% of their mothers were screened for GBS colonisation and amongst the prematurely born infants, only 44% of their mothers were screened (Stoll et al. 2011). Stoll et al also indicated that about 81% full term mothers of the babies diagnosed with early-onset GBS disease, had a negative GBS screen result (Stoll et al. 2011). A study from the 1980's discovered that the risk of GBS colonised pregnant woman delivering a baby with EOGBS is 25 times higher than those not being colonised by GBS (Boyer et al. 1985). It is therefore a clear indication that a correct and effective GBS screening procedure is of utmost importance. Universal screening for GBS, by the collection of a vaginal and rectal swab, at the gestational age of 35 – 37 weeks,

of all pregnant women recommended by the World Health Organisation (WHO) (Colleen et al. 2013), should be implemented in Windhoek to reduce the potential of EOGBS disease. Windhoek does not have a high GBS prevalence amongst the pregnant community that were screened in this study, but the prevalence could still be reduced, since EOGBS disease is preventable when a proper screening program is implemented.

4.2 Culture media.

The higher colonisation rate of 25% (49/169) obtained with the inclusion of the Granada agar in our study, is more in line with the colonisation rates from neighboring countries, with 32% reported in Zimbabwe and between 28.4% and 32.7% reported in South Africa (Moyo et al. 2000; Gaurav et al. 2014; Bolukaoto et al. 2015). Although in our study there were only four samples for which the GBS were detected on the Granada agar plate only, and not on the BCNA and/or Todd Hewitt broth. Therefore 2.4% (4/169) of the GBS isolates that were cultured on BCNA, Todd-Hewitt and Granada agar were detected on the Granada agar only. At the time the Granada agar was included in the study, the self-prepared BCNA agars were also replaced by pre-prepared BCNA (pBCNA) agars from bioMérieux. All GBS isolates in our study that were detected by the pBCNA were also detected on the Granada agar plates. The statistically significant (P value = < 0.0000) increase in prevalence of GBS colonisation yielded during the screening of the last 169 participants when the Granada agar and the pBCNA were included in the study could most probably be explained by the pre-prepared pBCNA plates that were used rather than the inclusion of the Granada agar, since there were only 4 isolates that were detected on the Granada agar and not on the pBNCA. When comparing the different media used in the thesis, the odds of testing positive are five times higher under the pBCNA, TH-pBCNA and Granada testing media as compared to the BCNA and TH-BCNA. In a study conducted by Gil et al, Granada agar plates presented with a 88.5% - 91.1% sensitivity for GBS isolation, the selective Columbia agar plates (5% human blood, Columbia agar, 10 µg colistin/ml and 15 µg nalidixic acid/ml) presented with a sensitivity ranging from 83.9% to 94.3% and the Lim broth (Todd Hewitt broth enriched with 1% yeast extract, 15 µg nalidixic acid/ml and 10 µg colistin/ml) showed a sensitivity of 63.5% - 75%. From this study it was clear that not one of these media showed 100% sensitivity, it was thus suggested that more than one type of media should be used for optimum detection of GBS (Gil et al. 1999). In another study evaluating the Granada agar plate, it was reported that 103 of the 105 GBS isolates were detected on the Granada agar plate and 50 GBS isolates were detected on the 5% Columbia sheep blood agar. In this study the Granada agar plates showed 98% sensitivity and a 100% specificity compared to the 47.6% sensitivity and 100% specificity shown by the 5% Columbia sheep blood agar (Tamayo et al. 2004). It is clear that the inclusion of more than one selective media for GBS screening definitely decrease the chance of a false negative culture. Although it is not clear if the inclusion of the

Granada agar from the start of the study would have increased the GBS isolation rate, since only 2.4% (4/169) of the GBS isolates were detected only on the Granada agar and not on the BCNA.

Studies by Gil et al. and Tamayo et al. indicated that the isolation rate of GBS has definitely increased by the use of more than one selective media in GBS screening (Gil et al. 1999; Tamayo et al. 2004). It is therefore important to use more than one selective media in GBS screening in order to reduce possible false negative cultures.

It might therefore be that the total prevalence result in our study could have been significantly higher if the pBCNA as well as the Granada agar had been used from the start of the study, since the odds of testing positive are five times higher under pBCNA and Granada media as compared to BCNA. The pBCNA and Granada agar was not included in the study from the beginning due to financial constraints.

4.3 Identification

The identification of all 117 GBS isolates was confirmed by the use of the Oxoid Streptococcal Group Kit (DR585). Random isolate identification that was confirmed by the VITEK, all presented with 96% - 99%, excellent identification confidence level. This indicates that the Oxoid Streptococcal Grouping Kit (DR585) has a high specificity and sensitivity, and could therefore be used to identify GBS isolates. Joachim et al used the Streptex agglutination test from Remel to confirm their GBS isolate identification (Joachim et al. 2009). Latex agglutination to confirm the identification of presumptive GBS isolates were performed by the use of Streptex—Slidex ® Strepto Plus—bioMérieux, Marcy l'Etoile, France by Bolukaoto et al in the South African study (Bolukaoto et al. 2015).

4.4 Risk factors

There was no statistical significance between the level of education and GBS colonisation. This is in agreement with the findings of Mavenyengwa et al that also reported that educational levels did not show any statistical significance associated with GBS colonisation (Mavenyengwa et al. 2010). Terry et al (Terry et al. 1999) reported that there was no statistical significance between low income and GBS colonisation, which could probably also be linked to level of education.

There was no statistical significance between employment status and GBS colonisation. It seems that there are a slightly higher percentage of single women, compared to married women that were colonised with GBS in this study, showing no statistical significance. This is

also in agreement with the findings from Zimbabwe where no statistical significance was found between employment status and GBS colonisation (Mavenyengwa et al. 2010).

In the current study no association was seen between occupation and GBS colonisation. Mavenyengwa et al also reported no statistical significant association between occupation and GBS colonisation (Mavenyengwa et al. 2010).

Since 87.7% of the participants in the current study were not married, the questions on their husband's employment and highest level of education had few responses for a meaningful analysis, which showed no statistical significance for either of these. Although very few participants from the current study were married, there was no association found between marital status and GBS colonisation. This is in agreement with Terry et al as well as Mavenyengwa et al who did not find any association between marital status and GBS colonisation (Terry et al. 1999; Mavenyengwa et al. 2010).

Participants that were rent-sharing had a higher GBS colonisation rate of 66.7%, where as those that own a house, showed a GBS colonisation rate of 25.9% and those that rented a house, showed 7.4% GBS prevalence. Once again this is in agreement with the Zimbabwean findings that there is no statistical significant association between type of accommodation and GBS colonisation (Mavenyengwa et al. 2010).

None of the participants, that took part in this study, indicated that they had fever and or lower abdomen pain during the pregnancy. Therefore no association was found between fever and or lower abdominal pain and GBS colonisation in our study. From the total number of participants, 2.5% of the colonised mothers indicated that they did experience trauma during the pregnancy.

4.5 Antimicrobial susceptibility

Only one isolate per participant were considered during the calculation of resistance rates amongst the GBS isolates from this study.

Benzylpenicillin, ampicillin, ceftriaxone, linezolid and vancomycin showed 100% sensitivity, all GBS isolates in our study tested sensitive to these six antimicrobial substances.

The benzylpenicillin, ampicillin and vancomycin presenting with uniform susceptibility in this study, is in agreement with the studies done in Brazil and Malaysia, Paris (France), Turkey as well as South Africa (Dutra et al. 2014; Eskandarian et al. 2014; Tazi et al. 2007; Arisoy et al. 2003; Bolukaoto et al. 2015). Moyo et al reported 98% fully sensitive penicillin GBS

isolates and 2% intermediate sensitive GBS isolates in Zimbabwe (Moyo et al. 2001).The finding that benzylpenicillin presented with 100% sensitivity against the tested GBS isolates in this study, show that penicillin is still the drug of choice (Hyde et al. 2002) in the Khomas region, Windhoek, Namibia.

Levofloxacin presented with 99.1% sensitivity. One out of the 117 GBS isolates showed intermediate resistance. Our sensitivity results for levofloxacin is very similar to that of studies from Brazil, Malaysia, Paris (France) and Turkey as well as South Africa (Dutra et al. 2014; Eskandarian et al., 2014; Tazi et al. 2007; Arisoy et al. 2003; Bolukaoto et al. 2015) all showed 100% sensitivity for Levofloxacin. Although the MIC presented for this isolate fall within the susceptible zone size to be sensitive, but the Advanced Expert System change the interpretation of it from sensitive to intermediate resistant.

GBS isolates in this study presented with 11.1% resistance (13/117) against erythromycin. The erythromycin resistance of GBS isolates in this study is a little less than most of the recently published data from elsewhere, except for the Brazilian study. Dutra et al reported a very low 4.1% erythromycin resistance in Brazil (Dutra et al. 2014). A Malaysian study presented with 23.3% erythromycin resistance (Eskandarian et al. 2014). Capanna et al detected a resistance rate amongst GBS isolates of 30% against erythromycin (Geneva, Switzerland) (Capanna et al. 2013). Both Frouhesh-Tehrani et al and Arisoy et al reported an erythromycin resistance rate of 21.2% amongst GBS isolates (Frouhesh-Tehrani et al. 2015; Arisoy et al. 2003). In Zimbabwe a 14% erythromycin resistance was reported (Moyo et al. 2001) and a 21.1% erythromycin resistance rate was reported recently from South Africa (Bolukaoto et al. 2015). The erythromycin resistance detected in this study is lower than the majority of studies done elsewhere.

Clindamycin susceptibility amongst GBS isolates in this study showed 18.8% (22/117) of intermediate resistance, 8.5% (10/117) fully resistant and 5.1% (6/117) inducible resistance. The clindamycin resistance rate amongst GBS isolates vary greatly from one study to the next. Our study presents with some of the lower resistance rates for clindamycin when compared to other studies. In Zimbabwe 7.2% resistance was reported against clindamycin (Moyo et al. 2001). South Africa recently showed 17.2% clindamycin resistance (Bolukaoto et al. 2015) and in Malaysia 17.5% clindamycin resistance was seen (Eskandarian et al. 2014). Clindamycin resistance was reported as low as 3% in Brazil (Dutra et al. 2014), 4.2% in Australia (Garland et al. 2011) and 9.1% in Izmir, Turkey (Arisoy et al. 2003). In Geneva, Switzerland a much higher resistance rate of 28% was reported against clindamycin (Capanna et al. 2013). Resistance rates amongst GBS isolates against clindamycin have been reported to be as high as 43% (DiPersio & DiPersio. 2006) and even 71% resistance

was reported in one study done in Norway (Bergsen et al. 2008). Inducible macrolide, lincosamide and streptogramin B ($iMLS_B$) resistance were reported in 15.6% (20/128) in a South African study (Bolukaoto et al. 2015). This is almost 3 fold the 4.3% inducible clindamycin resistance (ICR) that was detected in the current study. The 4.3% ICR is similar to the 3.4% cross-resistance against clindamycin that have been detected in an Australian study (Garland et al. 2011). The rate of ICR in this study was reported based on VITEK results. Kimberly et al as well as Frouhesh-Tehrani et al reported 9.5% ICR and Capanna et al reported 8.6% ICR (Kimberly et al. 2013), which is still higher than the 4.3% seen in this study. Both Bolukaoto and Kimberly used the D-zone test method for the detection of iMLSB, which is responsible for ICR, whereas in this study the ICR was determined by the VITEK analysis. The different test methods could have had an influence on the results. The erythromycin resistance in this study which is a little lower than in most of the other studies could also have contributed to the lower ICR rate seen in this study, since ICR could be detected amongst GBS strains that present with erythromycin resistance and clindamycin susceptibility (CLSI. 2006). The resistance rate of GBS against clindamycin varies amongst the majority of studies and this could probably be because clindamycin can be used to treat a variety of infections and can potentially develop resistance in some countries. Clindamycin, being a lincosamide, has been described in the Namibian antibiotic guidelines (MoHSS, 2011) as a good alternative treatment for uncomplicated skin and soft tissue infections in penicillin-allergic patients and is also recommended as treatment for bacterial skin infections, osteomyelitis and acute septic arthritis. Although in 2015 information from a Namibian study revealed that clindamycin was not a first, second or third line antibiotic choice by any of the doctors belonging to the medical associations that took part in the survey done by Pereko et al (Pereko et al. 2015). This could explain the low resistance seen amongst GBS isolates in this study. Amongst the sub-Saharan countries it is a pleasant finding that Namibia seems to present with the lowest clindamycin resistance for GBS isolates.

Group B *Streptococcus* isolates from this study presented with a resistance rate against tetracycline of 94.9% (111/117). This is in agreement with a recent South African study where 94.5% tetracycline resistance amongst GBS isolates were reported (Bolukaoto et al. 2015). In neighbouring Zimbabwe, Moyo et al found 100% tetracycline resistance (Moyo et al. 2000). In Switzerland and Malaysia 89% and 71.8% tetracycline resistance was detected respectively (Capanna et al. 2013; Eskandarian et al. 2014), which is lower than the resistance rates seen in our study as well as that of South Africa and Zimbabwe. Most studies detect tetracycline resistance in the majority of GBS isolates.

Trimethoprim/Sulfamethoxazole resistance of 6% amongst GBS isolates in this study was found. This is in lower than the 17.95% sulfamethoxazole/trimethoprim resistance that was

detected in Rio de Janeiro, Brazil (Soares et al. 2013). A much higher sulfamethoxazole/trimethoprim resistance of 46.8% was reported in Misiones, Argentina (Quiroga et al. 2008).

No linezolid resistance was detected in this study and this is in agreement with the 0% linezolid resistance that was reported from a Geneva, Switzerland study (Capanna et al. 2013) as well as from a study done in Misiones, Argentina (Quiroga et al. 2008).

In the current study ceftriaxone presented with 100% susceptibility against GBS isolates. Note that ceftriaxone susceptibility testing against GBS is not commonly reported. Our findings are not in agreement with the 30% resistance that was detected in Detroit, indicating that this 3rd generation cephalosporin is not a good alternative to penicillin (Simoes et al. 2004). It is however very good to see 100% susceptibility against ceftriaxone amongst Namibian GBS isolates, since it could be an indication that there is no abuse of this antibiotic and that it could be reserved for use when really needed.

Penicillin is still the drug of choice for GBS prophylaxis and still shows 100% susceptibility in GBS in this study. It is unfortunate but clear that from 1994 an increase in penicillin minimum inhibitory concentrations (MIC's) have been reported and it is therefore of utmost importance to monitor these MIC values through continual studies (Dutra et al. 2014). In a study by Janapatla et al. an elevation of erythromycin resistance from 30% to 44% were reported in Taiwan (Janapatla et al. 2008). An increase in both erythromycin and clindamycin resistance has also been reported (Eskandarian et al. 2013; Dutra et al. 2014). Due to the well-known increase of antibiotic resistance amongst bacteria, it is an indicator that susceptibility profiles of GBS cannot merely be assumed to be as expected, but rather have to be confirmed (if possible) before prophylaxis is being administered (especially in the Penicillin allergic patients where alternative drugs need to be used).

4.6 Antimicrobial resistance genes

Genes associated with antimicrobial resistance tested for in this study were *tet*(M), *tet*(O), *erm*(A/TR), *mef*(A/E), *aph*A-3 and *aad*-6. Genes usually associated with tetracycline resistance in Group B *Streptococcus* are known as *tet*(M) or *tet*(O) (Culebras et al. 2002). Inducible cross-resistance or constitutive resistance to macrolides streptogramin B antibiotics and lincosamine are encoded for by the *erm*(A/TR) gene. The *mef*(A or E) gene is usually responsible for antimicrobial resistance to erythromycin, due to the encoding of the efflux pump in bacteria (Zeng et al. 2006). Resistance to aminoglycosides are encoded for by both the *aph*A-3 and *aad*-6 genes (Zeng et al. 2006).

One hundred and fourteen fifteen of the 117 GBS isolates presented with a visible band for only 347 bp. The amplicon size 347 bp is associated with the *tet*(M) gene, which encodes for tetracycline resistance. Of these 114 presenting with the *tet*(M) gene, 111 of them presented tetracycline resistance according to the VITEK results. Six of these isolates did not present with tetracycline resistance according to the VITEK results. This could suggest, that while the *tet*(M) gene was detected, it was not active and was not conferring resistance.
Three isolates did not present with any visible bands on the electrophoresis. The VITEK susceptibility results of these isolates indicated that, two presented with tetracycline resistance only and the 3rd isolate presented with tetracycline resistance, erythromycin resistance, intermediate resistance for clindamycin as well as inducible clindamycin resistance. Zeng et al. have also reported MLS resistance genes were not detected in three of their isolates that showed iMLS phenotypes (see **Table 1.3**) (Zeng et al. 2006).

Another two isolates presented with amplicon sizes of 347 bp *tet*(M) and 548 bp *tet*(O). The *tet*(O) gene is known to have an amplicon size of 548 bp. These two isolates therefore presented with both genes associated with tetracycline resistance namely, *tet*(M) and *tet*(O).

One of the isolates presented with visible bands of amplicon sizes 209 bp, 347 bp and 498 bp. Amplicon size 209 bp is associated with the gene *mef* (A/E) which is known to encode for resistance against fourteen and 15-membered macrolides, which includes erythromycin.
These two isolates therefore presented with *tet*(O), *tet*(M) and *mef*(A/E) genes.

Two isolates presented bands with amplicon sizes of 209 bp and 347 bp. These isolates therefore presented with the *mef*(A/E) and *tet*(O) genes. The VITEK results for these isolates indicated that these isolates presented with erythromycin and tetracycline resistance.

Six of the 117 isolates were sensitive (according to the VITEK results) to tetracycline. All six of these isolates did also present with a visible band of approximately 347 bp, that is associated with the *tet*(M) gene which encodes for tetracycline resistance. The absence of tetracycline resistance in the presence of the *tet*(M) gene on these isolates could not be explained. This concurs with findings by Vickers Burdett (Burdett, V. 1993). In this study Burdett found that bacterial isolates with mutation in the *mia*A genes present as sensitive to tetracycline despite the presence of the *tet*(M) gene (Burdett, V. 1993). None of the isolates showed visible bands on the 1.5% agarose gel that is comparative to the *aphA*-3 (associated with HLR-KM) and *aad*-6 (associated with high level streptomycin resistance) genes.

CHAPTER FIVE: CONCLUSION

From the results obtained in this study, GBS prevalence amongst pregnant women at 35 weeks gestation onwards in the Windhoek, Khomas region, Windhoek, Namibia was 13.6% (80/588). Almost half the participant (46.25%) that tested positive for GBS colonisation tested positive for both the vaginal and rectal swabs, 36.25% tested positive for vaginal swabs only and 17.5% tested positive for rectal swabs only.

With the initial 419 swabs that were cultured on BCNA and in Todd Hewitt broth (with antibiotics), a prevalence rate of 7.4% (31/419) was obtained. For the last 169 swabs that were cultured on pBCNA, Granada agar and Todd Hewitt broth (with antibiotics), a prevalence rate of 29% (49/169) was seen. As suggested by Gil et al. and Tamayo et al. the isolation rate of GBS has definitely increased by the use of more than one selective media in GBS screening (Gil et al. 1999; Tamayo et al. 2004). It is therefore recommended to use more than one selective media for GBS isolation when a GBS screening procedure is implemented.

All the random GBS isolate identification that were confirmed with the VITEK identification cards presented with a 96% - 99%, excellent identification confidence level, that indicates the high sensitivity and specificity of the Oxoid Streptococcal Grouping Kit (DR585) that were routinely used for the identification of suspicious GBS colonies in this study.

Concerning marital status, it was seen that 17.8% of the GBS colonised women were single, 9.5% were married and 0% were divorced (although only two of the 588 participants indicated that they were divorced). According to the current study, single women seem to be slightly more prone to GBS colonisation than those that were married. This finding however was not statistically significant, with a P value of 0.315. With regard to education, there was not much difference in GBS colonisation rates in women with a below matric educational level and those with matric. Sixty five participants indicated that they had a tertiary education, only 2.2% of them were colonised with GBS. No statistical significance was seen. A slight decrease in GBS colonisation rates as the educational level increased was seen in this study, although this finding once again is not statistically significant, with a P value of 0.729. Employment status did not seem to influence the GBS colonisation rates with colonisation rates of 14.7%, 12.15% and 13.3% amongst women that are employed, unemployed and self-employed respectively. A total of 52% of participants from this study indicated that they were unemployed, contributing to the fact that only 55.4% of participants answered the question on occupation.

There doesn't seem to be an association between GBS colonisation rates and occupation. The different occupational categories revealed the following; women that work as professionals presented with 19.8% colonisation, skilled presented with 11% colonisation and semi-skilled presented with 13% colonisation (P = 0.318). Women that rent their own house or were rent sharing seem to have had more or less the same colonisation rate of 15% and 15.6% respectively. Women owning their own house seem to have had a slightly lower colonisation rate of 10.7%, but again with no statistical significance (p-value = 0.407). The median age of the GBS colonised women from this study were 28.

No antimicrobial resistance against benzylpenicillin, ampicillin, ceftriaxone, linezolid and vancomycin were seen amongst the GBS isolates in this study. Levofloxacin showed 99.1% sensitivity. Against trimethoprim/sulfamethoxazole, 6% resistance was seen and 94.9% resistance against tetracycline was seen. Erythromycin presented with 11.1% resistance whereas clindamycin presented with 8.5% full resistance and 18.8% intermediate resistance. Inducible clindamycin resistance (ICR) was detected in 5.1% of the isolates.

Genes associated with antimicrobial resistance tested for in this study were *tet*(M) and *tet*(O), *erm*(A/TR), *mef*(A/E), *aph*A-3 and *aad*-6. Genes associated with tetracycline resistance is *tet*(O) and *tet*(M). The gene *tet*(O) were detected in two samples. The resistance gene *tet*(M) were detected in 114 of the 117 samples.

The gene *mef*(A/E) associated with erythromycin resistance were detected in three isolates and the VITEK antimicrobial sensitivity test also showed these three isolates to be resistant to erythromycin. Nine of the isolates presented with erythromycin resistance on the VITEK but did not have the *mef*(A/E) gene present.

The gene *erm*(A/TR) were seen in four isolates. The VITEK antimicrobial sensitivity tests confirmed the presence of ICR which are encoded for by gene *erm*(A/TR). However two isolates that also showed ICR from the VITEK, did not show the presence of the *erm*(A/TR) gene.

Although six isolates tested sensitive against tetracycline, they did contain the *tet*(M) genes.

None of the isolates showed visible bands on the 1.5% agarose gel that is comparative to the *aphA*-3 and *aad*-6 genes, associated with high level kanamycin resistance and high level streptomycin resistance respectively.

LIMITATIONS AND RECOMMENDATIONS

This study presented with some limitations that could have an influence on the final outcomes.

Inducible Clindamycin Resistance (ICR) in this study was detected by means of the VITEK-2-Compact system and no D-test was performed. Performance of a manual D-test on all erythromycin resistant and clindamycin sensitive GBS isolates from this study could have confirmed the ICR rate reported from this study. This could have yielded a either higher or lower rate of ICR.

The pBCNA and Granada chromogenic agar was not included in the study from the start. Including both the pBCNA and Granada agar from the start of the study could possibly have increased the yield of GBS positive isolates.

Group B *Streptococcus* prevalence amongst pregnant women in this study is not a true reflection of the Khomas region. Due to limited private participants (5 participants from private Doctors took part in the study), the GBS prevalence would rather be a reflection of participants that visited the Windhoek Central Hospital antenatal clinic.

Initially the study protocol included the collection of umbilical cord swabs as well as ear and umbilical cord swabs from the new born babies. For the collection of these swabs we had to rely on the maternity ward staff to collect the swabs whenever a participant from the study (whose medical passport is marked with a coloured barcode) delivered a baby. Unfortunately a very limited number of these swabs (7/588) were collected and therefore no correlation between maternal colonisation and EOGBS or even GBS colonisation in infants could have been concluded from this study. The results from the seven babies that were swabbed were disregarded since this is not a representative number of babies from this study.

Different regions in Namibia need to be screened to obtain the GBS prevalence amongst pregnant women in the country.

Additional genes associated with GBS resistance could be analysed by the use of molecular techniques.

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APPENDICES

APPENDIX A: Group B *Streptococcus* participant questionnaire

GBS STUDY QUESTIONNAIRE.

PART I

Bar Code: ____

Date Attended |__|__|__|__|__|__|__|__|
(DD/ MM/ YY)

Hospital Number |__|__|__|__|__|__|__|__|

Date of birth |__|__|__|__|__|__|__|__|
(DD/ MM/ YY)

Socio-economic characteristics

	Married/	Single/	Divorced/	Windowed/	Cohabiting
Marital status	☐	☐	☐	☐	☐

	1. Below matric	2. Matric	3. Tertiary level
Highest level of education reached	☐	☐	☐
	Yes	No	Self-employed
Employed	☐	☐	☐
	Professional	Skilled	Semi-skilled
Occupation	☐	☐	☐
	Yes	No	Self-employed
Husband employed	☐	☐	☐
	1. Below matric	2. Matric	3. Tertiary level
Highest level of education reached	☐	☐	☐
	Own	Rent-whole	Rent-sharing
Accommodation	☐	☐	☐
Dwelling most during the year	Urban	Rural	Semi-urban
	☐	☐	☐

State the name of the place ………………………………………

Obstetric History

	Yes	No
Antibiotic treatment in the last 14 days	☐	☐
Parity	|__|__|	
No. of previous miscarriages	|__|__|	
No. of previous stillbirths	|__|__|	
No. of previous normal vaginal deliveries	|__|__|	

Any history of fever and pain in the lower abdomen during this pregnancy?

Yes ☐ No ☐

IF YES:
Treated ☐ Untreated ☐

When? ……………………………..
How many times? …………………………

Present pregnancy

Expected date of delivery (EDD) |__|__|__|__|__|__|__|__|
(DD/ MM/ YY)

Gestational age at current visit (weeks) |__|__|

	Yes	No
Trauma in pregnancy	□	□

Specify (If yes)________________________
____
____

Height (cm) |__|__|__|

Weight |__|__|__| (to nearest kg)

Mid-arm circumference (cm) |__|__|__|

	Yes	No
RPR test done	□	□
TPHA done	□	□
HIV test done	□	□

Bar Code:

APPENDIX B: Consent from

[MHSS Protocol number: R17/3/3

CONSENT FORM

Research Protocol: **The antibiotic susceptibility and gene based resistance of *Streptococcus agalactiae* (*Group B Streptococcus*), in pregnant women in Windhoek (Khomas region), Namibia.**

INVESTIGATORS:
Name: **F. Engelbrecht[1] BTech**, R.T. Mavenyengwa[1] DPhil, S. Khan[2] PhD and S.R. Moyo[2] DPhil
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Tel: 061-2072971
Investigator's Affiliation: Namibian University of Science and Technology (NUST)[1], Cape Peninsula University of Technology (CPUT)[2]

Procedure

This is a study focusing on investigating prevalence of *Group B Streptococcus* (GBS), an organism known to cause infections in women and babies.

1. Specifically it is a study that will be done over a six months period. Participants will include pregnant women between 35 weeks gestation, visiting Eros Family Practice, Windhoek Central Antenatal Clinic, Namibia. Participants will be screened for GBS colonisation either in the vaginal or rectum.
2. Three swabs, 2 vaginal and 1 rectal swabs will be collected from each participant by general practitioners, gynaecologists or registered nurses.
3. Venous blood will be collected from each pregnant women recruited and cord blood will also be collected at delivery.
4. A questionnaire will be used to collect demographic and clinical data. Voluntary informed and written consent must be obtained from you, for this information to be eligible for use in this study. A unique identification number will be allocated to you to protect your identity and all information will be handled with the strictest confidentiality and all data will be stored in a protected electronic data base.

Purpose and benefits

The study will contribute to valuable information that can be used to guide policy in group B *Streptococcus* therapy and prevention in the country.

Risks, Stress or Discomfort

If at any stage of the investigation you feel uncomfortable, you are free to withdraw. Finally, we would like to review your medical record for information about your health history and treatment.

Full name and surname..

Signed (signature of parent if a minor)..

Date..

APPENDIX C: Culture media on which the GBS have grown and a summary of the findings.

ID no	Direct BCNA		TH- BCNA		Granada	
	LVS	RS	LVS	RS	LVS	RS
27	1	1	1	1		
31	1	1	1	1		
40	1	1	1	1		
42		1		1		
44			1			
45			1			
49		1		1		
59	1		1			
65			1			
68		1		1		
70	1	1	1	1		
71	1		1			
73			1			
74			1			
79			1			
102			1			
212			1			
218			1			
224			1			
234			1			
238		1		1		
240	1		1			
244			1	1		
248	1	1	1	1		
249		1		1		
255	1		1			
261	1		1			
312				1		
314		1		1		
332	1		1			
333		1		1		
422						1
426		1	1	1	1	1
432				1		1
438	1	1	1	1	1	1
440	1	1	1	1	1	1
444					1	
446	1		1		1	
447	1	1	1	1	1	1

448						1
450	1	1	1	1	1	1
466	1	1	1	1	1	1
469			1	1	1	1
470			1	1	1	1
471			1	1	1	1
473	1	1	1	1	1	1
478			1	1	1	1
482	1	1	1	1	1	1
483	1	1	1	1	1	1
490	1	1	1	1	1	1
491	1		1			
508	1	1	1	1	1	1
525	1	1	1	1	1	1
530	1	1	1	1	1	1
535			1	1	1	1
538	1	1	1	1	1	1
539	1	1	1	1	1	1
548			1		1	
549	1		1		1	
552			1		1	
575	1	1	1	1	1	1
580	1	1	1	1	1	1
588			1			
596			1	1	1	1
595			1		1	
590			1	1	1	1
603			1		1	
606			1	1	1	1
613			1		1	
619			1	1	1	1
625				1		1
626			1		1	
628			1	1	1	1
641			1	1	1	1
644				1		1
665			1	1	1	1
670			1		1	
682			1	1	1	1
691			1	1	1	1
692						1
TOTAL	30	29	35	19	1	3

LVS=Vaginal swab; RS=Rectal swab; BCNA=Columbia sheep blood agar with 8 µg of gentamycin and 15 µg of nalidixic acid/ml; TH=Todd Hewitt broth with 8 µg of gentamycin powder and 15 µg of nalidixic acid; ID=Identification; No=number; 1=positive culture.

.

Summary of finding in table above:

Direct BCNA Positive for:	
V/S & R/S	5
V/S	6
R/S	6
TH-BCNA positives:	
V/S & R/S	1
V/S	11
R/S	1
Direct BCNA & Granada Positive for:	
V/S & R/S	17
V/S	2
R/S	0
TH-BCNA & Granada:	
V/S & R/S	14
V/S	9
R/S	3
Granada only:	
R/S	3
V/S	1
V/S & R/S	0

APPENDIX D: Summary of the MIC results, antibiotic interpretation (VITEK), gel lanes and genes identified for individual isolates.

			VITEK sensitivity results for the GBS positive isolates in this study																						
			Antibiotic tested																						
Isolate ID	Gel lane	Bands	PEN		AML		CTX		CRO		Levo		E		CD		LZD		VA		TE		SXT		ICR
			MIC	S/I/R	MIC	S/I/R	MIC	S/I/R	MIC	S/I/R	MIC	S/I/R	MIC	S/I/R	MIC	S/I/R	MIC	S/I/R	MIC	S/I/R	MIC	S/I/R	MIC	S/I/R	
27LVS	2	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	R*	>/=1	R	</=2	S	0.5	S	>/=16	R	</=10	S	-
27RS	1	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
31LVS	4	347	0.12	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
40LVS	5	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
42RS	7	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
42LVS	6	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
43LVS	3	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
44LVS	8	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
45LVS	9	347	</-0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
49LVS	11	347, 500	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	>/=320	R	-
49RS	10	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	160	R	-
59LVS	12	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
65LVS	13	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
68RS	14	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
70RS	15	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
70LVS		347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
71LVS	16	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	0.5	S	</=10	S	-
73LVS	17	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
74LVS	18	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-

79LVS	**19**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	2	I*	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
102LVS	**20**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
212LVS	**21**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
218LVS	**22**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
224LVS	**23**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
234RS		**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
234LVS	**25**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	>/=320	R	-
238RS	**26**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
238LVS	**27**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
240RS	**28**	**347, 500**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	2	R*	0.5	R*	</=2	S	0.5	S	>/=16	R	</=10	S	+
244LVS	**29**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.25	S	>/=16	R	</=10	S	-
244RS	**24**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
248LVS	**30**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
248RS	**31**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
255LVS	**33**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
261LVS	**34**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
312RS	**35**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
314RS	**36**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
332LVS	**37**	**347, 209, 500**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	>/=8	R	0.5	R*	</=2	S	0.5	S	>/=16	R	</=10	S	+
333RS	**38**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
422RS	**39**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
426LVS	**40**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
426RS	**41**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
432LVS		**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
432RS	**42**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
438LVS	**43**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-

438RS	**44**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
440LVS	**45**	**347, 209**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	2	R	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
440RS	**46**	**347, 209**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	2	R	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
444LVS	**47**	**347, 500**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	2	R	</=0.25	R*	</=2	S	0.5	S	>/=16	R	</=10	S	+
446RS		**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	160	R	-
446LVS	**48**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	160	R	-
447LVS	**56**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	>/=320	R	-
447RS	**57**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	160	R	-
448RS	**59**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
450LVS	**60**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
450RS	**60**	**None**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
466LVS	**61**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
466RS	**62**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
469LVS	**63**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
469RS	**64**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
470LVS	**66**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
471LVS	**69**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
471RS	**68**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
473LVS	**70**	**347 , 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
473RS	**71**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
478LVS	**50**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	160	R	-
478RS	**49**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
482LVS	**58**	**347**	0.12	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	2	S	</=10	S	-
482RS	**52**	**347**	0.12	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	2	S	</=10	S	-
483LVS	**53**	**347. 639**	0.12	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	2	S	</=10	S	-

483RS	**113**	**347**	0.12	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	2	S	</=10	S	-
490LVS	**54**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
490RS	**55**	**347**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
491LVS	**114**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
508LVS	**73**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
508RS	**72**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
525LVS	**74**	**347, 639**	0.12	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
525RS	**75**	**347, 639**	0.12	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
530LVS	**76**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
530RS	**77**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
535LVS	**32**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
535RS	**78**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
538LVS	**116**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
538RS	**79**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
539LVS	**117**	**347, 548, 639**	</=0.06	S	</=0.25	S	1		</=0.12	S	1	S	2	R	>/=1	R	</=2	S	0.5	S	>/=16	R	</=10	S	-
539RS	**80**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
548LVS	**81**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
549LVS	**82**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
552LVS	**115**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
575LVS	**118**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	2	R	0.5	R*	</=2	S	0.5	S	>/=16	R	</=10	S	+
575RS	**83**	**None**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	2	R	</=0.25	R*	</=2	S	0.5	S	>/=16	R	</=10	S	+
580LVS	**119**	**None**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
580RS	**84**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-

588LVS	85	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
588RS		347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
590LVS	86	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
590RS	87	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
595LVS	89	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
596LVS	90	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
603LVS	92	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	>/=8	R	>/=1	R	</=2	S	0.5	S	>/=16	R	</=10	S	-
613LVS	95	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	2	R	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
625RS	97	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
626LVS	98	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
626RS		347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
628LVS	99	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
628RS	100	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
641LVS	101	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	160	R	-
641RS	102	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	160	R	-
644RS	103	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
665LVS	105	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	0.5	I	</=2	S	0.5	S	>/=16	R	</=10	S	-
665RS	104	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
670LVS	106	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
670RS	67	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-
682LVS	108	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	>/=8	R	>/=1	R	</=2	S	0.5	S	>/=16	R	</=10	S	-
682RS	107	347	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	0.5	S	>/=8	R	>/=1	R	</=2	S	0.5	S	>/=16	R	</=10	S	-
691LVS	110	347, 639	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-

692RS	**11 1**	**347, 639**	</=0.06	S	</=0.25	S	</=0.12	S	</=0.12	S	1	S	</=0.12	S	</=0.25	S	</=2	S	0.5	S	>/=16	R	</=10	S	-	

ID=Identification; RS=Rectal swab; LVS=Lower vaginal swab; PEN=Penicillin; AML=Amoxicillin; CTX=Cefotaxime; CRO=Ceftriaxone; Levo=Levofloxacin; E=Erythromycin; CD=Clindamycin; LZD=Linezolid; VA=Vancomycin; TE=Tetracycline; SXT=Trimethoprim/sulfamethoxazole; ICR=Inducible Clindamycin Resistance; MIC=Minimum Inhibitory Concentration; S=Sensitive; I=Intermediate resistant; R=Resistant; - =negative; + =Positive.