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Molecular characterization of invasive *Streptococcus agalactiae* in South Africa, 2019 – 2020

Submitted by

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A Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Science in Medicine by Research (Clinical Microbiology and Infectious Diseases)

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Johannesburg

09 June 2023

Declaration

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In memory of my grandmother

Nowongile Ntozini

1939 – 2019

Presentations

Ntozini B. Molecular characterization of invasive Group B Streptococcus in South Africa, 2019-2020. GERMS-SA Principal Investigator's meeting. Virtual. 15 June 2021. (Oral)

Ntozini, B, von Gottberg, A, de Gouveia, L, Meiring, S, Quan, V, Walaza, S and Wolter, N (2021). Molecular characterization of invasive Group B Streptococcus in South Africa, 2019-2020. The International Conference on Group B Streptococcus (ICGBS). Virtual, 20 - 21 July 2021. (Oral)

Ntozini, B, von Gottberg, A, de Gouveia, L, Meiring, S, Quan, V, Walaza, S and Wolter N, (2022). Serotype distribution, antimicrobial resistance and molecular characteristics of invasive early onset and late onset group B streptococcal disease amongst infants in South Africa, 2019-2020. The International Conference on Group B Streptococcus (ICGBS). Virtual, 11-15 July 2022. (Oral)

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Ntozini B. Laboratory-based surveillance of Group A streptococcus (GAS) and Group B streptococcus (GBS) isolates causing invasive disease in South Africa. GERMS-SA surveillance officers meeting. Birchwood Hotel, Boksburg, Johannesburg, South Africa. 02-05 August 2022. (Oral)

Ntozini, B. Serotype distribution, antimicrobial resistance and molecular characteristics of invasive early onset and late onset group B streptococcal disease amongst infants in South Africa, 2019-2020. NICD 2022 Annual Influenza and Respiratory Disease Surveillance Symposium (AIRSS). Poliomyelitis Research Foundation (PRF) Auditorium, National Institute for Communicable Diseases (NICD), Johannesburg, South Africa, 17-18 August 2022. (Oral)

Ntozini, B, von Gottberg, A, de Gouveia, L, Meiring, S, Quan, V, Walaza, S and Wolter N. Serotype distribution, antimicrobial resistance and molecular characteristics of invasive early onset and late onset group B streptococcal disease amongst infants in South Africa, 2019-

2020. Wits Faculty of Health Sciences Biennial Research Day and Postgraduate Expo, Johannesburg, South Africa, 15 September 2022. (Poster)

Publications

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Abstract

Background: Group B streptococcus (GBS) is a leading cause of neonatal meningitis and sepsis. Capsular polysaccharide and protein-based GBS vaccines are currently in development. Therefore, it is important to understand the serotype and antigen distribution of GBS to assess potential vaccine coverage. We aimed to use whole genome sequencing (WGS) and phenotypic methods to assess the serotype and antigen distribution, antimicrobial susceptibility patterns, and the phylogeny of invasive GBS isolates in South Africa (SA).

Methods: As part of national laboratory-based surveillance, 661 invasive GBS isolates cultured from normally sterile-site specimens (e.g. blood and cerebrospinal fluid) from patients of all ages, were collected from 2019-2020. Phenotypic identification was based on colony morphology and β-hemolysis. Serotyping was performed using latex agglutination. Antimicrobial susceptibility testing was based on disc diffusion and broth microdilution methods. Whole genome sequencing (WGS) was performed using the NextSeq 550 System and the 2 x 100-bp paired-end mode. WGS based prediction of serotypes, surface protein genes, and resistance gene detection was performed using a validated GBS bioinformatics pipeline developed by the USA Centers for Disease Control and Prevention (CDC).

Results: A total of 658/661 isolates yielded good quality sequences from WGS and three isolates poor quality sequences. The isolates belonged to 6 major clonal complexes (CCs): CC1 (37/658, 5.6%), CC8/10 (69/658, 10.5%), CC17 (272/658, 41.3%), CC19 (45/658, 6.8%), CC23 (188/658, 28.6%) and CC24 (37/658, 5.6%). Five singletons (10/658, 1.5%) were identified. Excluding phenotypically and genotypically non-typeable isolates, concordance for serotyping using latex agglutination and WGS was 99.7% (647/649). Overall, 6 serotypes were detected: III (281/656, 42.8%), Ia (183/656, 27.9%), V (78/656, 11.9%), II (955/656, 8.4%), Ib (44/656, 6.7%), and IV (15/656, 2.3%). Three percent (20/658) of the isolates were non-susceptible to penicillin. Erythromycin and clindamycin resistance was detected in 16.1% (106/658) and 3.8% (25/657) of the isolates respectively. Majority (91.5%, 625/657) of the isolates were resistant to tetracycline. Fifty-five percent (11/20) of penicillin non-susceptible isolates had mutations in the PBP2x gene known to confer resistance, while no PBP2x resistance mutation were detected in the remaining resistant isolates. *ermTR* (34.9%, 37/106) and *mefA/E* (29.2%, 31/106) resistance genes were the most common determinants of erythromycin resistance. Clindamycin resistance was mediated by the *erm* (*ermB, T,* and *TR*) genes. Tetracycline resistance was driven by the presence of the *tet* genes (*tetM, tetO, and tetL*), with *tetM* being the most common (95.8%, 599/625). Nearly all

the isolates carried at least one of the 3 main pilus gene clusters (657/658, 99.8%), 1 of the 4 homologous alpha/Rib family determinants (656/658, 99.7%) and 1 of the serine-rich repeat (Srr) protein genes (626/658, 95.1%). The *hvgA* virulence gene was found exclusively in CC17 isolates.

Conclusion: Our results show that the majority of isolates circulating in SA belong to the 5 major GBS CCs (CC1, CC8/10, CC17, CC19, and CC23). This study suggests that vaccines currently under development should provide good coverage in our setting. We show that the GBS6 vaccine that targets serotypes Ia, Ib, II, III, IV, and V has the potential to cover 100% of invasive GBS disease in SA. A pilus protein-based vaccine has a potential coverage of 99.8% and the GBS-NN and the latch peptide vaccines have a potential coverage of 68.5% and 95.3% respectively. Beta-lactams remain appropriate for treatment and intrapartum antibiotic prophylaxis (IAP). However, detecting the emergence of non-susceptibility requires ongoing surveillance.

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List of abbreviations

aa	Auto-agglutination
AAP	American Academy of Pediatrics
adhP	Alcohol dehydrogenase gene
Alp (*alp*)	Alpha-like protein (gene)
AST	Antimicrobial susceptibility testing
atr	Amino acid transporter gene
BC	Blood culture
BibA	GBS immunogenic bacterial adhesin
CAMP (*cfb*)	Christie Atkinson Munch-Petersen (gene)
CC	Clonal complex
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CPS (*cps*)	Capsular polysaccharide (gene)
CSF	Cerebrospinal fluid
DLV	Double-locus variant
DNA	deoxyribonucleic acid
EOD	Early onset disease
erm	erythromycin ribosome methylase gene
Fbs	Fibrinogen-binding proteins
FN	False negative
FP	False positive
FQ	Fluoroquinolone
GBS	Group B Streptococcus
glck	Glucose kinase gene

glnA	Glutamine synthetase gene
GTR	Generalized time reversible
gyr	gyrase resistance gene
HvgA (*hvgA*)	Hypervirulent GBS adhesin (gene)
IAP	Intrapartum antibiotic prophylaxis
Isa	Macrolide resistance gene
iTOL	Interactive Tree of Life
linB	lincosamide nucleotidyltransferase gene
Lmb	Laminin-binding protein
LOD	Late onset disease
LPXTG	Leucine-proline-any amino acid-threonine-glycine
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
mef	macrolide efflux gene
MIC	Minimum inhibitory concentration
ML	Maximum-likelihood
MLST	Multilocus sequence typing
MS	Mass spectrometry
NGS	Next-generation sequencing
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
NPV	Negative predictive value
NT	Non-typeable
par	topoisomerase IV resistance mutation
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PCV	Polysaccharide conjugate vaccine

PFGE	Pulsed-field gel electrophoresis
pheS	Phenylalanyl tRNA synthetase gene
PI	Pilus island
PPV	Positive predictive value
PR-GBS	Reduced-penicillin susceptibility
ptsl	Sugar phosphotransferase gene
QC	Quality control
RPP	Ribosomal protection protein
SA	South Africa
ScpB	Group B streptococcal C5a peptidase
sdhA	Serine dehydratase gene
SfbA	Streptococcal fibronectin-binding protein A
sip	Surface immunogenic protein gene
SLV	Single-locus variant
SNP	Single nucleotide polymorphism
Srr	Serine rich repeat
ST	Sequence type
tet	Tetracycline resistance gene
tkt	Transkelotase gene
TLV	Triple-locus variant
TN	True negative
TP	True positive
TT	Tetanus toxoid
UK	United Kingdom
USA	United States of America
WGS	Whole genome sequencing

List of symbols

mm	Milimeter
℃	Degree celsius
β	Beta
%	Percentage
µl	Microlitre
rpm	Revolutions per minute
ml	Milliliter
ng	Nanogram

Chapter 1: Introduction

Streptococcus agalactiae, also referred to as Group B streptococcus (GBS), is a Gram-positive bacterium that resides as a common commensal of the genitourinary tract of humans. Eighteen percent of pregnant women worldwide are colonized with GBS, as reported in a meta-analysis conducted in 2017 (1). Even though it is part of the normal flora, GBS has the capacity to cause serious disease in newborns, pregnant women and non-pregnant adults (especially those with underlying medical conditions and adults who are 60 years and older) (2).

GBS is established as a leading cause of neonatal sepsis and meningitis globally (3). In newborns, GBS can be divided into early-onset disease (EOD: presenting from day 0 to 6 of life) and late-onset disease (LOD: presenting from day 7 to 89 of life). Colonization during pregnancy is the primary risk factor for invasive neonatal GBS disease, either during the pregnancy or after birth. Ascending infections during the pregnancy can lead to premature birth or stillbirth (4–6). In 2015, the burden of GBS disease in infants globally was estimated at 319 000 infants, resulting in 90 000 infant deaths, at least 57 000 fetal infections or stillbirths, and up to 3.5 million preterm births. Invasive GBS disease in pregnant women or postnatal infections were estimated to be at least 33 000 women (7).

In order to decrease the morbidity and mortality associated with GBS disease in newborns, the CDC, the American College of Obstetricians and Gynecologists, and the American Academy of Pediatrics (AAP) developed guidelines in 1996 (revised in 2010) to provide women who are at risk of delivering a GBS-infected child with intrapartum antibiotic prophylaxis (IAP) (8). Beta-lactam antibiotics are recommended for IAP and for treatment of GBS infections. For patients that are allergic to beta-lactams, alternative treatments such as clindamycin, erythromycin, and vancomycin are recommended (8). Beta-lactam antibiotics have been considered effective against GBS, however, there have been reports of cases of GBS with reduced beta-lactam susceptibility or non-susceptibility (9–14). Furthermore, cases of increased resistance to erythromycin and clindamycin have been reported globally (15, 16, 17).

IAP has reduced the incidence of EOD by more than 80% in the United States of America (USA) (18). Despite the success of IAP in high income countries, it is subject to high costs and is resource intensive, which limits the feasibility of this strategy in low income countries (9, 10). Furthermore, IAP is not effective in reducing the incidence of LOD (21). In light of

the challenges with feasibility of IAP in some countries and concerns about antibiotic resistance, maternal vaccination is considered a more effective strategy. An effective GBS vaccine has the potential to prevent disease across all groups at risk of infection, including pregnant women (the mother), the fetus, and the baby (7).

Various vaccine formulations against GBS are in development. This includes capsular polysaccharide (CPS)-based and protein-based vaccines (22). GBS expresses 10 different capsular types namely, serotypes Ia, Ib, and II-IX. Serotype specific polysaccharide-protein conjugate vaccines targeting serotypes Ia, Ib, II, III and V have been evaluated for safety and immunogenicity (22). A 6-valent (Ia, Ib, II, III, IV, and V) conjugate vaccine is currently in a phase Ib/II clinical trial. Due to the possibility of capsular switching after the introduction of CPS-based vaccines, protein-based vaccines are seen as more attractive and specific surface proteins are being explored as vaccine targets. This includes pilus proteins and the Alpha and Rib proteins of GBS.

Whole genome sequencing (WGS) is a powerful tool that can be used for molecular characterization of pathogens and its use is becoming increasingly popular. Population analysis made possible by WGS allows the identification of newly emerging GBS clusters, some of which may be pathogenic clades and have public health implications. The depth of information that is provided by WGS can also clarify the origins of disease. For example, Da Cunha *et al*. (2015) used WGS to show that the emergence of GBS in humans can be traced through genetic lineage as a result of tetracycline use (23).

A recent study by Metcalf *et al* (2017) (14) which aimed at evaluating and using a WGS bioinformatics pipeline for predicting antimicrobial resistance and capsular serotypes for invasive GBS, showed that WGS-based resistance and serotype detection is more effective than conventional testing due to the automated extraction of the data from a single source of permanently stored WGS data (14). Several studies have used WGS for the molecular characterization of GBS strains in order to determine serotypes and for antimicrobial resistance detection, however, there are limited data on WGS of GBS in South Africa (SA). To our knowledge, there is only a single study that conducted WGS on GBS in SA. In this study, Lukhele *et al* (2021) used WGS to investigate whether three invasive GBS cases caused by serotype III in babies that were born within 3 hours of each other in the same hospital had a common hospital source of infection.

1.1 Study rationale

With vaccines currently in development, it is important to know the serotype and antigen distribution of GBS strains causing invasive disease in SA to assess the potential coverage of these vaccines. Furthermore, antimicrobial resistance to antibiotics used against GBS is increasing, but there are limited data in SA. Different studies that have been conducted on GBS in SA to assess serotype distribution and antimicrobial resistance profiles have been limited to specific regions within SA and specific groups of individuals (e.g. EOD vs LOD or pregnant women). This study aimed to determine the serotype and genotype distribution, and the antimicrobial resistance patterns of invasive GBS causing disease nationally and in all ages in SA using phenotypic methods and WGS.

1.1.1 Aim and objectives

Aim: To describe the molecular epidemiology of GBS causing invasive disease in SA in 2019 and 2020.

Objectives:

i. Describe the demographic and clinical characteristics, serotype distribution of invasive GBS in all ages, and by early and late onset disease using phenotypic and genotypic (WGS) methods and compare serotype prevalence to serotypes contained in different vaccine formulations.
ii. Identify surface proteins in invasive GBS isolates in order to assess the potential coverage of different protein-based vaccines under development.
iii. Describe resistance to penicillin, ampicillin, erythromycin, clindamycin, tetracycline, rifampicin, and chloramphenicol using phenotypic and WGS methods.
iv. Describe the phylogeny of invasive GBS strains by time of disease onset (EOD vs LOD), age group, and geography and compare South African strains to globally circulating strains.

2 Chapter 2: Literature review

2.1 History of GBS

GBS was first identified by Nocard and Mollereau as a cause of bovine mastitis, which led to the lack of milk production in 1887 (24). It was later identified as a human pathogen in 1938 by Fry who described three cases of fatal puerperal sepsis (25). Human infections were not frequently identified until the 1960s, when an increase in reported GBS human infections occurred and GBS became recognized as the leading cause of sepsis in neonates in the USA (26). By the 1970s, GBS was the leading cause of meningitis and septicemia in newborns and young infants and led to a significant amount of pregnancy-related morbidity (27). GBS is also an important cause infections in non-pregnant adults, especially in adults who are 60 years and older and adults with underlying medical conditions (28).

2.2 Microbiology of GBS

GBS is a Gram-positive, facultative anaerobic, and catalase-negative coccus that grows in a variety of culture media. On blood agar, GBS appears as flat, slightly mucoid, grayish colonies surrounded by a zone of beta-hemolysis or complete hemolysis. This due to the pathogen's ability to produce hemolysin, a toxin that causes lysis of the hemoglobin in red blood cells. One to two percent of GBS isolates are non-hemolytic. GBS colonies on overnight cultures are typically 3 to 4 mm in diameter (2).

2.3 Identification of GBS

Definitive phenotypic identification of GBS is based on the detection of the group B-specific cell wall antigen which is common to all GBS strains. A number of serologic tests using hyperimmune group B-specific antisera can be used, however, the most widely used method is the latex agglutination test reacting with the group B antigen (27, 28). The Christie Atkinson Munch-Petersen (CAMP) test is one of the standard phenotypic tests for identification of GBS. The CAMP factor produced by GBS interacts synergistically with beta-hemolysin produced by most strains of *Staphylococcus aureus* resulting in enhanced hemolysis (29, 30). The combination of the CAMP test with bacitracin susceptibility testing and a bile esculin reaction provide an adequate presumptive differentiation of GBS from other β-hemolytic streptococci. Recently, chromogenic media have been introduced to improve the identification of GBS. It is based on colour change in the presence of GBS colonies (33). Polymerase chain reaction (PCR) based methods can also be used i.e. targeting the *cfb* gene encoding the CAMP factor (34), the *sip* gene (35) and the *pts1* gene (36). Matrix assisted laser

desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), a technique that uses laser energy and ionization for rapid identification of microorganisms can also be used for identification GBS.

2.4 Epidemiology of GBS

2.4.1 Maternal colonization and transmission

GBS resides as a common commensal of the gastrointestinal and genitourinary tract of healthy women. The gastrointestinal tract is the main reservoir and vaginal colonization with GBS is secondary to gastrointestinal colonization due to their proximity (37). GBS carriage rates among healthy women ranges from 10-30% globally. Colonization during pregnancy is the primary source of newborn infections and heavy colonization is an important risk factor for vertical transmission of GBS (38). Vertical transmission from mother to child can occur either by ascending infection from the vagina to the cervix and uterus, or during labor when the baby comes into contact with the bacterium (39). A study by Sensini *et al*. (38) conducted in Italy reported that neonates born from heavily colonized mothers had an increased rate of colonization (74/148, 50%) compared to those born from lightly colonized mothers (34/112, 30.4%) ($P<0.01$). Another important risk factor for GBS acquisition in infants is the prolonged rupture of the amniotic sac membrane. Hickman *et al*. reported a vertical transmission rate of 73.3% in cases of membrane rupture that occurred more than 12 hours before delivery compared to 38.4% in cases where the rupture occurred less than 12 hours before delivery ($P<0.05$) (40).

2.4.2 GBS disease

Even though GBS is a common commensal of the gastrointestinal tract, it has the capacity to cause severe disease. GBS can cause disease in pregnant women, newborns, children, and adults (especially those who are immunocompromised).

2.4.2.1 GBS infections in pregnant women

A 2017 systematic review and meta-analysis by Russell *et al*. estimated the global prevalence of maternal colonization to be 18%, with colonization rates from different countries varying from 11-35% (41). Colonization of the genital tract during pregnancy can lead to maternal infection during pregnancy or labor. GBS infections in pregnant women can cause bacteremia, endometritis, chorioamnionitis, urinary tract infection, and may lead to septic abortion. Colonization with GBS during pregnancy is also a risk factor for premature delivery

and stillbirths (10, 11, 12). Seale *et al*. (7) reported that world-wide in 2015, there were 33 000 cases of invasive GBS disease in pregnant or postpartum women and 57 000 fetal infections/stillbirths. The estimated worldwide number of preterm births attributed to GBS was estimated to be 3.5 million.

2.4.2.2 Disease in neonates

In newborns, GBS disease is classified into EOD, which manifests from 0 to 6 days of life and LOD, which manifests from 7 to 89 days of life. GBS infections in neonates may lead to pneumonia, sepsis, and meningitis. A systematic review and meta-analysis in 2017 estimated the global incidence of invasive GBS neonatal disease to be 0.49 cases per 1000 live births with the highest incidence reported in Africa (1.12 cases per 1000 live births) and lowest incidence reported in Asia (0.30 cases per 1000 live births) (45). In SA, the incidence of infant GBS disease has remained unchanged at 2 - 3 per 1000 live births over the past ~three decades and this is also associated with a high case-fatality ratio of between 13 - 18% (46–49).

2.4.2.2.1 Early-onset disease (EOD)

Newborns with EOD typically present with pneumonia and sepsis, and rarely meningitis. Approximately 85% of EOD cases occur within 24 hrs of birth and EOD has the highest proportion of fatal cases with a fatality ratio of 20% in neonates born before 33 weeks of gestation and 2-3% in full term neonates (8). The primary source of EOD is the vertical transmission of GBS from the colonized mother to the newborn. This can happen either *in utero* by ascending infection from the genital tract into the uterus or during passage through the vaginal canal. In addition to maternal colonization, there are other factors associated with an increased risk for EOD. This includes premature labour, prolonged rupture of membranes, maternal fever during labor, and previous pregnancy with GBS infection (8). The global incidence of EOD is reported to be 0.41 cases per 1000 live births, and in sub-Saharan Africa the incidence of EOD is recorded at 1.3 cases per 1000 births (50).

2.4.2.2.2 Late-onset disease (LOD)

LOD commonly presents as bloodstream infections (septicemia or bacteremia) or meningitis (14, 15). LOD patients present with meningitis in >80% of cases and results in approximately 20% mortality globally. Permanent neurological sequelae is a possibility in LOD patients (53). Acquisition of GBS in LOD is not well understood, however, studies have suggested a maternal source through acquisition of the bacterium during passage through the birth canal

(54). In a study conducted in the USA, 50% of babies with LOD were found to have the same GBS serotype as their mother (54). Breastfeeding has also been shown to be a source of LOD (55). LOD may also be acquired nosocomially through colonized hospital staff and equipment, as well as community acquired (56). The global incidence of LOD is estimated to be 0.26 cases per 1000 live births (45).

2.4.2.3 Disease in adults

Reports from various studies in high income countries suggest that the incidence of GBS infections in adults is increasing (15, 51–54). In England, the incidence of GBS infections in adults doubled from 1.48 to 2.99 per 100 000 population between 1991 and 2010 (58). In California (USA), the incidence increased from 5.8 per 100 000 population in 1995 to 8.3 per 100 000 population in 2012 (57). From 2008-2016, the incidence of GBS among adults in the USA was reported to have increased from 8.1 to 10.9 cases per 100 000 persons over the 8 years (15) and in Kentucky (USA), the annual incidence from 2014 to 2016 was reported to be 73 per 100 000 adults (60). The majority of adult invasive GBS infections occur in adults above 60 years of age as well as adults with underlying conditions such as diabetes, liver disease, malignancies, and cardiovascular disease. Adults with invasive GBS disease commonly present with skin and/or soft tissue infections, septic arthritis, urinary tract infections, or septicemia without focus (25, 28, 29).

2.5 Treatment and prevention

In order to prevent mother-to-child transmission of GBS, IAP can be used. Two strategies are used to identify women that are eligible for IAP: universal culture-based screening for GBS colonization, or identification of clinical risk factors for GBS transmission. The CDC recommends universal screening for maternal colonization routinely at 35-37 weeks' gestation. The risk-based approach depends on detection of risk factors such as prolonged rupture of membranes, bacteriuria, a previous child with EOD, preterm delivery less than 37 weeks' gestation, and maternal fever (63).

For both IAP and treatment of GBS infections, beta-lactam antibiotics (penicillin, ampicillin or cefozalin, but preferably penicillin) are the agents of choice. Alternative antimicrobial therapies such as clindamycin, erythromycin, fluoroquinolones and vancomycin are used for individuals who are allergic to beta-lactams (63).

Since the implementation of the universal screening strategy in the USA, the incidence of EOD has declined drastically (>80%) (18). However, this approach is not feasible in low-

middle-income countries where a large proportion of deliveries occur outside of healthcare settings. Furthermore, the cost of screening pregnant women, providing IAP and building the necessary infrastructure is high and therefore hinders the feasibility of the universal screening of pregnant women for GBS. In middle-income countries such as SA, the risk-based approach is used (64). The risk-based approach is also disadvantageous as risk factors do not identify all women at risk of transmitting the bacteria. Additionally, health care workers may fail to identify the risk factors and may therefore not administer IAP to women who actually need it. Other limitations of IAP are that it may not be possible for sudden deliveries, it may lead to increase in antibiotic resistance, and it does not prevent preterm delivery, stillbirths and LOD (65).

2.6 Antibiotic resistance and mechanisms of resistance

Table 2.1 shows a summary of the different antimicrobial resistance mechanisms known to occur in GBS.

2.6.1 Penicillin non-susceptibility

Penicillin remains effective against GBS, however, several studies from different countries including USA, Canada, Korea, and Japan have reported cases of reduced penicillin susceptibility, also known as PR-GBS (9, 14, 58-61). A 2021 study conducted by Kobayashi *et al*. reported a low (0.5%) but increasing rate of invasive PR-GBS in the USA from 1998 to 2018, and 0.1% of their isolates were non-susceptible to beta-lactams (63). In Japan, the prevalence of PR-GBS increased from 2.3% in 2005-2006 to 14.7% in 2012-2013 (68). In 2018, Africa *et al* reported that 4% (2/57) of their isolates were non-susceptible to penicillin G in a study conducted in pregnant women from the Western Cape Province, SA (69). Resistance to beta-lactam antibiotics in GBS is most commonly a result of mutations in genes that encode penicillin-binding proteins (PBPs) (70). PR-GBS are associated with mutations in PBPs, namely PBP1a, PBP2a, PBP2b, and PBP2x (11, 14, 61). The most frequent mutations detected in PR-GBS result in amino acid substitutions V405A and/or Q557E in the PBP2x transpeptidase (71). All PBP2x mutations are considered critical because of their association with being the first step of reduced susceptibility, and substitutions near the enzyme active sites of PBP2b, PBP1a, and PBP2a are also considered important (72).

2.6.2 Macrolide and lincosamide resistance

Erythromycin and clindamycin resistance in GBS has been rising in different countries including China, USA, and Italy. Erythromycin resistance in China was reported to be 74.1%

in both colonizing and invasive isolates in studies conducted in 2015-2016 (17, 63). In the USA in 2019, rates of erythromycin resistance in invasive neonatal and adult disease were reported to be 54.8% and 44.8% respectively (54, 64). In an Italian study conducted in 2016, the rate of erythromycin resistance was reported to be 42.8% in maternal colonizing GBS isolates. Macrolide and lincosamide antibiotics are chemically distinct and are structurally unrelated, however, these antibiotics share a similar mode of action (ribosomal modification). Macrolide and lincosamide resistance can occur as a result of various mechanisms. Macrolide resistance is commonly due to ribosomal modification mediated by the *erm* (*erm*B, *erm*A/*TR*, or *erm*C) genes (75). This mechanism confers cross resistance to macrolides, lincosamides, and streptogramin B (MLS_B), giving rise to the MLS_B phenotype which can be constitutive (c-MLS_B) or inducible (i-MLS_B). Another common mechanism is the macrolide efflux pump, which is regulated by the *mefA/E* gene and confers resistance to macrolides of members 14 and 15 only (76). This is referred to as the M phenotype. Resistance to lincosamides (*e.g.*, clindamycin) can also be due to ribosomal translocation conferred by *linB* genes and is referred to as the L phenotype (77). Another mechanism of resistance is the ribosomal protection of one of the ABC-F proteins, encoded by *mre* and *Isa* (*IsaC* and *IsaE*) genes. Drug inactivation through enzymatic modification is another type of mechanism for macrolide and lincosamide resistance. For lincosamides, this is facilitated by the *lnuB* gene (formally known as *linB*).

2.6.3 Tetracycline resistance

Tetracycline resistance is primarily associated with acquisition of resistance genes that are associated with mobile genetic elements (78). In GBS, it is commonly due to ribosomal protection proteins (RPPs) encoded by the *tetM* and *tetO* genes. Tetracycline resistance is also less frequently attributed to efflux proteins (encoded by *tetK* and *tetL*) (79). Most GBS strains isolated from humans are resistant to tetracycline (usually >80%) and tetracycline is therefore not a drug of choice for treatment of GBS infections (14).

Da Chuna *et al.* in 2014 reported that the acquisition of resistance elements, *tetO* and *tetM*, by a subset of GBS clones has led to their expansion. This, in turn, has led to the emergence of strains more adapted to colonizing and infecting humans, and has led to the dissemination of the ST-17 hypervirulent clone, which is associated with invasive neonatal infections (23).

2.6.4 Fluoroquinolone resistance

The first report of fluoroquinolone resistant (FQ) GBS was documented in Japan in 2003 (80) and since then FQ resistance in Japan has risen to 43.6% as reported by Nagano *et al.* in 2012 (81). A study conducted in China on isolates collected from 2012 to 2013 reported a resistance rate of 39.3% (82). In South Korea, the rate of FQ resistance has increased from 8.1% in 2008 (83) to 37.2% in 2014 (84). Fluoroquinolone resistance occurs due to either efflux proteins or mutations in the genes that encode the type II topoisomerase enzymes. This includes mutations in the gyrase (*gyrA/gyrB*) and topoisomerase IV (*parC/parE*) genes (36, 42). These mutations affect the binding of fluoroquinolone antibiotics and reduce their effectiveness. Common mutations in the *gyrA* gene lead to amino acid substitution Ser-81-Leu (S81L), while mutations frequently observed in the *parC* gene include those resulting in the Ser-79-Phe (S79K) substitution (76).

2.7 Virulence factors

GBS possesses several virulence factors that aid in colonization, adherence, invasion, and immune evasion. The CPS is the main pathogenic and virulence factor. The major adhesion factors in GBS include the fibrinogen-binding proteins (Fbs), Laminin-binding protein (Lmb), group B streptococcal C5a peptidase (ScpB), streptococcal fibronectin-binding protein A (SfbA) and the GBS immunogenic bacterial adhesin (BibA). Other surface antigens that facilitate virulence and colonization in GBS include the alpha-like protein (Alp) family, serine-rich repeat proteins (Srr), pilus proteins, and hypervirulent GBS adhesin (HvgA) (85). **Figure 2.1** shows the major surface proteins that mediate interaction of GBS with host cells.

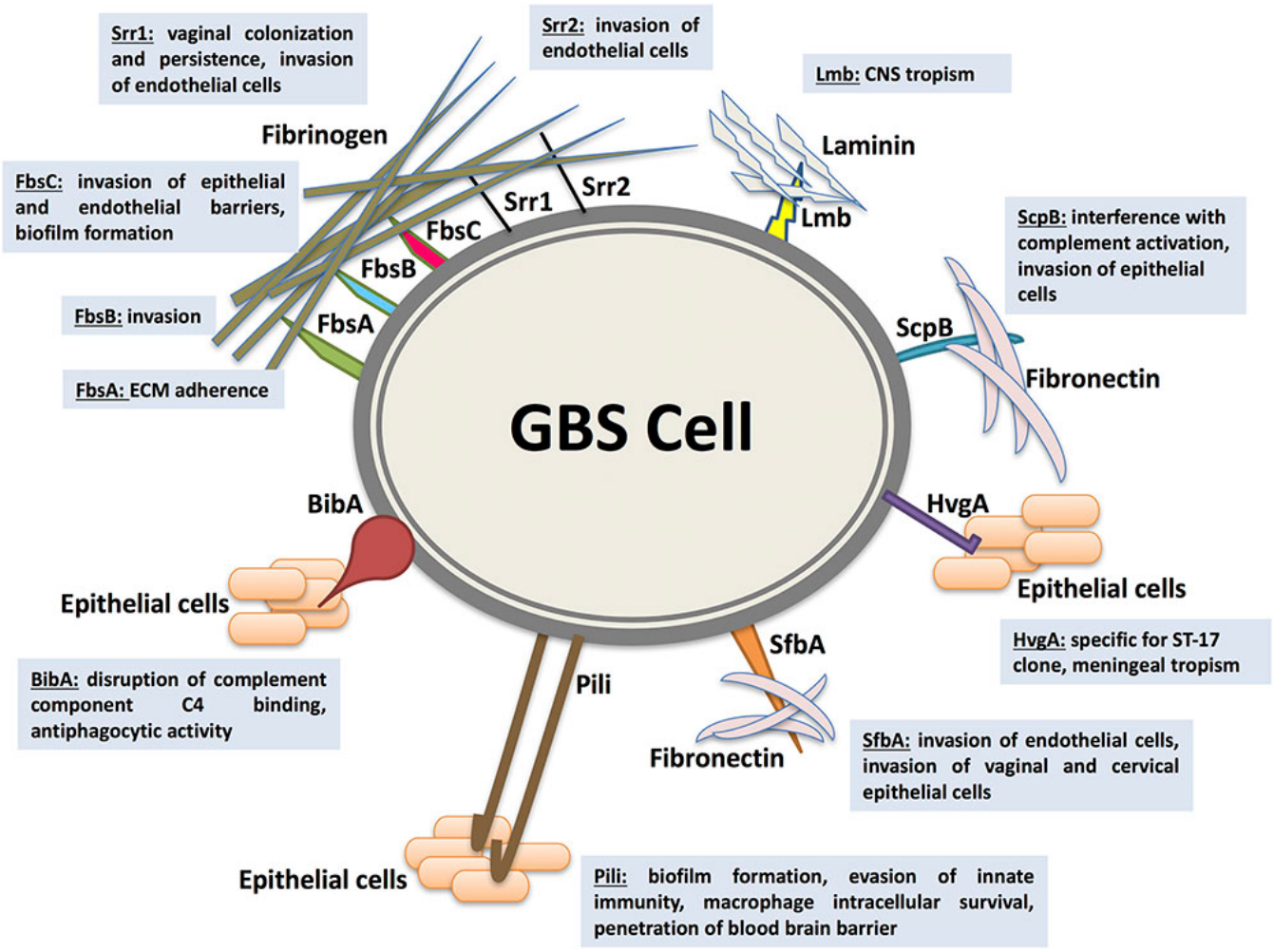


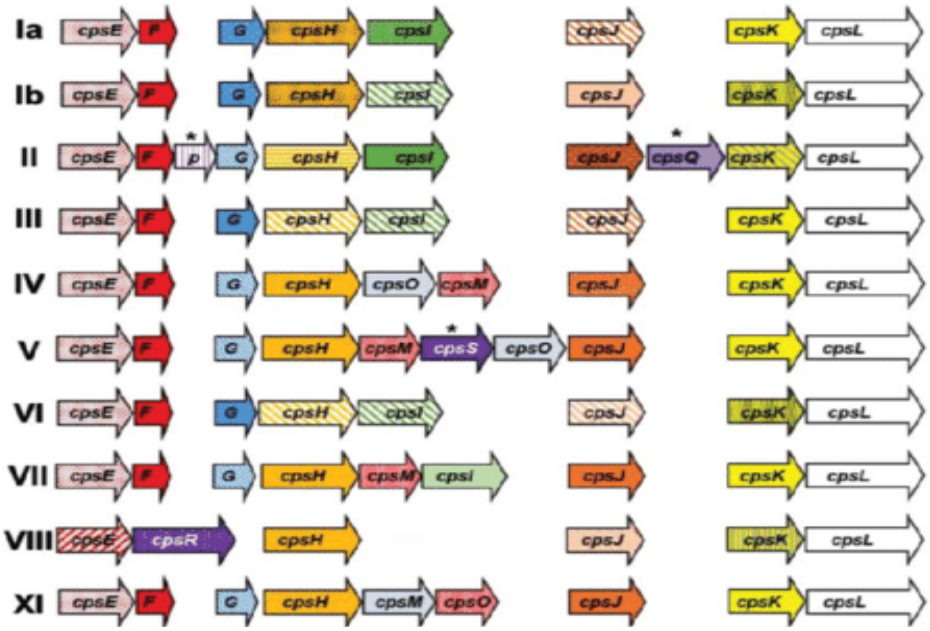
Figure 2.1 GBS antigens. Adapted from Shabayek and Spellerberg, 2018 (85).

2.7.1 Capsular polysaccharide (CPS)

The CPS is the most well studied virulence factor in GBS. The CPS of GBS is rich in sialic acid and plays a role in host immune defence evasion by inhibiting complement deposition and protecting the bacteria from opsonophagocytosis by immune cells. It also enhances biofilm formation, inhibits the binding of antimicrobial peptides and neutrophil extracellular traps (NET) thereby increasing the invasiveness of GBS (86). GBS is divided into ten antigenically distinct capsular types, known as serotypes. These serotypes are designated Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX based on the type-specific capsular polysaccharide (**Fig. 2.2**) (48, 49). All CPS types of GBS are high molecular weight polymers that contain a side chain that is capped with a sialic acid (N-acetylneuraminic acid) residue and they differ in their chemical composition, structure, and serological properties. Synthesis of the GBS CPS is controlled by genes located in the *cps* operon. This locus/operon contains a highly variable region (*cpsC-cpsK*) that determines the CPS type and this region is flanked by genes

that are conserved throughout the serotypes (cpsA-D) whose by-products are involved in the regulation of the synthesis of the capsule (**Fig. 2.2**) (50, 51).

A



B

Class	GBS gene	WZY nomenclature	Function
Regulatory genes	*cpsA*	*wzg*	Integral membrane regulatory protein
	cpsB	*wzh*	Protein-tyrosine phosphate
	cpsC	*wzd*	Chain length determinant protein
	cpsD	*wze*	Tyrosine-protein kinase
Repeating biosynthesis genes	*cpsE*	*wchA*	Initial glycotransferase
	cpsF	*wchJ*	Glycotransferase enhancer
	cpsG	*wchK*	Glycotransferase
	cpsH	*wzy*	Repeating unit polymerization
	cpsI		Glycotransferase
	cpsJ		Glycotransferase
	cpsK		Glycotransferase
	cpsL	*wzx*	Flipase. Repeating unit transportation
	cpsM		Glycotransferase
	cpsN		Glycotransferase
	cpsO		Glycotransferase
	cpsP		Glycotransferase
	cpsQ		Glycotransferase
	cpsR		Glycotransferase
	cpsS		Glycotransferase
CMP-sialic acid biosynthesis genes	*neuB*		N-acetylglucosaminine synthesis
	neuC		UDP-N-acetylglucosamine 2-epimerase
	neuD		Acertyltransferase
	neuA		CMP-NeuNAc synthetase

Figure 2.2 The genetic organization of the CPS locus of GBS in different serotypes (A) cps gene organization in the different GBS serotypes. Gene designations are indicated on each arrow. Colours indicate similarity between genes. (B) Predicted CPS functions based on the results of previous studies and sequence comparisons. Adapted from Song *et al*, 2018 (91).

2.7.1.1 GBS serotype epidemiology/distribution

Serotypes Ia, Ib, II, III, and V are the most common serotypes worldwide (92). While these serotypes predominate globally, local variation may affect the potential coverage of vaccines in each population. Furthermore, some serotypes are more commonly associated with colonization while some are associated with invasive disease (93).

2.7.1.1.1 Maternal colonization and maternal disease

Serotypes I – V account for 98% of colonizing GBS isolates worldwide (1). Based on a recent systematic review and meta-analysis which contained serotype data for 62 countries, the most common serotypes in maternal colonization globally were reported to be serotypes III (25%) and Ia (19%). There was variation of serotype observed by region. Serotypes III, Ia and V were the three most common serotypes in several regions (Europe and northern America, eastern Asia, southern, eastern/central Africa, and Australia/ New Zealand). Conversely, serotype III was less common in the Caribbean (11%), south-eastern Asia (11%), southern Asia (11%), and western Africa (11%). Serotype IV was more common in Europe and northern America (4%), and in southern Africa (3%) than other regions. The rarer serotypes VI-IX were more common in south-eastern Asia (16%), eastern Asia (11%), southern Asia (6%), and western Africa (15%), with most data from a Ghanaian study (n=83). An increase in serotype IV was reported from 1% pre-2001 to 7% in 2013-2018 (94). This serotype has also emerged as an important cause of both neonatal and adult infections and this emergence has been associated with capsular switching from *cps* type III to *cps* type IV. Serotype Ia is the most predominant serotype in maternal disease, accounting for 27% of cases, followed by serotype III (26%), and V (17%) (94).

2.7.1.1.2 Infant GBS disease

Serotype III is reported to be the most common serotype causing invasive infant disease, followed by serotypes Ia, Ib, II, and V (45). A recent systematic review on serotype distribution in infants with invasive GBS disease reported that serotypes Ia, Ib, II, III, and V accounted for more than 97% (6263/6500) of serotypes detected in all regions (45). Based on examination of serotypes by time of disease onset, nearly half (47.4%) of EOD cases were caused by serotype III compared to 73.0% LOD. In contrast, 22.8% of EOD was due to serotype Ia compared to 14.2% of LOD. Serotypes Ib and V accounted for 8.0% and 5.3%, as well as 10.6% and 4.0% of EOD and LOD respectively (45).

2.7.1.1.3 Invasive disease in older adults

Serotypes Ia, Ib, II, and V are most predominant in invasive disease in non-pregnant adults (24, 25). A world-wide systematic review published in 2020 reported that serotype V was the most common (25%) followed by serotype Ia (23%), and serotype III (11%) (94). In south-eastern Asia, serotypes VI to IX have a higher prevalence than in other regions, representing 31% of the isolates. In Europe, northern America and south-eastern Asia invasive disease in the elderly was caused less by serotype III, compared to invasive disease in the infant, mother or stillborn infant (94).

2.7.2 Surface proteins

2.7.2.1 Alp protein family

Members of the Alp protein family, including AlphaC, Alp1, Alp2/3, Alp4 and Rib, are the most predominant GBS surface proteins (96). These proteins contain an N-terminal secretion signal sequence (S), N-terminal conserved domain (N), a variable number of tandemly arranged repeats of 70–80 amino acids (R), and a C-terminal LPXTG cell-wall anchoring motif. The AlphaC, Alp3, and Rib proteins have undergone pre-clinical investigations as vaccine candidates and have been found to elicit immune response (22).

2.7.2.2 Serine-rich repeat proteins

The serine-rich repeat (Srr) proteins are also abundant surface proteins in GBS (97). In GBS, two allelic Srr proteins (Srr1 and Srr2) have been identified and GBS strains express either one of the two alleles and these have a highly conserved domain organization that includes a secretion signal sequence, two Srr domains that are glycosylated, a specialized fibrinogen binding domain between two Srr domains, and an LPXTG cell wall anchoring motif. These proteins bind to fibrinogen Aα using the dock, lock, and latch mechanism. This contributes to the pathogenesis of GBS meningitis and facilitates vaginal colonization (94, 95). These proteins have been shown to elicit an immune response and to provide serotype independent protection in mice (99).

2.7.2.3 GBS pili

Pili are long filamentous structures that protrude from the surface of bacteria and aid pathogenesis, adherence to host cells, colonization and invasion (99). Extensive genomic analyses of a large panel of GBS isolates showed that GBS has three pilus islands, PI-1. PI-

2a, PI-2b and these are named pilus type 1, 2a, and 2b respectively. Each pilus island encodes a pilus that is made up of three structural components, the major pilus unit (backbone protein, BP) which forms the shaft and two ancillary proteins (AP1 and AP2) that anchor the pilus to the bacterial cell wall (100). These proteins are of importance because antibodies against these have been shown to provide protective immunity against GBS in mouse models and in humans (101).

2.7.2.4 Hypervirulent group B streptococcus adhesion (*HvgA*)

The hypervirulent GBS adhesin (*HvgA*) is a surface protein that has been associated with a serotype III GBS clone, designated ST-17 within the CC17 clonal complex (102). This protein harbors an LPXTG motif that facilitates covalent attachment to the cell wall in a sortase dependent manner. The HvgA protein is encoded by genes located in the gbs2018 locus (allele C). The gbs2018 locus has three allelic forms or clusters (A, B, and C) and these are associated with different lineages. The HvgA protein which is encoded by allele C of the gbs2018 locus has been exclusively associated with ST-17 and cluster A comprised of isolates that belong to ST-19 and ST-23. Cluster B includes isolates from multiple STs (ST-1, ST-2, ST-6, ST-8, ST-9, ST-10 and ST-41) (102).

2.8 Vaccines

It has been proposed that a GBS vaccine given to pregnant women could be an effective way to prevent the types of invasive GBS diseases that cannot be prevented with IAP (e.g. LOD) and would be a better solution in countries where IAP is not feasible. There is currently no licensed GBS vaccine, however various polysaccharide conjugate (PCV) and surface protein vaccines are currently being tested and some have undergone clinical trials (91). In 1976, a study by Baker and Casper demonstrated that neonates born to mothers with low levels of serotype III maternal antibodies were more susceptible to EOD and LOD than those born to mothers with high levels of serotype III maternal antibodies (103). This study provided proof of concept that maternal vaccination could be a suitable preventative strategy for GBS infections in newborns and that the CPS of GBS could be a potential vaccine candidate. Transfer of maternal CPS-specific antibodies from mother to the newborn were able to confer protection to the newborns against GBS infections (103). Subsequent studies later confirmed the association between the levels of maternal antibodies against serotypes Ia and III and the susceptibility of newborns to GBS infections (99, 100).

The first generation of GBS vaccines used unmodified type-specific polysaccharides. While these vaccines were safe and triggered an immune response, the immunogenicity of these vaccines was low. Type Ia and III vaccines showed immune response in only about half of the recipients, indicating that these vaccines needed to be improved (101, 102). Second generation GBS vaccines incorporated glycoconjugates with the aim of increasing the immunogenicity of CPS-based GBS vaccines. GBS conjugate vaccines that were prepared with type-specific CPS and coupled to the tetanus toxoid (TT) and CRM_{197} did indeed increase the immunogenicity of GBS vaccines compared to non-conjugated vaccines (108–110). Monovalent conjugate vaccines based on the five most common disease-causing serotypes (Ia, Ib, II, III, and V) have been tested in phase I and II trials in healthy non-pregnant women (111–115). These trials have demonstrated that CPS conjugate vaccines were effective and could be safely administered to women early in the third trimester of pregnancy. Even though these monovalent conjugate vaccines showed increased immunogenicity, single serotypes do not confer cross-reactive immunity to other serotypes and therefore, multivalent vaccines had to be developed.

A trivalent (Ia, Ib, III) CRM_{197} GBS conjugate vaccine phase Ib/II clinical trial conducted in pregnant women showed that infants born to women who received the vaccine showed higher concentrations of serotype-specific antibodies compared to infants born to women in the placebo group (103). The Biovac Institute (Cape Town, SA) in collaboration with PATH is developing a multivalent conjugate GBS vaccine which targets the most dominant serotypes (116). In 2017, Pfizer began evaluating a pentavalent GBS PCV vaccine aimed at protecting against the common serotypes Ia, Ib, II, III, and V. However, more recently, serotype IV was added to the vaccine due to the recent increase in disease caused by this serotype (117). Vaccinating with these formulations may lead to capsular switching caused by selection of vaccine-escape recombinants. In 2012, Bellais *et al* (118) reported a capsular switch of a type III to IV, highlighting the need for ongoing surveillance. Therefore, it is important to understand serotypes and strains that are circulating in a country or region before these vaccines can be introduced. Currently, a phase I/II clinical trial is being conducted in SA to assess the safety, tolerability, and immunogenicity of a hexavalent vaccine in healthy non-pregnant and pregnant women (119).

Even though CPS is the main target for current vaccine formulations, CPS conjugate vaccines only cover serotypes included in the vaccines (i.e. they are not universal). Furthermore, these types of vaccines have the potential to interfere with other types of conjugate vaccines such as the *Haemophilus influenzae* type b, meningococcal and pneumococcal conjugate vaccines due

to interactions between the carrier proteins included in the different vaccines (115, 116). Additionally, there is a possibility of capsular switching and serotype replacement post vaccination (80,118,122,123). GBS surface antigens are also being investigated as alternative vaccine targets. These antigens are structurally conserved and can elicit a strong immune response against most GBS strains (124). Members of the Alp protein family, including AlphaC, Alp1, Alp2, Alp3, Alp4 and Rib, are the most predominant GBS surface proteins (96). In 2017, MinervaX Inc. revealed that in a phase I trial involving healthy women, their protein-based vaccine which was made by fusing highly immunogenic N-terminal domains of Alpha C and Rib (GBS-NN) resulted in a more than 30-fold increase in GBS-NN-specific antibodies (125). GBS pilus proteins have also been observed to elicit an immune response in pre-clinical and human studies (22). A vaccine based on the latch domain which contains the Srr1 and Srr2 proteins of GBS was shown to elicit serotype independent protection against GBS infection in a murine model (99). The development status of current GBS vaccine candidates in shown in **Table 2.2**.

Table 2.1 Antibiotic resistance mechanisms commonly found in Group B streptococcus for different antibiotic classes.

	Resistance mechanisms							
Antibiotic class	**Efflux pumps**	**Methylases**	**ABC transporters**	**Nucleotidyl transferases**	**Ribosomal protection proteins**	**Target mutations**	**Enzymes**	**Cell wall modifications**
Macrolide and lincosamides	*mefA/E* *mrsD*	*ermB* *ermTR* *ermT*	*IsaC* *IsaE*	*lnuB* *lnuC*				
Tetracycline	*tetK* *tetL*				*tetM* *tetO*			
Fluoroquinolone	Not yet characterized					Mutations in *parC* and *gyrA* genes		
Beta-lactams						Mutations in PBP genes (specifically PBP2x) and 23S rRNA mutations		
Aminoglycosides							AAC(60)-APH(200) bifunctional enzyme	
Glycopeptides								*vanG* insertion elements

Adapted from Hayes *et al*, 2020 (76)

Table 2.2 Summary of Group B streptococcus vaccine candidates in 2020.

Vaccine Candidate	Preclinical	Phase I	Phase II	Trials in pregnant women
Monovalent and bivalent conjugates (tetanus toxoid/ CRM197-CPS)	✓	✓	✓	✓
Trivalent CRM197-CPS conjugates	✓	✓	✓	✓
Hexavalent CRM197-CPS conjugates	✓	✓	✓	✓
N-terminal domains of the Rib and AlphaC proteins	✓	✓		
Pilus proteins	✓			
Other proteins	✓			
Biotinylated CPS conjugates	✓			

Adapted from Carrearas-Abad *et al*, 2020 (22)

2.9 Molecular characterization of GBS

Molecular typing is useful for the characterization of GBS because it can be used to infer genetic relationships between isolates and detect resistance genes. Studies have used several molecular techniques for the population analysis of GBS and these include pulsed-field gel electrophoresis (PFGE) (126), multilocus sequence typing (MLST) (127), and most recently WGS (19, 52).

2.9.1 Multilocus sequence typing (MLST)

MLST is a typing method that is used to classify GBS strains into sequence types (STs), which are further grouped into clonal complexes (CCs). This method is based on sequencing of seven housekeeping genes of GBS namely, alcohol dehydrogenase (*adhP*), phenylalanyl tRNA synthetase (*pheS*), amino acid transporter (*atr*), glutamine synthetase (*glnA*), serine dehydratase (*sdhA*), glucose kinase (*glcK*) and transkelotase (*tkt*) (127). Polymorphisms at each of the seven loci are classified and each allele is given a number. Combination of alleles makes up an ST and STs are grouped into CCs based on sharing four or more identical alleles (128).

MLST has been widely used to infer evolutionary relationships between bacterial strains. This method of inferring relationships is based on an eBURST algorithm that was developed to cluster related isolates based on MLST data by implementing a simple model of clonal expansion and diversification. Based on this model, the emergence of CCs is driven by an increase in the frequency of the founding genotype in a population as a consequence of either a fitness advantage or genetic drift, leading to it becoming a predominant clone. As this genotype increases in the population, it diversifies gradually due to mutations or recombination, leading to the descendants of the founder that differ in one or more of the seven housekeeping genes (89, 90). Genotypes that have allelic profiles that differ from that of the founder at only one of the 7 MLST loci are called single-locus variants (SLVs). When SLVs diversify further, they produce double-locus variant (DLVs) which have a profile that differs from that of the founder at 2 of the 7 loci, triple-locus variants (TLVs) that differ at 3 loci and so on (128).

MLST became the preferred typing method for GBS over restriction digest based methods such as PFGE due to its advantages. MLST data are stored on an open access database which means that data from different laboratories can be compared. Furthermore, MLST uses

standardized protocols which allows comparability of the results between laboratories and provides data on single nucleotide changes within the housekeeping genes.

2.9.1.1 Distribution of sequence types and clonal complexes among colonizing and invasive GBS isolates

Characterization of maternal and neonatal colonizing isolates using MLST has shown that four major sequence types (ST-1, ST-17, ST-19 and ST-23) are associated with colonizing strains regardless of geographical and epidemiological differences (91, 92). These STs belong to CC1, CC19, CC17 and CC23 respectively. Among colonizing isolates, these four major CCs accounted for 63.7% of isolates in Europe, 74.4% in the United States, 65.8% in Africa, 69.0% in Canada and 72.2% in the Middle East. This reflects that GBS are less diverse compared to other streptococcal pathogens including *S. pneumoniae* and *S. pyogenes* in terms of clonal diversity (116, 117).

In invasive disease, CC17 and CC23 have been reported to be responsible for the majority of EOD and LOD cases (134). CC17 has been consistently associated with invasive disease, possibly indicating intrinsic genetic properties to cause invasive disease. CC17 strains are responsible for the vast majority of meningitis cases in newborns, and for more than 80% of LOD cases. Within CC17 strains causing EOD and LOD, ST-17 is the dominant ST. The increased virulence of ST-17/CC17 has been associated with the fact that these strains possess the HvgA surface protein (85).

CC19 is more common in EOD than LOD (134, 135). This CC is dominated by a single ST (ST-19), which accounts for more than 72% of the sequence types that belong to CC19 (127, 131). Another CC that lacks diversity is CC23, which is made up of more than 87% ST-23 (127).

2.9.1.2 Distribution of clonal complexes among different GBS serotypes

Studies have shown that certain CCs among colonizing and invasive GBS strains (including CC17, CC19, and CC23) are associated with specific serotypes (115, 119–121). More than 90% of CC17 and CC19 isolates belong to serotype III, and 90% of all CC23 isolates belong to serotype Ia. CC17 was previously associated with only serotype III until recently, when Bellais *et al.* 2012 (118) reported serotype IV isolates to be CC17. Comparative analysis of the serotype IV isolates belonging to CC17 revealed that these isolates share the vast majority of their genes with serotype III, suggesting a capsular switch of the cps operon from type III to type IV. The proportion of colonizing vs invasive serotype III isolates differs between

CC17 and CC19. The majority of serotype III isolates belonging to CC17 are invasive, while the majority of serotype III isolates associated with CC19 are colonizing isolates (87, 96, 97). CC19 is predominantly serotype II. CC19 isolates from the Central Republic of Africa and Senegal were dominated by serotype II (95% and 66.7% respectively). CC23 isolates predominately belong to serotype Ia (87, 97, 98).

Some CCs are associated with multiple serotypes. For example, CC1 and CC10 are associated with a range of serotypes which have different invasive disease potential.

2.9.2 Whole-genome sequencing

Even though MLST has been widely used for the analysis of the population structure of GBS, this method has some disadvantages. MLST has limited ability to differentiate between closely related STs. Furthermore, housekeeping genes used for MLST typing may undergo recombination, which can skew the true phylogeny of GBS. Recombination of housekeeping genes has been reported in several pathogens including *E. coli*, *S. aureus*, and *S. enterica* (143–145). The cost of WGS has decreased due to the recent advances in next-generation sequencing (NGS), and the improved turnaround time and the fact that automated WGS analysis tools are readily available makes it possible to use this technology for molecular characterization of pathogens. Using WGS, one can extract all genes of interest from one data source and also allows comparison with other genomes. Comparison of the complete sequences within the species and with other species improves our understanding of the evolution and the virulence mechanisms of pathogens.

In 2017, Metcalf *et al* (14) established and validated a WGS bioinformatics pipeline for prediction of serotypes and antibiotic resistance in GBS isolates from the US. In 2020 McGee *et al* (12) used this pipeline to determine the serotype, ST, and surface protein distribution as well as resistance determinant genes among GBS circulating in the USA. Currently, there are no studies that have used WGS to characterize GBS in SA, therefore this study aims to fill this gap.

2.10 Epidemiology and molecular epidemiology of GBS in South Africa

2.10.1 Burden of GBS

The incidence of invasive GBS in SA has been estimated to be between 2-3 per 1000 live births over the past three decades (46–49). Globally, SA was reported to have highest incidence of invasive GBS disease in a study conducted in 2012 (146). This high incidence is

also associated with a high case-fatality ratio of between 13% to 18% and a high rate (24.0%) of neurological impairment (49). In 2015, Quan *et al* reported that the incidence of invasive GBS disease in infants in SA across the nine provinces ranged from 0.02 to 1.04 per 1,000 live births (147). The incidence was highest in Gauteng (1.04 cases per 1,000 live births) where the rates (per 1,000 live births) of EOD and LOD were 0.54 and 0.49 respectively (147).

In 2019, Mukesi *et al* reported that the prevalence of GBS carriage among pregnant women in SA was 37%. This study was conducted on samples among pregnant women between 35 and 37 weeks of gestation. The colonization rate in pregnant women in Garankuwa, Pretoria, was reported to be 30.9% (128/413) in 2015 (148). In another study conducted in pregnant women from the Western Cape Province, the colonization rate was reported to be 16.6% in 2018 (69). In the Eastern Cape, the colonization rate among pregnant women was reported to be 13.6% in 2019 (149).

2.10.2 Serotype distribution

The common serotypes reported in SA (Ia, Ib, II, III, and V) are the same ones that are reported to be common globally. In a study conducted on colonizing and disease isolates from mothers and newborns at Chris Hani-Baragwanath Hospital, SA in 2011, serotypes Ia, Ib and III were the most common among pregnant women (74.1%, 401/541) and newborns (69.6%, 275/395). The most common serotypes responsible for EOD and LOD were serotype III (57.7%, 79/137 and 84.3%, 91/108 respectively) and Ia (22,6%, 31/137 and 13,9%, 15/108 respectively), based on serotyping by latex agglutination and PCR (150). Another study conducted in Garankuwa in 2015 in pregnant women showed the following colonization rates: III (29.7%, 38/128), Ia (25.8%, 33/128), II (15.6%, 20/128), V (10.9%, 14/128), IV (8.6%, 11/128), Ib (8.6%, 11/128), and nontypeable (NT) (0.7%) based on latex agglutination (148). In a study conducted in pregnant women (colonizing isolates) from the Western Cape in 2018 (N=57), serotype V was reported to be the most predominant, accounting for 66.7% of the isolates, followed by serotype III, which accounted for 21.1%. Serotypes Ia, II, IV, and IX accounted for 1.75% each and three isolates were NT (69). In 2019, Mukesi *et al*. reported the following serotype distribution in a study conducted on pregnant women (N=67) living in the Eastern Cape: II (52.2%), III (17.9%), V (16.4%), Ia (9.0%), IV (1.5%), and Ib (3.0%) (149).

2.10.3 Molecular epidemiology of GBS in South Africa

Very little is known about the molecular epidemiology of GBS in SA. PCR is the main method used in the few studies that have investigated some molecular characteristics of GBS isolates circulating in different geographic regions in SA. Mukesi *et al*, 2019 (149), Chukwu *et al*, 2015 (148), and Madzivhandila *et al*, 2011 (150) used PCR for the molecular determination of serotypes. Currently, there are no published data on the population structure of GBS in SA.

There is also paucity of data on GBS antimicrobial susceptibility profiles and mechanisms of resistance in SA. In a study conducted in Garankuwa in 2015, GBS isolates (N=128) showed 100% susceptibility to penicillin, ampicillin, vancomycin, and gentamicin. Erythromycin resistance was 21.1% and clindamycin resistance was 17.2%. Methylation encoded by the *ermB* gene was the most common mechanism of erythromycin resistance (55.0%). Thirty-eight percent of the isolates harboured a combination of the *ermB* and *linB* genes. The *mefA* and *ermTR* genes were each detected in 3.4% of the isolates (151). A 2018 study conducted in the Western Cape (N=57) reported 98.0% susceptibility to clindamycin and erythromycin, 96.0% susceptibility to penicillin G, and 94.5% resistance to tetracycline and trimethoprim (69). This study assessed the prevalence of resistance to relevant antibiotics, however, the study did not investigate any resistance mechanisms.

In a study conducted in Garankuwa, Pretoria in 2015, multiplex PCR performed to assess the prevalence of GBS surface antigens showed that the *rib*, *bca*, *alp2/3*, epsilon and *alp4* protein determinants were harboured by all the isolates of different capsular types. The *rib* gene was the most common (44.5%, 57/128), followed by *bca* (24.7%, 31/128). Alp2/3, epsilon, and alp4 were detected in 17.9% (23/128), 8.6% (10/128), and 4.7% (6/128) of the study isolates respectively (148). In 2013, Madzivhandila *et al* reported that all isolates (N=825 colonizing and N=284 invasive) collected in a study conducted in a hospital in Soweto harbored at least one of the three pilus island determinants. These were identified alone or in combination and they were detected in the following frequencies: PI-2a (29.8%), PI-2b (0.2%), PI-1+PI-2a (24.8%), and PI-1+PI-2b (45.1%) (152).

3 Chapter 3: Materials and Methods

3.1 Ethical approval

Ethics approval for the GERMS-SA national-laboratory based surveillance system (protocol number: M1809107) and this sub study (M210476) was obtained from the Human Research Ethics Committee of the University of the Witwatersrand, Johannesburg, SA.

3.2 Source of isolates

The National Health Laboratory Service (NHLS) provides laboratory services to all South African public sector hospitals, which serve about 80% of the population of SA. The GERMS-SA program, which is coordinated by the National Institute for Communicable Diseases (NICD) of the NHLS performs active, national, laboratory-based, surveillance for bacterial and fungal infections of public health importance in SA, including GBS. This study was conducted on invasive GBS isolates collected from 2019-2020 from patients of all ages in SA. An invasive GBS case was defined as an individual with GBS isolated from a normally sterile site (e.g., blood, cerebrospinal fluid). From January 2019 through December 2020, 1748 cases of invasive GBS were reported to GERMS-SA. Of the 1748 cases, 714 (40.8%) had isolates (95.1%, 679/714) or specimens (4.9%, 35/714) sent to the NICD and 1035 (59.2%) were audit cases (cases that were identified when a surveillance audit was performed, no specimens/isolates were received for these cases). Of the 714 isolates and specimens, 661 (92.6%) were confirmed as GBS by phenotypic methods at the NICD and 53 (7.8%) were non-viable (**Figure 3.1**). Non-viable isolates were confirmed to be GBS by PCR using the Fast Track Diagnostics Neonatal Meningitis multiplex real-time PCR assay (Fast Track Diagnostics, Luxembourg). Only the viable isolates were included in this study.

3.3 Patient characteristics

Demographic and clinical data are obtained from the NHLS laboratory information system and in enhanced surveillance sites, these data are collected by surveillance officers through patient interview and medical record review after identification of cases through laboratory surveillance. In enhanced surveillance hospital sites, surveillance officers also collect additional data such as clinical characteristics and final outcome of hospitalization.

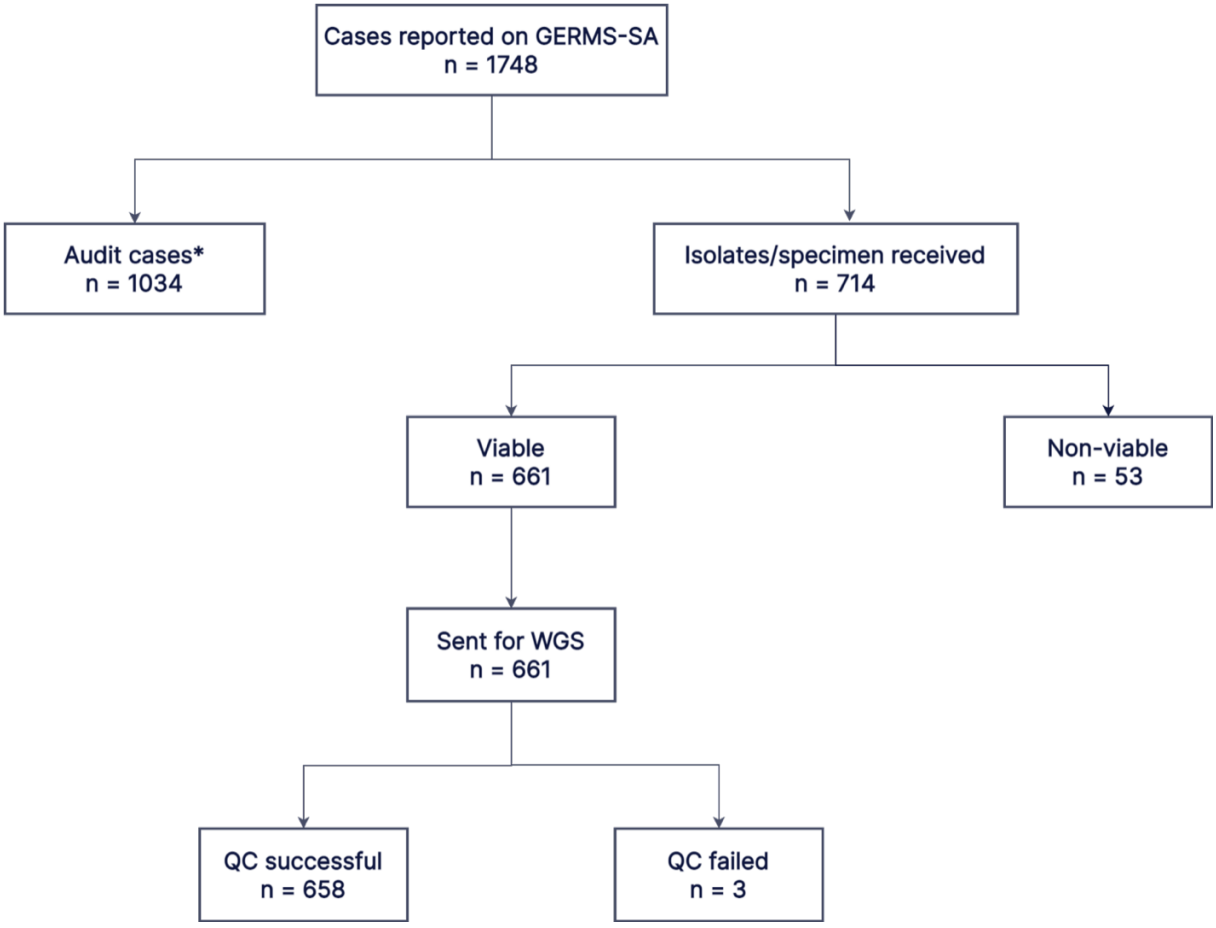


Figure 3.1 Flowchart showing the samples included in the study. * Cases that were identified when a surveillance audit was performed. No isolates or specimen was received for these cases. QC = quality control.

3.4 Bacterial culture and identification of GBS

Upon receipt at NICD, the isolates were cultured overnight on 5% blood agar (Diagnostic Media Products, Johannesburg, SA) and incubated at 37°C for 18-24 hours in the presence of 5% CO_2. Isolates were stored in skimmed milk at -70°C. The presumptive isolates were confirmed as GBS based on colony morphology, and β-hemolysis on blood agar containing 5% sheep blood (Diagnostic Media Products). Isolates that morphologically appeared to not be GBS were confirmed using MALDI-TOF MS and/or real-time PCR using the Fast Track Diagnostics Neonatal Meningitis multiplex real-time PCR assay (Fast Track Diagnostics, Luxembourg).

3.5 Strain serotyping by latex agglutination

Capsular serotyping was performed using the Immulex^{TM} Group B Streptococcus antisera; Ia, Ib, and II to IX (SSI Diagnostica A/S, Hillerød, Denmark) as per manufacturer's instructions. Briefly, a suspension of the isolate was prepared by inoculating a single colony of GBS into 3ml of Brain Heart Infusion (BHI) broth and incubated at 37°C in the presence of 5% CO_2 for 1 hour. A drop of the culture was placed onto a reaction card, a drop of the antiserum was added and mixed with the bacterial culture. A positive reaction was indicated by agglutination within 30 seconds for that particular serotype. Isolates that tested negative with all antisera were sub-cultured and confirmed as GBS using MALDI-TOF MS. If these isolates were confirmed as GBS but produced a negative reaction with all type specific antisera, they were designated as serologically non-typeable (NT).

3.6 Antimicrobial susceptibility testing (AST)

3.6.1 Disc diffusion method

Antibiotic susceptibility testing using the disc diffusion method was performed by preparing an inoculum by making a direct saline solution of the isolates selected from 18-24-hour agar cultures. A suspension was required to contain approximately 1 to 2 x 10^8 CFU/ml and this was measured either visually or using a photometric device and a 0.5 McFarland standard was used for comparison of turbidity. The suspension was then used to inoculate fresh Muller-Hinton agar plates supplemented with 5% defibrinated sheep blood for each isolate tested and the agar surface was allowed to dry for 15 minutes. Using forceps, the following discs were placed on the plates; a systematic Gram positive ring (MASTRINGS-S®, Mast Group, UK) containing oxacillin, erythromycin, clindamycin, chloramphenicol, tetracycline, and rifampicin, as well as separate discs for ofloxacin, linezolid, vancomycin, and cotrimoxamole. The plates were incubated overnight at 37°C in the presence of 5% CO_2. The plates were then examined, ensuring that the lawn of growth was even and confluent and no obstructed zones were detected. The zone of inhibition was measured for each disc (to the nearest mm) including the diameter of the disc which is 6 mm and the zones, and interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (153).

3.6.2 Broth microdilution method

Minimum inhibitory concentrations (MIC) for isolates were determined by broth microdilution using commercially customised TREK panels (Trek Diagnostics Inc., Ohio, United States) following manufacturer's instructions. Each Sensititre® HPB susceptibility

plate was coated with erythromycin, penicillin, clindamycin, linezolid, gentamicin, meropenem, cotrimoxazole, tetracycline, ceftriaxone, amoxicillin, ceftaroline, quinupristin/dalfopristin, levofloxacin, rifampicin, chloramphenicol and vancomycin. A 0.5 McFarland standard suspension of GBS in Mueller-Hinton broth was prepared. 100µl of the suspension was transferred into Mueller-Hinton broth with TES/lysed horse blood and mixed. The suspension was poured into a sterile trough and 100µl of the suspension was transferred into each well of Sensititre® susceptibility plates. The susceptibility plates were sealed and incubated at 37°C for 18-24 hours in an O_2 incubator. To check for purity, the lysed horse blood isolate suspensions were also inoculated onto 5% blood agar plates and incubated at 37°C for 18-24 hours in a CO_2 incubator. MIC breakpoints were interpreted using CLSI guidelines (154). For penicillin, isolates with MICs of ≥0.12mg/L were defined as non-susceptible. Isolates that were intermediately resistant or resistant to any of the antibiotics were regarded as non-susceptible.

3.7 DNA extraction

GBS pure overnight cultures were inoculated into 3 ml of BHI broth (Diagnostic Media Products) and incubated for 18-24 hours at 37°C in 5% CO_2. Prior to the extraction, a pre-lysis step was performed in order to lyse the cells in preparation for DNA extraction. Briefly, 1.3 ml of the suspension was aliquoted into a sterile 1.5 ml Eppendorf tube and centrifuged for 3 minutes at 13,000 rpm to pellet the cells. The bacterial pellets were suspended in 200µl of 10 mg/ml lysozyme (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for 1 hour.

Genomic DNA was extracted manually using the QIAamp® DNA Mini kit (QIAGEN, Hilden, Germany) as per manufacturer's instructions. 50 µl of AL lysis buffer and 20 µl of Proteinase K were added to the resuspended pellets and this was incubated at 56°C for 1 hour. 150 µl of lysis buffer AL was added and this was incubated at room temperature for 10 minutes. 250 µl of 100% molecular grade ethanol was added and the samples (700 µl) were transferred to Qiagen columns and centrifuged at 8000 rpm for 1 minute. Columns were then transferred to new collection tubes and 500 µl of buffer AW1 was added to each column and this was centrifuged at 8000 rpm for 1 minute. Columns were transferred to new collection tubes and 500 µl of buffer AW2 was added and this was centrifuged at 14,000 rpm for 3 minutes. Columns were transferred to 1.5 ml sterile Eppendorf tubes, 60 µl of pre-warmed elution buffer was added to each column and this was incubated for 5 minutes at room

temperature, followed by centrifugation at 12,000rpm for 2 minutes. The columns were discarded and the 1.5 ml sterile Eppendorf tubes with 60 µl of eluted DNA were retained.

3.8 DNA quantification

DNA was quantified using the Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA) and the Qubit™ dsDNA BR assay kit (Invitrogen) according to manufacturer's instructions. For each sample, a working solution was prepared by mixing 199 µl BR buffer with 1 µl BR reagent. 195 µl of the working solution was mixed with 5 µl of extracted DNA and this was incubated at room temperature for 2 minutes. The concentration for each sample was measured on the Qubit® 2.0 fluorometer (Invitrogen). DNA samples with a minimum concentration of 10 ng/µl were sent for whole genome sequencing to the NICD Core Sequencing Facility. Samples with a concentration of less than 10 ng/µl were re-extracted and quantified.

3.9 Whole genome sequencing and assembly

Library preparation was performed using the Illumina Nextera DNA Flex Library Preparation Kit. Briefly, the DNA samples were diluted to a starting concentration of 4 ng/µl and tagmented at 55°C for 5 minutes to generate adapter-ligated DNA fragments. A post tagmentation step was performed for washing the adapter-tagged DNA before PCR amplification. The tagmented DNA was amplified using a limited-cycle PCR to Illumina sequencing adapters and to create dual indexed adapters (index 1, i7 adapters and index 2, i5 adapters) for the different samples. The amplified libraries were purified and size-selected using Agencourt AMPure® XP beads (Beckman Coulter Inc., Brea, California, USA). Purified DNA libraries were quantified and assessed for quality on an Agilent Bioanalyser (Agilent Technologies, Santa Clara, California, USA). Genomic sequencing was performed using the NextSeq 550 System (Illumina Inc., San Diego, United States) and the 2 x 100-bp paired-end mode. Quality control (QC), removal of adaptors/trimming, and *de novo* assembly was performed using the CDC GBS bioinformatics pipeline (14) (https://github.com/BenJamesMetcalf). QC using the pipeline relied on FastQC and MultiQC. To evaluate the quality of the sequences, the following was taken into consideration: the genome size (~2MB), GC content (35%). Trimming of adapters and removal of low quality bases relied on CutAdapt v1.6. The '-q 20' option was used to remove low quality bases at the end of each read, trimming of adapters was performed using the '-b', and filtering of reads less than 50 bases was done using the '--minimum-length' option. *De novo* assembly relied on VelvetOptimiser v1.2.10 (155). The CDC GBS bioinformatics pipeline used in this study was

installed and set up in a high performance computing cluster (HPCC) located at the University of Witwatersrand. The nextflow implementation of the original pipeline used in this study is found on Github (https://github.com/shaze/GS). Sequencing was successful for 658 of the 661 viable isolates.

3.10 Bioinformatics analysis

The CDC GBS bioinformatics pipeline was used to determine the serotypes, antibiotic resistance determinants and predicted MICs, sequence types, and the presence/absence of genes encoding important GBS surface proteins.

3.10.1 Serotype determination from WGS

Serotypes were determined from the WGS data using the validated GBS bioinformatics pipeline (14) (https://github.com/BenJamesMetcalf), which relies upon BLASTn similarity search and uses the nucleotide sequences of the ten *cps* loci that correspond to the ten known GBS serotypes (Ia, Ib, and II-IX) as query sequences and the results were compared to results obtained from latex agglutination tests. Samples that produced discrepant results when comparing the two methods were retested, first through latex agglutination and if the results remained discrepant, WGS was also repeated.

3.10.2 Detection of resistance genes

Sequences were analysed to identify mutations in the PBP genes (*pbp1a* and *pbp2x*), presence of macrolide resistance genes (*erm*B, *erm*A/*TR*, or *erm*C, and *mef*), fluoroquinolone resistance genes (*gyrA/gyrB* and *parC/parE*), *linB*, and *tetM* genes using the GBS bioinformatics pipeline as previously described (14). In addition, the ARG-ANNOT and ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/) databases were incorporated in order to detect additional resistance determinants. The results were then compared to the antibiotic susceptibility patterns from the phenotypic characterization. For samples with discordant results, phenotypic characterization was repeated, as well as sequencing if needed.

Positive and negative predictive values (PPV and NPV respectively) of WGS for prediction of phenotype based on absence/presence of resistance mechanisms were calculated to evaluate its performance. The phenotypic results (broth microdilution and disc diffusion methods) were used as gold standard. For PPV and NPV calculations; a true positive (TP) was considered as an isolate that was phenotypically resistant to a specific antibiotic and had resistance genes or mutations detected and true negative (TN) as an isolate that was

phenotypically susceptible to a specific antibiotic and had no resistance genes or mutations detected. A false positive (FP) was considered as an isolate that was phenotypically susceptible to a specific antibiotic and had resistance genes or mutations detected. A false negative (FN) was considered as an isolate that were phenotypically resistant to a specific antibiotic and had no resistance genes or mutations detected. The PPV was calculated as TP/TP+FP. Similarly, the NPV was calculated as TN/TN+FN.

3.10.3 Identification of genes that encode surface proteins

The WGS pipeline (14) was also used to detect the presence/absence of surface protein genes that encode the alpha protein family (*alpha, Rib, Alp1, Alp2/3*), hypervirulent GBS adhesion (*hvgA*), pilus islands (*PI1, PI2A, PI2B*), and Srr proteins (*Srr1* and *Srr2*) (https://github.com/BenJamesMetcalf). The pipeline relies on BLAST and uses the nucleotide sequences of the target genes as query. An identity of ≥ 95% was required for a positive result for each sequence query.

3.10.4 MLST typing

MLST typing using the pipeline relied upon the read mapping tool SRST2 and the PubMLST database (https://pubmlst.org/) and determined sequence types by extracting the nucleotide sequences of the seven MLST housekeeping genes (*adhP, pheS, atr, glnA, sdhA, glcK,* and *tkt*) and comparing them to the known STs found in the GBS MLST web server (https://pubmlst.org/organisms/streptococcus-agalactiae/). In a few instances, the pipeline could not confidently assign a ST due to insufficient quality during base-calling on one of the 7 housekeeping genes. In such cases, a putative ST was assigned based on the portion of the gene that had sufficient quality for base calling. STs not previously described were submitted to and assigned a ST by the *S. agalactiae* MLST database curator.

The STs and putative STs were grouped into CCs based on the Global Optimal eBURST (goeBURST) algorithm. These were grouped into CCs based on sharing at least 6 of the 7 MLST loci, otherwise an ST was considered a singleton. The relationships between STs and different parameters was assessed using PHYLOViZ software v2.0 (PHYLOViZ team, Lisbon, Portugal) based on the Global Optimal eBURST (goeBURST) and illustrated by a minimum spanning tree.

3.10.5 Phylogenetic analysis

For WGS based phylogenetic analysis, we selected samples that produced high quality sequences (N=435) by filtering for those that had ≤ 150 contigs and N50 ≥ 30 000. This ensured that the tree was not skewed by the lower quality sequences. Single nucleotide polymorphisms (SNPs) detection was performed using the kSNP v3 (http://ksnp.sourceforge.net/), an alignment-free tool for SNP identification and phylogenetic analysis of complete microbial genome sequences based on k-mer analysis. A core SNPs matrix was generated from the assemblies and the derived SNPs were used to perform all the phylogenetic analyses. We used a k-mer length of 19 and a requirement that 100% of the genomes have a nucleotide at a given SNP position in order for the SNP to be considered to be a core. A maximum-likelihood (ML) phylogenetic tree of the study isolates was constructed using the generalized time reversible (GTR) substitution model and gamma distribution in the RAxML tool using 100 bootstrap (https://github.com/stamatak/standard-RAxML). An ascertainment bias correction for the SNP analysis was performed using the felsenstein correction. Phylogenetic trees were visualized with metadata using the Interactive Tree of Life online software (iTol version 6) (https://itol.embl.de/). Subclades were defined as a subgroup of isolates within the major CCs that share the same surface antigens, which differ from another subgroup in the same CC.

3.10.6 Representative global database

In order to compare SA isolates to global isolates, WGS data contained within the PubMLST database was accessed on 31 March 2022 (https://pubmlst.org/organisms/streptococcus-agalactiae). The database contained 12 185 submissions and a genome collection of 10 179 genomes. Data for the genome collection were exported and filtered for only invasive isolates that had data available for country of isolation, ST, disease type, source, and year. The corresponding 1 122 genomes for these were downloaded and SNP analysis was performed using kSNP as mentioned above and a maximum-likelihood tree was constructed using RAxML tool. Visualization was done on iTOL.

3.11 Statistical analyses

Distribution of serotypes with respect to age group and sex was assessed using the chi-squared test (X^2). A P-value of <0.05 was considered significant. All statistical analyses were performed in R version 4.1.2.

4 Chapter 4: Results

4.1 Bacterial isolates

The isolates analysed in this study comprised of 661 invasive GBS isolates recovered from patients of all ages in SA, from 1 January 2019 through 31 December 2020. Basic patient characteristics are shown in **Table 4.1**. Patients were divided into six age categories, 0-6 days (EOD), 7-89 days (LOD), 3 months to less than 5 years, 5-<18 years, 18-45 years, and >45 years. Majority of the cases were EOD (250/661, 37.8%) and LOD (183/661, 27.7%) cases and patients in the age group of 5 to less than 18 years accounted for the least number of cases (8/661, 1.2%) (**Table 4.1**). Gauteng and Western Cape provinces accounted for most of the cases; 36.9% (244/661) and 30.7% (203/661) respectively (**Table 4.1**). Blood cultures represented 80.6% (533/661) of the specimen types collected followed by cerebrospinal fluid (CSF). Data on race and outcome of disease were collected for 114/661 (17.3%) and 122/661 (18.5%) cases from enhanced surveillance sites, respectively. Enhanced surveillance was started in 2020, therefore active collection of data on outcome and race began in this year.

Table 4.1 Characteristics of patients with invasive GBS in South Africa, 2019-2020 (N = 661)

Characteristic	2019 (N=316) n (%)	2020 (N=345) n (%)	Total (N=661) n (%)
Province			
Gauteng	123 (38.9)	121 (35.1)	244 (36.9)
Western Cape	105 (33.2)	98 (24.8)	203 (30.7)
Kwa-Zulu Natal	36 (11.4)	56 (16.2)	92 (13.9)
Eastern Cape	19 (6.0)	25 (7.2)	44 (6.7)
Limpopo	14 (4.4)	20 (5.8)	34 (5.1)
Mpumalanga	12 (3.8)	9 (2.6)	21 (3.2)
Free State	4 (1.3)	11 (3.2)	15 (2.3)
North West	2 (0.6)	3 (0.9)	5 (0.8)
Northern Cape	1 (0.3)	2 (0.6)	3 (0.5)
Sex			
Female	164 (51.9)	161 (46.7)	325 (49.2)
Male	139 (44.0)	178 (51.6)	327 (48.0)
Unknown	13 (4.1)	6 (1.7)	19 (2.9)
Age group			

0-6 days (EOD)	107 (33.9)	143 (41.4)	250 (37.8)
7-89 days (LOD)	86 (27.2)	97 (28.1)	183 (27.7)
3 months – <5 years	11 (3.5)	14 (4.1)	25 (3.8)
5 – <18 years	3 (0.9)	5 (1.4)	8 (1.2)
18-45 years	55 (17.4)	32 (9.3)	87 (13.2)
> 45 years	46 (14.6)	48 (13.9)	94 (14.2)
Unknown	8 (2.5)	6 (1.7)	14 (2.1)
Specimen type			
Blood culture	245 (77.5)	288 (83.5)	533 (80.6)
Cerebrospinal fluid	34 (10.8)	37 (10.7)	71 (10.7)
Joint fluid	9 (2.8)	2 (0.6)	11 (1.7)
Pleural fluid	0 (0.0)	2 (0.6)	2 (0.3)
Other specimen*	28 (8.9)	16 (4.6)	44 (6.7)
Race			
Black	2 (0.6)	97 (28.1)	99 (15.0)
Coloured	0 (0.0)	13 (3.8)	13 (2.0)
Asian	0 (0.0)	1 (0.3)	1 (0.2)
White	0 (0.0)	1 (0.3)	1 (0.2)
Unknown	314 (99.4)	233 (67.5)	547 (82.7)
Outcome			
Died	0 (0)	29 (8.4)	29 (4.4)
Recovered	2 (0.6)	91 (26.4)	93 (14.1)
Unknown	314 (99.4)	225 (65.2)	539 (81.5)

EOD = early-onset disease, LOD = late onset disease.

* includes isolates from products of conception, placenta, intrauterine death, tissue (uterus, cervical, placenta, lung, liver), bone

4.2 Serotype results

4.2.1 Concordance of GBS serotyping by latex agglutination and whole-genome sequencing

All 661 isolates were sent for WGS and three isolates produced poor quality sequences. These three isolates were excluded from subsequent analysis. A pairwise comparison of results from conventional serotyping and molecular serotyping from WGS is shown in **Table 4.2**. Based on phenotypic serotyping by latex agglutination, a serotype could be determined for 98.8% (650/658) of the isolates. Eight isolates (1.2%, 8/658) were non-typeable (NT). Based on WGS prediction, a serotype could be determined for 99.8% (657/658) of isolates and could not be determined for 0.2% (1/658) and this isolate was designated ND (not determined). Excluding the NT and ND isolates, 99.7% (647/649) of the serotyping results were concordant between the two methods. Two isolates (0.3%, 2/647) isolates showed discordant results between the two methods. These included one isolate which was designated serotype II phenotypically and Ib by WGS, as well as one isolate which was designated serotype III phenotypically and II by WGS

Table 4.2 Pairwise comparison of conventional serotyping and molecular serotyping of 649 invasive GBS isolates that yielded serotyping results from South Africa, 2019-2020. Concordance calculations excluded the nontypeable (NT) isolates and samples where WGS could not determine the serotype (ND) shaded in grey (n=9).

	Latex agglutination								**Concordance**	
	Sero-type	Ia	Ib	II	III	IV	V	NT	Number	%
WGS prediction	Ia	181						2	181/181	100%
	Ib		43	1				1	43/44	97.7%
	II			52	1			3	52/53	97.1%
	III				280			1	280/280	100%
	IV					15			15/15	100%
	V						76	1	76/76	100%
	ND						1			
	Total								**647/649**	**99.7%**

NT = nontypeable, ND = not determined

4.2.2 Serotype distribution

For serotype description, the isolates with discrepant results were excluded, and for NT and ND isolates, phenotypic combined with WGS predicted serotype (whichever was available) was used as the final result. Overall, a total of six serotypes namely: Ia, Ib, II, III, IV, and V were detected in this study. Serotype III was the most common, accounting for 42.8% (281/656) of all isolates, followed by Ia (183/656, 27.9%), V (78/656, 11.9%), II (55/656, 8.4%), Ib (44/656, 6.7%), and IV (15/656, 2.3%).

When serotype distribution was stratified by age group, more than two-thirds (68.0%, 124/183) of LOD cases were caused by serotype III compared to 38.6% (95/249) of EOD cases (P<0.001). In contrast, 30.9% (77/249) of EOD was due to serotype Ia compared to 23.5% (43/183) of LOD (P<0.001). In children aged 3 months to <18 years, serotype III accounted for 42.4% (14/33) of cases and Ia accounted for 39.4% (13/33) of cases (P<0.001). All other ages had an almost uniform distribution of serotypes Ia, II, III, and V compared to EOD, LOD, and age <18 years (P=0.279 for 18-45 years and P=0.262 for >45 years) (**Figure 4.1**).

Stratification of serotype by sex showed no significant difference in serotype distribution between males and females [P = 0.229] (**Supplementary Fig.1**).

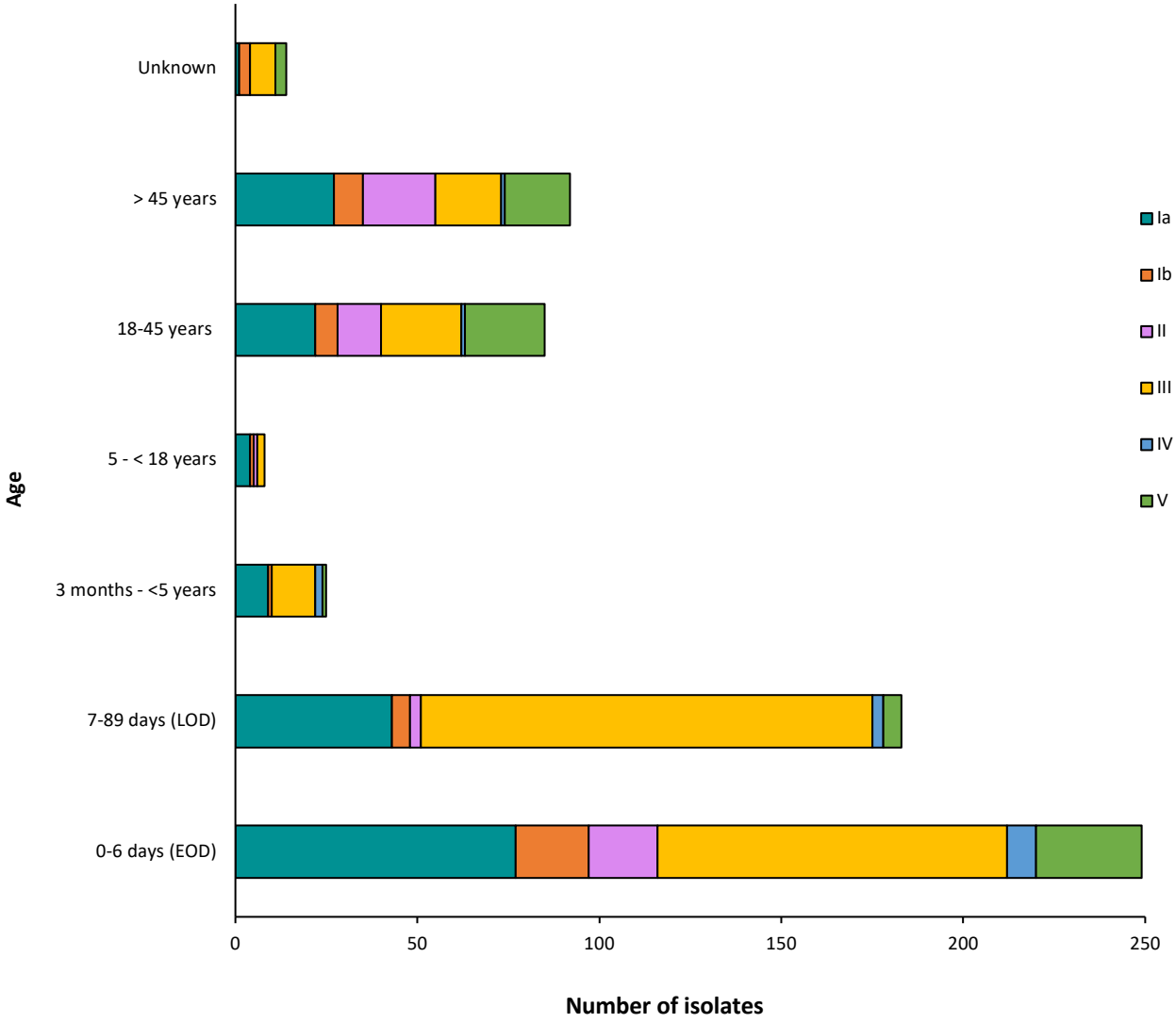


Figure 4.1 Distribution of invasive group B Streptococcus (GBS) serotypes by age group in South Africa, 2019-2020 (n = 656). EOD is early-onset disease and LOD is late-onset disease.

4.3 MLST typing

STs could be determined for 95.3% (627/658) of the isolates. For 28/658 (4.3%), a putative ST was assigned (see supplementary Table 1). For three isolates (0.4%), the pipeline could not determine the sequence of one of the housekeeping genes and therefore could not determine the ST and these were designated NF (not found) (supplementary Table 1).

MLST typing identified 32 different STs, of which 9 were predominant: ST17 (178/627, 28.4%), ST23 (151/627, 24.1%), ST109 (59/627, 9.4%), ST8 (41/627, 6.5%), ST1 (37/627, 5.9%), ST24 (28/627, 4.5%), ST10 (25/627, 4.0%), and ST28 (24/627, 3.8%). Five STs (ST1956, ST1957, ST1958, ST1981, ST1982) identified were novel. The STs and putative STs were grouped into 6 major CCs (CC1, CC8/10, CC17, CC19, CC23, and CC24) and five

singletons based on goeBURST analysis (**Figure 4.2**). CC17 was the most common, comprising 41.3% (272/658) of all isolates and CC23 was the second most common (188/658, 28.6%). Each CC was represented by a dominant serotype. CC17 isolates were almost exclusively serotype III (96.3%, 262/272), and the remaining CC17 isolates were serotype IV. Majority (90.4%, 170/188) of CC23 isolates were serotype Ia. Sixty-six percent (44/67) of CC8/10 isolates were serotype Ib. Fifty-four percent of CC24 (20/37) isolates were serotype V and 60.0% (27/45) of CC19 were serotype II (**Figure 4.2**).

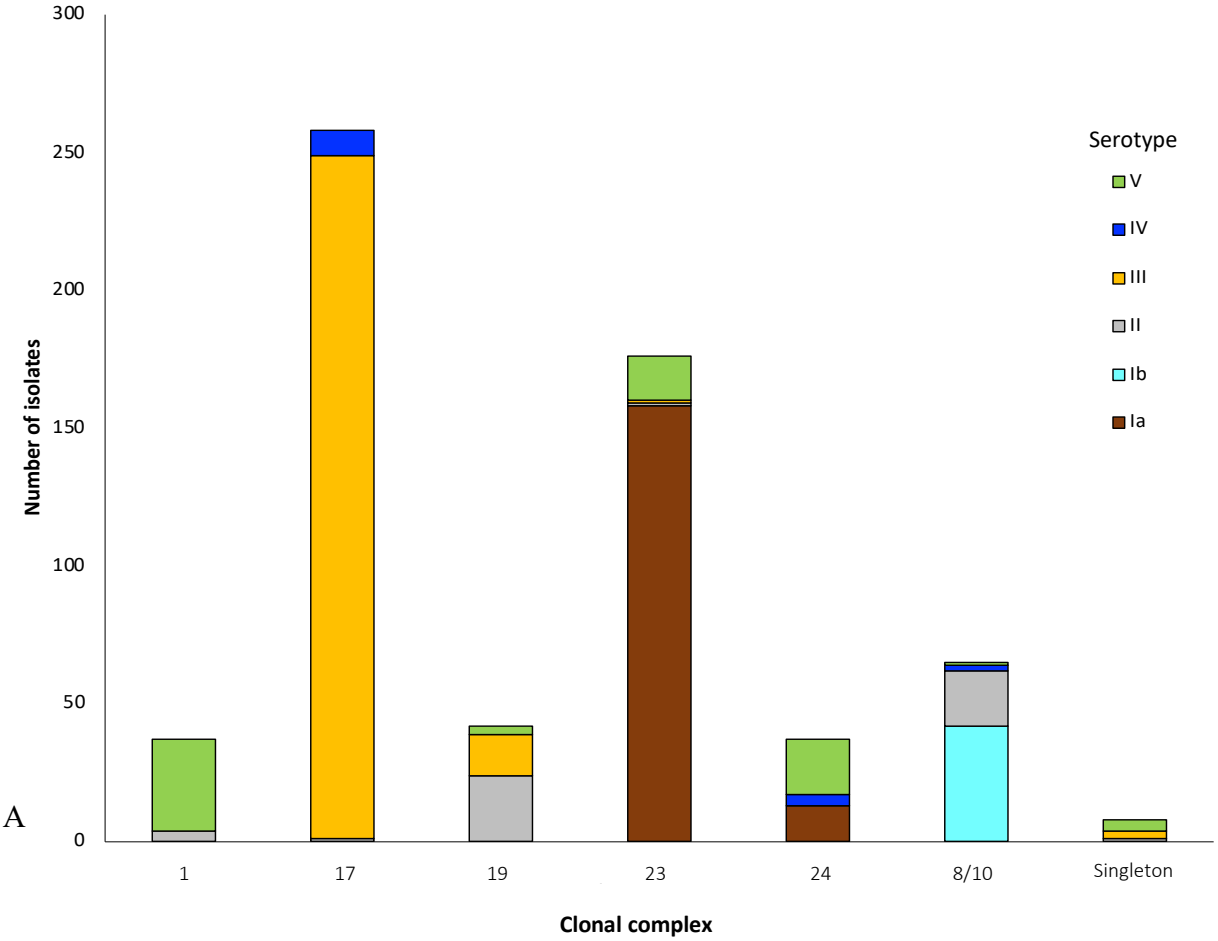
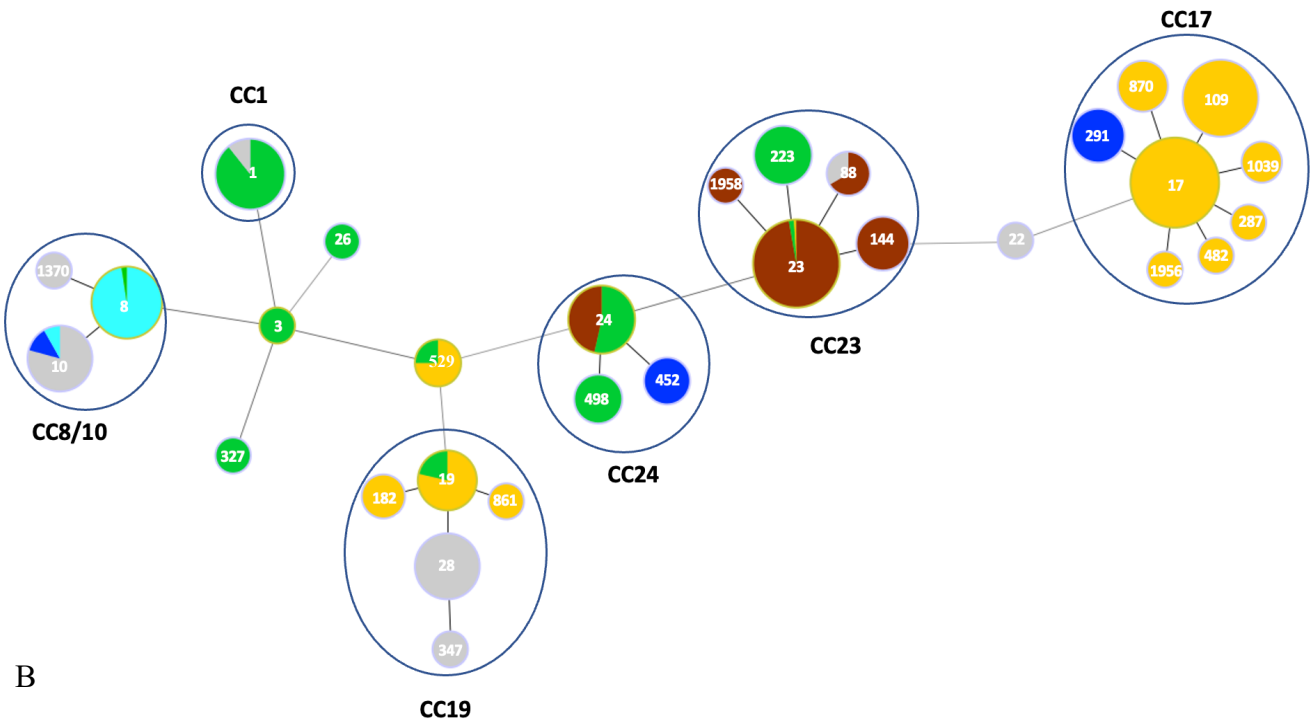


Figure 4.2 (A) Serotype distribution by clonal complex (CC) among GBS causing invasive disease in all ages in South Africa, 2019-2020.



B

Figure 4.2 (B) PHYLOViZ diagram depicting individual clonal complexes and associations with the six different serotypes detected among GBS causing invasive disease in South Africa, 2019-2020. In the eBURST diagram, each sequence type (ST) is displayed as a circle and the size of the circle is representative of the number of isolates detected.

4.4 Distribution of surface proteins

4.4.1 Pilus proteins

Almost all isolates (99.8%, 657/658) harbored at least one of the 3 pilus determinants. The most common pilus genes detected were a combination of PI-1 and PI-2b (41.1%, 270/657), followed by PI-2A (33.3%, 219/657), a combination of PI-1 and PI-2A (24.4%, 160/657), and PI-2B (1.2%, 8/657). One (0.2%) isolate was negative for all pilus island types. Majority (95.6%, 258/270) of isolates that had a combination of PI-1 and PI-2B were serotype III, while PI-2a was identified in 81.7% (179/219) of serotype Ia isolates. Isolates that harbored a combination of PI-1 and PI-2a were distributed across all serotypes while PI-2B isolates were distributed equally among serotype III and IV (**Table 4.3**). CC17, CC23 and CCC24, were dominated by the PI-1 and PI-2b combination (98.9%, 269/272) and PI-2a (97.9%, 184/188 and 86.5%, 32/37) respectively. CC19, CC1, and CC8/10 were all dominated by a combination of PI-1 and PI-2a (**Supplementary figure 2**).

4.4.2 Alpha protein family

Majority of the isolates (99.7%, 656/658) tested positive for at least 1 of the 4 homologous genes of the alpha protein family determinants. These were detected in the following frequencies: *rib* (60.0%, 341/656), *alp1* (24.9%, 163/656), *alpha* (16.8%, 110/656), and *alp2/3* (6.1%, 40/656). Two isolates contained a combination of *alp2/3* + *alpha* and *alpha* + *rib* respectively, while two other isolates were negative for these genes (**Table 4.3**). Most of *alp1* positive isolates (96.9%, 158/163) were serotype Ia, and 80.0% (32/40) of *alp2/3* positive isolates were serotype V, while majority of *rib* positive isolates (81.8%, 279/341) were serotype III. *alpha* positive isolates were distributed across all serotypes except for serotype III: Ia (12.0%, 13/108), Ib (40.7%, 44/108), II (20.4%, 22/108), IV (6.5%, 7/108), V (20.4%, 22/108) (**Table 4.4**). CC17 and CC19 isolates were dominated by the *rib* protein determinant (99.6%, 271/272 and 93.3%, 42/45 respectively), CC23 by *alp1* (86.2%, 162/188), CC1 by *alp2/3* (97.3%, 36/37), and all CC24 (n=37) and CC8/10 (n=69) isolates had the *alpha* determinant (**Supplementary figure 2**).

4.4.3 Serine-rich repeat glycoproteins

Serine-rich repeat glycoprotein determinants, *srr1* and *srr2* were identified in 52.8% (349/658) and 41.8% (276/658) of isolates respectively. Thirty-two (4.8%) of the isolates were negative for the *srr* determinants and one isolate (0.2%) had a combination of *srr1* and *srr2* (**Table 4.3**). Most (94.6%, 262/276) of *srr2* positive isolates were serotype III. Most *srr1* positive isolates were serotype Ia (52.7%, 185/347). All (15/15) serotype IV isolates were *srr2* positive. Isolates that were negative for *srr* genes were mostly serotype II (75.0%, 24/32), followed by serotype III (21.9%, 7/32) and serotype V (3.1%, 1/31) (**Table 4.4**). CC17 isolates were almost exclusively *srr2* while all the other CCs were dominated by *srr1* (**Supplementary figure 2**).

4.4.4 HvgA

A total of 263/658 (40.0%) isolates were positive for *hvgA*. All the *hvgA* positive isolates belonged to CC17 with 255 (97.0%) of these isolates being serotype III and the remaining 8 (3.0%) were serotype IV. Seven serotype III/CC17 isolates were *hvgA* negative and these all belonged ST870 (**Figure 4.3 and Table 4.3**).

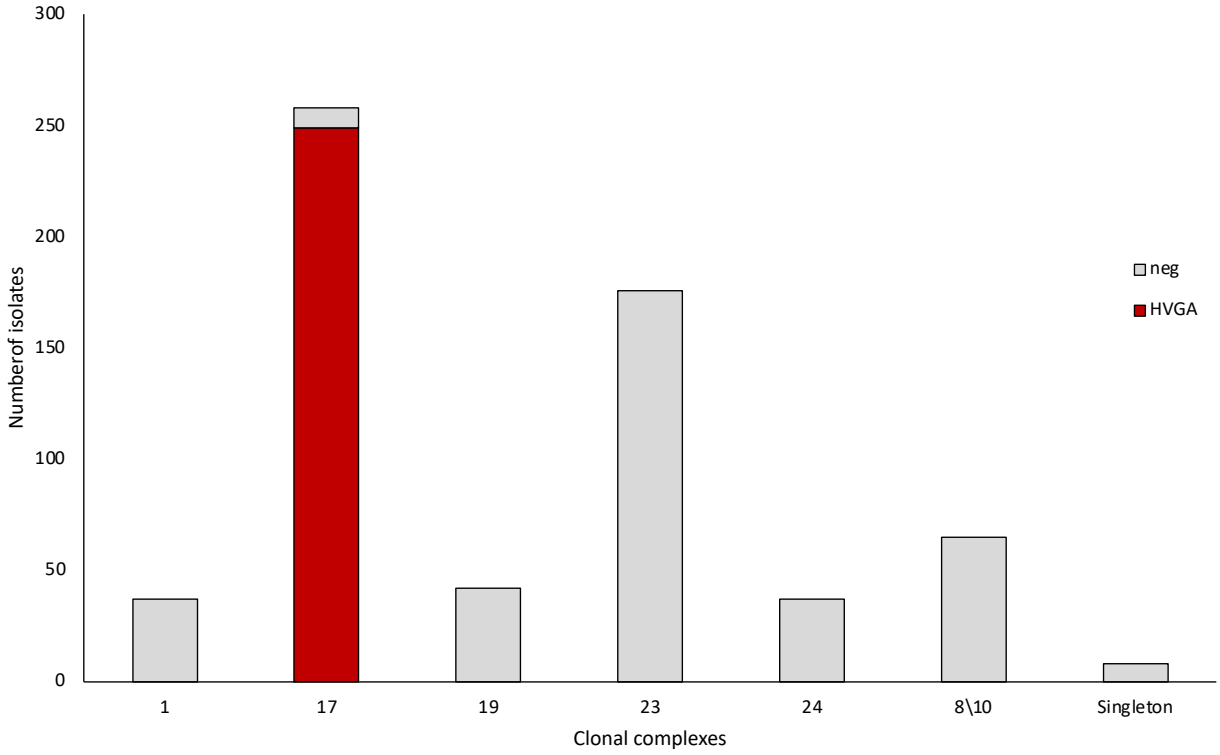
Table 4.3 Distribution of surface protein determinants amongst invasive GBS isolates from all age groups in South Africa, 2019-2020 (n=658)

Protein	Number of isolates	Percentage (%)
Alpha-like (Alp) protein family		
alp1	163	24.8
alp2/3	40	6.1
alpha	110	16.7
alp2/3:alpha	1	0.2
alpha:rib	1	0.2
rib	**341**	**51.8**
neg	2	0.3
Pilus islands (PI)		
PI-2a	219	33.3
PI-2b	8	1.2
PI-1:PI-2a	160	24.3
PI-1:PI2-b	**270**	**41.0**
neg	1	0.2
Serine-rich repeat proteins		
srr1	**349**	**52.8**
srr2	276	41.8
srr1:srr2	1	0.2
neg	32	4.8
Hypervirulent GBS adhesin		
hvgA	263*	40.0
neg	395	60.0

*all CC17

Results that are in bold highlight the most common protein determinants for each class

A



B

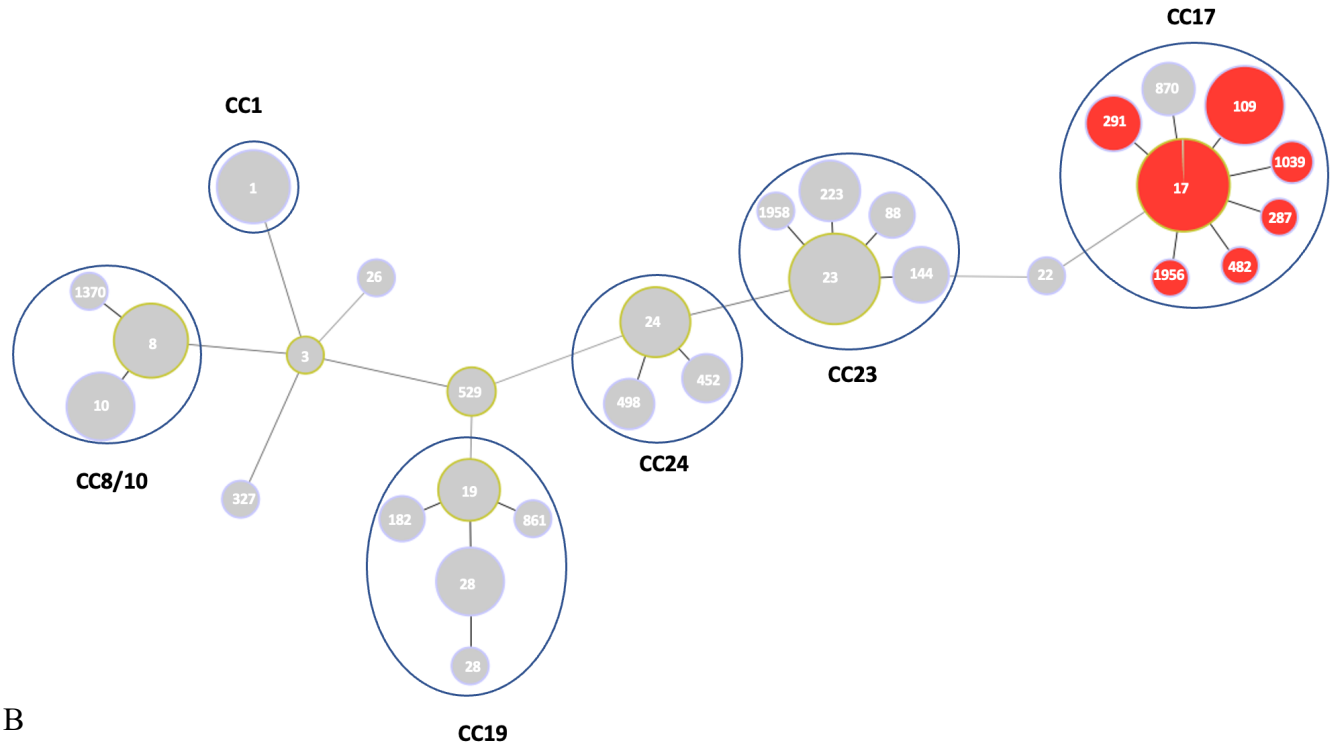


Figure 4.3 (A) Proportion of isolates with the hypervirulent GBS adhesin protein gene determinant (*hvgA)* by clonal complex among GBS isolates causing invasive disease in SA, 2019-2020. (B) Phyloviz diagram depicting associations of clonal complexes with the *hvgA*. Neg = *hvgA* gene not detected. HVGA = *hvgA* gene detected.

Table 4.4 Detection of surface protein determinants by serotype in invasive group B streptococcus isolates collected from patients of all age groups in South Africa, 2019-2020 (n = 656)

			Serine-rich repeat protein				**Alp protein family**							**Pilus island**				
Serotype	**No. of isolates (%)**	*hvgA*	*srr1*	*srr2*	*srr1: srr2*	neg	*alp1*	*alp2/3*	*alpha*	*rib*	*alpha: rib*	*alp2/3 :alpha*	neg	PI2a	PI2b	PI1:PI 2a	PI1:PI 2b	neg
Ia	183 (27.9)	-	183 (52.7)	-	-	-	158 (96.9)	2 (5.0)	13 (12.0)	10 (2.9)	-	-	-	179 (81.7)	-	3 (1.9)	-	1 (100)
Ib	44 (6.7)	-	44 (12.7)	-	-	-	-	-	44 (40.7)	-	-	-	-	-	-	44 (27.8)	-	-
II	55 (8.4)	-	31 (8.9)	-	-	24 (75.0)	-	5 (12.5)	22 (20.4)	28 (8.2)	-	-	-	2 (0.9)	-	52 (32.9)	1 (0.4)	-
III	281 (42.8)	255 (97.0)*	12 (3.5)	261 (94.6)	1 (100)	7 (21.9)	-	1 (2.5)	-	279 (81.8)	1 (100)	-	-	-	4 (50.0)	19 (12.0)	258 (95.6)	-
IV	15 (2.3)	8 (3.0)*	-	15 (5.4)	-	-	-	-	7 (6.5)	8 (2.3)	-	-	-	-	4 (50.0)	-	11 (4.1)	-
V	78 (11.9)	-	77 (22.2)	-	-	1 (3.1)	5 (3.1)	32 (80.0)	22 (20.4)	16 (4.7)	-	1 (100)	2 (100)	38 (17.4)	-	40 (25.3)	-	-
Total	**656**	**263**	**347**	**276**	**1**	**32**	**163**	**40**	**108**	**341**	**1**	**1**	**2**	**219**	**8**	**158**	**270**	**1**

*all CC17. Dashes represent where specific surface protein determinants were not detected.

hvgA = hypervirulent GBS adhesin gene, *srr* = serine rich repeat protein determinant, alp = alpha protein family determinants, PI = pilus island determinant, neg = negative, no. = number

4.5 Antimicrobial susceptibility results

4.5.1 Susceptibility patterns

Antimicrobial susceptibility results from phenotypic methods and WGS predictions are shown in **Tables 4.5 and 4.6**. Based of phenotypic/conventional testing, all of the GBS isolates tested showed 100% susceptibility to linezolid, and synercid. High tetracycline resistance rates (91.5%, 625/658) were detected. The prevalence of erythromycin and clindamycin resistance was 16.1% (106/658) and 3.8% (25/658) respectively. Eighty-eight percent (22/25) of the clindamycin resistant isolates also showed cross resistance to erythromycin. GBS isolates showed 97.0% (638/658) susceptibility to penicillin and 3.0% (20/658) of the isolates showed penicillin nonsusceptibility. Levofloxacin nonsusceptibility was detected in 0.3% (2/658) of isolates. The frequencies of antimicrobial susceptibility for all antibiotics tested are summarized in **Table 4.5**.

Based on WGS, 559/658 (85.0%) isolates yielded susceptibility results for the β-lactam antibiotics: penicillin, ceftriaxone, and meropenem and these isolates were predicted to be 100% susceptible to these three antibiotics (**Tables 4.6**). Susceptibility results in the remaining 15% of the isolates could not be predicted due to low depth of coverage of the targets. Phenotypic testing showed a slightly lower frequency of susceptible isolates compared to WGS: 97.0% (638/658), 96.2% (633/658), and 97.4% (641/658) for penicillin, ceftriaxone, and meropenem respectively. The higher susceptibility rate detected by WGS is likely due to the presence of PBP2x 1-6 in non-susceptible isolates, and the presence of new PBP2x mutations that have not been associated with resistance.

The pipeline predicted erythromycin resistance to be 15.2% (100/658), which was similar to that observed phenotypically (16.l%, 106/658) (**Tables 4.5** and **4.6**). The six isolates that were phenotypically resistant to erythromycin but were predicted to be susceptible by the pipeline lacked resistance genes targeted in the study. The isolates that showed intermediate resistance (15/658, 2.3%) to erythromycin phenotypically were predicted to be susceptible by the pipeline. Fourteen of these isolates did not have any resistance genes, which is most likely the reason why the pipeline predicted them as susceptible. This may also be due to other resistance mechanisms not examined through the pipeline used in our study. Some of these were at the cusp of resistance and susceptibility. All isolates that produced discrepant results for all antibiotics tested results had phenotypic resistance testing repeated from a fresh isolate.

Clindamycin resistance predicted by the pipeline was much higher (10.8%, 71/658) compared to what was seen phenotypically (3.8%, 25/658) (**Tables 4.5** and **4.6**). All the isolates (50/71) predicted to be resistant by the pipeline but were phenotypically susceptible to clindamycin harboured either the *ermTR*, *ermT*, or *lsa* resistance genes which are known to be responsible for clindamycin resistance. All (13/658, 2.0%) isolates that showed intermediate resistance to clindamycin were predicted to be susceptible by the pipeline and these lacked the resistance mechanisms investigated in the study.

WGS predictions and phenotypic results were in agreement for levofloxacin, synercid, and linezolid. Isolates were 100% susceptible to these three antibiotics (**Tables 4.5** and **4.6**). The pipeline predicted 100% susceptibility to vancomycin and chloramphenicol, however, phenotypic testing showed 99.5% (654/658) and 98.8% (650/658) susceptibility for these two antibiotics respectively.

Table 4.5 Phenotypic antibiotic susceptibility of invasive Group B streptococcus isolates recovered from patients of all age groups in SA, 2019-2020.

Antimicrobial agent	**Total no. of isolates**	**Sensitive n (%)**	**Intermediate n (%)**	**Resistant n (%)**
Penicillin	658	638 (97.0)	-	20 (3.0)*
Ceftriaxone	658	633 (96.2)	-	25 (3.8)*
Erythromycin	658	537 (81.6)	15 (2.3)	106 (16.1)
Clindamycin	658	620 (94.2)	13 (2.0)	25 (3.8)
Vancomycin	658	655 (99.5)	0 (0.0)	3 (0.5)
Tetracycline	658	31 (4.7)	2 (0.2)	625 (91.5)
Chloramphenicol	658	650 (98.8)	0 (0.0)	8 (1.2)
Rifampicin	658	653 (99.2)	1 (0.2)	4 (0.6)
Levoflaxacin	658	654 (99.4)	2 (0.3)	2 (0.3)
Meropenem	658	641 (97.4)	-	17 (2.6)*
Linezolid	658	658 (100)	0 (0.0)	0 (0.0)
Synercid	658	658 (100)	0 (0.0)	0 (0.0)

Results were interpreted based on the CLSI guidelines for disc diffusion and broth micro-dilution. In cases where results were not in agreement, the MIC from broth-microdilution was used for final result.

Table 4.6 WGS predictions of antibiotic susceptibility of invasive Group B streptococcus isolates recovered from patients of all age groups in SA, 2019-2020.

Antimicrobial agent	Total no. of isolates that yielded susceptibility results	Sensitive n (%)	Intermediate n (%)	Resistant n (%)
Penicillin	559	559 (100)	-	0 (0.0)*
Ceftriaxone	559	559 (100)	-	0 (0.0)*
Erythromycin	658	558 (84.8)	0 (0.0)	100 (15.2)
Clindamycin	658	587 (89.2)	0 (0.0)	71 (10.8)
Vancomycin	641	641 (100)	0 (0.0)	0 (0.0)
Tetracycline	658	26 (4.0)	0 (0.0)	632 (96.0)
Chloramphenicol	641	641 (100)	0 (0.0)	0 (0.0)
Levofloxacin	658	654 (99.4)	2 (0.3)	2 (0.3)
Meropenem	559	559 (100)	-	(0.0)*
Linezolid	658	658(100)	0 (0.0)	0 (0.0)
Synercid	658	658 (100)	0 (0.0)	0 (0.0)

*non-susceptible

4.5.2 Resistance genes and/or mutations

4.5.2.1 Penicillin non-susceptibility

Of the 658 isolates subjected to disc diffusion and broth microdilution as well as WGS methods, 17 (2.5%) did not yield PBP2x types due to assembly errors. This is associated with low depth of coverage of individual targets. All of these isolates were phenotypically susceptible to penicillin and the pipeline yielded results for serotype, and macrolide and lincosamide results that were consistent with phenotypic results (data not shown). Excluding the 17 penicillin susceptible isolates that did not yield PBP2x types, 88.6% (550/621) of the remaining susceptible isolates were associated with the most common PBP2x types 1-5, which have not been associated with β-lactam nonsusceptibility or reduced β-lactam susceptibility. The remaining isolates (71/621, 11.4%) contained PBP2x >6 (see **Table 4.7**) which is associated with penicillin non-susceptibility or reduced penicillin susceptibility.

Of the 20 isolates that showed phenotypic non-susceptibility to penicillin, 6 (30.0%) contained PBP2x-39, 1 (5.0%) contained PBP2x-101, 4 (20.0%) had new PBP2x types which had not been added to the CDC PBP2x database. The isolates with new PBP2x types had sequences sent to the CDC for assignment and these will later be named and added to the database. Nine (45.0%) had PBP2x types 1-5 (**Table 4.7**) which so far have not been associated with resistance. The PPV of WGS for penicillin was low (13.4%), while NPV was high (98.3%). This means that there is a high probability that an isolate will be phenotypically susceptible given that it had no resistance mutations detected. However, the probability that an isolate will be phenotypically resistant given the detection of resistance mutations (PPV) is low.

4.5.2.2 Macrolide and lincosamide resistance

Erythromycin non-susceptibility was detected in 18.4% (121/658) of the isolates; where 106 were resistant and 15 showed intermediate resistance. Macrolide resistance genes were identified for 78.5% (95/121) of the erythromycin non-susceptible isolates, and the remaining twenty six (21.5%) isolates were negative for resistance determinants examined (**Table 4.8**). The most prevalent resistance genes detected in erythromycin resistant isolates were *ermTR* (39.0%, 37/95), followed by *mefA/E* (32.6%, 31/95), *ermT* (13.7%, 13/95), and *ermB* (8.4%, 8/95). Two isolates (1.9%) harbored a combination of *ermTR* + *mefA/E* and one isolate (0.9%) had a combination of *lsaC* and *mefA/E*. Another two isolates (1.9%) had a combination of *ermB* + *lsa.* The isolates with dual resistance mechanisms did not have higher levels of resistance (i.e. MIC values) compared to those with a single type of resistance mechanism. Almost all isolates (13/14, 93.3%) that showed intermediate resistance to erythromycin tested negative for the resistance genes examined and the remaining isolate had a combination of the *ermB* + *lsa* genes (see **Table 4.8**). Furthermore, ten (1.8%) erythromycin susceptible isolates harbored some of the known resistance genes/mutations (see **Table 4.9**). The PPV and NPV of WGS for erythromycin was high (90.5% and 95.3% respectively), which means a 90.5% probability that an isolate would be phenotypically resistant given the presence of a resistance gene and a 95.3% probability of an isolate being phenotypically susceptible given that it had no resistance genes detected.

Among the twenty-five clindamycin-resistant isolates, eight (32.0%) of the isolates harbored the *ermB* gene, four (16.0%) isolates were positive for the *ermTR* determinant, four (16.0%) had *mefA/E* (however this mechanism is known to only confer resistance to macrolides i.e. erythromycin) , two (8.0%) had a combination of *ermB*+*lsa*, one (4.0%) had a combination of

mefA/E+lsa, one (4.0%) isolate had the *ermT* gene, and the remaining five (20.0%) isolates did not harbour any of the examined genes (**Table 4.7**). Of the ten isolates with intermediate clindamycin resistance, seven (70.0%) did not have any of the genes we screened for and the remaining three had the *mefA/E, lsaC*, and a combination of *ermB+lsa* respectively (**Table 4.8**). Additionally, 13.1% (81/620) of the clindamycin susceptible isolates had known resistance genes detected (see **Table 4.9**). NPV and PPV of WGS for clindamycin susceptibility were 91.3% and 80.0% respectively

4.5.2.3 Tetracycline resistance

The majority (625/657, 95.1%) of the isolates were phenotypically resistant to tetracycline. Most (95.5%, 599/625) of the observed tetracycline resistance was associated with the *tetM* resistance determinant (**Table 4.5**). Additionally, 11 (1.8%) isolates which contained the *tetM* determinant were susceptible to tetracycline, suggesting that *tetM* was inactive in these isolates. There were no discrepancies among the 13 isolates that were positive for the *tetL* and *tetO* determinants (5 of which occurred in combination with *tetM*). Six isolates (1.0%) tested positive for the presence of a *tet* determinant, however, the pipeline could not determine which type (*tetM, L*, or *O*) it was (**Table 4.8**). Seven (1.1%) isolates that were resistant to tetracycline did not contain any *tet* determinants. Two isolates (0.2%) showed intermediate resistance to tetracycline and these isolates had the *tetM* resistance determinant. One tetracycline susceptible isolate contained a *tet* gene with an undetermined type (see **Table 4.8**). NPV and PPV of WGS for tetracycline were 73.1% and 98.1% respectively.

4.5.2.4 Fluoroquinolone resistance

Two (0.3%) isolates were resistant to levofloxacin and two (0.3%) showed intermediate resistance (Table 4.4). No resistance mutations in the *gyrA* and *parC* genes were detected in the two isolates with intermediate resistance. Of the two levofloxacin-resistant isolates, one had a combination *gyrA*-S11 and *parC*-S6F substitutions and the other had no resistance mutations detected in the two genes. Six isolates that contained mutations in either the gyrase or topoisomerase genes alone were susceptible to levofloxacin (see **Table 4.8).** NPV and PPV of WGS for levofloxacin were 73.1% and 98.1% respectively.

Table 4.7 Penicillin binding protein (PBP) 2x types identified in invasive Group B streptococcus isolates in South Africa and associated phenotypic susceptibility profiles, 2019-2020

		PBP2x type								
	Number of isolates tested (N = 658)	**1** **n (%)**	**2** **n (%)**	**4** **n (%)**	**5** **n (%)**	**39** **n (%)**	**101** **n (%)**	**178** **n (%)**	**New[a]** **n (%)**	**NF[b]** **n (%)**
Non-susceptible	20	4 (20.0)	1 (5.0)	0 (0.0)	4 (20.0)	6 (30.0)	1 (5.0)	0 (0.0)	4(20.0)	0 (0.0)
Susceptible	638	144 (22.6)	203 (31.8)	3 (0.5)	200 (31.3)	61 (9.6)	0 (0.0)	8 (1.3)	2 (0.3)	17 (2.7)

[a]New: PBP2x types that have not yet been defined by the CDC. Sequences for these were sent to the CDC for assignment.

[b]NF: PBP2x unassigned due to low coverage in the PBP2x region

Table 4.8 Macrolide, lincosamide, and fluoroquinolone resistance determinants and associated phenotypic non-susceptibility among invasive South African Group B streptococci recovered during 2019-2020 from patients of all age groups.

	Antibiotic							
	Clindamycin		**Erythromycin**		**Tetracycline**		**Levofloxacin**	
Resistance mechanism	**R (N = 25)**	**I (N = 13)**	**R (N = 106)**	**I (N = 15)**	**R (N = 625)**	**I (N = 2)**	**R (N = 2)**	**I (N = 2)**
Macrolide and lincosamide								
ermB	8 (32.0%)	-	8 (7.5%)	-				
ermT	1 (4.0%)	-	13 (12.3%)	-				
ermTR	4 (16.0%)	-	37 (34.9%)	-				
mef	4 (16.0%)	1 (7.7%)	31 (29.2%)	-				
ermB:Isa	2 (8.0%)	1 (7.7%)	2 (1.9%)	1 (6.7%)				
ermTR:mef	-	-	2 (1.9%)	-				
lsaC	-	1 (7.7%)	-	-				
IsaC:mef	1 (4.0%)	-	1 (0.9%)	-				
neg	5 (20.0%)	10 (76.9%)	12 (11.3%)	14 (93.3%)				
Tetracycline								
TetM					599 (95.8%)	2(100%)		
TetO					8 (1.3%)	-		
TetL:TetM					2 (0.3%)	-		
TetM:TetO					3 (0.5%)	-		
Tet					6 (1.0%)	-		
neg					7 (1.1%)	-		
Levofloxacin								
gyrA-S81L:*parC*-S83F							1 (50.0%)	-
neg							1 (50.0%)	2 (100%)

R = resistant, I = intermediate, N = number of isolates

Table 4.9 Macrolide, lincosamide, and fluoroquinolone resistance determinants and associated phenotypic susceptibility among invasive South African Group B streptococci recovered during 2019-2020 from patients of all age groups.

	Clindamycin	**Erythromycin**	**Tetracycline**	**Levofloxacin**
Resistance mechanism	**S (n = 620)**	**S (n = 537)**	**S (n = 31)**	**S (n = 654)**
Macrolide and lincosamide				
ermB	1 (0.2)	1 (0.2)	-	-
ermT	13 (2.1)	1 (0.2)	-	-
ermTR	34 (5.5)	1 (0.2)	-	-
mef	27 (4.4)	1 (0.2)	-	-
ermB:Isa	1 (0.2)	1 (0.2)	-	-
ermTR:mef	2 (0.3)	1 (0.2)	-	-
lsaC	0 (0.0)	1 (0.2)	-	-
lsa	2 (0.3)	2 (0.4)	-	-
23S3-C4T	1 (0.2)	1 (0.2)	-	-
neg	539 (86.9)	528 (98.3)	-	-
Tetracycline				
TetM	-	-	11 (35.5)	-
TetO	-	-	0 (0.0)	-
TetL:TetM	-	-	0 (0.0)	-
TetM:TetO	-	-	0 (0.0)	-
Tet	-	-	1 (3.2)	-
neg	-	-	19 (61.3)	-
Levofloxacin				
gyrA-S81L:*parC*-S83F	-	-	-	1 (0.2)
parC-D87N	-	-	-	1 (0.2)
parC-S83F	-	-	-	8 (1.2)
parC-S83Y	-	-	-	2 (0.3)
neg	-	-	-	642 (98.2)

S = susceptible, neg = no resistance gene/mutation present, dashes represent cases where the genes were not applicable to the antibiotics

4.6 Phylogenetic relationships

4.6.1 Phylogeny of isolates

Maximum-likelihood phylogenetic analysis based on core SNPs showed that the isolates clustered into 5 major clades/lineages which correspond to 6 CCs as defined by MLST (**Figure 4.4**). Four of these clades matched 4 CCs as defined by MLST perfectly, however, two of the CCs were mixed and fell into one lineage based on WGS (i.e. isolates assigned as CC24 by MLST clustered with CC23 isolates based on SNP based phylogeny). The phylogenetic analysis showed no clustering of isolates by geography or by age group (see **supplementary figure 3)**

The CC17 clade was the largest CC and phylogeny of this CC indicated a mix of SLVs of ST17 clustering with the ST17 isolates of serotype III, a cluster of ST291 isolates which were all serotype IV, and a cluster of ST870 (serotype III) isolates. All CC17 isolates, except cluster ST870 isolates and a single ST17 isolate (which clustered with ST870) were *hvgA* positive. All isolates in this clade harboured *srr2* and the *rib* protein determinant. Majority of isolates in this clade had a pilus island combination of PI-1:PI-2b while a few had PI-2b and which formed a small sub-clade (**Figure 4.4**)

The CC23 clade was the second largest CC. Isolates in this clonal complex formed four major subclades, two of which were clustering close to each other and two clades which clustered with isolates of CC24 (**Figure 4.4**). These CC23 clades differed in the combination of proteins/ virulence factors they possessed. The two largest subclades consisted of ST23 isolates which all belonged to serotype Ia, and these haboured *alp1* protein and PI2A. The third subclade contained two ST23 isolates and ST144 isolates which possessed the *rib* protein instead of the *alp1* protein that was found in the largest clade of ST23 isolates and belonged to serotype Ia. The fourth subclade which was found to cluster with CC24 isolates was a cluster of ST223 isolates and two ST23 isolates. All the isolates in this cluster were serotype V, as opposed to serotype Ia which dominated in CC23 and these were clustering closely with CC24 isolates of the same serotype (serotype V). The ST23/serotype V isolates were *alp1* positive and possessed surface proteins similar to those of ST144/serotype Ia isolates. The ST223/serotype V isolates had the *rib* protein and other proteins similar to those of ST23/serotype Ia.

Three subclades were observed within the CC24 clade, one subclade was serotype Ia (ST24) isolates which contained the alpha protein determinant, the second cluster was serotype V

isolates (ST24 and ST498) that also possessed the alpha protein and clustered closely with the CC23 isolates of serotype V. The third subclade was ST452 isolates that were serotype IV. These isolates also contained the alpha protein determinant but had PI2B and *srr2* determinants which were found in CC17 isolates instead of the PI2A and *srr1* found in CC23 isolates.

CC8/10 isolates formed three subclades. One subclade consisted of isolates of serotype Ib, the second one consisted of serotype II isolates, and the third subclade was a small cluster of serotype IV isolates. All CC8/10 isolates contained the alpha protein. The serotype IV isolates contained *srr2* and a combination of PI-1+PI-2b as opposed to *srr1* and a combination of PI-1+PI-2a which was found in all the other CC8/10 isolates (except a single isolate that contained PI-2a).

CC1 had two main subclades, one consisting of serotype II isolates and a larger one of serotype V isolates. All isolates in this CC were *srr1* positive and possessed a combination of PI-1+PI-2a. Furthermore, all the isolates except one had *alp2/3*; the single isolate had a combination of *alp2/3* and alpha.

CC19 phylogeny showed three subclades which were dominated by ST28 isolates of serotype II forming a cluster, a cluster of serotype III isolates, and a cluster of serotype V isolates that clustered with singleton ST529 (also serotype V). All but the cluster of serotype V isolates tested positive for the *rib* protein determinant. All (except one) of the CC19 isolates had a combination of PI-1 and PI-2a. CC19 harboured all of the *srr* negative isolates and the majority of these formed a cluster and constituted most of the serotype II ST28 isolates. The rest formed two smaller clusters and were serotype III.

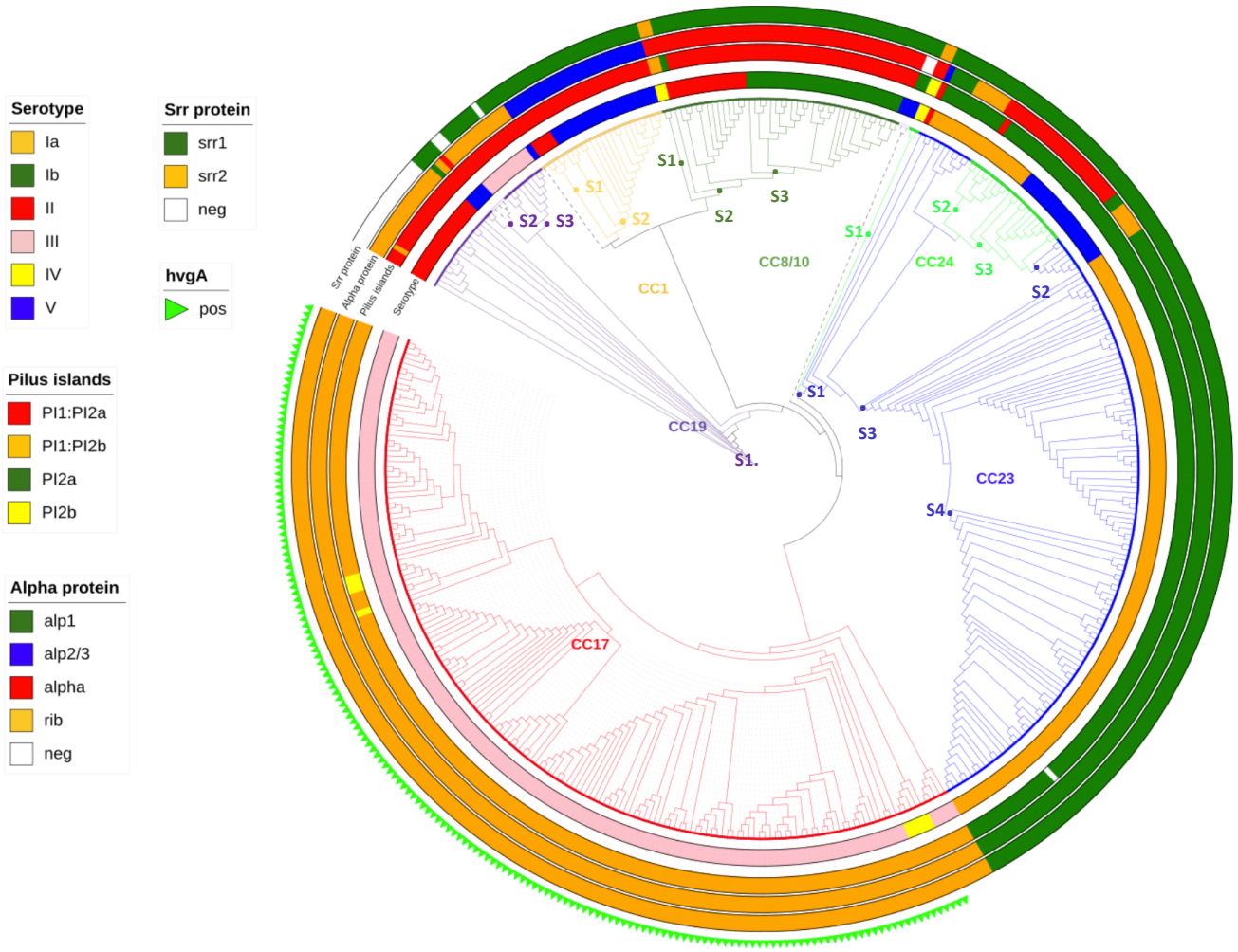


Figure 4.4 A core SNP based maximum likelihood phylogeny of 435* invasive GBS isolates collected from South African patients of all age groups from 2019-2020. Branch colours in the tree and the inner thin strip correspond to clonal complexes (CCs) and are labelled with the same colour. The subsequent strips (from inner to outer strip) indicate serotype (Ia – V), alpha protein family determinants, pilus island (PI) determinants, serine-rich repeat (*srr*) protein determinants, and the hyper-virulent GBS adhesion (*hvgA*) protein. Dotted branches represent singletons STs that to do not fall within a specific CC. *This tree only included isolates that produced sequences with ≤ 150 contigs and N50 ≥ 30 000. Major subclades discussed within each CC are indicated (e.g. S1, and S stands for subclade)

4.6.2 Comparison of South African isolates to global isolates

In order to compare the genetic profiles of SA isolates to global isolates, maximum likelihood core SNPs based and MLST based was constructed phylogeny from the combined global and SA WGS data (**Figure 4.5** and **Supplementary figure 4**). SA isolates were related to those from North America, other parts of Africa, and Europe. Even though the phylogenetic tree shows apparent cladal groupings, isolates from SA were distributed amongst these clades and this was the same for isolates from North America, Europe, Oceania, and other parts of Africa.

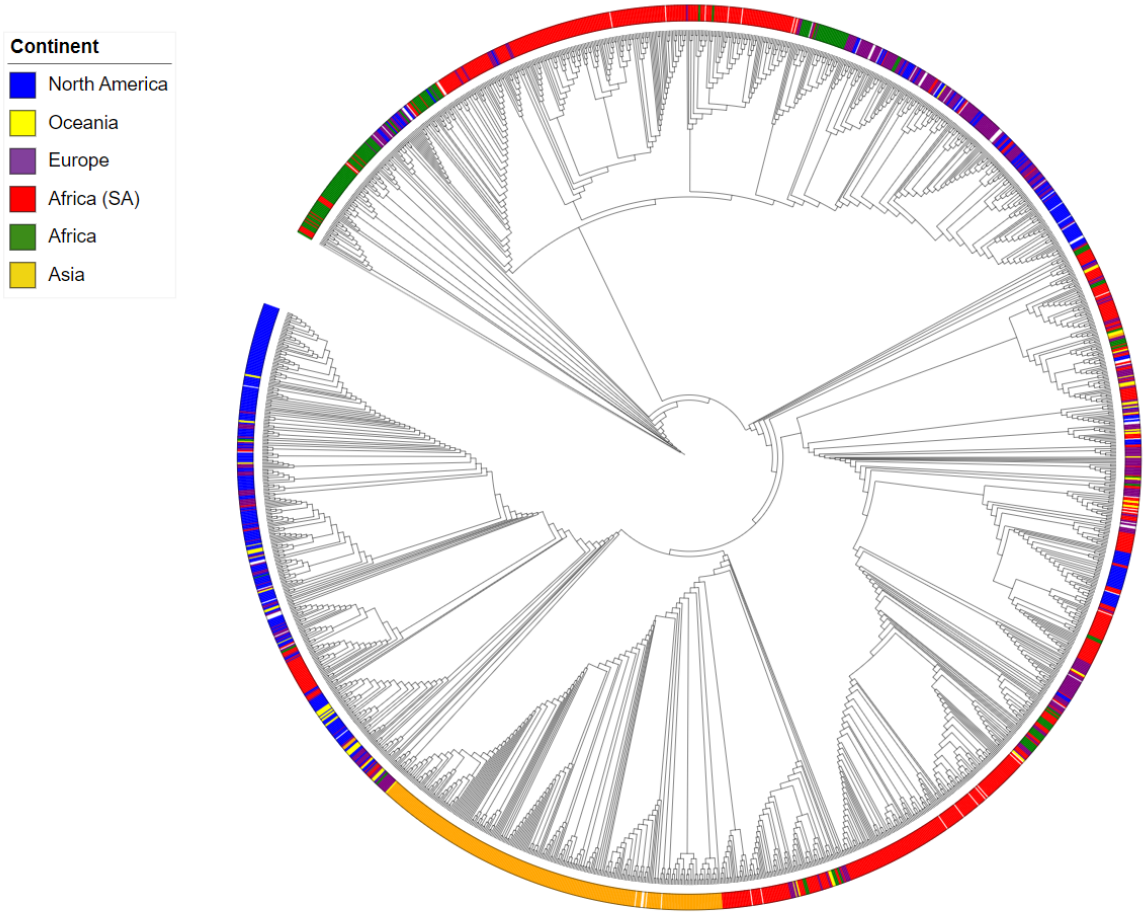


Figure 4.5 Maximum likelihood phylogenetic tree comparing invasive GBS isolates from South Africa (n = 654, collected from 2019-2020) to a representative global dataset (n=1112) extracted from the PubMLST database (for all isolates that had data available for country of isolation, ST, disease type, source, and year of collection). PubMLST database accessed 31 March 2022. The colour strip indicates the continent of isolate origin.

5 Chapter 5: Discussion

GBS remains a leading cause of neonatal sepsis and meningitis, as well as an important cause of invasive disease in adults. With a number of GBS vaccines currently under development, it is important to understand the distribution of serotypes and surface proteins in order to assess the potential coverage of capsular polysaccharide and protein-based vaccines. Assessment of antibiotic susceptibility profiles is crucial in informing which antibiotics should be used for treatment and to monitor for emerging resistance. The current study aimed to determine the serotype and surface protein distribution, antimicrobial susceptibility and the phylogeny of invasive GBS infections reported in patients of all age groups in SA between 2019 and 2020.

This study included 658 invasive GBS isolates from all age groups and from all provinces in SA, collected from 2019-2020. Gauteng and the Western Cape province accounted for majority of the cases (67.6%), which may be due to these two provinces having better capacity and resources for testing than others. Blood cultures accounted for more than 80% of disease isolates and we report that GBS polysaccharide and protein based vaccines currently in development would offer protection against invasive disease in SA. Additionally, our results showed no decrease in GBS cases during the COVID-19 pandemic in the year 2020, which is in line with what was reported by Brueggemann *et al* who reported that they observed no significant changes in the incidence of invasive GBS infections coinciding with the introduction of COVID-19 containment measures (156).

5.1 Serotypes of invasive GBS isolates

The concordance between phenotypic serotyping and WGS-based prediction of serotypes was 99.7% (647/649). This concordance is higher than that of three recent studies that described a concordance of 87%-94% (157–159). However, it is slightly lower than the 100% concordance described in a study conducted in the USA (14). Serotyping was repeated for the two discordant isolates, however, latex agglutination and WGS were performed from cultures grown on different days, which may be the reason for the discordance. These samples may also be contaminated and contain two different serotypes. In order to resolve this, these samples will be re-sequenced and WGS and phenotypic serotyping will be done from the same culture and from a single colony.

Our results suggest that WGS is sufficiently accurate to use for serotyping of GBS, especially in view of the increasing availability and affordability of WGS. In addition, automated

extraction of the data for other genes can be done from a single source of permanently stored WGS data.

WGS based prediction of serotypes yielded fewer non-typeable isolates (0.2%, 1/658) than phenotypic serotyping (1.2%, 8/658). This suggests that the *cps* gene in the phenotypically non-typeable isolates was not expressed or expressed at low levels which were not detectable by the latex agglutination assay. Previous studies have shown that if an isolate is non-typeable, it may be due to low or no expression of the capsular gene or the presence of a capsular variant that does not react to any of the antisera for the 10 known serotypes (160, 161). In this study, WGS was able to determine the sequences for all of the non-typeable isolates. Further analysis is required to identify if these isolates had any mutations in the *cps* region that may affect or inactivate the expression of this gene.

This study provides the first comprehensive examination of GBS serotypes causing invasive disease in all provinces in South Africa as well as across all age groups. Overall, we detected 6 serotypes (Ia, Ib, II, II, IV, and V). A hexavalent capsular polysaccharide conjugate vaccine (GBS6) that targets serotypes Ia, Ib, II, III, IV, and V is currently being tested in a phase Ib/II clinical trial. All the serotypes detected in our study are included in the GBS6 vaccine. Therefore, should this vaccine be introduced in SA, it has the potential to cover 100% of GBS disease serotypes.

In infants with EOD and LOD, serotype III dominated infections with 38.6% and 68.0% respectively. In the same age groups, serotype Ia was the second most common, accounting for 30.9% and 25.3% of EOD and LOD respectively. The data are comparable to what was reported in a systematic review of global GBS serotypes in 2020. This review reported that serotype III accounted for 46% and 77% of EOD and LOD respectively (94). In patients older than 5 years in our study, there was a larger distribution of serotypes and serotype V was more common in adults compared to individuals less than 18 years of age. This is similar to previous reports. A 2021 systematic review and meta-analysis reported that serotype V was the most common serotype in non-pregnant adults aged 15 years and older (162). Another systematic review conducted in 2020 reported serotype V as the most common serotype in the elderly population (aged 60 years and older) (94).

5.2 Distribution of surface proteins

5.2.1 Pilus islands

In this study, a combination of PI-1 and PI-2b (41.0%) was the most common pilus islands detected, followed by PI2a alone (33.3%), a combination of PI-1 and PI-2a (24.3%), and the detection of PI2-b alone was uncommon in the study (1.2%). PI-1 alone was not detected in this study. These results are similar to those in a study previously conducted in 2013 on colonizing and invasive isolates from individuals in Soweto using PCR where the PI frequencies were: PI-1+PI-2b, 45.1%; PI-2a, 29.8%; PI-1+PI-2a, 24.8%; and PI-2b, 0.2%. However, these results are different from what was reported in the USA in 2017. McGee *et al.* reported a combination of PI-1+PI2-a being the most common in the USA, with a frequency 58.3%, followed by PI-2a alone (30.8%), PI-1 + PI-2b (7.4%), PI-2b (3.2%), and PI-1 (0.3%) in a study conducted on individuals of all ages (12).

Previous investigations of pilus islands as targets for vaccine development indicated that a vaccine containing the 3 known pilus types of GBS would prevent against disease caused by all GBS (141, 142). Results observed in this study suggest that a pili-based vaccine that includes components of PI-1, PI-2a, and PI-2b could provide coverage for 99.8% of infections causing invasive disease in our setting.

5.2.2 Alp protein family

About half (51.8%) of our study isolates harboured the *rib* protein determinant. The *alp1* protein determinant was the second most common (24.8%), followed by *alpha* (16.7%), and then *alp2/3* (6.1%). This is different from what was found in the USA where the frequencies of the different *alp* protein family determinants were evenly distributed between 2015-2017 (*alp1*: 28.1%, *alpha*: 26.3%, *alp2/3*: 23.4%, and *rib*: 22.2%) (12). In a study conducted on isolates collected from pregnant women in Garankuwa, SA in 2015, the *rib* protein determinant was also the most common (44.5%) as detected by PCR, and *alp2/3* was detected at 17.7%, which is similar to our study. This study however did not examine the presence of the *alpha* protein determinant which was the second most predominant in our study.

One of the GBS protein-based vaccines currently being investigated is a GBS-NN vaccine that was engineered by fusing the N-terminus of the AlphaC and Rib proteins. Our data suggests that this vaccine would have 68.5% coverage in South Africa.

5.2.3 Srr proteins

The *srr1* gene was detected in 52.8% of the isolates and *srr2* in 41.0%. There are currently no available data on the distribution of *srr* protein determinants in SA or other African countries for comparison. In the USA, 96.4% of isolates harboured *srr1*, which is different from what we detected in this study (12). Based on our results, the latch peptide GBS vaccine that targets Srr1 and Srr2 could protect against 95.3% of invasive GBS disease in SA.

5.3 Antimicrobial susceptibility profiles and resistance genes

Determination of antimicrobial resistance profiles is crucial for informing which antibiotics to use for treatment and to monitor for emerging resistance. Even though penicillin is still appropriate as the first line treatment for GBS infections and IAP, several studies from different countries have reported a reduction in susceptibility of GBS to beta-lactam antibiotics (9,11,66,163–165). In our study, 97.0% of the isolates were susceptible to penicillin. This is lower from what was previously reported by two studies conducted in Soweto and Garankuwa, SA in 2015. These two studies reported 100% susceptibility. The study conducted in Soweto was conducted on HIV-exposed infants while the one in Garankuwa was conducted on pregnant women (151). The difference in study populations may be the reason for the observed variation in penicillin susceptibility. Furthermore, resistance may have increased over time since 2015 in SA. A recent study in Japan reported an increase in the incidence of isolates with reduced penicillin susceptibility from 2.3% in 2005-2006 to 14.7% between 2012 and 2013 (68,166). Therefore the prevalence of penicillin non-susceptible isolates in our study was lower compared to Japan. A study conducted on invasive isolates from infants in the UK in 2019 showed 100% susceptibility to penicillin, which is higher than observed in our study. Metcalf *et al.* reported that in the USA in 2017 (14), 0.7% of the invasive isolates (collected from all age groups) showed reduced susceptibility to penicillin and a 2020 USA study by McGee *et al*, also from patients of all age groups reported an incidence of 0.1% (12).

Of the twenty penicillin non-susceptible isolates found in this study, almost half (45.0%) did not have any PBP2x mutations known to cause β-lactam non-susceptibility or reduced susceptibility. Fifty-five percent of the penicillin-resistant isolates harboured mutations in the PBP2x gene that are known to be associated with reduced β-lactam susceptibility. Phenotypic resistance detection was repeated for all these isolates that produced discordant results in order to confirm that the results. In a study conducted in the USA in 2021, Kobayashi *et al* reported that >10% (15/136) of isolates with reduced β-lactam susceptibility in their study

contained PBP2x 1-5, which have not been previously associated with reduced β-lactam susceptibility. This percentage is significantly lower than what was found in our study. The absence of mutations in the PBP2x gene in penicillin non-susceptible isolates in our study may suggest that there are other mechanisms involved in β-lactam non-susceptibility and future research should focus on exploring these. Furthermore, this study reported that a small fraction (16/9180, 0.17%) of isolates containing PBP2x ≥6 were phenotypically susceptible to β-lactam antibiotics tested. In our study, 13.9% of isolates with PBP2x ≥6 were phenotypically susceptible to penicillin, which is substantially higher than what was reported in the USA by Kobayashi *et al*. The majority of these isolates were PBP2x type 39. Future work should focus on determining whether there are additional mutations that may lead to hindering the action of the PBP2x 39. Additionally, these samples that produced discrepant results will be sent to collaborators at the USA CDC for further investigation.

Erythromycin resistance was detected in 17.1% of the study isolates. This resistance rate is lower than most of the recently published data. McGee *et al*. reported an erythromycin resistance rate of 55.2% in invasive isolates the USA in 2020 (12). A 2021 study conducted on colonizing isolates in Vietnam found that 76.2% of their isolates were resistant to erythromycin (167). Erythromycin resistance is reported to be high in China, with rates of 74.1% reported for both colonizing and invasive strains (73, 82). In SA in 2015, Bolukaoto *et al* reported 21.1% erythromycin resistance in a study conducted on pregnant women in Garankuwa (151). Countries that have reported lower erythromycin resistance levels include Ghana (1.0% in 2015), Iceland (9.0% in 2012), and Lithuania (4.1% in 2017) (168–170).

Our isolates showed a prevalence of clindamycin resistance of 4.6%. Like erythromycin, other countries have reported higher rates of clindamycin resistance compared to what we found. A clindamycin resistance prevalence of 43.5% was reported in the USA in 2020 in a study conducted McGee *et al* on invasive GBS isolates (12). Van Du *et al* reported a clindamycin resistance rate of 58.2% among colonizing isolates from Vietnam in 2021(167). Over the past decade, different countries have reported high rates of clindamycin resistance including Taiwan (65.9%), Portugal (34.0%), Algeria (43.2%), and Ireland (21.3%) (112,113,171,172). Ghana has reported a similar resistance rate (3.1%) to ours and Brazil and Argentina have reported lower clindamycin resistance rates (2% and 1% respectively) (169,173,174). In 2015, Bolukaoto *et al* reported that clindamycin resistance in Garankuwa was 17.2%, which is higher than what was detected in our study (151). This may be due to the fact that these isolates were collected from one hospital and therefore likely clonal. These

isolates were also colonizing isolates compared to invasive isolates examined in the current study.

We found that the presence of *ermTR* and *mef* genes were the most common mechanisms of erythromycin resistance (31.6 and 26.3% respectively). Twenty four percent of clindamycin resistant isolates carried the *ermB* gene. Interestingly, 51.5% of erythromycin and 20.2% of clindamycin resistant isolates did not carry the known resistance genes. This suggests that there are other mechanisms responsible for clindamycin and erythromycin resistance. Additionally, it was observed that a small number of susceptible isolates carried resistance genes for tetracycline, clindamycin, and erythromycin. The presence of resistance genes in these phenotypically susceptible isolates maybe due to these genes being inactive. In a study conducted in Garankuwa, SA on pregnant women, 38% (11/29) of clindamycin resistant isolates carried the *linB* gene as determined by PCR. However, this gene was not detected in our study.

Tetracycline resistance is reported to be high (>80%) in human GBS isolates (76). In this study, tetracycline resistance was detected in 96.8% of the isolates which is similar to what was reported in the Western Cape, SA in 2018 (94.8%). These results are also comparable to what has been reported in France (95.5%) (175), Italy (93.3%) (176), and China (93.5%) (177). Da Chuna *et al* reported that tetracycline resistance in GBS was driven by the expansion of certain clones of GBS that acquired resistance elements *tetO* and *tetM*. The selection and expansion of these clones resulted in the emergence of GBS strains that are better adapted for colonization and infection. The study reported that this has led to the spread of the hypervirulent ST17 clone that is responsible for majority of neonatal invasive infections (23).

In this study, tetracycline resistance was mainly mediated by *tetM*, which was identified in 95.5% of resistant isolates. This is similar to what was reported in the USA (92.8%) (12) and in European countries such as France (95.0%) (175) and Italy (97.1%) (176). Contrarily a study in mainland China reported that *tetO* was the most common (74.7%) gene detected in their tetracycline resistant isolates (177).

In our study, levofloxacin resistance was detected in 0.3% of the isolates. This is contrary to the recent reports that FQ resistance is increasing globally and the fact that several countries have reported high FQ resistance (76). FQ resistance in South Korea is reported to have increased from 8.1% in 2008 (83) to 37.2% in 2013 (84). In Japan FQ resistance has increased from a report of three isolates in 2003 (178) to a resistance rate of 43.6% reported in

2012 (81). A USA study published in 2019 reported a FQ resistance rate of 2.3% in invasive isolates from non-pregnant adults (15). Compared to other countries, France (179), Italy (180), and Spain (111) reported lower FQ resistance rates (1.5%, 3.0%, and 1.2% respectively), even though these rates were still higher than what we reported. FQ resistance is mediated by mutations in the *gyrA* and *parC* genes. We found that one (50%) of the two levofloxacin resistant isolates had a mutation in the *gyrA* gene and another in the *parC* gene occurring together. The other resistant isolate had no mutations detected in both genes, suggesting the action of other resistance mechanisms.

Even though beta-lactam and FQ non-susceptibility are still rare, monitoring remains essential for emerging resistance to guide empiric therapy. WGS for resistance gene detection showed high PPV and NPV values for erythromycin, clindamycin, tetracycline and levofloxacin which means implies that WGS it is highly likely that WGS will detect resistance genes in phenotypically resistant isolates and correctly show absence of resistance mechanisms in phenotypically susceptible isolates. This however, was not the case for the penicillin susceptibility prediction where WGS showed a low PPV value and a high NPV. Even though WGS showed high PPV and NPV for erythromycin, clindamycin, tetracycline and levofloxacin which means that if resistance genes are detected, there is a high probability that the isolate is phenotypically resistant. If resistance mechanisms are not detected, there is a high probability of the isolates being phenotypically susceptible. This however, was not the case for the penicillin where WGS showed a low PPV value and a high NPV. Even though WGS showed high PPV and NPV values for the other antibiotics, there were some phenotypically resistant isolates that lacked the targeted resistance mechanisms and phenotypically susceptible isolates that had resistance mechanisms detected; which may be a result of inactivation of resistance genes as well as the presence of other mechanisms not targeted by our pipeline. Our calculations of PPV and NPV were very restricted since we only examined known/specific resistance targets. However, based on the results, genotypic resistance may not be a great indicator of phenotypic resistance i.e. phenotypic resistance testing is still needed and cannot be replaced with WGS, especially if all possible resistance determinants have not been identified. WGS however, will still be a useful tool for understanding the prevalence of known resistance mechanisms.

5.4 Population structure and phylogeny of South African GBS isolates

Our study reports the population structure of invasive GBS isolates in patients of all age groups and across all provinces of SA. Our data shows that the South African GBS population

is similar to that from the USA, Europe, and other African countries (181–184). MLST based characterization revealed that invasive GBS isolates in SA belong to six CCs (CC1, CC8/10, CC17, CC19, CC23, and CC24). CC17 and CC23 being were dominant, accounting for 41.1% and 21.1% respectively. CC1, CC8/10, CC17, CC19, and CC23 are the most common clonal complexes circulating globally, reaffirming that there is limited genetic diversity among GBS compared to other pathogens including *S. pneumoniae* and *S. pyogenes* (125, 126).

CC24 has been reported in the UK in recent studies, however, in our study it was detected at slightly higher prevalence compared to the UK. The frequency at which CC24 isolates were detected in our study was almost the same as that of CC1 and CC19 (i.e. CC24: 5.9% vs CC1: 5.9% and CC19: 6.5%). In a study published from the UK in 2022, CC24 (designated CC498/CC24 in the study) was detected at 3.6% (7/19) and the other 5 CCs (CC1, CC8/10, CC17, CC19, and CC23) all accounted for more than 17.0% each (145).

Maximum likelihood phylogeny based on core SNPs revealed 5 major lineages/clades. Four lineages matched CC1, CC8/10, CC17, and CC19 perfectly. CC23 and CC24 isolates were mixed and fell within a single lineage within the tree. During the past decade, MLST has been considered the gold standard for characterization of bacterial isolates based on examining the sequences of housekeeping genes. However, MLST based phylogeny is likely to be interrupted by recombination. The housekeeping genes used by the MLST scheme have been shown to be prone to recombination in various organisms including *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella enterica* (141, 142, 182). Therefore, MLST based phylogeny is not entirely free from noise created by recombination, and WGS based phylogeny is used as a way to minimize the interference from recombination.

From maximum likelihood phylogeny (core SNPs based), our study showed that CC23 and CC24 isolates were very closely related and may form a single lineage or clonal complex. The results that we found are similar to what was found in a UK study published early in 2022 by Khan *et al* on strains causing disease in adults between 2014 to 2015 (186). In this UK study, CC24 (designated CC498/CC24) isolates were re-assigned as CC23 based on the fact that the seven isolates of CC498/CC24 (assigned by MLST alone) were clustering clearly within the CC23 cluster in the core SNPs-recombination free phylogenetic tree. Similar to this UK study, our ST498/ST24 (CC24) isolates clustered closely with CC23 isolates. In addition to the two STs (ST498 and ST24) that made up CC498/CC24 in the UK study, we detected a third ST (ST452) that formed part of CC24 in our study. Isolates of ST452 formed their own

subclade which branched distantly compared to the other CC24 isolates but still very closely with other CC23 isolates and closely with CC17 isolates. These ST452 isolates all belonged to serotype IV and shared a similar profile of surface proteins with CC17 isolates (i.e were *hvgA* positive and possessed *srr2* while all other CC23/CC24 isolates possessed *srr1*). Campisi *et al* (2016) reported that ST452 serotype IV arose from large recombination events between ST291 (CC17) and ST24 (CC24, then used to be classified as part of CC23) and genomes of ST452 isolates are composed of two parts that are nearly identical to the corresponding regions of ST24 and ST291 (187). Similar to the UK study, our MLST based CC24 isolates were re-assigned to CC23 based on WGS. The close relatedness of these two CCs (as assigned by MLST) may not be that surprising based on the fact that ST24, which is the founder ST of CC24 is a DLV of ST23, the founder ST of CC23. Based on our results, a combination of both WGS clustering and MLST is expected to provide a more accurate assessment of the phylogeny of GBS.

The lineages revealed by maximum likelihood phylogeny showed that isolates in each cluster were associated with specific serotypes and surface proteins. Some of the CCs were more clonal while some were diverse in terms of serotypes and surface proteins.

Almost all (97%) CC17 isolates belonged to serotype III. The remaining isolates (3%) were serotype IV (ST291). Previous studies have reported that CC17 was exclusively associated with serotype III and that ST291/serotype IV isolates arose as a result of a capsular switch from cps III to cps IV (188). CC17 isolates of both serotype III and IV have been reported to be hypervirulent, causing the majority of invasive GBS disease, especially in newborns and infants. The increased virulence of the CC17 clone has been associated with the presence of the HvgA protein, which is encoded by the *hvgA* gene. In our study, the *hvgA* gene was detected exclusively in CC17 isolates and was carried by 99% of the isolates in this CC. CC17 isolates all contained the srr2 determinant and the majority carried *rib* and PI-1+PI-2b. Studies conducted in the UK (186) and the USA (12) reported similar results.

CC19 showed more diversity in terms of serotypes. ST529 which was classified as a singleton by MLST was shown to cluster within CC19 based on maximum likelihood phylogeny. This CC showed subclades that belonged to serotypes II, III, and V. This CC also harbored all of the isolates that did not contain the *srr* gene. The latch peptide vaccine that is under development targets Srr1 and Srr2, and vaccination with this formulation could potentially result in an increase in disease caused by the *srr* negative isolates of CC19. All isolates in this

CC harboured PI-1+PI-2a and majority of the isolates were *rib* positive. McGee *et al* reported similar results in isolates from the USA (12).

CC1 was dominated by serotype V. This has been reported by other studies globally (94). The *alp2/3* gene was mainly found in this CC and almost all isolates in this CC harboured PI-1+PI2a and *srr1* gene determinants.

Phylogenetic comparison of our isolates to a representative global database from PubMLST revealed that GBS isolates causing disease in SA are similar to those responsible for invasive GBS disease in other countries. This suggests that GBS strains circulating globally are similar and there is no association with circulating strains and geography. While SA isolates were distributed among isolates from the different continents, isolates from Asia clustered into a single cladal group.

5.5 Limitations

Our study had some limitations. First, not all of the cases reported to the GERMS-SA surveillance had isolates received at the NICD for further analysis. Viable isolates were available for about half of the reported cases. This implies that genotypic and phenotypic results may not be a full representation of cases in the GERMS-SA database. This may also imply that the results in this study may not be representative of all cases in the South African population. Secondly, data on disease presentation or clinical syndrome was not collected for the majority of the individuals for whom isolates were available. Therefore, were unable to determine whether there was an association between genotypes and clinical syndrome. Additionally, little to no data were collected on underlying conditions and other risk factors for comparison, especially for the year 2019. Furthermore, this study only described invasive GBS isolates, which leaves a gap in understanding GBS carriage in SA. We were also unable to identify all mechanisms of antibiotic resistance in the isolates. Certain resistant isolates tested negative for the investigated mechanisms of resistance. Another limitation was that NPV and PPV calculations of WGS for prediction of phenotype based on absence/presence of resistance mechanisms were very restricted because the study only looked at known resistance mechanisms. Moreover, PPV and NPV are also influenced by prevalence and therefore may differ in different settings. Lastly, this study only included isolates collected over a two-year period. This means that we were unable to monitor changes in reported GBS cases and surface protein distribution over time.

5.6 Conclusions

Our study describes comprehensive, population-based examination of strain characteristics of invasive GBS in SA across all age groups. This study has contributed valuable data on the serotype epidemiology of invasive GBS in SA, which may help in prediction of the potential coverage and usefulness of vaccines currently under development. We show that the hexavalent (GBS6) conjugate vaccine that includes serotypes Ia, Ib, II, III, IV, and V has the potential to cover 100% of invasive GBS disease in SA. Of the same significance, we also showed that a pilus protein-based vaccine has the potential of protecting against 99.8% of GBS disease. Furthermore, the GBS-NN and the latch peptide vaccines have a potential coverage of 68.5% and 95.3% respectively.

This study also provides valuable data on antimicrobial susceptibility patterns of GBS in SA. Even though penicillin remains appropriate for treatment of GBS infections, our data adds to the global evidence that penicillin resistance and emergence of GBS isolates with reduced penicillin susceptibility is increasing and therefore ongoing monitoring is needed. Antibiotic resistance genes/mutations were detected in 95.0% of phenotypically resistant isolates. We report that there may be other resistance mechanisms responsible for resistance in the remaining 5.0% of isolates.

Majority of our isolates clustered into the 5 most common CCs of GBS (CC1, CC8/10, CC17, CC19, and CC23), which is similar to what has been reported globally. This shows that GBS isolates circulating in SA are not different from what is circulating globally and also shows that the diversity of GBS strains is limited.

High concordance between the phenotypic serotype and WGS serotype prediction as well as the detection of resistance genes in phenotypically resistant isolates shows that WGS can be used as a prediction for the phenotype. Furthermore, WGS is advantageous compared to phenotypic tests as it allows the extraction of multiple data from one source that can be permanently stored. WGS also allows comparison of genomic data with global data to examine relatedness of isolates from different parts of the world. WGS was however was did not predict phenotypic resistance well in our study as some resistant isolates did not harbor known resistance genes or mutations and some phenotypically susceptible isolates had resistance predictors detected.

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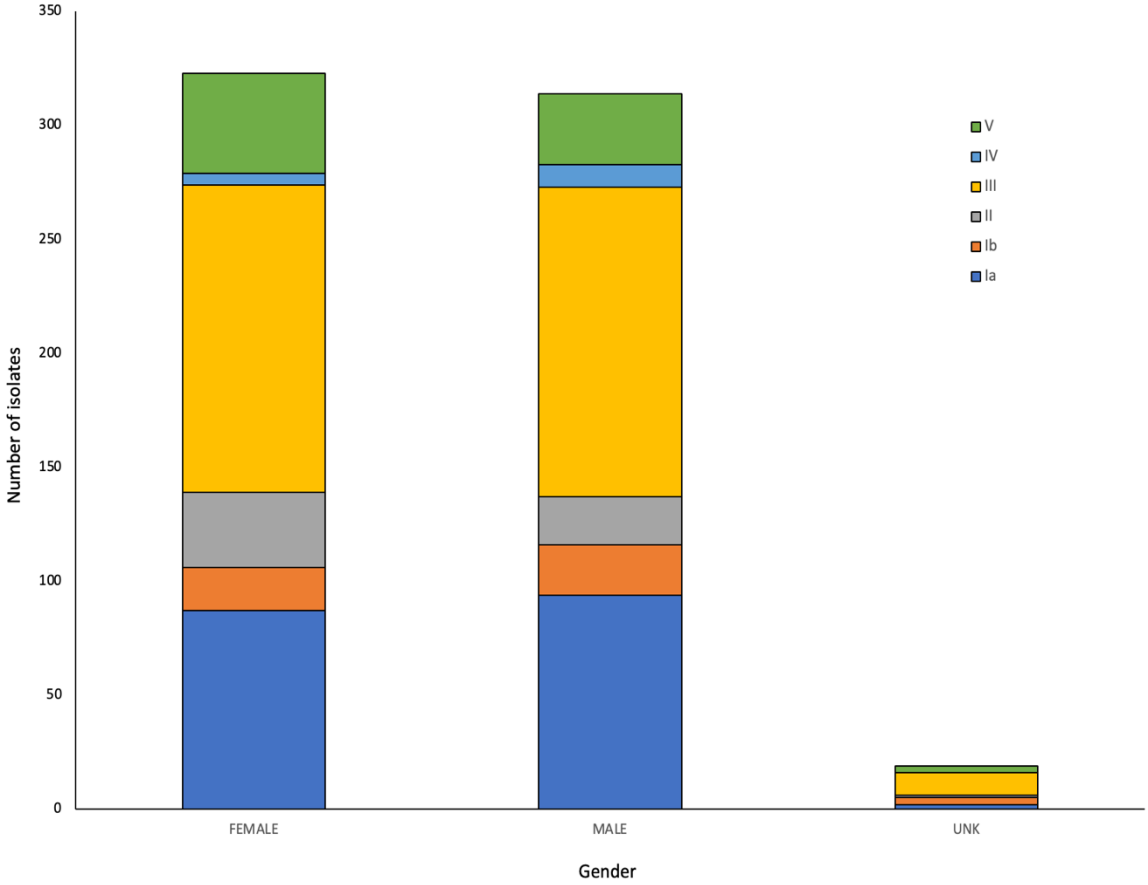
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7 Supplementary figures and tables

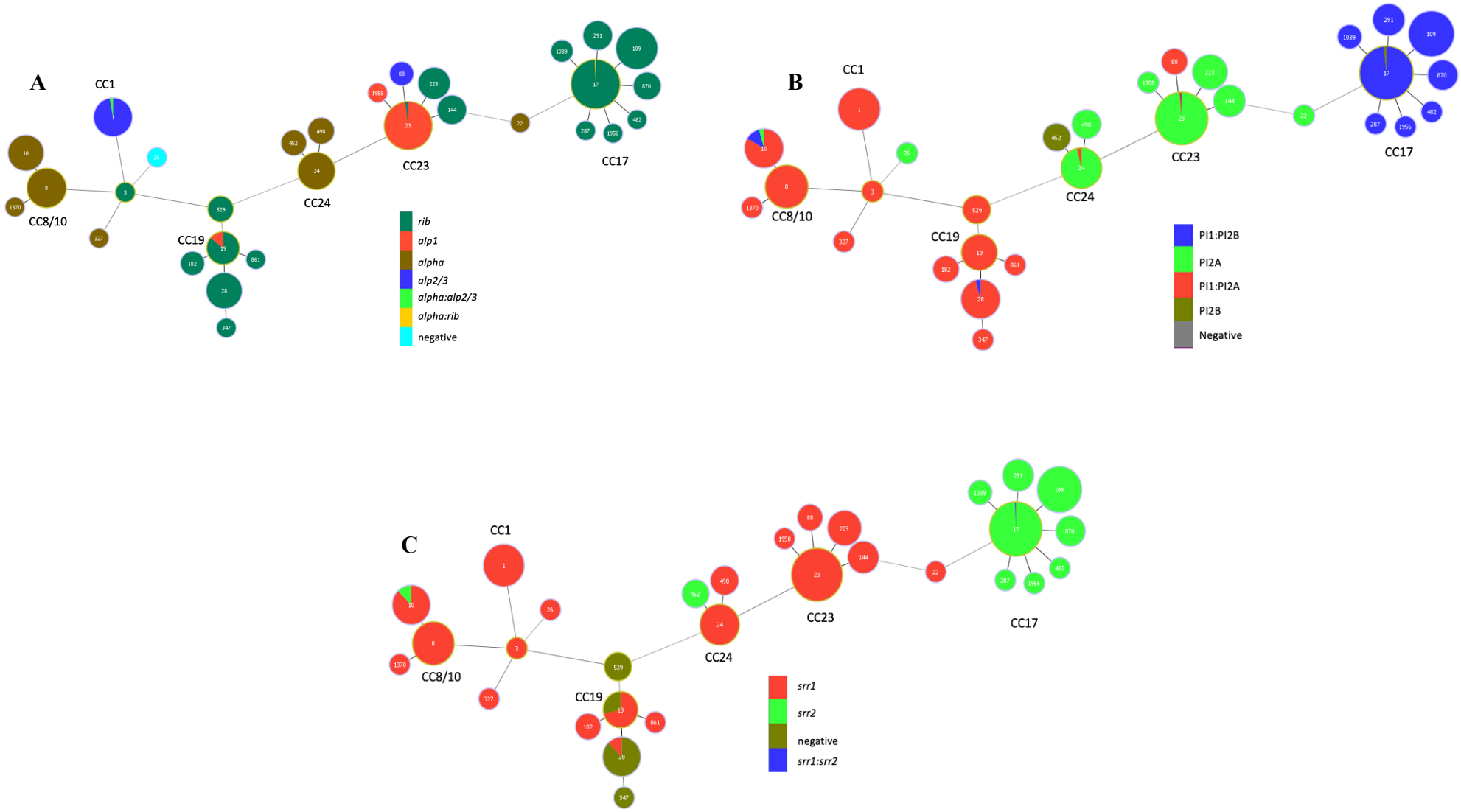
Supplementary table 1 Clonal complexes and sequence types detected in invasive Group B streptococcus isolates from patients of all ages in South Africa, 2019-2020

CC	ST	Number of isolates
1	1	37
17	17	178
	1956	1
	1981	1
	1982	1
	10	1
	1039	2
	109	59
	109*	8
	17*	4
	287	1
	291	9
	482	1
	870	6
19	182	3
	19	14
	28	23
	28*	3
	347	1
	861	1
23	1958	1
	144	8
	223	13
	23	151
	23*	11
	88	3
	NF	1

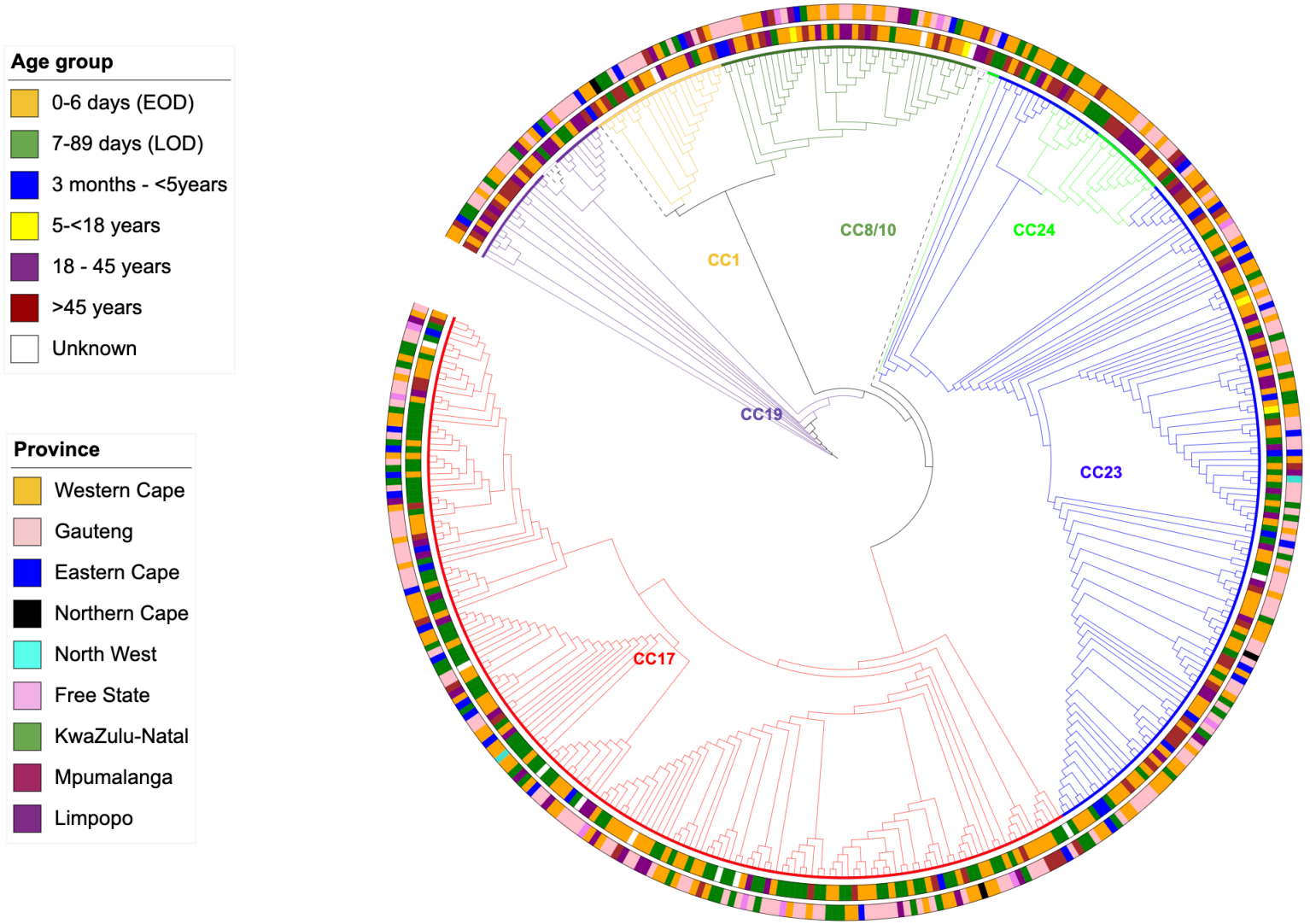
24	24	28
	452	4
	498	5
8\10	1957	1
	10	24
	1370	1
	8	41
	8*	1
	NF	1
Singletons	22	1
	26	1
	3	1
	327	1
	529	4
	538*	1
	NF	1
	Grand Total	**658**



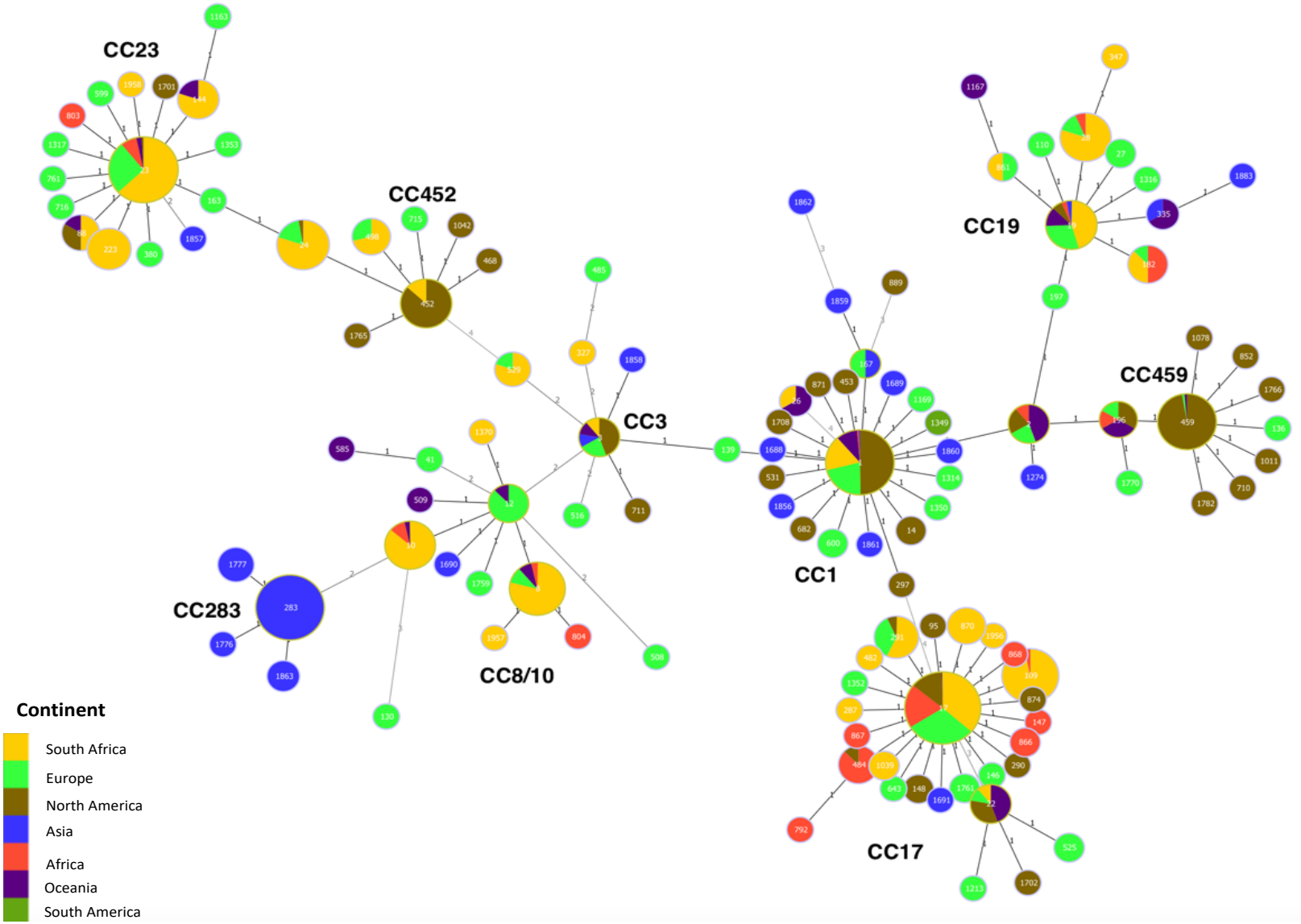
Supplementary figure 3: Distribution of invasive GBS serotypes in South Africa by sex, 2019-2020



Supplementary Figure 2 PHYLOViZ diagram depicting individual clonal complexes and associations with a) alpha protein, b) pilus island, and c) SRR protein determinants among GBS causing invasive disease in South Africa, 2019-2020. In the diagram, each sequence type (ST) is displayed as a circle and the size of the circle is representative of the number of isolates detected.



Supplementary Figure 3 A core SNP based maximum likelihood phylogeny of 435* invasive GBS isolates collected from South African patients of all age groups from 2019-2020. Branch colours in the tree and the inner thin strip correspond to clonal complexes (CCs) and are labelled with the same colour. The subsequent strips (from inner to outer strip) indicate age group, and the province. Dotted branches represent singletons STs that to do not fall within a specific CC. *This tree only included isolates that produced sequences with ≤ 150 contigs and N50 ≥ 30 000.



Supplementary Figure 4 PHYLOViZ tree comparing invasive GBS isolates from South Africa (n = 654, collected from 2019-2020) to a representative global dataset (n=1112) extracted from the PubMLST database (for all isolates that had data available for country of isolation, ST, disease type, source, and year of collection). PubMLST database accessed 31 March 2022.

8 Appendix

Appendix A: Project ethics clearance certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R49 Ms B Ntozini

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M210476

NAME: (Principal Investigator)	Ms B Ntozini
DEPARTMENT:	School of Pathology Dept of Clinical Microbiology & Infectious Diseases Medical School University
PROJECT TITLE:	*Molecular characterization of invasive Streptococcus agalactaie in South Africa*
DATE CONSIDERED:	
DECISION:	Approved unconditionally
CONDITIONS:	Sub-study under M1809107
SUPERVISOR:	Drs N Wolter and S Walaza
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in and therefore reports and re-certification will be due in the month of each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator Date

Appendix B: Turnitin report

Buhle Ntozini (2213934)_dissertation-2.docx

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