NAMIBIA UNIVERSITY
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THE EFFECT OF GROUP A STREPTOCOCCUS ON MATERNAL AND NEONATAL MORBIDITY IN WINDHOEK

By

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Thesis presented in fulfilment of the requirements for the degree of Master of Health Sciences, Faculty of Health and Applied Sciences, Namibia University of Science and Technology, Windhoek, Namibia

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October 2019

DECLARATION

I, Azaria Amadea Vries…… hereby declare that the work contained in the thesis entitled "The Effect of Group A Streptococcus on Maternal and Neonatal Morbidity in Windhoek" is my own original work and that I have not previously, in its entirety or in part, submitted it at any university or other higher education institution for the award of a degree.

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ABSTRACT

Purpose of the research: Globally, puerperal infections cause morbidity in 5-10% of all pregnant women each year. To date, there is very little data in Namibia on the colonization and antibiotic susceptibility patterns of *S. pyogenes* in pregnant women and neonates. This study aimed at determining the prevalence, antimicrobial susceptibility patterns and genetic based resistance of the isolates from pregnant women at 35 weeks to 37 weeks gestation and neonates at 6 week post-delivery follow up at the Windhoek Central Hospital (WCH) maternity ward.

Objectives: To determine the prevalence of GAS in pregnant women at 35-37 weeks gestation and neonates in Windhoek, and assess the genetic basis of resistance to antibiotics in those isolates.

Results: Out of the 165 screened pregnant women, 15 women (9.1%) were colonized by *S. pyogenes.* The prevalence of vaginal *S. pyogenes* in pregnant women was therefore 5.5% and rectal colonization was 3.6% with a median age of 28.9Resistance was observed against clindamycin (93.3%) and penicillin (66.7%). The *TetO* resistance gene was detected in 100% of the *S. pyogenes* isolates. Four (26.7%) isolates possessed the *TetM* resistance gene. No isolates possessed the *ErmA, ErmB or MefA* resistance genes.

Conclusions: This study aimed at determining the prevalence of GAS in pregnant women at 35-37 weeks gestation and the resistance pattern of *S. pyogenes* isolates. The study concluded that compared to 0.03% found by Mead & Winn, 2000 and 4.8% by Verkaeran *et al.,* 2014 the maternal colonization with GAS in pregnant women at the WCH was 9.1%. The women colonized by GAS were between the ages of >20-≤39, single, from a low income area and interestingly employed. The prevalence of *s. pyogenes* infections in neonates post delivery,it was found to be 0.0%. Only 18 of the 40 parents approached for consent agreed, the other 22 refused as they argued that the sample collection procedure was invasive and would be traumatic for the neonates. The study found that out of the 15 *S. pyogenes* isolates tested against erythromycin, penicillin G, tetracycline, clindamycin and vancomycin, resistance to clindamycin was 93.3% and 66.7% with penicillin. Although it was found that all the isolates were sensitive to tetracycline with the disk diffusion test this current study found a low resistance to tetracycline by GAS isolates in the genotypic testing. In conclusion the study showed that vaginal-rectal colonization with GAS was an uncommon finding in pregnant women and neonates at the WCH maternity ward. In conclusion these findings do not necessitate the screening of women in pregnancy and neonate's post-partum.

Recommendations: Further studies with a larger population should be conducted as this might have a more favourable outcome. This in turn would advocate the need to screen pregnant women for GAS

strongly. Also, addititonal genes encoding resistance associated with *S. pyogenes* resistance should be analyzed using molecular techniques.

Educating the recruited patients on the sample collection in both the women and neonates might have aided in more of them consenting to have swabs taken.

A nutrient broth could have been used for the cultivation of the organism after thawing of the isolates. It supports the growth of microorganisms that are not very nutritionally demanding. Gelatin peptone and beef extract provide nitrogen, vitamins, minerals and amino acids essential for growth. Furthermore a DNA extraction kit which would have yielded a higher quality of DNA could have been used instead of the boiling method.

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ABBREVIATIONS

AMR	Antimicrobial resistance
ANC	Antenatal care
AST	Antimicrobial susceptibility testing
CDC	Centres for Disease Control and Prevention
CLSI	Clinical Laboratory Standards Institute
DNA	Deoxy Ribonucleic Acid
GAS	Group A Streptococcus
Hb	Haemoglobin
HIV	Human Immunodeficiency Virus
MIC	Minimum Inhibitory Concentration
MoHSS	Ministry of Health and Social Services
NET	Neutrophil Extracellular Trap
NUST	Namibia University of Science and Technology
PCR	Polymerase Chain Reaction
RT	Room temperature
S. pyogenes	*Streptococcus pyogenes*
SIC	Streptococcal Inhibitor of Complement
SLO	Streptolysin O
SLS	Streptolysin S
SPSS	Statistical Package for Social Sciences
UNICEF	United Nations Children's Emergency Fund
WHO	World Health Organisation

CHAPTER ONE:

1.1 Introduction

Streptococcus pyogenes is a common human pathogen that can induce a wide spectrum of diseases, ranging from non-invasive diseases, such as pharyngitis, scarlet fever, and impetigo, to invasive diseases such as erysipelas, cellulitis, pneumonia, bacteraemia, necrotizing fasciitis, and toxic shock syndrome (Yang *et al.,* 2013). *S. pyogenes* is a β haemolytic bacterium that belongs to the Lancefield serogroup A, classified by its haemolytic capacity and antigenicity of the carbohydrate in its cell wall (Al-Ameri & Al-Kolaibe, 2015). Group A Streptococcus infects individuals of all ages with symptoms alternating from a carrier state to mild or acute pharyngo tonsillitis and invasive disease (Creti *et al.,* 2007). Group A Streptococcus (GAS) is a historically notable cause of puerperal infections and sepsis but the initiation of hand-washing and upgraded hospital hygiene reduced the incidence of puerperal sepsis drastically. Puerperal Sepsis is defined in the International Classification of Diseases (ICD-10), as a "temperature rise above 38 °C maintained over 24 hours or recurring during the period from the end of the first to the end of the tenth day after childbirth or abortion" (Chisembele, 2014).

According to global disease burden figures, the World Health Organization (WHO) ranked GAS as the ninth leading cause of human mortality, with the majority of deaths attributable to invasive GAS infections (Ralph & Carapetis, 2013). GAS is documented as one of the principal infectious agents in human puerperal fever (Busowski *et al.*, 2013). While the incidence of many diseases have declined in first world countries, third world countries continue to suffer due to an increased burden of GAS infections resulting in millions of deaths annually (Carapetis *et al.*, 2005). *S. pyogenes* persists in having devastating effects on public health and on economies (Thenmozhi *et al.,* 2010). An assessment by Carapetis *et al.,* 2005 of the group A streptococcal diseases burden approximated that 18.1 million people were suffering from invasive *S. pyogenes* diseases, with a further 1.78 million incidents arising each year. Globally, puerperal infections cause morbidity in 5-10% of all pregnant women each year (Van Dillen *et al*., 2010).

Invasive GAS infections constitute a significant epidemiological problem. The majority of cases occur in women during the postnatal period (Busowski *et al.,* 2013). *S. pyogenes* is a significant cause of puerperal infections and sepsis. Sub-Saharan Africa experiences an elevated maternal mortality rate (MMR) with 920 maternal deaths per 100 000 live births (UNICEF, 2009). Puerperal sepsis is the principal source of maternal mortality at Mbarara Regional Referral Hospital in Uganda (Ngonzi *et al.,* 2016). Therefore, additional investigation into puerperal sepsis to report the microbiology and epidemiology of sepsis is

suggested (Ngonzi *et al.,* 2016). *S. pyogenes'* postpartum infections persist in being the predominant source of serious maternal postpartum infections and mortality as reported in Asia (11.6%), Africa (9.7%), and Latin America/Caribbean (7.7%) (Say *et al*., 2014).

According to the Namibia Demographic and Health Survey (NDHS) 2006-2007, almost every month a woman dies due to pregnancy related complications in Namibia. Namibia's maternal mortality ratio has increased from 271 deaths per 100 000 live births in 2000 to 449 deaths per 100 000 live births in 2006. The Namibia Statistics Agency (2014) reports that 22 668 maternal deaths were reported during the 2001 census. Mulama (2015), found that puerperal sepsis was the leading cause of direct maternal death in Namibia. From literature, it is obvious that maternal death is a burden and very little information is available on puerperal sepsis in Namibia. Determining the prevalence of GAS in pregnant women at 35-37 weeks gestation and the resistance pattern of isolates are crucial steps in the development of clinical policies and procedures for the management of GAS infections, ultimately reducing maternal and neonatal morbidity and mortality. Due to lack of scientific data to this effect, there are currently no policies in Namibia for screening of GAS in mothers and babies before and after delivery. This study aimed to provide scientific evidence on prevalence of GAS in mothers and babies in Windhoek.

In spite of the use of antibiotics and hospital sanitation efforts, GAS infections are recurring globally. It is highly likely for obstetricians to come across streptococcal infections in the puerperium, whereby the organism that colonizes the lower genital tract often triggers ascending infections (Vivekanantham *et al.,* 2014). The ability of GAS to initiate infection in postpartum patients is determined by a number of factors, including a mother with compromised immunity, administration of antibiotics during and after labour, a delay in diagnosis, environmental exposures of the mother, and particular virulence factors utilized by *S. pyogenes* (Mason & Aranoff, 2013). Neonatal sepsis is a common and deadly disease. It is broadly defined as a systemic inflammatory response occurring in the first four weeks of life as a result of a suspected or proven infection (Du Pont-Thibodeau *et al.,* 2014). Currently, in Namibia pregnant women are not screened for *S. pyogenes* during antenatal care (ANC) visits.

The capability of GAS strains to express numerous virulence factors. Protein M is considered as the main virulence factor, limiting phagocytosis, disturbing the function of complement, and being responsible for adhesion (Golińska *et al.*, 2016). Clinical isolates of *S. pyogenes* were classically differentiated into M serotypes, based on structural differences of the M protein (encoded by the *emm* gene), which is a fibrillary cell-wall protein involved in adherence to human cells (Lynskey, Lawrenson & Sriskandan, 2011).

The *emm* gene encodes the cell surface M virulence protein presumably responsible for at least 100 known M serotypes of *S. pyogenes (CDC*, 2008). Creti *et al*., (2007) demonstrated that *emm*1 strains were consistently responsible for approximately 20% of invasive GAS infections, while variations in the frequencies of the other types were noted, although the cause of most cases of invasive infections were restricted to *emm*1, *emm*3, *emm*4, *emm*6, *emm*12, and *emm*18 (Hamilton *et al*., 2013). *Emm* sequence typing has been widely used in many regions of the world as the preferred method to study and define the molecular epidemiology of GAS strains (Barth *et al*., 2015).

According to the World Health Organisation approximately 830 women succumb to pregnancy- or childbirth-related complications around the world every day (WHO, 2018). Puerperal sepsis caused by GAS remains a significant source of maternal and infant mortality globally, this includes nations with rigorous care measures, modern antibiotic regimens and practices to regulate infections (Hamilton *et al.,* 2013). Sepsis is among the leading causes of preventable maternal death in Europe and the western countries. It continues to be a major contributor to maternal deaths even though the rate of maternal deaths due to other causes has drastically gone down. In Poland, 462 maternal deaths were recorded over a 10-year period, and sepsis accounted for 27.3% of the direct maternal deaths and was the second leading cause of death (Troszynski, 2003).

Maternal death during pregnancy and labour constitutes the principal problem of prenatal medicine, and still a major public health topic (Troszynski, 2003). A major cause of maternal death worldwide is sepsis and very little is known on the incidence of severe maternal morbidity related to sepsis. An estimated 75 000 maternal deaths are caused by puerperal sepsis every year, the majority being in developing countries (van Dillen *et al.,* 2010). When infection develops within 4 days of delivery, maternal mortality is highest with a nearly 60% death rate once streptococcal toxic shock syndrome develops (Shinar *et al.,* 2016).

GAS is the same organism that causes streptococcal throat infection. However, when the infection occurs in the uterus, it can be deadly (Anderson, 2014). Sepsis is a significant pathological condition because of its gravity on both fetal and maternal health outcomes. Some of the immediate consequences for the mother include septicaemia, endotoxic shock or the development of peritonitis or abscess formation leading to surgery. In the fetus, some of the consequences include a reduced five-minute Apgar Score, neonatal septicaemia, and pneumonia. Sepsis significantly affects morbidity and mortality (Chisembele, 2014). The single most important risk factor for postpartum infection seems to be caesarean section, and prophylactic antibiotics during the procedure substantially reduce the infection risk (Smaill & Gyte, 2010).

Group A streptococcus can cause invasive infections in the form of endometritis, necrotizing fasciitis, or streptococcal toxic shock syndrome (Stevens *et al*., 2016). These infections, when associated with sepsis, have been associated with mortality rates of 30 to 50%. When a pregnant woman presents with GAS infection, her symptoms are often atypical, with extremes of temperature, unusual and vague pain, and pain in the extremities (Anderson, 2014).

Maternal mortality is highest in sub-Saharan Africa and direct causes of mortality accounted for 77.7% while indirect causes contributed 22.3% (Ngonzi *et al.,* 2016). Low resource countries account for 99% (286 000) of the global maternal mortalities with sub-Saharan Africa responsible for the bulk of the maternal deaths and accounting for 62% followed by southern Asia at 24% (Ngonzi *et al.,* 2016). The most frequent cause of maternal mortality was puerperal sepsis with 30.9% (Ngonzi *et al.,* 2016). This is in contrast with results from other studies and reports that show postpartum haemorrhage as the commonest cause of maternal deaths globally (Ngonzi *et al.,* 2016). According to the WHO (2018), maternal sepsis is the third most common direct cause of maternal mortality. Infections that are not treated with haste and managed improperly can lead to sepsis, death/disability for the mother and an increased possibility of early neonatal infection and other adverse outcomes (WHO, 2018). A study by Chiwuze *et al.,* 1998 reported that in the state of Anambra of Nigeria, sepsis was the fourth leading cause of death and contributed 12.1% to the maternal deaths. Another study by Melah *et al.,* 2003 that puerperal sepsis was the most persistent morbidity on obstructed labour in the State of Gombe, Nigeria. Sepsis is detailed to be a serious complication of induced abortion in Nigeria. The incidence and case fatality rates in West Africa indicate severe maternal morbidity from direct obstetric causes, it showed sepsis to have a case fatality rate of 33.3%. The incidence and prevalence of both invasive and non-invasive GAS infections in developing countries are largely unknown (Barth *et al.*, 2018). In South Africa, puerperal sepsis remains one of the leading causes of maternal deaths and a substantial number of these deaths are avoidable. Puerperal sepsis is one of the main indications for emergency peripartum hysterectomy (O'Callaghan, 2009).

CHAPTER TWO

2.1 Literature Review

Streptococci are nonmotile, nonsporeforming, catalase negative, and Gram positive cocci that present in chains or pairs. The majority of *Streptococci* are facultative anaerobes while some are obligate anaerobes (Todar, 2008). Streptococcus consists of a variety of species, some of which cause disease in humans and animals, while others are significant in the manufacture of certain fermented products (Encyclopaedia Brittanica, 2019). Several species of Streptococci are important ecologically as part of the normal microbial flora of animals and humans; but some can also cause diseases that range from acute to sub-acute or even chronic (Patterson, 1996). Streptococcus infections are still one of the important problems facing contemporary medicine (Krzyściak *et al*., 2013). One of the most invasive groups of bacteria is the Streptococcus genus. It is divided into 49 species and eight subspecies, from which as many as 35 have been identified as sources of invasive infections in humans. Microorganisms considered to be the cause of common infections include: *S. pneumoniae*, *S. pyogenes*—group A, *S. agalactiae*—group B, and *S. mutans* (Krzyściak *et al*., 2013). Bacteria of the *Streptococcus* genus include a large number (> 100 species) of microorganisms colonizing human and animal mucous membranes. They occur as physiological flora in the oral cavity and intestines of humans and animals (Canny & McCormick, 2008). In addition, they often inhabit the skin, throat, and upper respiratory tract. However, numerous streptococci occur as opportunistic pathogens, causing infections in the case of weak immunological response of the host body they occupy (Avila, Ojcius & Yilmaz, 2009).

2.1.1 Classification of streptococci

Streptococci are classified on the basis of colony morphology, hemolysis, biochemical reactions, and serologic specificity. They are divided into three groups by the type of hemolysis on blood agar: β hemolytic which is the complete lysis of red cells, α hemolytic which is incomplete, green hemolysis, and ɣ hemolytic which is no hemolysis. Serologic grouping is based on antigenic differences in cell wall carbohydrates and in the polysaccharide capsule in group B streptococci (Patterson, 1996). Streptococcal species have been separated into distinct groups such as pyogenic, mitis, *mutans,* and *bovis*. Among these, the pyogenic group comprises multiple human and animal pathogens such as *Streptococcus agalactiae* (Lancefield group B), *Streptococcus equi* (group C), *Streptococcus dysgalactiae* (group C), as well as GAS (Hamada *et al.,* 2015).

The classification of streptococci into major categories has been based on a series of observations over many years namely the colony morphology and haemolytic reactions on blood agar, secondly the serologic specificity of the cell wall group-specific substance (Lancefield antigens) and other cell wall or capsular antigens, thirdly the biochemical reactions and resistance to physical and chemical factors, and lastly the ecologic features. Molecular genetics have also been used to study streptococci (Jawetz, Melnick & Adelberg, 2013). The combination of the above mentioned methods have solidified the classification of streptococci for purposes of clinical and epidemiologic benefit, but as knowledge evolves, new methods have been introduced with the result that several additional classification systems have been described (Spellerberg, 2016). Alternatively, different specie names have been used to describe similar organisms. In other instances, some members of the same species have been included in another species or classified separately. The genus *Enterococcus*, for example, now includes some species previously classified as group D streptococci (Jawetz, Melnick & Adelberg, 2013).

The traditional Lancefield classification system, which is based on serotyping, has been replaced by *emm* typing, which has been used to characterize and measure the genetic diversity among isolates of *S pyogenes* (Khan, 2018). This system is based on a sequence at the 5' end of a locus (*emm*) that is present in all isolates. The targeted region of *emm* displays the highest level of sequence polymorphism known for *S. pyogenes* gene, with more than 150 *emm* types having been described to date (Mcgregor *et al.,* 2004). The *emm* gene encodes the M protein. There are 4 major subfamilies of *emm* genes, which are defined by sequence differences within the 3' end, encoding the peptidoglycan-spanning domain. The chromosomal arrangement of *emm* subfamily genes reveal 5 major *emm* patterns, designated as *emm* patterns A through to E. An example of the usefulness of *emm* typing is described by (McGregor *et al.,* 2004). Figure 2.1 shows the biochemical characteristics of streptococci. Streptococci are classified on the basis of colony morphology, haemolysis and serologic specificity. They are divided into three groups by the type of haemolysis on blood agar: β-haemolytic (clear, complete lysis of red cells), α haemolytic (incomplete, green haemolysis), and γ haemolytic (no haemolysis).

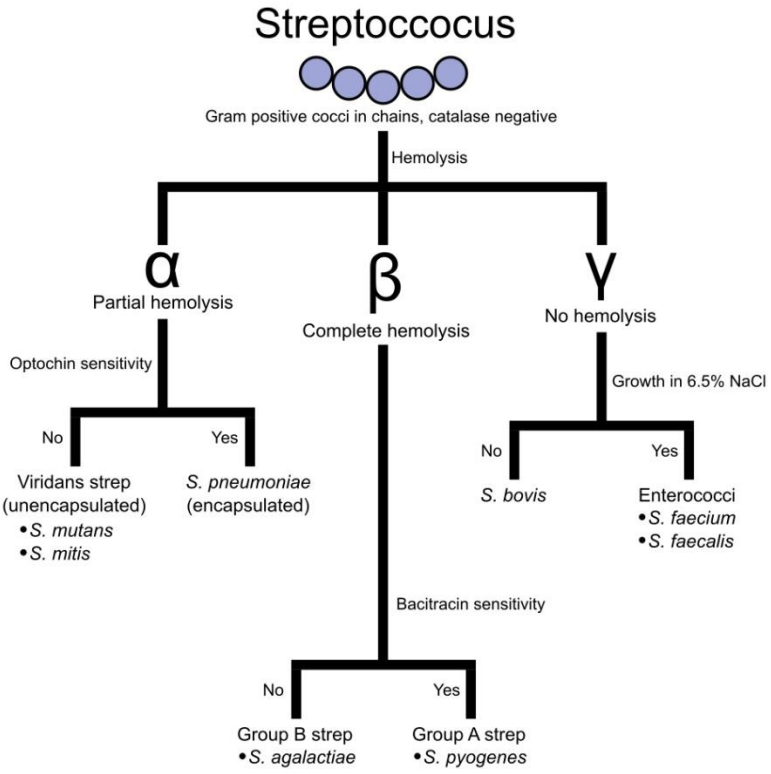

Figure 2.1 [Classification of Streptococcus] (2016). Retrieved from https://step1.medbullets.com/microbiology/104175/introduction-to-streptococcus

2.1.2 Lancefield grouping of streptococci

Created by Rebecca Lancefield, the system was historically used to organize the various members of the family *Streptococcaceae,* which includes the genera *Lactococcus* and *Streptococcus,* but now is largely redundant due to enormous growth in the number of streptococcal species identified since the 1970s (Isenberg, 1992). However, it has remained clinically useful even after the taxonomic changes, and as of 2018, Lancefield grouping is often still used to communicate medical microbiological test results in the United States (Facklam, 2003). As originally described by Lancefield, β-haemolytic streptococci can be divided into many groups based on the antigenic differences in group-specific polysaccharides located in the bacterial cell wall. More than 20 serologic groups have been identified and designated by letters (Khan, 2018). Of the non–group A streptococci, group B is the most important human pathogen (the most common cause of neonatal sepsis and bacteraemia), although other groups (particularly group G) have

occasionally been implicated as causes of pharyngitis (Tiemstra & Miranda, 2009). It is distinguished from other groups of β-haemolytic streptococci by a group-specific polysaccharide (Lancefield antigen) in the cell wall (Patterson, 1996).

Lancefield grouping, hemolysis, and colony morphology considered together enable a clinically useful subdivision of the genus. For the most part the organisms of the Lancefield groups are β-hemolytic and associated with pyogenic infections (Nizet & Arnold, 2018). Organisms that cannot be assigned to a Lancefield group usually go with the viridans streptococci; they generally are of lower virulence but may be associated with bloodstream infection, endocarditis, and a variety of diseases in a compromised patient (Haslam *et al.,* 2015). The Lancefield grouping system cannot be used in itself for accurate identification of specific βhaemolytic species, but it can be a useful part of the identification procedure. Lancefield grouping defines catalase-negative, coagulase-negative bacteria based on the carbohydrate composition of bacterial antigens found on their cell walls (Todar, 2008). *Enterococcus*, formerly known as Group D Streptococcus, was believed to be a member of the genus Streptococcus until 1984, after the Lancefield criteria were devised, and so were included in the original Lancefield grouping (Lancefield, 1933). Many but not all species of streptococcus are beta-haemolytic. Notably, *Enterococcus* and *Streptococcus bovis* (Lancefield Group D) are not βhaemolytic (Jankoska *et al.,* 2008). Though there are many groups of streptococcus, only five are known to commonly cause disease in immune-competent human beings: Group A, Group B, both members of Group D, and two groups that lack the Lancefield carbohydrate antigen: *Streptococcus pneumoniae* and *viridans streptococci* (Isenberg, 1992).

2.1.3 Group A *Streptococcus*

Group A *Streptococcus* (GAS) is the only species in this group of β-haemolytic streptococci. GAS is one of the leading pathogenic bacteria that infect children and adolescents, and it is associated with a wide spectrum of infections and diseases (Khan, 2016). Since the mid 1980s, GAS have been identified as a re-emerging cause of severe invasive infections, such as septic shock, puerperal sepsis, soft tissue infections (including necrotizing fasciitis), and streptococcal toxic shock syndrome (STSS). Despite increased awareness and improved treatment of the most severe disease manifestations, the mortality rate remains high (Stevens, 2016).

Group A streptococci typically have a capsule composed of hyaluronic acid and exhibit β (clear) haemolysis on blood agar (Todar, 2008). Group A streptococci or *S. pyogenes* has been recognized as an important

human pathogen, since early days of modern microbiology, and it remains among the top ten causes of mortality from an infectious disease (Ralph & Carapetis, 2013).

Figure 2.2 represents a cutaway view of group A streptococcus. (a) The outer edge is made up of fimbriae partly consisting of M- protein antigens. The capsule, protein antigens, and C carbohydrate antigens make up the other components of the cell envelope. (b) Details the position of the M protein in the cell envelope.

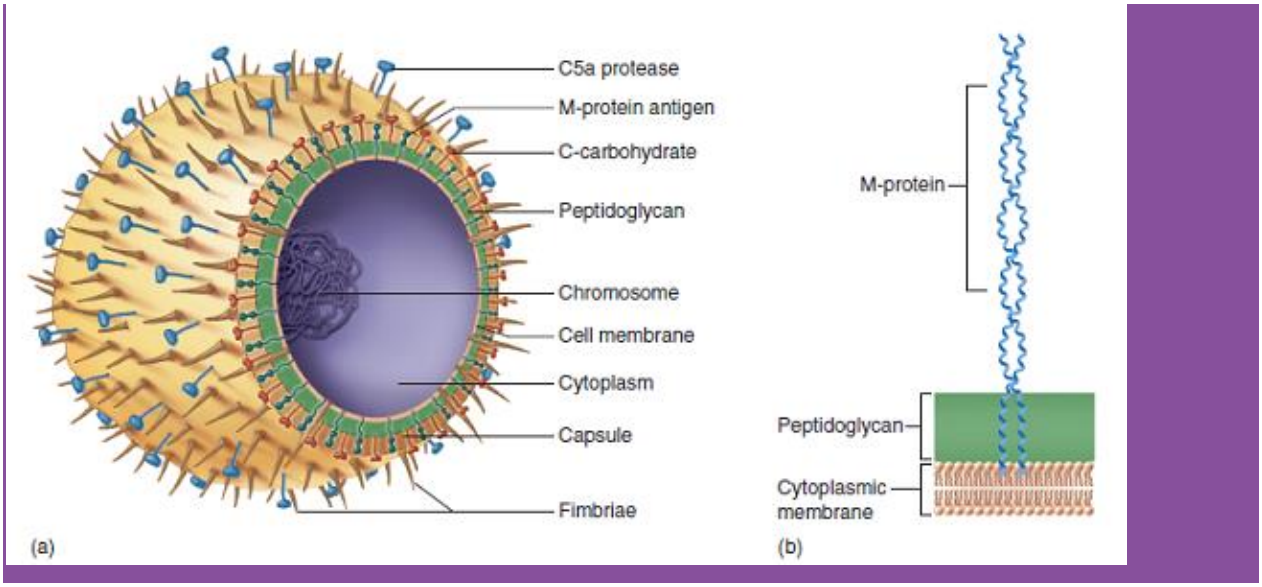

Figure 2.2 [Cutaway view of Group A streptococcus]. Retrieved from https://www.chegg.com/homework-help/critical-thinking-ability-reason-solve-problems-using-facts-chapter-18-problem-9-solution-9780077967420-exc.

2.1.4 Pathogenicity of GAS

S. pyogenes induces serious human disease by at least three mechanisms: pharyngitis and pyoderma (puss forming); toxin elaboration as in toxic shock syndrome; and immune-mediated inflammation in acute rheumatic heart failure (Stevens, 2016). No absolute explanation is feasible for the propensity of certain body sites for GAS infection, nor for the ability of certain M-type strains to produce pharyngitis and of others to produce pyoderma. The first step in the pathogenesis of GAS disease in humans is successful colonization of the upper respiratory mucosa or skin (Nizet, 2018).

GAS disease pathogenesis involves colonization of the upper respiratory tract or the skin of a human host. GAS biofilm formation facilitates persistence within the human host. Both M protein and fibronectin-

binding proteins are vital for subsequent phagocytic uptake of GAS into respiratory epithelial cells. This process of intracellular invasion allows GAS access to an intracellular niche, and represents a proximal step in the pathogenesis of systemic infection (Walker *et al.,* 2014).

Figure 2.3 is a representation of *S. pyogenes* infections initiated by adhesion of the bacterial organism to human epithelial cells, inclusive of oral, nasal cavities and the skin. Bacterial pathogens express various molecules that are anchored in the cell wall as fimbrial-like structures. Several surface fimbrial-like proteins also bind to human extracellular matrix proteins, including fibronectin (Fn), laminin, and collagen. Several surface fimbrial-like proteins also bind to human extracellular matrix proteins, including fibronectin. After initial invasion of human tissues, it is reported that *S. pyogenes* can spread rapidly to various organs. *S. pyogenes* escapes from the human immune system, enabling the organisms to initiate systemic and severe infections (Terao, 2012).

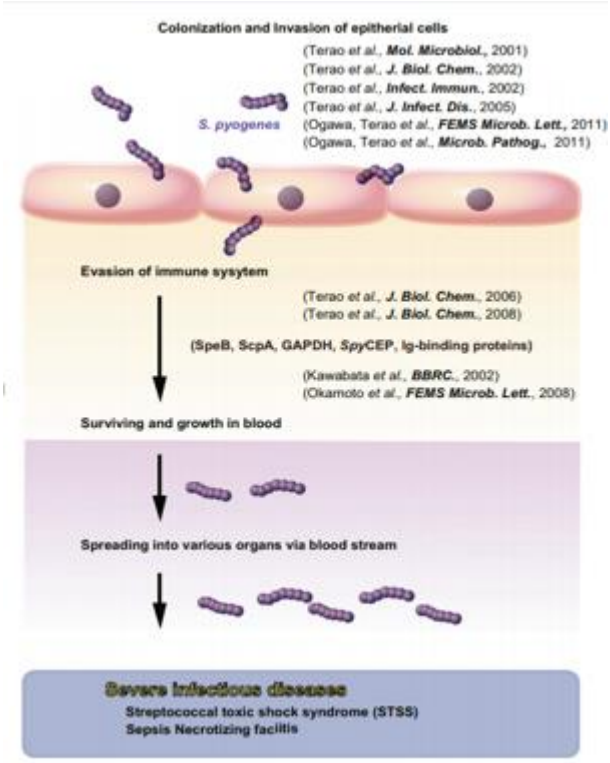

Figure 2.3 [Proposed pathogenesis of severe *S. pyogenes* infection] (2012). Retrieved from https://www.sciencedirect.com/science/article/pii/S1349007912000497

2.1.5 Disease in the general population

S. pyogenes is a major pathogen capable of causing a variety of diseases in different age groups of people (Khan, 2018). In a study by Felsenstein (2014) that included 100 patients that complained of a sore throat, all divided into different age groups, *S. pyogenes* colonies were confirmed on the basis of β-haemolysis, bacitracin sensitivity test, and latex agglutination test for group A. Forty two out of a total of 100 samples, were confirmed as group A Streptococcus. From the study, it was reported that all age groups, with a maximum occurrence in the 5-15 years of age, were suffering from group A Streptococcal pharyngitis (Felsenstein, 2014). Therefore, every case of sore throat especially affecting children should be investigated to detect the causative agent for initiation of proper therapy (Ray *et al.*, 2010).

An understanding of the diverse nature of infectious disease complications attributable to this organism is an important cornerstone of paediatric medicine (Khan *et al.,* 2018). In addition to infections of the upper respiratory tract and the skin, *S pyogenes* can cause a wide variety of invasive systemic infections (Wong & Yuen, 2012). Along with *Staphylococcus aureus*, group A streptococcus is one of the most common pathogens responsible for cellulitis. Infection with this pathogen is also linked to other potentially serious nonsuppurative complications: acute rheumatic fever (ARF) and acute glomerulonephritis. In addition, infection with *S. pyogenes* has re-emerged as an important cause of toxic shock syndrome (TSS) and of life-threatening skin and soft-tissue infections, especially necrotizing fasciitis (Khan *et al.,* 2018).

Streptococcal throat symptoms may include a swollen red sore throat and tonsils, tonsil exudate, high fever, headache, neck lymphadenopathy. Abdominal pain, nausea, and vomiting might be present especially in children. Individuals with scarlet fever may experience a quickly spreading red rash that feels similar to sandpaper on the body (Khan *et al.,* 2018). The rash begins on the trunk, then spreads outward, usually sparing the palms, soles, and face but worsens under the arm and in the groin, swollen lips and red spots on the tongue (red papillae), strawberry tongue may be present as the disease resolves. A red skin rash that looks like a group of small blisters or red bumps occurs in impetigo. When the blisters burst and fluid seeps out, the fluid dries and the blisters become coated with a yellow or grey crust. The sores usually occur around the nose and mouth but can be spread to other areas of the body by fingers, clothing and towels. Itching and soreness are generally mild (CDC, 2018).

Invasive Group A Streptococcus infections may present as any of several clinical syndromes, including pneumonia, bacteremia in association with cutaneous infection (e.g., cellulitis, erysipelas, or infection of

a surgical or non-surgical wound), deep soft tissue infection (e.g., myositis or necrotizing fasciitis), meningitis, peritonitis, osteomyelitis, septic arthritis, postpartum sepsis (i.e., puerperal fever), neonatal sepsis, STSS or bacteremia (NCCID, 2018). Skin and soft tissue infections tend to be the most common invasive GAS manifestations (Seidler & Seidler, 2013). Streptococcal Toxic Shock Syndrome results in a swift drop in blood pressure and organ failure. Symptoms may include fever, skin redness, dizziness, flu like symptoms, confusion, shock, diarrhea, vomiting and extreme muscle pain. This disease is the most serious manifestation of invasive GAS disease (NCCID, 2018). Necrotizing Fasciitis (flesh eating bacteria) is a deep-seated infection of the subcutaneous tissue that results in rapid destruction of the fascia and fat, but may spare the skin itself. Symptoms may include fever and intense pain, redness and swelling in the affected area. Often the pain is disproportionate to (much worse than) the appearance of the infection (CDC, 2018). Post-Streptococcal Glomerulonephritis is a kidney disease that can develop as a result of the immune system fighting off the group A strep throat or skin such as strep throat, scarlet fever, and impetigo. It usually takes about 10 days after streptococcal throat infection or scarlet fever and about 3 weeks after a group A streptococcus skin infection for it to develop. Some people may have no symptoms or symptoms that are so mild that they don't seek medical help (INH, 2019). Rheumatic fever is an inflammatory disease that can develop as a complication of inadequately treated streptococcal throat infection or scarlet fever. Rheumatic fever can cause permanent damage to the heart, including damaged heart valves and heart failure (CDC, 2018).

2.1.6 Disease in women including pregnant women

Puerperal sepsis is a postpartum pelvic bacterial infection contracted by women after vaginal or abdominal delivery. The condition is identified by fever at 1 day postpartum, although more rapid and severe infection leading to death may occur (Zakour *et al.,* 2012). Puerperal sepsis has been recognized as a major contributor to maternal and new born morbidity since age-old times. The introduction of maternity hospitals in the 1600s triggered a steep rise in puerperal sepsis cases and deaths, which remained unchanged until the late 1800s (Zakour *et al.,* 2012). Epidemics of puerperal sepsis were common in the 1600s to 1800s as side effects of hospital practices in the days before infection control and antimicrobial therapy (Zakour *et al.,* 2012).

Puerperal Sepsis symptoms include chills, soreness in the womb, fever, cold sweats and excessive thirst. Symptoms that present to a lesser extent include headache, back pain, nausea and

vomiting. Puerperal Sepsis is diagnosed based on accumulation of the symptoms listed above and an examination of the body systems involved. In the case of puerperal sepsis, the body system involved is the uterus, although other systems such as the lungs, brain, urinary tract and bowels may also be involved. Serious puerperal GAS infections and other invasive GAS infections have reappeared over the past 25 years, and these infections must now be considered in the differential diagnosis of postpartum sepsis. Though still infrequent, such infections must be recognized and treated aggressively to prevent severe morbidity or mortality (Busowski, 2013).

Hamilton, Stevens and Bryant (2013) state that infection with GAS during pregnancy does not originate from vaginal colonization but rather that upper respiratory infection/colonization precedes haematogenous seeding of the placenta/uterus, suggesting an important tropism of GAS for these tissues during pregnancy. Infections during this period result in disastrous outcomes for both mother and foetus.

Group A Streptococcus is a well-known cause of vulvovaginitis in prepubescent girls but genital infections that include endometritis and vulvovaginitis are rarely described in adult women (Verkaeren *et al.,* 2014). GAS vulvovaginitis has also been linked to chronic dermatological conditions, vaginal foreign body, sexual abuse, and anatomical abnormalities (Verkaeren *et al.,* 2014). GAS is not always symptomatic, and the genital and rectal tracts were found to be colonized by GAS in 0.03% of 6944 women at 35–37 weeks of pregnancy in 2000 (Mead & Winn *et al.,* 2000). Familial contamination has also been reported in a mother with GAS-associated vulvovaginitis and perineal cellulitis and her child with GAS pharyngitis (Serban, 2018).

2.1.7 Disease in neonates and prevalence of GAS

Lancefield group A streptococci (GAS) was the predominant cause of neonatal sepsis in the 1930s and 1940s (Miyairi *et al.,* 2004). Neonatal sepsis is a prevalent and life-threatening disease. Neonatal refers to the first four weeks postnatal, whether born prematurely or at term. Neonatal sepsis is generally defined as a systemic inflammatory response, manifesting in the first four weeks of life, as a result of a suspected or proven infection (Shah and Padbury, 2014). Worldwide, 360 346 neonates succumbed from sepsis and infections in 2011 (Du Pont-Thibodeau *et al.,* 2014). *S. pyogenes* is an uncommon cause of infection in the neonatal period but description of cases of severe infection in newborns is infrequent (Salto *et al.,* 2011).

Group A β-hemolytic streptococcus is a prevalent cause of a wide range of infections in infants, children, and adults. Group A streptococcal infections have long been associated with serious disease and death,

but toward the middle of the 20th century, a marked reduction in the prevalence and intensity of such infections occurred (Tuner *et al.,* 2013).

S. pyogenes has presented a major cause of neonatal and puerperal sepsis. This association has become less frequent during the past three decades. *S. pyogenes* has been cultured from the anus and vagina of pregnant women, from umbilical stumps and the hands and nasopharynx of neonates, mothers and nursery staff, but most of the *S. pyogenes* infections were asymptomatic or cases were associated with omphalitis (Greenberg *et al.,* 1999).

Group A Streptococcus was a major cause of neonatal sepsis before the antibiotic era, but its incidence declined with the availability of antibiotics and improved perinatal infection prevention measures. It is now a rare cause of neonatal sepsis. *S. pyogenes* sepsis in early infancy can be associated with a variety of complications such as a pulmonary infection, often with rapid progression to necrotizing pneumonia. Although an unusual cause of sepsis in the first months of life, GAS remains an important pathogen for the delivering mother and new born infant (Janampet *et al.,* 2014).

During the past decade there have been an increased number of reports of severe *S. pyogenes* infections in children and adults associated with fulminant course (Greenberg *et al.,* 1999). However, the number of cases of severe *S. pyogenes* neonatal sepsis reported in the literature during the past 30 years is small (Greenberg *et al.,* 1999).

2.1.8 Disease burden in the world

Carapetis *et al.,* 2015 estimated that there was a minimum of 517 000 deaths each year as a result of severe GAS diseases. The global prevalence of severe GAS disease is at least 18.1 million cases, with 1.78 million new cases annually (Walker, 2014). Rheumatic heart disease is the greatest burden, with a global prevalence of at least 15.6 million cases, with 282 000 new cases and 233 000 deaths yearly (Walker, 2014). The burden of invasive GAS diseases is astonishingly high, with at least 663 000 recent cases and 163 000 deaths each year (Nelson *et al.,* 2016). Furthermore, there are an additional 111 million prevalent cases of GAS pyoderma, and over 616 million incident cases per year of GAS pharyngitis (Walker, 2014).

Various clinical manifestations of *S. pyogenes* are more common in different parts of the world. Streptococcal caused pharyngitis is more common in temperate areas and peaks in late winter and early spring whereas impetigo is more prevalent in a warm humid climate. School-aged children carry *S. pyogenes* in their throats and are more at risk of having the disease (NCCID, 2018).

Worldwide, there are estimated to be over 600 million cases of streptococcal throat infection and more than 100 million cases of GAS pyoderma annually. Although uniformly susceptible to penicillin and still impeccably susceptible to many other antimicrobial agents, GAS infection continues to present serious clinical and public health challenges. The majority of *S. pyogenes* infections are of short duration and are relatively quick to resolve. However, invasive disease can be serious and life-threatening. In addition, GAS differs from other pyogenic bacteria in their potential to produce delayed sequelae, e.g., post streptococcal acute glomerulonephritis (PSAGN) and acute rheumatic fever (ARF) following uncomplicated infections (Efstratiou & Lamagni, 2017).

Despite being in existence for hundreds of years, *S. pyogenes* remains a significant cause of global disease and death, with a particular impact in low income settings. The majority of cases of acute rheumatic fever (ARF), rheumatic heart disease (RHD), acute post streptococcal glomerulonephritis (APSGN), and invasive *S. pyogenes* cases occur in these low-resource settings (Carapetis, 2005).

2.1.9 Disease burden in Africa

The true burden of group A streptococcal disease in Africa is not known. GAS is a significant cause of mortality and morbidity on the global scale and in developing countries. According to global disease burden figures, the WHO (2005) ranked GAS as the ninth leading cause of human mortality, with the majority of deaths attributable to invasive GAS infections and rheumatic heart disease. Developing countries, which account for 80% of the world's population, continue to experience high cases of acute rheumatic fever/rheumatic heart disease affecting some 2.4 million children aged 5–14 years old residing in developing countries (Barth *et al.,* 2015). On the global scale, GAS is an important cause of morbidity and mortality primarily in developing countries, with >500 000 deaths annually (Nizet, 2018). According to the WHO (2005) estimates suggest that GAS caused a substantial burden of disease and death on a global scale, mainly in children and young adults and in less developed countries.

Improved living conditions and access to healthcare during the last century are credited for the considerable decline in the prevalence of GAS in developed countries. However, a few studies have been reported from within Africa, and in these GAS carriage ranged around 9.0%. Studies in South African reported GAS carriage isolation rates, which ranged from 1.62% to 16.8% (Engel & Mayosi, 2013). The

prevalence of GAS pharyngitis, is often higher in developing countries and impoverished communities within industrialised nations. The most recent data from South Africa was collected more than 30 years ago with rates then ranging from 23.2% to 45.5% (Engel & Mayosi, 2013). There are no incidence data on GAS pharyngitis in Africa. Data on GAS carriage from countries in Africa remain scarce with only a few studies reporting on carriage (Engel & Mayosi *et al.,* 2017).

Timely and targeted antibiotic treatment of GAS infections constitute the backbone of prevention of complications (Karthikeyan & Mayosi, 2009). The delivery of preventive interventions has been difficult in settings with fragile health systems and limited access to care, and insufficient to show major impacts (Watkins *et al.,* 2017).

2.1.10 Disease prevention and management

Hand contact is generally considered the primary route of GAS transmission, and GAS vulvovaginitis has been associated with a household or personal history of GAS-related skin or respiratory infection (Verkaeren *et al.,* 2014). GAS pharyngitis is most commonly spread through direct person-to-person transmission. Typically transmission occurs through saliva or nasal secretions from an infected person (CDC, 2019). People with group A strep pharyngitis are much more likely to transmit the bacteria to others than asymptomatic pharyngeal carriers. Crowded conditions such as those in schools, daycare centres, or military training facilities facilitate transmission (Efstratiou, 2017).

Penicillin or amoxicillin is the antibiotic of choice to treat GAS pharyngitis. There has never been a report of a clinical isolate of group A streptococcus that is resistant to penicillin. However, resistance to azithromycin and clarithromycin is common in some communities (Brook, 2017). For patients with a penicillin allergy, recommended regimens include narrow-spectrum cephalosporin's (cephalexin, cefadroxil), clindamycin, azithromycin, and clarithromycin (CDC, 2019). One method of minimizing antibiotic use while maximizing its benefit would be to recommend prophylaxis only for those household contacts who are at the highest risk of subsequent invasive GAS infection and/or for those at the highest risk of death from invasive infection. However, epidemiological studies of invasive GAS infection have identified several risk factors for sporadic disease (CDC, 2002).

2.1.11 GAS virulence factors

S. pyogenes causes over 600 million infections annually making it a predominant human pathogen. GAS throat infections are common in children between 4 and 7 years and pose several clinical and public health challenges (Dharmapalan *et al.,* 2018). M proteins, pili, leukocidins, streptolysins (O & S), complement inhibiting proteins, immunoglobulin-degrading enzymes, and superantigens are genome-encoded virulence factors that have been well characterized in *S. pyogenes*, where efflux pumps and leukocyte evasion strategies stay as integral factors (Dharmapalan *et al.,* 2018). High genomic plasticity is seen in *S. pyogenes* due to the prophage integration and horizontal gene transfer (Dharmapalan *et al.,* 2018).

Super antigens are significant virulence factors in the pathogenesis of invasive disease caused by *S. Pyogenes*. Super antigen genes were distinguished to determine the prevalence, occurrence and genetic restriction amongst different *emm* types of GAS. Superantigen genes are not randomly distributed amongst GAS isolates (Commons, 2008). In cultures of *S. pyogenes* isolated from patients and carriers in different territories of the Russian Federation, the genes of erythrogenic toxins A, B and C (*SpeA, SpeB* and *SpeC*) were detected. The prospect of the identification of *S. pyogenes* by method of polymerase chain reaction (PCR) on the basis of primers to erythrogenic toxin B was determined by Dmitrieva *et al.,* 2002 in a study looking at the frequency of genes in GAS strains and identification by PCR. The *SpeB* gene was identified in the *S. pyogenes* cultures included in the study and demostrated species specificity (Dmitrieva *et al.,* 2002).

GAS secretes several pyrogenic exotoxins including *SpeA, SpeB, SpeC,* and *SpeD.* These are responsible for pyrogenic activity, superantigenic activation of T cells of specific repertoire, and augmentation of endotoxic shock. *SpeB* degrades a wide variety of host and bacterial cellular and extracellular components including matrix glycoproteins (FN, vitronectin, laminin, and integrin), IL-1, chemokines, immunoglobulins, and complement components (Hamada *et al.*, 2015).

The ability of GAS to cause diseases with multiple clinical manifestations results from virulence factors which are variably expressed among GAS strains and result in different host susceptibilities (Lynskey *et al.,* 2011). Upon GAS detection, the immune system launches a complex response which critically depends on the recruitment and activation of neutrophils, macrophages, and dendritic cells (Mishalian *et al.,* 2011). These processes are dependent on the activation of innate immune responses by interactions between pattern recognition receptors (PRRs) with GAS-derived pathogen-associated molecular patterns (PAMPs) (Fieber & Kovarik, 2014).

Figure 2.4 below is a representation of the evasion of innate immunity by GAS

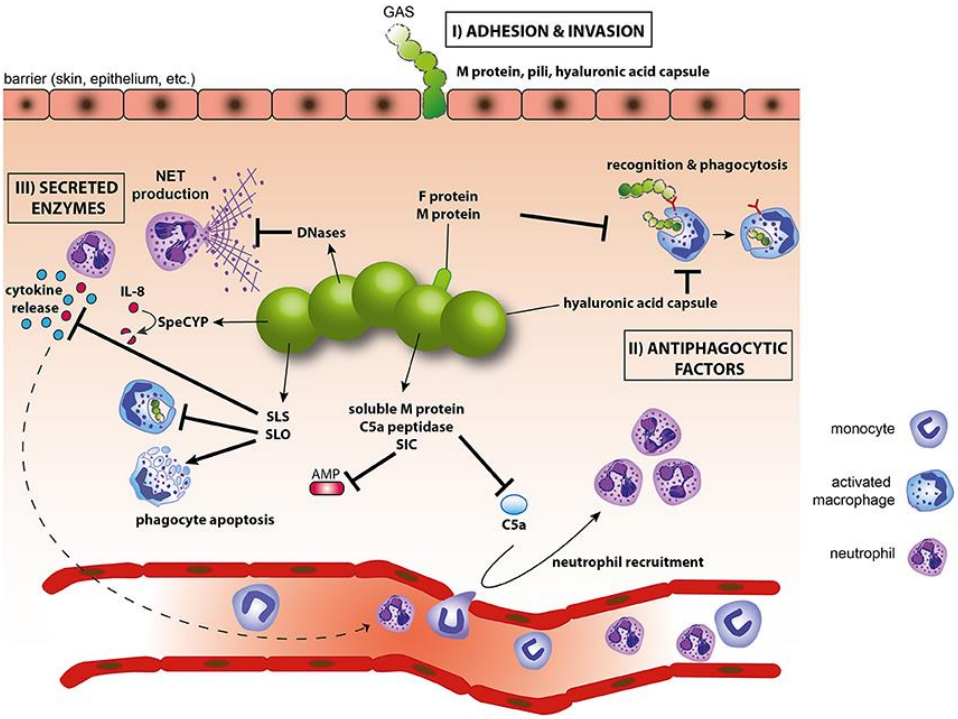

Figure 2. 4 [Responses of innate immune cells to Group A *Streptococcus]* (2014). Retrieved from https://www.researchgate.net/figure/Evasion-of-innate-immunity-by-GAS-I-M-protein-F-proteins-of-pili-and-the-hyaluronic_fig1_267045271

Figure 2.4 describes the evasion of innate immunity by GAS: (I) M protein employs antiphagocytic virulence factors to avoid the first line of defence, i.e., phagocytic cells. (II) Streptolysin O (SLO), also inhibits phagocytosis and aids in the killing of GAS within phagolysosomes. (III) C5a peptidase inhibits complement activation and antimicrobial peptides and prevents phagocyte recruitment. SLO induces apoptosis of phagocytes and Streptolysin (SLS) interferes with cytokines or cytokine production. Neutrophil extracellular trap (NET) is also a streptococcal inhibitor of complement.

2.2 Methods of isolation and identification of GAS

Laboratory diagnosis of streptococcal infections relies upon the efficient isolation of β-haemolytic streptococci and its identification as GAS. Streptococci are commonly grown on an agar medium supplemented with blood. This allows the detection of β-haemolysis, which is paramount in identification

steps, and enhances the growth of streptococci by the addition of an external source of catalase. Serological grouping by the Lancefield method is the criterion standard for differentiation of pathogenic streptococcal species but group A organisms can be identified more cost effectively by numerous latex agglutination, co agglutination, or enzyme immunoassay procedures (Spellerberg, 2016).

2.2.1 Blood agar

Historically, *S. pyogenes* was first cultured and identified as the cause of erysipelas by Friedrich Fehleisen in 1883, and it received its species designation from Rosenbach in 1884. Today, laboratory diagnosis of group A streptococcal infections still largely relies on culturing bacteria from clinical specimens. To detect *streptococci* in clinical samples (and especially *S. pyogenes*), the material is most often cultured on blood agar plates, which facilitates an easy preliminary screen for β-haemolytic colonies (Spellerberg & Brandt, 2016). Blood agar is an enriched, bacterial growth medium. Fastidious organisms, such as *streptococci*, do not grow well on ordinary growth media (http://www.oxoid.com). When cultured on blood agar plates, the production of a characteristic zone of complete hemolysis (β-haemolysis) is an important clue to the classification of *S pyogenes.* Media for culturing Gram positive bacteria such as Columbia agar with colistin and nalidixic acid also provide adequate culturing conditions for *S. pyogenes*. Optimal incubation conditions for the vast majority of streptococcal strains include a temperature range of 35°C to 37°C in the presence of 5% CO_2 or under anaerobic conditions (Skariyachan, 2014).

2.2.2 Bacitracin sensitivity

Group A strains can also be distinguished from other streptococci by their sensitivity to bacitracin. A disc that contains 0.04U of bacitracin inhibits the growth of more than 95% of group A strains, whereas 80-90% of non–group A strains are resistant to this antibiotic. The bacitracin disc test is simple to perform and interpret and is sufficiently accurate for presumptive identification of GAS. Although many laboratories use bacitracin sensitivity as a presumptive identification test for GAS, it may give false positive results with Group C and Group G *streptococci* (Brahmadathan & Gladstone, 2006).

2.2.3 Catalase test

The enzyme catalase breaks down hydrogen peroxide, yielding water and oxygen. This results in visible formation of bubbles of oxygen. The test is performed to distinguish between catalase positive organisms e.g. *Staphylococcus* species, and catalase negative organisms e.g. *Streptococcus* species (Encyclopaedia Brittanica, 2017).

2.2.4 Pyrolidonyl aminopeptidase detection test

Presumptive identification of a strain as a group A streptococcus can also be made on the basis of production of the enzyme L-pyrrolidonyl-beta-naphthylamide (PYRase). Among the β -hemolytic streptococci isolated from throat culture, only group A isolates produce PYRase, which can be identified on the basis of the characteristic color change (red) after inoculation of a disk on an agar plate followed by overnight incubation (Khan, 2018). A negative catalase and agglutination with Lancefield group A antiserum (Slidex, Streptokit; bioMe´rieux, Marcyl'Etoile, France) can also be used to confirm GAS (Facklam, 2002).

2.3 Molecular methods

The advances in Polymerase Chain Reaction (PCR) technology targeting transcriptional regulator genes provide the most reliable and rapid method for detection of pathogenic bacteria (Kumar *et al.,* 2011). Transcriptional regulators are specialized DNA binding proteins which play a crucial role in directing gene expression within bacteria for their adaptation and survival in different environmental conditions. S*py*1258 is a putative transcriptional regulator gene (*TetR/AcrR* family) which is specific for *S. pyogenes* and can be used as a marker for its detection (Liu *et al.,* 2005).Molecular mechanisms mediating GA*S* host interactions remain poorly understood but are crucial for diagnostic, therapeutic, and vaccine development (Graham *et al.,* 2006). Streptococcal pyrogenic exotoxins A (*SpeA*) and C (*SpeC*) are members of a family of superantigens produced by group A streptococci that appear to play a key role in the pathogenesis of streptococcal toxic shock syndrome (Christ, Meals & English, 1997). The *SpeB* gene is chromosomally encoded, whereas the exotoxin-encoding genes *SpeA* and *SpeC* are carried by lysogenic phages, and are easily disseminated to other GAS (Baidya *et al.,* 2017).

2.3.1 Antimicrobial susceptibility of GAS (Drug classes and sub classes)

Antibiotics are antimicrobial agents produced by microorganisms that inhibit the growth or kill other microorganisms while being harmless to the host cells (Yang *et al*., 2014). The determination of the susceptibility of pathogens to antibiotics is necessary for the selection of the most appropriate one for treating microbial infections. Antibiotics which kill bacteria are said to be bactericidal, while those that only prevent their multiplication are referred to as bacteriostatic. However, some antibiotics can act as both bacteriostatic and bactericidal depending on their concentration. Antibiotics are evaluated for their inhibitory potentials (Owuama, 2017).

One approach to treat severe invasive *S. pyogenes* infections has been to utilize a combination of penicillin and clindamycin. The rationale is that penicillin provides coverage against 100% of *S. pyogenes* strains and clindamycin has demonstrated greater efficiency in experimental models of necrotizing fasciitis (Ikebe *et al.,* 2005).

Most of the *S. pyogenes* strains are susceptible to penicillin (Bassetti *et al.,* 2000), and hence penicillin is universally recommended for treatment of *S. pyogenes* infections (Bowen *et al.,* 2012). However, macrolides are used as an alternative in penicillin-allergic patients (Camara *et al.,* 2013). Further, increased macrolide-resistance and asymptomatic oropharyngeal colonization of *S. pyogenes* have been reported in different countries (Chang *et al.,* 2011). An increase of up to 50% in *streptococci* resistance to penicillin has been observed (Nunes *et al.,* 2005). Surveillance studies have shown temporal changes in drug resistant *streptococci*, predominantly due to environmental factors, and this could be a major reason for drug-resistant strains causing pediatric and adult diseases (Camara *et al.,* 2013).

According to a study by Camara *et al.,* 2013 in Senegal 40 isolates were sensitive to β-lactam antibiotics including penicillin, amoxicillin, and cephalosporins. Out of the 40 isolates, 100% of the isolates were resistant to tetracycline.

Erythromycin is an effective macrolide antibiotic for treating GAS respiratory tract infections. However, increasing erythromycin resistance in GAS isolates was noted in the 1990s, and in some countries, this resistance peaked in the early 2000s. Several resistance genes, *mef(A), erm(B)*, and *erm(A)* subtype *erm(TR)*, are associated with erythromycin resistance in GAS. *mef(A)* encodes an efflux pump which selectively removes 14- and 15-membered macrolides from the bacterial cell and is commonly associated with the M phenotype resistant to macrolides but susceptible to lincosamides and streptogramin. In

addition, the erythromycin resistance genes often co-occur with the tetracycline resistance genes *tet(O)* and *tet(M)* (Huang *et al*,.2014).

Table 2. 1 Susceptibility rates of *Streptococcus pyogenes* below shows the result of susceptibility testing of *S. pyogenes* isolates against 17 antibiotics with disk diffusion method.

ANTIBIOTICS	MEAN VALUES ± SD (mm)	R (%)	I (%)	S (%)
β-lactamins				
Penicillin G	30.43 ± 2.3	0	5	95
Amoxillin	30.93 ± 2.9	0	0	100
Cefixime	23.7 ± 2.76	0	0	100
Cefpodoxine	28.5 ± 3.2	0	0	100
Cefotaxime	28.73 ± 2.6	0	0	100
Ceftriaxone	29.43 ± 2.9	0	0	100
Macrolides				
Erythromycin	24.7 ± 2.1	0	2.5	97.5
Spiramycine	20.33 ± 3.2	37.5	40	22.5
Azythromycin	20.36 ± 1.66	0	0	100
Lincosamines				
Clindamycin	21.64 ± 2.45	0	2.6	97.4
Streptogramines				
Pristinamycin	25.53 ± 2.55	2.5	0	97.5
Ketolides				
Telithromycin	25.28 ± 2.34	0	7.5	92.5
Phenicols				
Chloramphenicol	24.03 ± 2.85	0	17.9	82.1
Glycopeptides				
Teicoplanin	18.28 ± 2.72	0	0	100
Vancomycine	18.85 ± 1.73	0	0	100
Fluoroquinolones				
Levofloxacin	19.05 ± 1.65	0	0	100
Tetracyclines				
Tetracycline	9.53 ± 2.36	100	0	0

*Susceptibility rates have been interpreted according to CLSI breakpoints.
Abbreviations: R, resistant; I, intermediate; S, susceptible.

Table 2.1 [Susceptibility rates of *S. pyogenes* (Disk diffusion)] (2013). Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3987753/pdf/mbi-6-2013-071.pdf

Table 2. 2 Susceptibility rates of *S. pyogenes* and MIC values (E-test)is a presentation of MIC values of 10 antibiotics tested against *S. pyogenes* isolates.

ANTIBIOTICS	R (%)	I (%)	S (%)	MIC_{50}	MIC_{90}	GEOM MEAN	MIC RANGE
β-lactamins							
Penicillin G	0	0	100	0.016	0.023	0.012	0.004–0.032
Cefpodoxime	0	0	100	0.016	0.016	0.016	0.0016–0.023
Cefixime	0	0	100	0.094	0.094	0.071	0.016–0.125
Cefotaxime	0	0	100	0.023	0.023	0.021	0.008–0.047
Ceftriaxone	0	0	100	0.023	0.023	0.023	0.012–0.064
Macrolides							
Erythromycin	0	0	100	0.094	0.125	0.079	0.032–0.019
Azythromycin	0	2.5	97.5	0.38	0.5	0.355	0.125–0.75
Phenicols							
Chloramphenicol	0	0	100	3	4	0.723	2–4
Fluoroquinolones							
Levofloxanine	0	0	100	0.75	0.075	0.738	0.38–2
Glycopeptides							
Teicoplanin	0	0	100	0.094	0.094	0.206	0.023–2

*Susceptibility rates have been interpreted according to CLSI breakpoints.
Abbreviations: R, resistant; I, intermediate; S, susceptible; MIC, minimal inhibitory concentration (mg/L).

Table 2.2 [Susceptibility rates of Streptococcus pyogenes and MIC values (E-test)] (2013). Retrieved form https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3987753/pdf/mbi-6-2013-071.pdf

Table 2.1 shows the results of susceptibility testing of 40 *S. pyogenes* isolates against 17 antibiotics with disk diffusion method, while MIC range, geometric means, and the calculated MIC_{50} and MIC_{90} values of 10 antibiotics tested are shown in table 2.2 (Camara *et al*., 2013). According to a nationwide laboratory based study conducted in Germany from 2003-2013 by Imöhl & van der Linden (2015), isolates were susceptible to penicillin, cefotaxime and vancomycin. The highest rate of resistant or intermediate resistant isolates was with tetracycline with 9.8%, macrolides (4.0%) following, trimethoprim/sulfamethoxazole (SXT) (1.9%), levofloxacin (1.3%), chloramphenicol (0.9%) and clindamycin (0.7%) (Imöhl & van der Linden, 2015). The resistance rates of *S. pyogenes* to several antibiotics vary considerably worldwide. Resistance rates from 2% to 98% have been reported for macrolides (Imöhl & van der Linden, 2015). While in several European countries, an increase of macrolide resistance has been described during the last 10–20 years, recently a decrease has been noted in some of these countries (Imöhl & van der Linden, 2015). In a study by Camara *et al.,* 2013 in Dakar Senegal a total of 40 strains were isolated and their susceptibility to 17 antibiotics was tested using a standard disk

diffusion method. The minimum inhibitory concentrations (MICs) were determined using the E-test. All isolates were sensitive to β-lactam antibiotics including penicillin, amoxicillin, and cephalosporins. Macrolides remained active with the exception of spiramycin, which showed reduced susceptibility (Camara *et al.,* 2013).

2.3.2 Susceptibility to β-lactams

In a study by Camara *et al.,* (2013) the β-lactam antibiotics showed high activity with low MIC_{90} ranging from 0.016 to 0.094 mg/L. Penicillin remains effective with an MIC_{90} value of 0.023 mg/L, although two strains showed intermediate susceptibility to this molecule. All isolates were found to be susceptible to amoxicillin, cefixime, cefpodoxime, cefotaxime, and ceftriaxone (Camara *et al.,* 2013). *S. pyogenes* has persisted in being universally susceptible to β-lactams since the 1940s. However, a compelling number of treatment failures have been reported (Gillespie, 1998). According to Bonofiglio *et al.*, 2018 following 70 years of use, penicillin is still globally active against GAS, GCS and GGS. However, therapeutic failures have been recorded in 2–28% of pharyngitis cases (median: 12%) attributable to different causes (Bonofiglio *et al.*, 2018).

2.3.3 Susceptibility to macrolides, lincosamins, streptogramins-B, and ketolids (MLSB K)

A study by Camara *et al.,* 2013 in Senegal demonstrated that in isolates of *S. pyogenes* obtained from respiratory tract infections that erythromycin showed good activity with 97.5% of displaying susceptibility and only 2.5% with intermediate susceptibility. Azythromycin remains fully active as all 40 isolates are completely susceptible. Erythromycin and azythromycin had MIC_{90} values of 0.0125 mg/L and 0.5 mg/L, respectively. In contrast, more than half of the isolates were resistant to spiramycin with 37.5% of resistance and 40% intermediate susceptibility. Clindamycin showed high activity with 97.4% of the strains susceptible. Only 2.5% of the isolates were resistant to pristinamycin. 92.5% of the strains were susceptible to telithromycin, and 7.5% showed reduced susceptibility (Camara *et al.,* 2013).

According to Brown & Ryback (2004), a total of 4508 isolates of *S. pyogenes* were collected during 2001–2002 and, of the 4496 isolates with culture source information, 4159 (92.5%) were from throat cultures. All isolates were susceptible to penicillin. Overall, 5.7% of isolates were resistant (MIC >_1 mg/L) and 0.3% were intermediate (MIC 0.5 mg/L) to erythromycin, with similar results for clarithromycin (5.5% resistant, 0.1% intermediate) and azithromycin (5.6% resistant, 0.3% intermediate). The MIC_{90} of telithromycin was

0.03 mg/L, which was lower than those of azithromycin (0.25 mg/L) and erythromycin (0.12 mg/L). The MIC range for telithromycin against *S. pyogenes* was 4 mg/L for 12 isolates.

2.4 Methods used for antimicrobial susceptibility testing (AST)

2.4.1 Kirby Bauer disk diffusion method

A few methods used for evaluating antibiotics include the filter paper disc (Kirby-Bauer) method (Bauer *et al.,* 1966). According to Kirby-Bauer disk diffusion procedures, the density of bacterial strains solutions are adjusted to 0.5 McFarland standard (bioMerieux) by suspending the inoculum in sterile saline (0.9 % NaCl) which is equivalent to an optical density (OD) of 0.08 to 0.1 at 600 nm. Mueller-Hinton agar (BioMaxima) plates are swabbed with a lawn of the bacterium suspension and within 15 minutes the sterile cotton antimicrobial susceptibility test discs (BioMaxima) are placed onto plates. Each isolate is subjected to incubation at 35 °C and its optimal temperature, i.e. 22°C or 37°C (Schwalbe, Steele-Moore & Goodwin, 2007). Results can be read after 24-48 hours and interpretation of sensitive, intermediate and resistant is made according to the zone sizes and in accordance to the published Clinical & Laboratory Standards Institute (CLSI) guidelines.

2.4.2 E-test

The E-test has been developed to provide a direct quantification of antimicrobial susceptibility of microorganisms (Reller *et al.,* 2009). The device consists of a predefined, continuous, and exponential gradient of antibiotic concentrations immobilized along a rectangular plastic test strip. After 48 hours incubation, a drop-shaped inhibition zone intersects the graded test strip at the inhibitory concentration (IC) of the antibiotic. A predefined stable antimicrobial gradient is present on a thin inert non-porous plastic carrier strip 5 mm wide, 60 mm long known as E-test strip. When this E test strip is applied onto an inoculated agar plate, there is an immediate release of the drug and establishment of an antimicrobial concentration gradient in an agar medium. After overnight incubation, the tests are read by viewing the strips from the top of the plate, a symmetrical inhibition ellipse is produced. The intersection of the lower part of the ellipse shaped growth inhibition area with the test strip indicates the MIC value (Narayanan *et al.,* 2019).

2.4.3 Vitek- 2 identification and sensitivities method

The VITEK system originated in the 1970s as an automated system for identification and AST and has evolved today into the VITEK 2 system, which automatically performs all of the steps required for identification and AST after a primary inoculum has been prepared and standardized (Ligozzi *et al.,* 2002).

2.4.4 Genes coding for resistance

In the treatment of infectious diseases the usage of antibiotics is vital in the defence of public health. The critical escalation in the use and misuse of antimicrobial drugs in clinical and agricultural environments has presented a corresponding increase in the concentration of compounds found in waste streams and the environment in general (WHO, 2001). The macrolide resistance genes *mef*(A), *erm*(A) and *erm*(B), and the tetracycline resistance gene *tet*(M) were searched for by PCR as described (Pires *et al.*, 2005). Erythromycin resistance in staphylococci is encoded by the *erm* genes. All isolates found to be resistant to erythromycin by phenotypic methods contained at least 1 erythromycin resistance gene (Graham *et al.,* 2006). In *Streptococcus pyogenes*, efflux-mediated erythromycin resistance is associated with the *mef* gene, represented mostly by *mef*(A), although a small portion of strains carry different *mef* subclasses (Del Grosso *et al.,* 2011). A global sample of group A streptococci (GAS) revealed ≥ 80 separate acquisitions of tetracycline resistance. Of 244 clones, 38 and 25% displayed resistance to tetracycline and erythromycin, respectively; and a relatively high proportion (15%) were resistant to both classes of drugs *Tet*(M) displayed a highly significant association with *erm*(B) (Ayer *et al.,* 2007). A high level of resistance to erythromycin and tetracycline was seen in β haemolytic streptococci in India although discordance between genotypic and phenotypic results was reported (Bhardwaj *et al.,* 2018). Absence of *erm(A)* and *tet(O)* with high prevalence of *tet(M)* and *erm(B*) was observed (Bhardwaj *et al.,* 2018).

2.5 Problem Statement

According to global disease burden figures, the WHO ranked GAS as the ninth leading cause of human mortality, with the majority of deaths attributable to invasive GAS infections (Ralph & Carapetis, 2013). Reproductive tract infections are one of the most serious public health issues in both developed and developing countries. In Africa, around 65 000 000 cases of genital tract infections occur annually (Mombasheri *et al.,* 2014). Puerperal fever is a devastating disease affecting women within the first three days after childbirth and progressing rapidly, causing acute symptoms of severe abdominal pain, fever and debility (Hallett, 2005). Group A streptococci is documented as one of the principal infectious agents in human puerperal fever (Busowski *et al.*, 2013). Group A βhaemolytic streptococcus is the infectious cause of puerperal fever. *S. pyogenes* is strictly a human pathogen usually found in the skin and throat and less frequently in the rectum and the female genital tract (Zakour *et al.,* 2012). GAS is a major cause of maternal and neonatal morbidity and mortality in the world and the problem is more pronounced in resource limited countries (Fillipi *et al.,* 2016). Antibiotic resistance is increasing to commonly available drugs and treatment options have become limited (Ventola, 2015). According to Indongo, (2014) A retrospective audit of maternal records was conducted during the period of 2008-2012 with detailed analysis of 154 maternal deaths recorded in Namibia. It was found that 53% of women who lived in rural areas suffered from puerperal sepsis (Indongo, 2014). Deaths due to sepsis were higher in Africa, with an odds ratio of 2:71 (Khan *et al.,* 2006). The most frequent causes of maternal deaths in Africa (for 1997-2002) were uncontrolled bleeding (haemorrhage), sepsis or infections inclusive of HIV. In Namibia, the maternal mortality rate has increased almost two fold since 2000 and is now 200 deaths per 100 000 live births (UNICEF, 2008). Neonatal mortality is also elevated (19 deaths per 1000 live births) accounting for 52% of under 5 mortality. HIV AIDS is implicated in 59% of maternal deaths and 14% of infant deaths (UNICEF, 2008). Currently, there are no statistics on GAS prevalence in pregnant women in Namibia and antimicrobial susceptibility patterns. Determining the prevalence of GAS in pregnant women at 35-37 weeks gestation and the resistance patterns of isolates are crucial steps in the development of clinical policies and procedures for the management of GAS infections ultimately reducing maternal and neonatal morbidity and mortality. Due to lack of scientific data to this effect, there are currently no policies in Namibia for screening of GAS in mothers and babies before and after delivery. This study aims to provide scientific evidence on prevalence of GAS in mothers and babies in Windhoek.

2.6 Research objectives

Main objective: To determine the prevalence of GAS in pregnant women at 35-37 weeks gestation and neonates in Windhoek, and assess the genetic basis of resistance to antibiotics in those isolates.

Specific objectives:

1. To determine the prevalence of Group A *Streptococcus* in women at 35-37 weeks gestation.
2. To establish the prevalence of Group A *Streptococcus* in babies with infections post-delivery.
3. To evaluate the antimicrobial susceptibility patterns and gene based resistance of the Group A *Streptococcus* isolates.

CHAPTER THREE:

3. Methodology

3.1 Study design

This was a quantitative descriptive cross sectional study which was done at the antenatal and postnatal clinic at the Windhoek Central Hospital (WCH) maternity ward. A consecutive sampling method was used to collect a total of 165 samples using consecutive sampling. Patients that attended antenatal care at the clinic and that met the inclusion criteria were asked to participate in the study. Participants were asked to complete a consent form and samples were then collected from the participants.

3.2 Study population

This study targeted pregnant women at 35 - 37 weeks of gestation that attended antenatal care at the Windhoek Central Hospital and neonates at WCH. A population of approximately 165 pregnant woman and 18 neonates participated.

3.3 Inclusion Criteria

Pregnant women at 35-37 weeks gestation who attended antenatal care at the Windhoek Central Hospital and also mothers and neonates that attended the postnatal clinic at the Windhoek Central Hospital who had infections suggestive of GAS such as pelvic pain, fever and foul smelling vaginal discharge.

3.4 Exclusion Criteria

Pregnant women below 35 weeks gestational age or above 37 weeks gestation and those who received antibiotic treatment for GAS or who had been treated with antibiotics in the previous 3 months were excluded from the study.

3.5 Study setting

This study was conducted at the Windhoek Central Hospital (WCH) maternity ward situated in the Khomas Region of Namibia. Specimens were collected form women who attended antenatal screening as well as neonates who attended post-natal screening with their mothers at the selected hospital. Khomas Region is the most populous region in Namibia with an estimated population of 342 141 (Khomas Census Regional Profile, 2011) and Windhoek Central Hospital is the referral hospital for regional hospitals. Laboratory analysis was carried out at the Namibia University of Science and Technology (NUST), Department of Health Sciences.

3.6 Sample Size

Equation 1.1below, adopted from Mead & Winn., 2000 was used to calculate the number of participants who were included in the study, whereby **n** is number of participants, **z** is confidence interval 95% (1.96), **p** is proportion of *S. pyogenes* colonization among pregnant women and **d** was margin of error (5%) = 0.05. Ten percent for non-response rate was added.

Equation 1.1

$$n = [\frac{(z)2 \times P(1-P)}{\sqrt{d}}]$$

3.7 Sampling technique

A consecutive sampling method was used to recruit participants which included selection of participants based on their availability. This technique was suitable for this study because it allowed inclusion of participants based on their availability and voluntary participation. The participants who consented were recruited consecutively.

3.8 Materials and methods

3.8.1 Specimen collection and transportation

Questionnaires were administered to participants (Appendix C), which included questions relating to socio economic status, eligibility criteria, signs and symptoms and medical history including gravidity, parity and gestational age. All participants were required to give written consent to participate in the study (Appendix A). The study used the collection criteria set according to the Centres for Disease Control and Prevention (CDC) morbidity and mortality weekly report of May 1996, which proposes that a vaginal swab from a pregnant woman be collected between the 35th and 37th week of gestation. A vaginal and rectal swab was collected by a registered nurse from each of the 165 patients. A swab was carefully inserted into the vagina about 5 cm and gently rotated for 10 to 30 seconds. It was ensured that the swab touched the walls of the vagina so that moisture was absorbed by the swab and then withdrawn without touching the skin. The swab was inserted into the rectal sphincter, rotated, and withdrawn. The swab was then examined for fecal staining. Ear, eye and wound (if present) swabs were collected from neonates by a registered nurse. A sterile swab was placed into the outer ear and gently rotated to collect secretions. If a purulent discharge was present this was also sampled. For wound collection, a sterile swab was gently rotated on the area to collect the exudate which was then placed into transport medium. The swabs were transported in Amie's transport medium at room temperature (RT). Amie's transport medium preserves

the microbiological specimens and maintains viability of microorganisms without causing significant increase in growth or compromising the quality of the specimens (Gumede *et al.,* 2017). GAS will remain viable on plates for only 5–7 days after streaking if stored at room temperature (Gera & McIver, 2013). Specimens were kept in the 2-8 °C fridge prior to culture.

3.8.2 Preparation of culture media

Manufacturer instructions were followed to ensure a high quality medium which supported the growth of bacteria was used in the study.

3.8.2.1 Preparation of Columbia blood agar

As per manufacturer recommendation, 39 g of blood agar base was weighed out and added to 1 liter of distilled water. The mixture was transferred to a sterile 1000 mL Schott bottle. Once the mixture was dissolved completely it was sterilized by autoclaving at 121 °C for 15 minutes. The citrated blood was then brought to room temperature while the autoclaved liquid was kept at 50 °C to prevent solidification. The 50 mL blood was mixed before transferring it to the agar mixture and then poured into standard (100 mm X 15 mm) petri dishes, flamed over to remove bubbles and then left to solidify. Plates were then labelled and dated and stored in the 2-8 °C fridge.

3.8.2.2 Preparation of nutrient agar

Nutrient Agar is used to support growth of a wide variety of microorganisms. The medium contains gelatin and beef extract which provide nitrogen, carbon, vitamins, and amino acids in Nutrient Agar. Agar is the solidifying agent (http://www.oxoid.com). As per manufacturer recommendation, 28 g of agar was mixed with 1 litre of distilled water. The mixture was then autoclaved at 121°C for 15 min, cooled and before solidifying it was poured into standard sterile petri dishes on a level, horizontal surface to give uniform depth. The plates were then labelled and dated and stored in the 2-8 °C fridge.

3.8.2.3 Quality control of media

Culture media plays an important role in any microbiology laboratory. It is extensively used for isolation, identification and sensitivity testing of many pathogenic organisms. To ensure that the media is of high standard yielding satisfactory results, a proper quality management system is crucial (Basu, Pal & Desai, 2004). The media quality is determined directly upon the quality of the raw materials used in preparation.

Water was an essential raw material used in the preparation of the culture media. The pH of water should not be less than 5.5 (http://www.oxoid.com). There were various additives used in the preparation of the media. Blood was one of the most important additives used in the production of the media plates. Sterility, uniformity, viscosity and colour of the blood was observed before it was used for media production (WHO, 2003). Sterilization plays an important role in the quality of the media. Autoclaving at 121 °C for the sterilization of the media was used in this study. Overall the physical appearance of media often suggests the quality. The media prepared was also screened for physical characteristics such as excessive bubbles, unequal filling of plates, freezing and contamination. Control organisms for *S. pyogenes* was used in this study (ATCC 19615).

3.8.2.4 Isolation and identification of presumptive *S. pyogenes* isolates

All 366 swabs collected in this study were cultured on Columbia agar supplemented with 5% horse blood and incubated in 5% CO_2 for 24 to 48 hours at 37 °C for the isolation of GAS*. S. pyogenes* isolates were phenotypically identified by bacteriological characteristics. When properly performed and interpreted, culturing swabs on a 5% sheep blood agar with trypticase soy base incubated in air remains the gold standard and reference method for the diagnosis of *S. pyogenes* acute pharyngitis (Shulman *et al.,* 2012). When cultured on blood agar plates, the production of a characteristic zone of complete haemolysis (beta haemolysis) is used for presumptive identification of *S. pyogenes.* Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. A small inoculum of presumptive bacterial isolate was mixed into a hydrogen peroxide solution (3%) and observed*. S. pyogenes* is catalase negative. The lack of catalase is noted by a lack of or weak bubble production. Presumptive isolates were cultured on blood agar again with a disk containing 0.04U of bacitracin. Group A strains can also be distinguished from other groups by their sensitivity to bacitracin. A disc that contains 0.04U of bacitracin inhibits the growth of more than 95% of group A strains (Khan *et al.,* 2018).

3.8.2.5 Preservation of presumptive *S. pyogenes* isolates

The presumptive isolates were preserved in a 50% glycerol solution. Bacterial glycerol stocks are important for long-term storage of plasmids. The addition of glycerol stabilizes the frozen bacteria, preventing damage to the cell membranes and keeping the cells alive. A glycerol stock of bacteria can be stored stably at -80 °C for many years (Engelbrecht, 2015). All presumptive *S. pyogenes* isolates from the participants were labelled and stored at -80 °C for further processing.

3.9 Molecular confirmation of presumptive isolates

3.9.1 Preparation of fresh isolates prior to molecular analysis

Isolates preserved in 50% glycerol were resuscitated by thawing and inoculating on nutrient agar followed by incubation at 37 °C for 24 to 48 hours.

3.9.2 DNA extraction

DNA was extracted from all sixteen *S. pyogenes* isolates obtained in this study. An ATCC 19615 control strain was included in this study. Sterile colonies were picked up with a sterile loop and emulsified into 2 ml Eppendorf tubes with 200 uL of nuclease free water. The tubes were then loaded onto a heating block and boiled at 100 °C for 15 min. After boiling, the suspensions were cooled and centrifuged for 10 min at 8000 revolutions per minute (rpm). The supernatant containing DNA was transferred to a sterile labeled Eppendorf tube and preserved at -20°C prior to use.

3.9.3 Preparation of primers

The primers were prepared according to the manufacturer's instructions (Inqaba Biotec, Pretoria, South Africa)

Table 3.1 Oligonucleotide primers used for molecular confirmation of presumptive *S. pyogenes* isolates.

Primer	Sequence (5' -3')	Fragment size (bp)	References
SpeA-F	ATGGAAAACAATAAAAAAGTATTG		
SpeA-R	TTACTTGGTTGTTAGGTAGACTTC	755	Duran *et al., (*2012)
SpeB-F	ATGAATAAAAAGAAATTAGGTGTCAG		
SpeB-R	CTAAGGTTTGATGCCTACAA	1200	Duran *et al., (*2012)
SpeC-F	GCAGGGTAAATTTTTCAACGACACACA		
SpeC-R	TGTGCCAATTTCGATTCTGCCGC	407	Duran *et al., (*2012)

3.9.4 Confirmation of *S. pyogenes* using PCR

Twelve microliters of One Taq^{R} Master Mix with standard buffer (Ingaba Biotechnical Industries , Pretoria, South Africa) containing: 10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 5% glycerol, 0.08% IGEPAL^{R} CA-630, 0.05% Tween^{R} 20, 0.024% Orange G, 0.003% Xylene Cyanol and 33 units/mL One Taq DNA polymerase, was mixed with 10.5 µL of nucleic acid free water, 1 uL each of 10 pMol of reverse and forward primers for the genes, 5 uL of DNA template to make a final reaction volume of 28.5 uL.

The cycling conditions were as follows: 94 ˚C for 4 min as an initial denaturation followed by 35 cycles of denaturation at 93 ˚C for 1 min adapt in all, annealing at the respective annealing temperature for 1 min and extension at 72 ˚C for 1 min with a final elongation step of 72 ˚C for 7 min followed by a hold at 4 ˚C. Amplification was verified in a 2% agarose gel electrophoresis at 110 volts for 45 min in a 1X TBE buffer and thereafter viewed in a transilluminator and photographed. *S. pyogenes* ATCC 19615 was used a positive control.

3.9.5 Antimicrobial susceptibility testing

The antimicrobial susceptibility on all sixteen isolates was determined using the Kirby Bauer disk diffusion method. The disk diffusion method was performed according to the CLSI guidelines (CLSI, 2018). A colony of each confirmed *S. pyogenes* isolate was used to prepare an inoculum equivalent to a 0.5 McFarland standard (CLSI, 2018). A sterile cotton swab was dipped into the broth culture of each isolate and streaked evenly all over the surface of a 5% blood agar plate. Antibiotic sensitivity testing disks were placed on the inoculated plates using a sterile needle. The plates were then incubated at 37 ˚C for 18-24 h. The zones of inhibition were measured using a sliding Vernier calliper and were considered as sensitive, intermediate or resistant to an antibiotic based on CLSI, 2018. The *S. pyogenes* isolates were tested against the following antibiotics: Erythromycin 15ug, Tetracycline 30ug, Clindamycin 2ug and Penicillin 10ug and Vancomycin 2ug.

3.9.6 List of primers used in the study

Table 3.2 Oligonucleotide primers used for molecular screening of resistant determinants

Primer	Sequence (5' -3')	Fragment size (bp)	References
ErmA-F	CCGCAAGGAGAAGGTTATAATGA		
ErmA-R	GCATTCACCCGTTGACTCATTTCC	190	Ouba *et al.* (2008)
ErmB-F	GCTCTTGCACACTCAAGTCTCGAT		
ErmB-R	ACATCTGTGGTATGGCGGGTAAGT	117	Ouba *et al., (*2008)
mefA-F	GACCAAAAGCCACAATTGTGGA		
mefA-R	CCTCCTGTCTATAATCCCATG	1432	Ouba *et al., (*2008)
tetO-F	AACTTAGGCATTCTGGCTCAC		
tetO-R	TCCCACTGTTCCATATCGTCA	515	Ouba *et al., (*2008)
tetM-F	TTCCAACCATACAATCCTTG		
tetM-R	ATGGAAAACAATAAAAAATATTG	1080	Ouba *et al., (*2008)

3.9.6.1 Multiplex PCR for gene based resistance testing

Twelve microliters of One Taq^{R} Master Mix with standard buffer (Ingaba Biotec , South Africa) containing: 10 mM Tris-HCl, 1.5 mM MgCl2, 50 mM KCl, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.2 mM dTTP, 5% glycerol, 0.08% IGEPAL^{R} CA-630, 0.05% Tween^{R} 20, 0.024% Orange G, 0.003% Xylene Cyanol and 33units/mL One Taq DNA polymerase, was mixed with 10.5 µL of nucleic acid free water, 1 uL each of 10 pMol of reverse and forward primers for the respective capsular types, 5 uL of DNA template to make a final reaction volume of 28.5 uL.

The cycling conditions were as follows: 94 ˚C for 4 min as an initial denaturation followed by 35 cycles of denaturation at 93 ˚C for 1 min adapt in all, annealing at the respective annealing temperature for 1 min and extension at 72 ˚C for 1 min with a final elongation step of 72 ˚C for 7 min followed by an infinite hold at 4 ˚C. Amplification was verified in a 2% agarose gel electrophoresis at 110 volts for 45 min in a 1X TBE buffer and thereafter viewed in a transilluminator and photographed. *S. pyogenes* ATCC 19615 was used as a positive control.

3.10 Data analysis

All statistical analysis and data obtained from the study questionnaires and experimental results were imported from excel into the Statistical Package for Social Sciences (SPSS) version 24 for data analysis. Data was analysed to establish the prevalence of GAS in women at 35-37 weeks gestation and also to

determine whether GAS infection was a complication in neonates. Additionally, data was processed to unravel the susceptibility of GAS to antibiotics commonly used for treatment in Namibia.

3.11 Ethical considerations

Ethical approval of the study was obtained from NUST's research ethics committee and the Ministry of Health and Social Services of Namibia (MoHSS). Written, informed consent was sought and obtained from the participants in languages that they understood. Confidentiality was maintained regarding participants information. Direct identifiers such as the patient name appeared on the consent form and was separated from the main questionnaire within 24 hours. Study IDs matching the samples collected were assigned to the participants. Information was stored securely in a file and locked in a file cabinet when not in use and handled only by the researcher when actively used during research. The data will be stored for 3 years after the study has completed and thereafter be destroyed.

CHAPTER FOUR

4. Results

4.1 Prevalence of *S. pyogenes* carriage among pregnant women and neonates

The age range of the population was 18 – 45 years and the average age was 28.9 years with a median of 28. The average gravidity was 2.52 and the average parity was 2.26. A total of 165 vaginal and rectal swabs were collected from 165 women while 16 women declined to take part in the study. A total of 18 ear and eye swabs were collected from neonates at their 6 week follow up. Table 4.1 presents the prevalence of *S. pyogenes* in pregnant women and neonates recruited in the study

Table 4.1

			Frequency (n)	Percentage (%)	Total
Neonate culture results	Ear	+	0	0	18 (100%)
		-	18	100	
	Eye	+	0	0	18 (100%)
		-	18	100	
Pregnant women culture results	Vaginal	+	9	5.5	165 (100%)
		-	156	94.5	
	Rectal	+	6	3.6	165 (100%)
		-	159	96.4	
					(n=165)

Key: -= sample where *S. pyogenes* was not isolated += sample where *S. pyogenes* was isolated

Out of the 165 screened pregnant women, 15 women (9.0%) were colonized by *S. pyogenes.* The prevalence of vaginal *S. pyogenes* in pregnant women was therefore 5.4% and rectal colonization was 3.6%. A total of 18 neonates who attended their 6 week (post birth) follow up at the Windhoek Central Hospital Antenetal Clinic were also screened for *S. pyogenes* colonization. No colonization was found.

In table 4.2 below the association of S. pyogenes colonization with demographic and socio-economic characteristics is shown. The pregnant women who were colonized by *S. pyogenes* were between the ages of >20-≤39, single, employed and from a low income area.

Table 4.2

Factors	Colonized (n)	Percentage (%)	Not colonized (n)	Percentage (%)
Age (Years)				
≤20	0	0	9	5.5
>20-≤39	15	9.1	132	80.0
>40	0	0	9	5.5
Educational Level				
<Matric	0	0	9	6.0
Matric	8	53.3	105	70.0
Tertiary	7	46.7	36	24.0
Employment				
Employed	9	60	59	39.3
Un employed	6	40	91	60.7
Marital Status				
Single	9	60	120	80.0
Married	6	40	30	20.0
HIV Status				
Negative	14	93.3	141	94.0
Positive	1	6.7	9	6.0
Geographical Location				
Low Income	12	80	116	77.3
Middle Income	3	20	34	22.7

The statistical significance of the association was not established due to the low numbers of isolates under each parameter.

The Frequency distribution of the presumptive identification and confirmation of *S. pyogenes* using oligonucleotide primers for *Spec A, B* and *C* genes is dispalyed in table 4.3 below. All 15 presumptive *S. pyogenes* isolates were confirmed using the *SpeB* gene.

Table 4.3

Confirmation gene		**Frequency (n)**	**Percentage (%)**
SpeA	Present	0	0
	Absent	15	100
SpeB	Present	15	100
	Absent	0	0
SpeC	Present	0	0
	Absent	15	100

(n= 15)

In table 4.4 below shows the susceptibility patterns of the *S. pyogenes* isolates obtained from the study.There was high resistance to clindamycin and penicillin by the *S. pyogenes* isolates. The isolates were sensitive to tetracycline, vancomycin and erythromycin.

Table 4.4

Antimicrobial agent	**Percentage of GAS antibiotics sensitive, intermediate and resistant**					
	Sensitive (S)		**Intermediate (I)**		**Resistant (R)**	
	Frequency (n)	**Percentage (%)**	**Frequency (n)**	**Percentage (%)**	**Frequency (n)**	**Percentage (%)**
Erythromycin	15	100	0	0	0	0
Clindamycin	1	6.7	0	0	14	93.3
Penicillin	5	33.3	0	0	10	66.7
Tetracycline	15	100	0	0	0	0
Vancomycin	15	100	0	0	0	0
						(n=15)

The Frequency distribution of genes coding for drug resistance in *S. pyogenes* isolates can be seen in table 4.5 below. Genes coding for resistance to antibiotics were screened among *S. pyogenes* isolates and Although all the isolates were sensitive to tetracycline, 26.7% presented with the *tet M* gene and 100% presented with the *TetO* gene. While most of the isolates with 93.3 % were resistant to clindamycin there no genotypic resistance noted.

Table 4.5

Resistant genes		**Frequency (n)**	**Percentage (%)**
ErmA	Present	0	0
	Absent	15	100
ErmB	Present	0	0
	Absent	15	100
MefA	Present	0	0
	Absent	15	100
TetO	Present	15	100
	Absent	0	0
TetM	Present	4	26.7
	Absent	11	73.3

(n=15)

CHAPTER 5

5. Discussion

This study provides the first population-based estimate of the incidence of GAS colonization among pregnant women and neonates in Windhoek. The study aimed at determining the prevalence of *S. pyogenes* infections in neonates post delivery. As shown in Table 4.1 the prevalence in neonates was 0%. *S. pyogenes* was not detected when the samples from the neonates were cultured. *S. pyogenes* infections may be observed in persons of any age, although the prevalence of infection is higher in children, presumably because of the combination of multiple exposures (in schools or nurseries, for example) and weak host immunity (Martin, *et al.,* 2004). Disease in neonates is uncommon, which may reflect a protective, transplacentally-acquired immunity (Efstratiou & Lamagni, 2014). While the incidence of many diseases has declined in developed countries, regions of the world with low income and poor infrastructure continue to suffer a high burden of *S. pyogenes* diseases with millions of deaths yearly (Carapetis *et al.*, 2005). In a study by Hamilton *et al.*, 2013 infant mortality was 0%, implying once more that maternal acquisition of GAS took place well after delivery. The low prevalence rate found in this study may be attributed to the small number of neonates sampled. Mothers were reluctant to consent to sample collection as they felt it was invasive and traumatic.

The study also aimed at determining the prevalence of *S. pyogenes* colonization in pregnant women at 35 to 37 weeks gestation as well as colonization in neonates at the Windhoek Central Hospital's Antenatal Clinic. According to Verkaeren *et al.,* 2014 GAS infection involving endometritis and vulvovaginitis is an infrequent occurrence and is more frequently reported in adolescent girls than in adult women. GAS is not commonly symptomatic, and the genital and rectal tracts were found to be colonized by GAS in 0.03% of 6944 women at 35–37 weeks of (Mead & Winn *et al.*, 2000). The prevalence of *S. pyogenes* in pregnant women in this current study was 9.1% (Table 4.1). Fifteen isolates were recovered from 165 swabs collected from pregnant women. Vaginal colonization with *S. pyogenes* in pregnant women was 5.4% and rectal colonization was 3.6%. A study by Chuang *et al.,* 2002 in the United States shows that the occurrence of GAS postpartum infection yearly is 6 per 100 000 live births, with maternal mortality estimated at 2%. Puerperal sepsis causes approximately 75 000 maternal deaths annually worldwide (Maharaj, 2007) with maternal mortality peaking in Asia (11.6%), Africa (9.7%), and Latin America/Caribbean (7.7%) Compared to studies done by Verkaeren *et al.,* 2014 (4.8%) and Mead & Winn, 2000 (0.03%), the colonization in pregnant women in this study was higher (9%) but was not significant enough to motivate for screening of GAS in pregnant women during ANC.

A study done by The American College of Obstetricians and Gynecologists, 2014 found that, compared with a base pregnancy rate per cycle of approximately 20% at age 30–31 years, pregnancy colonization rates decline steadily beginning at age 34–35 years, with average rates of 13.2% at age 38–39 years and 6.6% at age 42–44 years. In this study, the median age of women with a gestational age of 35-37 weeks was 28.9 years as shown in Table 4.2. The relation between age and fertility cuts across many intertwined domains: biologic, social, political, economic, scientific, medical, and technologic (The American College of Obstetricians and Gynecologists, 2014). The age group most colonized in this study was 25-30 years. According to Chuang *et al.*, 2002 the ages of case patients were 14 to 42 years with a mean of 29 years. A Pubmed database search done by Hamilton *et al.,* 2013 indicated that GAS infections occurring either during pregnancy or postpartum had a mean age of 28.7 years.

A study done by Bello *et al.,* 2010 in South Africa on time to pregnancy and pregnancy outcomes reported a mean gravidity of 2.3 while the mean parity was 2.0. There were a total of 2467 pregnancies included in the study (Bello *et al.,* 2010). This study had a mean gravidity of 2.52 and the average parity was 2.26, with a total of 165 pregnancies in this study.

In this study, 12 (80%) of the women colonized with *S. pyogenes* were from low income settings. The occurrence of invasive *S. pyogene*s infection differs by time and geographical region, which presumptively imitates a population's susceptibility to selective strains, but also the natural diversity in the predominant types (O'Brien *et al.,* 2002). Considerably higher rates are observed in developing countries and within indigenous populations in developed countries, such as the USA and Australia, which range from 12 to 83/100,000 (Efstratiou & Lamagni, 2014).

The presumptive identification of GAS is commonly done by colonial morphology and haemolysis on blood agar and testing for sensitivity of isolates to bacitracin even though there are reports about the occurrence of bacitracin resistant strains (Olender *et al.,* 2012). Many routine clinical laboratories use this criteria for diagnosing GAS infections (Menon, Lloyd & Jacob, 2005). In this study the *SpeB* gene was used to confirm that the isolates were *S. pyogenes* in a method adopted from Ma *et al.,* 2006. The *SpeB* gene is carried by all strains of *S. pyogenes*, but the degree of expression varies from strain to strain (Ma *et al.,* 2006). All presumptive isolates were confirmed as GAS by molecular techniques as shown in Table 4.3.

Penicillin still remains the treatment of choice for infections due to GAS. For mild to moderate infections including pharyngitis and skin and soft tissue infections, oral penicillin V at a dose of 500 mg two to three times a day for 10 days is recommended (Stevens *et al.,* 2016). A first-generation cephalosporin is an

acceptable alternative unless there is a history of immediate hypersensitivity to a β-lactam antibiotic. Macrolides (erythromycin, clarithromycin and azithromycin) and lincosamides (clindamycin) are commonly used first-line drugs against GAS infections in patients with beta-lactam allergies (Ashurst & Edgerley-Gibb, 2019). A combination of penicillin and clindamycin has been utilized to treat invasive *S. pyogenes* infections Tadayoshi *et al.,* 2005. There was a high resistance to clindamycin and penicillin by the *S. pyogenes* isolates in this study as shown in Table 4.4. This is in agreement with studies done by Tadayoshi *et al.,* 2005 in Japan (1.4% clindamycin).

The reappearance of *S. pyogenes* as a source of significant human infections in the USA, Europe, and elsewhere has been comprehensively recorded in recent years and has intensified public awareness about this organism (Efstratiou & Lamagni, 2016). An overall resurgence of disease, combined with the absence of a certified *S. pyogenes* vaccine and existing concern about the acquisition of penicillin resistance, remains a significant concern and highlights the seriousness of heightening global surveillance of this pathogen (Efstratiou & Lamagni, 2014).

The study also aimed at determining the antimicrobial susceptibility patterns and gene based resistance of the isolates. The rapid emergence of resistant bacteria is occurring worldwide, endangering the efficacy of antibiotics, which have transformed medicine and saved millions of lives (Ventola, 2015). Tetracycline is an antibiotic that has been utilized considerably in human and veterinary medicine for decades. Although its usage in human therapy has reduced in recent years, its consumption in the agricultural environment is still extensive (Miranda *et al.,* 2018). Resistance to tetracycline is due to numerous genes that code for one of three mechanisms: efflux pumps, ribosomal protection proteins, or enzyme degradation. Many of these genes are found on mobile genetic elements that carry resistance to other antibiotics (Roberts, 2005; Zhang *et al.*, 2011). GAS has maintained a reasonable level of susceptibility to tetracycline, being an alternative for penicillin-allergic patients infected with macrolide-resistant strains. Resistance to tetracycline is due to genetic acquisition of ribosomal protection genes - *tet(O)* and *tet(M) (Bharwaj, 2018).* This current study found a low resistance to tetracycline by GAS isolates (as presented in Table 4.5).This is in agreement with studies done by Dundar *et al.,* 2010 in Turkey and Lu *et al.,* 2017 in China who also reported low resistance of GAS to tetracycline. However, studies by Bhardwaj *et al.*, 2018 and Abraham *et al.,* 2018 in India reported an increased resistance of GAS to tetracycline. A high level of resistance to erythromycin and tetracycline was observed in β haemolytic streptococci in India. Inconsistency in the genotypic and phenotypic results was reported a lack of *erm(A)* and *tet(O)* with high prevalence of *tet(M)* and *erm(B)* was observed (Bharwaj *et al.,* 2018)

5.1 Conclusion

Infection with *S. pyogenes* causes a wide variety of diseases, it is an ubiquitous organism which can cause both non-invasive and invasive infections (Khan *et al.,* 2016). Understanding the nature and diversity of its complications is an integral part of medicine (Khan *et al.,* 2016). Invasive GAS infections constitute a significant epidemiological problem. The majority of cases occur in women during the postnatal period (Busowski *et al.,* 2013). Maternal mortality is highest in sub-Saharan Africa and direct causes of mortality accounted for 77.7% while indirect causes contributed 22.3% (Ngonzi *et al.,* 2016). Low resource countries account for 99% (286 000) of the global maternal mortalities with sub-Saharan Africa responsible for the bulk of the maternal deaths and accounting for 62% followed by southern Asia at 24% (Ngonzi *et al.,* 2016). The most frequent cause of maternal mortality was puerperal sepsis (30.9%).

This study aimed at determining the prevalence of GAS in pregnant women at 35-37 weeks gestation and the resistance pattern of *S. pyogenes* isolates. Determining the latter would assist in the development of clinical policies and procedures for the management of GAS infections in Namibia. Therefore reducing maternal and neonatal morbidity and mortality. This study concluded that compared to 0.03% found by Mead & Winn, 2000 and 4.8% by Verkaeran *et al.,* 2014 the maternal colonization with GAS in pregnant women at the WCH was 9.1%. The women colonized by GAS were between the ages of >20-≤39, single, from a low income area and interestingly employed.

According to Janampet *et al.,* 2014 *s. pyogenes* sepsis in early infancy can be associated with a variety of complications such as a pulmonary infection, often with rapid progression to necrotizing pneumonia. Thus the study also looked at determining the prevalence of *s. pyogenes* infections in neonates post delivery,it was found to be 0.0%. Only 18 of the 40 parents approached for consent agreed, the other 22 refused as they argued that the sample collection procedure was invasive and would be traumatic for the neonates.

According to Bassetti *et al.,* 2000 most *S. pyogenes* strains are susceptible to penicillin, hence its universal recommendation for treatment of these infections (Bowen *et al.,* 2012). Further, increased macrolide-resistance and asymptomatic oropharyngeal colonization of *S. pyogenes* have been reported in different countries (Chang *et al.,* 2010). The study found that out of the 15 *S. pyogenes* isolates tested against erythromycin, penicillin G, tetracycline, clindamycin and vancomycin, resistance to clindamycin was 93.3% and 66.7% with penicillin. Although it was found that all the isolates were sensitive to tetracycline with the disk diffusion test this current study found a low resistance to tetracycline by GAS isolates in the

genotypic testing. , 26.7% presented with the *tet M* gene and 100% presented with the *TetO* gene. While most of the isolates (93.3 %) were resistant to clindamycin there was no genotypic resistance noted.

Infection with GAS can have a significant implication on the health of a pregnant women and neonates if left untreated. The main aim of this study was to determine the prevalence of GAS in pregnant women at 35-37 weeks gestation and neonates in Windhoek, and assess the genetic basis of resistance to antibiotics in those isolates. The study showed that vaginal-rectal colonization with GAS was an uncommon finding in pregnant women and neonates at the WCH maternity ward. In conclusion these findings do not necessitate the screening of women in pregnancy and neonate's post-partum.

5.2 Limitations

The prevalence for this study cannot be generalized to the entire Namibian population of pregnant women, the samples collected were only from the Windhoek Central Hospital's Maternity Antenatal Clinic. The calculated sample size was also not met due to financial constraints and a time limit. Therefore the results obtained in this study are only applicable to the WCH's maternity ward that was included in the study.

The study also aimed at determining the prevalence of *S. pyogenes* infections in neonates post delivery. The prevalence of GAS in neonates was not found because no colonization was noted when the neonatal samples were cultured. Also, only 18 of the neonates parents consented to taking ear and eye swabs. The majority of the parents refused to give consent as they felt the swabbing would be invasive and cause discomfort to their babies. This is a contributing factor to the low number of samples collected from neonates.

Sample collection took longer than expected as there was trouble finding a registered nurse to do the swabbing. Also, the completion of the questionnaires was done by the investigator as the nurses refused to take time out to inform and assist the participants.

Nutrient agar instead of blood agar was used to culture colonies of *S. pyogenes* for use in DNA extraction. *S. pyogenes* is most often cultured on blood agar plates. It is a fastidious organism, and does not grow well on ordinary growth media. Therefore fine colonies were observed on the Nutrient agar. Blood plates were not used as contamination from the sheep blood (DNA & Hb) would have interfered in the polymerases activity during PCR. Due to financial constraints a boiling method to extract DNA from the

isolates was used. Light bands were observed on the gels that were done and this could be due to the minimal concentration of DNA extracted.

5.3 Recommendations

Further studies with a larger population should be conducted as this might have a more favourable outcome. This in turn would advocate the need to screen pregnant women for GAS strongly. Also, addititonal genes encoding resistance associated with *S. pyogenes* resistance should be analyzed using molecular techniques.

Educating the recruited patients on the sample collection in both the women and neonates might have aided in more of them consenting to have swabs taken.

A nutrient broth could have been used for the cultivation of the organism after thawing of the isolates. It supports the growth of microorganisms that are not very nutritionally demanding. Gelatin peptone and beef extract provide nitrogen, vitamins, minerals and amino acids essential for growth. Furthermore a DNA extraction kit which would have yielded a higher quality of DNA could have been used instead of the boiling method.

5.4 Significance and contribution

Increasing the knowledge on the prevalence in mothers and babies with infections related to Group A *Streptococcus* postpartum will help in the clinical management of these patients. This will likely reduce maternal and infant morbidity and mortality. Antibiotic profiling in this study facilitates the choice of antibiotic prophylaxis and treatment options for pregnant women, mothers and neonates with GAS colonization.

5.4.1 Capacity building

The research is important for capacity building and it will be published in a peer reviewed journal. This is a leading study in this area in Namibia and other aspects of this research can be investigated by other students for their Honours, Masters and PhD studies.

5.4.2 Contribution to policy

The findings of this study will assist in establishing whether GAS should be screened in pregnant women routinely or only in suspected cases. Investigation of drug resistance in GAS will inform the MoHSS on treatment options for GAS in Namibia**.**

REFERENCES

Al-Ameri, G., Al-Kolaibe, A. (2015). Identification and genotypic analysis of Streptococcus pyogenes isolated from pharyngitis and tonsillitis infected children in IBB city in Yemen. *Journal of Microbiology and Antimicrobials, Vol. 7(3), pp. 21-27, DOI:10.5897/JMA2013.0277* Anderson, B. (2014). Puerperal Group A Streptococcal Infection. *Obstetrics & Gynecology*, 1 DOI: 10.1097/AOG.0000000000000175

Ashurst, J. V., Edgerley-Gibb, L.(2019) Streptococcal Pharyngitis. *StatPearls Publishing*.

Avila, M., Ojcius, D. M., & Yilmaz, O. (2009). The oral microbiota: living with a permanent guest. *DNA and cell biology*, *28*(8), 405-11.

Ayer, V., Tewodros, W., Manoharan, A., Skariah, S., Luo, F., Bessen, B. E. (2007). Tetracycline Resistance in Group A Streptococci: Emergence on a Global Scale and Influence on Multiple-Drug Resistance. *Antimicrobial Agents and Chemotherapy*. 51 (5) 1865-1868; DOI: 10.1128/AAC.01341-06

Baidya, A., Das Bimal K, Kapil Arti, Ghosh Arnab, Mathur Purva, Kabra Sushil K, Chandra Dinesh, Juneja Rajnish (2017). Characterisation of *Streptococcus pyogenes* on the basis of pyrotoxin exotoxin genes in north India Volume: 19 Issue Number: 2Page: 104-109

Barth, D. D., Mayosi, B. M., Jabar, A., & Engel, M. E. (2015). Prevalence of group A streptococcal disease in North and Sub-Saharan Africa: a systematic review protocol. *BMJ open*, *5*(8), e008646. doi:10.1136/bmjopen-2015-008646

Barth, D., Mayosi, B., Badri, M., Whitelaw, A., & Engel, M. (2018) Invasive and non-invasive group A β-haemolytic streptococcal infections in patients attending public sector facilities in South Africa: 2003–2015, *Southern African Journal of Infectious Diseases*, 33:1, 12-17, DOI: 10.1080/23120053.2017.1376546

Bassetti, M., Manno, G., Collidà, A., Ferrando, A., Gatti, G., Ugolotti, E., … Bassetti, D. (2000). Erythromycin resistance in Streptococcus pyogenes in Italy. *Emerging infectious diseases*, *6*(2), 180–183. doi:10.3201/eid0602.000212

Bello, B., Kielkowski, D., Heederik, D., & Wilson, K. (2010). Time-to-pregnancy and pregnancy outcomes in a South African population. *BMC public health*, *10*, 565. doi:10.1186/1471-2458-10-565

Bhardwaj, N., Mathur, P., Behera, B., Mathur, K., Kapil, A., & Misra, M. C. (2018). Antimicrobial resistance in beta-haemolytic streptococci in India: A four-year study. *The Indian journal of medical research*, *147*(1), 81–87. doi:10.4103/ijmr.IJMR_1517_16

Bonofiglio, P., Gagetti, G., Gabarrot, G., Kaufman, S., Mollerach, M., Toresani, I., Vigliarolo, L., von Specht, M., Lopardo, H. (2017). Susceptibility to β-lactams in β-hemolytic streptococci. *Revista Argentina de Microbiología,* Volume 50, Issue 4, October–December 2018, Pages 431-435

Bowen, A. C., Lilliebridge, R. A., Tong, S. Y., Baird, R. W., Ward, P., McDonald, M. I., Currie, B. J., Carapetis, J. R. (2012). Is Streptococcus pyogenes resistant or susceptible to trimethoprim-sulfamethoxazole? *J Clin Microbiol.;50(12):4067-72. doi: 10.1128/JCM.02195-12. Epub 2012 Oct 10*

Brahmadathan, K. N., Gladstone, P. (2006). Microbiological diagnosis of Streptococcal Pharyngitis: Lacunae and other implications. *Indian Journal of Medical Microbiology,24 (2):92-6*

Brook I. (2017). Treatment Challenges of Group A Beta-hemolytic Streptococcal Pharyngo-Tonsillitis. *International archives of otorhinolaryngology*, *21*(3), 286–296. doi:10.1055/s-0036-1584294

Brook, I. (2013). Penicillin failure in the treatment of streptococcal pharyngo-tonsillitis. *Current Infectious Disease Reports.* 15(3):232–235.

Brooks, G., Carroll, K., Butel, J., Morse, S., Timothy A., (2013). *Jawetz, Melnick, & Adelberg's Medical Microbiology, 26e.* The McGraw-Hill Companies, Inc. All rights reserved. ISBN 978-0-07-179031-4 MHID 0-07-179031-4 ISSN 1054-2744

Brown, S. D., Rybak, M. J. (2004). Antimicrobial susceptibility of Streptococcus pneumoniae, Streptococcus pyogenes and Haemophilus influenzae collected from patients across the USA, in 2001–2002, as part of the PROTEKT US study. *Journal of Antimicrobial Chemotherapy* 54, Suppl. S1, i7–i15 DOI: 10.1093/jac/dkh313

Busowski, M.T., Lee, M., Busowski, J.D., Akhter, K., Wallace, M.R. (2013). Case Report Puerperal Group A Streptococcal Infections: A Case Series and Discussion. *Hindawi Publishing Corporation Case Reports in Medicine* Volume 2013, Article ID 751329, 4 pages http://dx.doi.org/10.1155/2013/751329

Camara, M., Dieng, A., Boye, C. S. (2013). Antibiotic Susceptibility of Streptococcus Pyogenes Isolated from Respiratory Tract Infections in Dakar, Senegal. *Microbiology Insights*. 6 71–75 doi:10.4137/MBI.S12996.

Canny, G. O., & McCormick, B. A. (2008). Bacteria in the intestine, helpful residents or enemies from within? *Infection and immunity*, *76*(8), 3360-73.

Carapetis, J. R., Steer, A. C., Mulholland, E. K., Weber M. (2005). The global burden of group A streptococcal diseases. *The Lancet Infectious Diseases, 5(11)685-94. doi:10.1016/51473-3099(05)70267-x.*

Centres for Disease Control and Prevention. (2008). Streptococcus Laboratory. Retrieved from https://www.cdc.gov/streplab/m-proteingene-typing.html on 10th of July 2017.

Chang, J. C., LaSarre, B., Jimenez, J. C., Aggarwal, C., Federle, M. J. (2011). Two group A streptococcal peptide pheromones act through opposing Rgg regulators to control biofilm development. *PLoS Pathogens*; 7(8):e1002190. doi: 10.1371/journal.ppat.1002190. Epub 2011 Aug 4.

Chiwuze, J., Oronsaye, A., Okolocha, C., Onoguwe, B., (1998) Factors associated with high maternal morbidity and mortality in Ekpoma, Nigeria. African J Med Pract 1998, 5(4):187-93

Christ, E. A., Meals, E., English, B. K. (1997). Streptococcal pyrogenic exotoxins A (SpeA) and C (SpeC) stimulate the production of inducible nitric oxide synthase (iNOS) protein in RAW 264.7 macrophages. PMID: 9421860

Chuang, I., Van, B.C., Beall, B., Schuchat, A. (2002). Population-based surveillance for postpartum invasive group A streptococcus infections, 1995-2000. Clinical Infectious Diseases: an official publication of the Infectious Diseases Society of America.35:665–670.

Cogen, A. L., Nizet, V., Gallo, R. L. (2008). Skin Microbiata: A Source of Disease or Defense. *British Journal of Dermatology*, doi: 10.1111/j.1365-2133.2008.08437.

Commons, R., Rogers, S., Gooding, T., Danchin, M., Carapetis, J., Robins-Browne, R., Curtis, N. (2008). Superantigen genes in group A streptococcal isolates and their relationship with. *Journal of medical microbiology. 57. 1238-46. 10.1099/jmm.0.2008/001156-0.*

Creti, R., Imperi, M., Baldassarri, L., Pataracchia, M., Recchia, S., Alfarone, G., Orefici, G. (2007). *emm* Types, Virulence Factors, and Antibiotic Resistance of Invasive Streptococcus pyogenes Isolates from Italy: What Has Changed in 11 Years? *Journal of Clinical Microbiology*, p. 2249–2256 Vol. 45, No. 7 doi:10.1128/JCM.00513-07

Del Grosso, M., Camilli, R., Barbabella, G., Blackman Northwood, J., Farrell, D. J., & Pantosti, A. (2011). Genetic resistance elements carrying mef subclasses other than mef(A) in Streptococcus pyogenes. *Antimicrobial agents and chemotherapy*, *55*(7), 3226–3230. doi:10.1128/AAC.01713-10

Demirgül, F., & Tuncer, Y. (2017). Detection of Antibiotic Resistance and Resistance Genes in Enterococci Isolated from Sucuk, a Traditional Turkish Dry-Fermented Sausage. *Korean journal for food science of animal resources*, *37*(5), 670–681. doi:10.5851/kosfa.2017.37.5.670

Dharmapalan, D., Inbanathan, F. Y., Kharche, S., Patil, A., Joshi, S., Yewale, V., Daniel, J., Walia, K., Veeraraghavan, B. (2018). Whole genome shotgun sequences of Streptococcus pyogenes causing acute pharyngitis from India. *Data in brief*, *18*, 1340-1349. doi:10.1016/j.dib.2018.03.129

Dmitrieva, N., Trofimov, D., Eshchina, A., Riapis, L., Pavlova, O., Petrova, T., Skorkina, I., Gerasimov, A., Alekseev, L., Zhuravlev, M., Briko, N. (2002). Frequency of genes speA, speB, and speC in Streptococcus pyogenes strains and the identification of the infective agent by polymerase chain reaction. *Zhurnal mikrobiologii, epidemiologii, i immunobiologii. 3-6.*

Du Pont-Thibodeau, G., Joyal, J. S., & Lacroix, J. (2014). Management of neonatal sepsis in term newborns. *F1000prime reports*, *6*, 67. doi:10.12703/P6-67

Dundar, D., Sayan, M., Tamer, G.S. (2010). Macrolide and Tetracycline Resistance and emm Type Distribution of Streptococcus pyogenes Isolates Recovered from Turkish Patients. *Microbial Drug Resistance Volume 16*. DOI: 10.1089/mdr.2010.0021

Duran, N., Ozer, B., Duran, G. G., Onlen, Y., & Demir, C. (2012). Antibiotic resistance genes & susceptibility patterns in staphylococci. *The Indian journal of medical research*, *135*(3), 389–396.

Efstratiou, A. (2017). WHO Global Collaborating Centre for Reference and Research on Diphtheria and Streptococcal Infections, Public Health England, London, UK Healthcare-Associated Infections and Antimicrobial Resistance Department, Public Health England.

Efstratiou, A., & Lamagni, T. (2016). Epidemiology of Streptococcus pyogenes. Streptococcus pyogenes. *Basic Biology to Clinical Manifestations.*

Engel, M. E. and Mayosi, B. M. (2013). Clinical and epidemiological aspects of streptococcus pyogenes pharyngitis and carriage in Africa. *SA Heart Journal,* DOI: https://doi.org/10.24170/10-2-1802

Engel, M. E., Cohen, K., Gounden, R., Kengne, A. P., Barth, D. D., Whitelaw, A. C., Francis, V., Badri, M., Stewart. A., Dale, J. B., Mayosi, B. M., Maartens, G. (2017). The Cape Town Clinical Decision Rule for Streptococcal Pharyngitis in Children. *Pediatr Infect Dis J.*;36(3):250-255. doi: 10.1097/INF.0000000000001413

Engelbrecht, F. (2015). The antimicrobial susceptibility and gene-based resistance of Streptococcus Agalactiae (group B Streptococcus) in pregnant women in Windhoek (Khomas region), Namibia. Cape Peninsula University of Technology.

E-TEST (Epsilometer test): Principle, purpose, procedure, results and interpretations (2015). *Microbe Online*. Retrieved from https://microbeonline.com/e-test-epsilometer-test-principle-purpose-procedure-results-and-interpretations/

Facklam, R. (2002). What Happened to the Streptococci: Overview of Taxonomic and Nomenclature changes. Clinical Microbiology Reviews. 15 (4) 613-630; DOI: 10.1128/CMR.15.4.613-630.2002

Felsenstein, S., Faddoul, D., Sposto, R., Batoon, K., Polanco, C. M., & Dien Bard, J. (2014). Molecular and clinical diagnosis of group A streptococcal pharyngitis in children. *Journal of clinical microbiology*, *52*(11), 3884-9.

Ferretti, J., Stevens, D., Fischetti, V., (2016). Streptococcus pyogenes : Basic Biology to Clinical Manifestations [Internet]. Oklahoma City (OK): University of Oklahoma Health Sciences Center; 2016-.

Fieber, C., & Kovarik, P. (2014). Responses of innate immune cells to group A Streptococcus. *Frontiers in cellular and infection microbiology*, *4*, 140. doi:10.3389/fcimb.2014.00140

Filippi, V., Chou, D., Ronsmans, C., Graham, W., Say, L. (2016). Levels and Causes of Maternal Mortality and Morbidity. In: Black, R. E., Laxminarayan, R., Temmerman, M., et al., editors. Reproductive, Maternal, Newborn, and Child Health: Disease Control Priorities, Third Edition. London School of Hygiene and Tropical Medicine. Washington (DC): The International Bank for Reconstruction and Development / The World Bank https://www.ncbi.nlm.nih.gov/books/NBK361917/ doi: 10.1596/978-1-4648-0348-2_ch3

Gera, K., & McIver, K. S. (2013). Laboratory growth and maintenance of Streptococcus pyogenes (the Group A Streptococcus, GAS). *Current protocols in microbiology*, *30*, 9D.2.1–9D.2.. doi:10.1002/9780471729259.mc09d02s30

Golińska, E., van der Linden, M., Więcek, G., Mikołajczyk, D., Machul, A., Samet, A., Piórkowska, A., Dorycka, M., Heczko, P., Strus, M. (2016). Virulence factors of Streptococcus pyogenes strains from women in peri-labor with invasive infections. European Journeal of Clinical Microbiology & Infectious Diseases: official publication of the European Society of Clinical Microbiology, 35(5), 747–754. Doi:10.1007/s10096-016-2593-O10.1007/s10096-016-2593-O

Graham, M. R., Virtaneva, K., Porcella, S. F., Gardner, D. J., Long, R. D., Welty, D. M., Barry, W. T., Johnson, C. A., Parkins, L. D., Wright, F. A. (2006). Analysis of the transcriptome of group A Streptococcus in mouse soft tissue infection. *The American Journal of Pathology*. 169(3):927-42. doi: 10.2353/ajpath.2006.060112
Greenberg, D., Leibovitz, E., Shinnwell, E. S., Yagupsky, P., Dagan, R. (1999). Neonatal sepsis caused by Streptococcus pyogenes: resurgence of an old etiology? *Pediatric Infectious Disease Journal.* 18(5):479-81.

Gumede, L., Radebe, F., Nhlapo, D., Maseko, V., Kufa-Chakezha, T., Kularatne, R. (2017) Evaluation of the Copan eSwab®, a liquid-based microbiology transport system, for the preservation of Neisseriagonorrhoeae at different temperatures. *Southern African Journal of Infectious Diseases* 32:3, 96-99, DOI: 10.1080/23120053.2017.1313935

Hallett C. (2005). The attempt to understand puerperal fever in the eighteenth and early nineteenth centuries: the influence of inflammation theory. *Medical history*, *49*(1), 1–28.

Hamada, S., Kawabata, S., Nakagawa, I. (2015). Molecular and genomic characterization of pathogenic traits of group A Streptococcus pyogenes. *Proceedings of the Japan Academy, series B.* https://doi.org/10.2183/pjab.91.539

Hamilton, M. S., Stevens, D. L., Bryant, A. E. (2013). Pregnancy-Related Group A Streptococcal Infections: Temporal Relationships Between Bacterial Acquisition, Infection Onset, Clinical Findings, and Outcome. Clinical Infectious Diseases. 57(6):870-6. doi:10.1093/cid/cit282.

Haslam, D., St. GemeIII, J., (2018). Classification of Streptococci in Principles and Practice of Pediatric Infectious Diseases (Fifth Edition).

http://www.jmedtropics.org/viewimage.asp?img=JMedTrop_2017_19_2_104_218399_t2.jpg Journal of Medicine in the Tropics.

https://www.cdc.gov/streplab/groupa-strep/index.htm (2018)

https://www.gfmer.ch/Medical_education_En/PGC_RH_2004/chisembele_review.htm

Huang, C., Lai, J., Huang, I., Chen, P., Wang, H., Shiau, Y., Cheng, Y., Hsieh, L., Chang, S., Yang, T., Lauderdale (2014) Epidemiology and Molecular Characterization of Macrolide-Resistant *Streptococcus pyogenes* in Taiwan. *Journal of Clinical Microbiology Jan 2014, 52 (2) 508-516; DOI: 10.1128/JCM.02383-13*

Ikebe, T., Endoh, M., Watanabe, H. (2005). Increased expression of the ska gene in emm49-genotyped Streptococcus pyogenes strains isolated from patients with severe invasive streptococcal infections. *Japanese Journal of Infectious Diseases;58(5):272-5.*

Imöhl, M,, van der Linden, M. (2015). Antimicrobial Susceptibility of Invasive *Streptococcus pyogenes* Isolates in Germany during 2003-2013. *PLOS ONE* 10(9): e0137313.https://doi.org/10.1371/journal.pone.0137313

Indongo, N. (2014). Risk factors and causes of Neonatal deaths in Namibia. *European Scientific Journal*. /SPECIAL/ edition ISSN: 1857 – 7881 (Print) e - ISSN 1857- 7431

Isenberg, H. D., (1992). Clinical microbiology procedures handbook. Washington, D.C.: *American Society of Microbiology.*

Janampet, B., Giridhar, V., Dumpa, V., Tolan Jr, R. (2014). A 31-Day-Old Infant with Late-Onset Sepsis and Complicated Pneumonia Due to Streptococcus pyogenes. *Infectious Disease in Clinical Practice*. 22. e58-e59. 10.1097/IPC.0b013e3182a0e94e.

Jankoska, G., Trajkovska-Dokic, E., Panovski, N., Popovska-Jovanovska, K., Petrovska, M. (2008). Virulence factors and antibiotic resistance in Enterococcus faecalis isolated from urine samples. *Microbiology and Parasitology Institute, Medical Faculty, Skopje, R. Macedonia.* 29(1):57-66.

Karthikeyan, G., Mayosi, B. M. (2009) Is Primary Prevention of Rheumatic Fever the Missing Link in the Control of Rheumatic Heart Disease in Africa? Circulation ; 120: 709–13.

Khan, K. S., Wojdyla, D., Say, L., Gulmezoglu, A. D., Van Look, P. V. A. (2006). WHO analysis of causes of maternal death: a systematic review. *The Lancet*.

Khan, Z. Z., (2018). Group A Streptococcal (GAS) Infections. American College of Physicians, Infectious Diseases Society of America, International Society for Infectious Diseases Group A Streptococcal (GAS) Infections.

Krzyściak, W., Pluskwa, K. K., Jurczak, A., & Kościelniak, D. (2013). The pathogenicity of the Streptococcus genus. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology*, *32*(11), 1361-76.

Kumar, A., Bhatnagar, A., Gupta, S., Khare, S. (2011). sof gene as a specific genetic marker for detection of Streptococcus pyogenes causing pharyngitis and rheumatic heart disease. *Cell Mol Biol (Noisy-le-grand)*. 57(1):26-30.

L. Barth Reller, Melvin Weinstein, James H. Jorgensen, Mary Jane Ferraro, Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices, *Clinical Infectious Diseases*, Volume 49, Issue 11, 1 December 2009, Pages 1749–1755, https://doi.org/10.1086/647952

Lancefield RC (1933). "A serological differentiation of human and other groups of hemolytic streptococci". J Exp Med. 57 (4): 571–95. doi:10.1084/jem.57.4.571. PMC 2132252. PMID 19870148.

Levin, K. A. (2006). Study design III: Cross-sectional studies. *Evidence-Based Dentistry* 7, 24–25. doi:10.1038/sj.ebd.6400375

Liang, Y., Liu, X., Chang, H., Ji, L., Huang, G., Fu, Z., Zheng, Y., Wang, L., Li C., Shen, Y., Yu, S., Yao, K., Ma, L., Shen, X., Yang, Y. (2012). Epidemiological and molecular characteristics of clinical isolates of Streptococcus pyogenes collected between 2005 and 2008 from Chinese children. *Journal of Medical Microbiology*. 61, 975–983 DOI 10.1099/jmm.0.042309-0

Ligozzi, M., Bernini, C., Bonora, M. G., De Fatima, M., Zuliani, J., & Fontana, R. (2002). Evaluation of the VITEK 2 system for identification and antimicrobial susceptibility testing of medically relevant gram-positive cocci. *Journal of clinical microbiology*, *40*(5), 1681-6.

Liu, D., Hollingshead, S., Swiatlo, E., Lawrence, M. L., Austin, F. W. (2005). Rapid identification of Streptococcus pyogenes with PCR primers from a putative transcriptional regulator gene. *Res Microbiol.* 156(4):564-7.

Lu, B., Fang, Y., Fan, Y., Chen, X., Wang, J., Zeng, J., Li,Y., Zhang, Z., Huang, L., Li, H., Li, D., Zhu, F., Cui. Y., Wang, D. (2007). High Prevalence of Macrolide-resistance and Molecular Characterization of Streptococcus pyogenes Isolates Circulating in China from 2009 to 2016. Department of Laboratory Medicine, Civil Aviation

Lynskey, N. N., Lawrenson, R. A., and Sriskandan, S. (2011). New understandings in *Streptococcus pyogenes*. *Curr. Opin. Infect. Dis*. 24, 196–202. doi: 10.1097/QCO.0b013e3283458f7e

Ma, Y., Bryant, A. E., Salmi, D. B., Hayes-Schroer, S. M., McIndoo, E., Aldape, M. J., & Stevens, D. L. (2006). Identification and characterization of bicistronic speB and prsA gene expression in the group A Streptococcus. *Journal of bacteriology*, *188*(21), 7626–7634. doi:10.1128/JB.01059-06

Maharaj D. (2007). Puerperal pyrexia: a review. Part I. *Obstetrical and Gynecological Survey*. 2007; 62:393–399.

Mancilla-Díaz, J. M. (2015). Systematic review of disordered eating behaviors: Methodological considerations for epidemiological research. *Revista Mexicana de Trastornos Alimentarios*, *6*(1), 51-63.

Martin J. M., Green M., Barbadora K. A., Wald E. R. Group A streptococci among school-aged children: clinical characteristics and the carrier state. *Pediatrics*;114(5):1212–1219.

Mason K. L., Aronoff D. M. Postpartum group A Streptococcus sepsis and maternal immunology. *American Journal of Reproductive Immunology*;67(2):91–100

Maternal mortality https://www.who.int/news-room/fact-sheets/detail/maternal-mortality

McGregor, K. F., Spratt, B. G., Kalia, A., Bennett, A., Bilek, N., Beall, B., & Bessen, D. E. (2004). Multilocus sequence typing of Streptococcus pyogenes representing most known emm types and distinctions among subpopulation genetic structures. *Journal of bacteriology*, *186*(13), 4285-94.

Mead, P. B., Winn, W. C. (2000). Vaginal-rectal colonization with group A streptococci in late pregnancy. *Infectious Diseases in Obstetrics and Gynecology.* 2000;8(5-6):217-9.

Melah, G. S., El-Nafaty, A. U., Massa, A. A., & Audu, B. M. (2003) Obstructed labour: a public health problem in Gombe, Gombe State, Nigeria. *Journal of Obstetrics and Gynaecology,* 23:4, 369-373, DOI: 10.1080/0144361031000119510

Menon, T., Lloyd, C., Jacob, S. (2005). Should bacitracin sensitivity be used in the presumptive identification of group A streptococci? *Indian J Med Microbiol*. 23(4):275.

Ministry of Health and Social Services (MoHSS) [Namibia] and Macro International Inc. 2008. Namibia Demographic and Health Survey 2006-07. Windhoek, Namibia and Calverton, Maryland, USA: MoHSS and Macro International Inc.

Mishalian, I., Ordan, M., Peled, A., Maly, A., Eichenbaum, M. B., Ravins, M., et al. (2011). Recruited macrophages control dissemination of group A Streptococcus from infected soft tissues. *J. Immunol*. 187, 6022–6031. doi: 10.4049/jimmunol.1101385

Miyairi, I., Berlingieri, D., Protic, J., Belko, J., (2004). Neonatal Invasive Group A Streptococcal Disease: Case Report and Review of the Literature. *Pediatric Infectious Disease Journal*. 2004;23(2)
Morse, Stephen & Brooks, G. & Carroll, Karen & Butel, J. & Mietzner, Timothy. (2010). Jawetz, Melnick, & Adelberg's Medical Microbiology, 25th edition.

Mulama, L. (2015). Analysis of causes and risk factors associated with maternal deaths in Namibia.

Namibia Statistics Agency. (2014). Namibia 2011 Census Mortality Report. Windhoek

Narayanan, S., Sabarigirinathan, C., Vinayagavel, K., Srinidhi, L., BalaSiddharth, S., Scindia, N., Guguloth, A., ArunPrasadh, G. (2019). Antibacterial Assay- A Review. *Journal of Science* Vol 9 Issue 1 18-23.
National Collaborating Centre for Infectious Diseases. 2018. Disease Debrief: Group A Streptococcus. Retrieved from https://nccid.ca/debrief/group-a-streptococcus/

Nelson D. C., Garbe J., Collin M. (2011). Cysteine proteinase SpeB from *Streptococcus pyogenes*. A potent modifier of immunologically important host and bacterial proteins. *Biol. Chem.* 392 1077–1088. 10.1515/BC.2011.208

Nelson, G. E., Pondo, T., Toews, K. A., Farley, M. M., Lindegren, M. L., Lynfield, R., Van Beneden, C. A. (2016). Epidemiology of Invasive Group A Streptococcal Infections in the United States, 2005-2012. *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America*, *63*(4), 478–486. doi:10.1093/cid/ciw248

Ngonzi, J., Tornes, Y., Mukasa, P., Salongo W., Kabakyenga, J., Sezalio, M., Wouters, K., Jacquym, Y., Van Geertruyden, J. (2016) Puerperal sepsis, the leading cause of maternal deaths at a Tertiary University Teaching Hospital in Uganda. *BMC Pregnancy and Childbirth*BMC series – open, inclusive and trusted201616:207 https://doi.org/10.1186/s12884-016-0986-9

NHS inform Scarlet Fever (2019). Retrieved from https://www.nhsinform.scot/illnesses-and-conditions/infections-and-poisoning/scarlet-fever on 23rd of April 2019

Nizet, V., Arnold, J. C., (2018). Streptococcus pyogenes (Group A Streptococcus). *Principles and Practice of Pediatric Infectious Diseases* (Fifth Edition), Elsevier, 2018, Pages 715-723.e2, ISBN 9780323401814, https://doi.org/10.1016/B978-0-323-40181-4.00118-3.

Nunes S., Sá-Leao R., Carric J., Alves C. R., Mato R., Brito Avo A., et al. (2005). Trends in drug resistance, serotypes, and molecular types of *Streptococcus pneumoniae* colonizing preschool-age children attending day care centers in lisbon, portugal: a summary of 4 years of annual surveillance. *J. Clin. Microbiol.* 43 1285–1293. 10.1128/JCM.43.3.1285-1293.2005

O'Callaghan, K. (2009). How do we reduce deaths due to puerperal sepsis in South Africa? *Obstetrics and Gynaecology Forum Vol 19, No 4*

O'Brien K. L., Beall B., Barrett N. L., Cieslak P. R., Reingold A., Farley M. M., Danila, R., Zell, E. R., Facklam, R., Schwartz, B., Schuchat, A. (2002). Epidemiology of invasive group A streptococcus disease in the United States, 1995-1999. *Clinical Infectious Diseases*;35(3):268–276.

Olender, A., Łetowska, I., Karyński, M., Kiernicka-Ciekot, K., Pels, K. (2012). Problems with identification of beta-hemolytic streptococcus resistant to bacitracin isolated from patients with pharyngitis.

Owuama, C. (2017). Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using a novel dilution tube method. *African Journal of Microbiology Research.* 11. 977-980. 10.5897/AJMR2017.8545.

Patterson, M. J., (1996). Streptococcus In: Baron S, editor. Medical Microbiology. 4th edition. Galveston (TX): University of Texas Medical Branch at Galveston; 1996. Chapter 13. Available from: https://www.ncbi.nlm.nih.gov/books/NBK7611/

Pires, R., Rolo, D., Mato, R., de Almeida, J., Johansson, C., Henriques-Normark, B., Morais, A., Brito-Avô, A., Gonçalo-Marques, J., Santos-Sanches, I. (2009) Resistance to bacitracin in *Streptococcus pyogenes* from oropharyngeal colonization and noninvasive infections in Portugal was caused by two clones of distinct virulence genotypes, *FEMS Microbiology Letters*, Volume 296, Issue 2,Pages 235–240, https://doi.org/10.1111/j.1574-6968.2009.01642.x

Population and Housing Census Regional Profile, Khomas Region. (2011). Basic analysis and highlights. Retrieved from https://cms.my.na/assets/documents/p19dptss1ri5r1f2kt6i5931i31g.pdf

Priloska, G. "Virulence factors and antibiotic resistance in Enterococcus faecalis isolated from urine samples". Prilozi. 29 (1): 57–66. PMID 18709000.

Ralph, A., Carapetis, J. (2013). Group A streptococcal diseases and their global burden. Host pathogen interactions in streptococcal disease. University of Western Australia, Perth, Australia Currenyt topics in microbiology and immunology. 2013;368:1-27. doi: 10.1007/82_2012_280.

Ray, D., Sinha, S., Saha, S., Karmakar, S., Dutta, R. N., Bhattacharya, S., Pal, N. K., & Bhattacharya, B. (2010). A Preliminary Sentinel Surveillance Report on Antibiotics Resistance Trend of Streptococcus Pyogenes in Kolkata Region, India. *Al Ameen J Med S ci* 3 (2):1 4 6 -1 5 1 I S S N 0 9 7 4 - 1 1 4 3

Salto, F., De la Huerga López, M., Menéndez Hernando, A., Ester Sanz, C. L. (2011). Late neonatal sepsis caused by Streptococcus pyogenes. *Archivos argentinos de pediatría*, *109*(4), e85-e87.

Say, L., Chou, D., Gemmill, A., Tuncalp, O., Moller, A., Daniels, J., Gulmezoglu, A. M. (2014). Global causes of maternal death: a WHO systematic analysis. *The Lancet Global Health,* doi: http://dx.doi.org/10.1016/S2214-109X(14)70227-X.

Schwalbe, R., Steele-Moore, L., Goodwin, A.C. (2007). Antimicrobial Susceptibility Testing Protocols CRC Press, Boca Raton. 12. Z. Markiewicz, Z.A.

Seidler, K. A., & Seidler, N. W. (2013). Role of extracellular GAPDH in Streptococcus pyogenes virulence. *Missouri medicine*, *110*(3), 236–240.

Serban, E. D. (2018). Perianal infectious dermatitis: An underdiagnosed, unremitting and stubborn condition. *World journal of clinical paediatrics*, 7 (4), 89-104. Doi:10.5409/wjcp.v7.i4.89

Shah, B. A., Padbury, J. F. (2014). Neonatal sepsis: an old problem with new insights. *Virulence.* 1;5(1):170-8. doi: 10.4161/viru.26906.

Shinar, S., Fouks, Y., Amit, S., Pauzner, D., Tarabeia, J., Schechner, V., Many, A. (2016). Clinical characteristics of and preventative strategies for peripartum group A streptococcal Infections. The American College of Obstetricians and Gynaecologists. ISSN: 0029-7844/16

Shulman S. T., Bisno A. L., Clegg H. W., Gerber M. A., Kaplan E. L., Lee G., Martin, J. M., Van Beneden, C. (2012). Clinical practice guideline for the diagnosis and management of group A streptococcal pharyngitis: 2012 update by the Infectious Diseases Society of America. *Clinical Infectious Diseases*. 55:1279–1282.

Skariyachan, S., Narayan, S., Aggimath, S., Tejaswini, N., Reddy, S., Rajeswari, M. & N. (2014). Molecular Modeling on Streptolysin-O of Multidrug Resistant Streptococcus Pyogenes and Computer Aided Screening and In Vitro Assay for Novel Herbal Inhibitors. Current computer-aided drug design. 10. 10.2174/15734099106661404011039 10.

Smaill, F., Gyte, G. (2010). Antibiotic prophylaxis versus no prophylaxis for preventing infection after cesarean section. *The Cochrane database of systematic reviews*, (1), CD007482. doi:10.1002/14651858.CD007482.pub2

Spellerberg, B., Brandt, C. (2016). Laboratory Diagnosis of Streptococcus pyogenes (group A streptococci). Institute of Medical Microbiology and Hygiene, University of Ulm, Albert Einstein Allee 11, 89081 Ulm, Germany.

Stevens, D. L., Bisno, A. L., Chambers, H. F., Dale Everett, E., Dellinger, P., Goldstein, J. C., Sherwood, L., Gorbach, Hirschmann, J. V., Kaplan, E., Montoya, J. G., Wade, J. C. (2005). Practice Guidelines for the Diagnosis and Management of Skin and Soft-Tissue Infections.

Stevens, D., Bryant, A., Ferretti, J., Fischetti, V. (2016). Severe Group A Streptococcal Infections. Streptococcus pyogenes: Basic Biology to Clinical Manifestations [Internet]. Oklahoma City (OK): University of Oklahoma Health Sciences Center; 2016-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK333425/

Tadayoshi, I., Hirasawa,K., Suzuki, R., Isobe, J., Tanaka, D., Katsukwa, C., Kawahara, R., Tomita, M., Ogata, K., Endoh, M., Okuno, R., Watanabe, H. (2005). Antimicrobial Susceptibility Survey of *Streptococcus pyogenes* Isolated in Japan from Patients with Severe Invasive Group A Streptococcal Infections. The Working Group for Group A Streptococci in Japan. *Antimicrobial Agents and Chemotherapy*. 49 (2) 788-790; DOI: 10.1128/AAC.49.2.788-790.2005

Terao, Y. (2012). The virulence factors and pathogenic mechanisms of Streptococcus pyogenes. *Journal of Oral Biosciences.* Volume 54, Issue 2, Pages 96-100. ISSN 1349-0079. https://doi.org/10.1016/j.job.2012.02.004.

The American College of Obstetricians and Gynecologists Committee on Gynecologic Practice. (2014). Female Age-Related Fertility Decline. Retrieved from https://www.acog.org/Clinical-Guidance-and-Publications/Committee-Opinions/Committee-on-Gynecologic-Practice/Female-Age-Related-Fertility-Decline?IsMobileSet=false

The Editors of Encyclopaedia, (2017). Catalase. *Encyclopaedia Brittanica, inc*. Retrieved from: https://www.brittanica.com/science/catalase on 25 June 2019

The Prevention of Invasive Group A Streptococcal Infections Workshop Participants; Prevention of Invasive Group A Streptococcal Disease among Household Contacts of Case Patients and among Postpartum and Postsurgical Patients: Recommendations from the Centres for Disease Control and Prevention, *Clinical Infectious Diseases*, Volume 35, Issue 8, 15 October 2002, Pages 950–959, https://doi.org/10.1086/342692

Tiemstra, J., Miranda, R., (2009). Role of non-group a streptococci in acute pharyngitis. Family Medicine Center, Department of Family Medicine, University of Illinois at Chicago, Chicago, IL 60607, USA. J Am Board Fam Med. 2009 Nov-Dec;22(6):663-9. doi: 10.3122/jabfm.2009.06.090035.

Todar, K. (2008). Todar's Online Textbook of Bacteriology. Retrieved from http://www.textbook of bacteriology.net on 4th of May 2017.

Toxic Shock Syndrome Signs and Symptoms (2017). Retrieved from https://www.mayoclinic.org/diseases-conditions/toxic-shock-syndrome/symptoms-causes/syc-20355384 on 23rd April 2019.

Troszyński, M., Chazan, B., Kowalska, B., Jaczyńska, R., Filipp, E. (2003). Four main reasons of maternal death in Poland between 1991-2000. Ginekologia polska. 74. 1306-15.

Turner, C. E., Dryden, M., Holden, M. T. G, Davies F. J., Lawrenson, R. A., Bentley L. S. D., Efstratiou, A., Sriskandan, S. (2013). Molecular Analysis of an Outbreak of Lethal Postpartum Sepsis Caused by *Streptococcus pyogenes. Journal of Clinical Microbiology.* 51 (7) 2089-2095; DOI: 10.1128/JCM.00679-13

Van Dillen, J., Zwart, J., Scutte, J., Van Roosmalen, J. (2010). Maternal sepsis: epidemiology, etiology and outcome. *Current Opinion in Infectious Diseases British Medical Journal. Doi:10.1097/QC.obo13e328339257c.*

Ventola, C. L. (2015). The Antibiotic Resistance Crisis: Part 1: Causes and Threats. *Pharmacy and Therapeutics*, *40*(4), 277–283.

Verkaeren, E., Epelboin, L., Epelboin, S., Boddaert, N., Brossier, F., Caumes, E. (2014). Recurrent Streptococcus pyogenes genital infection in a woman: test and treat the partner. *International Journal of Infectious Diseases,* Volume 29, Pages 37-39, ISSN 1201-9712. doi: 10.1016/j.ijid.2014.07.026.

Vivekanantham, S., Mudalige, N., Suri, V., Kamaledeen, A., Law, P. (2014). Maternal and Fetal Death following Group A Streptococcal Meningitis in Mid-Term Pregnancy. Hindawi Publishing Corporation Case Reports in Obstetrics and Gynecology Volume 2014, Article ID 268693, 4 pages http://dx.doi.org/10.1155/2014/2686

Walker, J., Barnett, T., McArthur, J., Cole, J., Gillen, C., Henningham, A., Sriprakash, K., Sanderson-Smith, M., Nizet, V., (2014). Disease Manifestations and Pathogenic Mechanisms of Group A *Streptococcus*. Clinical Microbiology Reviews Apr 2014, 27 (2) 264-301; DOI: 10.1128/CMR.00101-13

Watkins, D. A., Johnson, C. O., Colquhoun, S. M., (2017). Global, regional, and national burden of rheumatic heart disease, 1990–2015. *New England Journal of Medicine*, 377: 713-22

WHO, UNICEF, UNFPA, World Bank. Trends in maternal mortality: 1990 to 2010. Geneva: World Health Organization; 2012.

Women & Infants Hospital. (2014, March 6). Group A streptococcus in pregnancy: Expert contributes to research. *ScienceDaily*. Retrieved January 30, 2019 from www.sciencedaily.com/releases/2014/03/140306130423.htm

Wong, S. S., & Yuen, K. Y. (2012). Streptococcus pyogenes and re-emergence of scarlet fever as a public health problem. *Emerging microbes & infections*, *1*(7), e2.

World Health Organisation. WHO Global Principles for the Containment of Antimicrobial Resistance in Animals Intended for Food. World Health Organisation; Geneva, Switzerland: 2000. WHO/CDS/CSR/APH/2000.4

World Health Organization. (2005). The current evidence for the burden of group A streptococcal diseases. WHO reference number: WHO/FCH/CAH/05.07. Retrieved from https://www.who.int/maternal_child_adolescent/documents/fch_cah_05_07/en/

World Health Organization. (2018). Retrieved from https://www.who.int/news-room/fact-sheets/detail/maternal-mortality

Yang, P., Peng, X., Zhang, D., Wu, S., Liu, Y., Cui, S., Wang, Q. (2013). Characteristics of Group A Streptococcus Strains Circulating during Scarlet Fever Epidemic, Beijing, China, 2011. *Emerging Infectious Diseases*, *19*(6), 909-915. https://dx.doi.org/10.3201/eid1906.121020

Zakour, N. L., Venturini, C., Beatson, S. A., Walker, M. J., (2012). Analysis of a *Streptococcus pyogenes* Puerperal Sepsis Cluster by Use of Whole-Genome Sequencing. *Journal of Clinical Microbiology*. 50 (7) 2224-2228; DOI: 10.1128/JCM.00675-12

Zhang, Y., Shao, Z.,Wang, J., Wang, L., Li, X., Wang, C., Tang, J., Pan, X. (2013). Prevalent distribution and conservation of *Streptococcus suis* Lmb protein and its protective capacity against the Chinese highly virulent strain infection. *Microbiological Research*, Volume 169, Issues 5–6, Pages 395-401. doi.org/10.1016/j.micres.2013.09.007

APPENDICES

APPENDIX A: Consent form

NAMIBIA UNIVERSITY
OF SCIENCE AND TECHNOLOGY

13 Storch Street
Private Bag 13388
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NAMIBIA

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Dear participant my name is Azaria A. Vries, student number 200851021 and contact number +264812839110. I am a final year Master of Health Sciences student at the Namibia University of Science and Technology (NUST). I would like to invite you to participate in a research study entitled 'The Effect of Group A Streptococcus on Maternal and Neonatal Morbidity in Windhoek'. Group A Streptococcus is a major cause of maternal and neonatal disease and death in the world and the problem is more pronounced in resource limited countries such as Namibia. Currently there are no statistics on GAS prevalence in pregnant women in Namibia. Determining the prevalence of GAS in pregnant women at 35-37 weeks gestation and the resistance pattern of isolates are crucial steps in the development of clinical policies and procedures for the management of GAS infections. Due to lack of scientific data to this effect there are currently no policies in Namibia for screening of GAS in mothers and babies before and after delivery. This study aims to provide scientific evidence on prevalence of GAS in mothers and babies in Windhoek.

APPENDIX B: Study questionnaire for the pregnant women

Study Title:	**The Effect of Group A Streptococcus on Maternal and Neonatal Morbidity in Windhoek**

Visit Date:	

Patient Details

Age:	18-24		25-31		32-38		38-44		44-48	
Marital status:	Single		Married		Divorced		Widowed			
Area/Location:										

Language:	Afrikaans		English			
Participant educational level:					Yes	No
				Primary School Education		
				High School Education		
				Tertiary Education		
				No Schooling Completed		

Socio-economic Status

Are you employed?	Yes		No	

If Yes, approximately how much do you earn? (per month)	N$				
Do you currently receive a government grant for support?	Yes		No		[redacted]

Eligibility Criteria

Are you currently on an antibiotic treatment regime:	Yes I am currently on treatment	
(If currently on treatment or in last 3 months please EXCLUDE):	Yes I took treatment in last 3 months	
Anibiotics include: Penicillin, erythromycin, clindamycin and tetracycline	No I have not taken treatment in the last 3 months	
	Don't know	
Does participant know HIV status	Yes, HIV positive	
	Yes, HIV negative	
	Never tested or unknown	

Signs & Symptoms

Have you felt feverish in the past two weeks:	Yes		No	
Have you experienced pain in the lower abdomen or pelvis:	Yes		No	
Have you had foul-smelling vaginal discharge:	Yes		No	
Have you experienced chills:	Yes		No	
Have you had any feelings of discomfort or illness:	Yes		No	
Have you experienced headache/s:	Yes		No	
Does it happen daily that you do not feel like eating:	Yes		No	
Have you experienced an increased heart rate:	Yes		No	

Medical History

Gestational period:	35 weeks		36 weeks		37 weeks	
Number of pregnancies (gravidity)						
Number of pregnancies carried to above 24 weeks (parity)						

Physical Examination

Auxiliary temperature reading in degrees Celsius?	

APPENDIX C: Study questionnaire for the neonates

Study Title:	**The Effect of Group A Streptococcus on Maternal and Neonatal Morbidity in Windhoek**

Visit Date:	

Patient Details

Date of birth:	
Area/Location:	

Eligibility Criteria

Are you currently on an antibiotic treatment regime:	Yes I am currently on treatment	
(If currently on treatment or in last 3 months please EXCLUDE): Anibiotics include: Penicillin, erythromycin, clindamycin and tetracycline	Yes I took treatment in last 3 months	
	No I have not taken treatment in the last 3 months	
	Don't know	
Does participant know HIV status?:	Yes, HIV positive	
	Yes, HIV negative	
	Never tested or unknown	

Infection Signs & Symptoms

Have you felt feverish in the past two weeks:	Yes		No	
Have you had any feelings of discomfort (irritable):	Yes		No	
Does it happen daily that you do not feel like eating:	Yes		No	
Are swollen neck glands present:	Yes		No	
Is there a thickened or bloody nasal discharge present:	Yes		No	

Physical Examination

Axilliary temperature reading in degrees Celsius?	

APPENDIX D: Permission letter from the Ministry of Health and Social Services

REPUBLIC OF NAMIBIA

Ministry of Health and Social Services

Private Bag 13198
Windhoek
Namibia

Ministerial Building
Harvey Street
Windhoek

Tel: 061 – 2032150
Fax: 061 – 222558
Email: shimenghipangelwa71@gmail.com

OFFICE OF THE PERMANENT SECRETARY

Ref: 17/3/3 **AV**
Enquiries: Mr. J. Nghipangelwa

Date: 07 December 2017

Ms. Azaria A. Vries
Namibia University of Science and Technology
Windhoek

Dear Ms. Vries

Re: The effect of Group A Streptococcus on Maternal and Neonatal Morbidity in Windhoek.

1. Reference is made to your application to conduct the above-mentioned study.
2. The proposal has been evaluated and found to have merit.
3. **Kindly be informed that permission to conduct the study has been granted under the following conditions:**

3.1 The data to be collected must only be used for academic purposes;
3.2 No other data should be collected other than the data stated in the proposal;
3.3 Stipulated ethical considerations in the protocol related to the protection of Human Subjects' should be observed and adhered to, any violation thereof will lead to termination of the study at any stage;
3.4 A quarterly report to be submitted to the Ministry's Research Unit;
3.5 Preliminary findings to be submitted upon completion of the study;

3.6 Final report to be submitted upon completion of the study;

3.7 Separate permission should be sought from the Ministry of Health and Social Services for the publication of the findings.

Yours sincerely,

Andreas Mwoombola (Dr.)
Permanent Secretary

"Your Health Our Concern"

APPENDIX E: Permission letter from the Windhoek Central Hospital (WCH)

MINISTRY OF HEALTH AND SOCIAL SERVICES

Private Bag 13215 Windhoek Namibia	Harvey Street Windhoek Central Hospital	Tel. No: (061) 203 3024 Fax No: (061) 222886
Enquiries: Ms. S.Iipinge	Ref.	Date: 27 March 2018

OFFICE OF THE CHIEF MEDICAL SUPERINTENDENT

Ms.Azara A. Vries
Namibia University of Science and Technology
0812839110

Dear Ms. Vries

RE: PERMISSION TO RESEARCH ON THE EFFECT OF GROUP A STREPTOCOCCUS ON MATERNAL AND NEONATAL MORBIDITY IN WINDHOEK, NAMIBIA

This letter serves to inform you that permission has been granted for you to conduct a study at Windhoek Central Hospital on the above mentioned subject as you have requested and does not include any remuneration.

The patients/Clients information should be kept confidential at all times.

Yours sincerely

.....

DR.D.I.UIRAB
CHIEF MEDICAL SUPERINTENDENT

27-03-2018
Windhoek Namibia
WINDHOEK CENTRAL HOSPITAL

APPENDIX F: Ethical clearance from the Faculty of Health Sciences (NUST)

NAMIBIA UNIVERSITY OF SCIENCE AND TECHNOLOGY

13 Storch Street
Private Bag 13388
Windhoek
NAMIBIA

T: +264 61 207 9111
F: +264 61 207 2444
W: www.nust.na

FACULTY OF HEALTH AND APPLIED SCIENCES

DECISION/FEEDBACK ON RESEARCH PROPOSAL ETHICAL CLEARANCE

Dear ~~Prof/Dr/Mr~~/Ms/Other(s): Azaria Vries

Student No (if applicable): 200851021

Research Topic:	The Effect of Group A Streptococcus on Maternal and Neonatal Morbidity in Windhoek
Supervisor (if applicable):	Mr Martin Mukesi
Co-supervisor(s): if applicable	Ms VF Tjijenda
Qualification registered for (if applicable):	Master of Health Sciences

Re: Ethical screening application No: REC - 00018/2017

The Research Ethics Screening Committee has reviewed your application for the above-mentioned research project. Based on the recommendation of the expert reviewer, the research as set out in the application is hereby:

(Indicate with an X)

Approved: i.e. may proceed with the project		
Approved provisionally: i.e. may proceed but subject to compliance with recommendation(s) listed below	**X**	
Not approved: Not to proceed with the project until compliance with recommendation(s) listed below and resubmit ethics application for consideration		
IS MINISTRY OF HEALTH & SOCIAL SERVICES (MoHSS) APPROVAL REQUIRED?	YES: **X**	NO:

It is important to note that as a researcher, you are expected to maintain ethical integrity of your research, strictly adhere to the ethical policy of NUST, and remain within the scope of your research proposal and supporting evidence as submitted to the REC. Should any aspect of your research change from the information as presented, which could have an impact or effect on any research participants/subjects/environment, you are to report this immediately to your supervisor or REC as applicable in writing. Failure to do so may result in withdrawal of approval. Kindly consult your supervisor or HoD if you need further clarification.

We wish you success in your research endeavour and are of the belief that it will have positive impact on your career as well as the development of NUST and the society in general.

Ethical issues that require compliance/ must be addressed		
No.	**Ethical issues**	**Comment/recommendation**
1.		To obtain approval from the MoHSS and submit copy to FHAS-REC secretariat*.
2.		To provide consent form attached to the research questionnaire.

NB: May attach additional page as required; * Failure to do so will invalidate research outcome

Full Name (reviewer): PROF YAPO G ABOUA_ Signature: .....Date: 04/09/2017

Full Name: PROF.....OMOTAYO AWOFOLU...Signature: .....Date: 2/9/2017
Chair: Ethics Screening Committee