Characterization of Group A Streptococci Isolated from Throat of Healthy School Children in Ethiopia

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Medical Microbiology

By

Alemseged Abdissa, B. Sc

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To my father, the late Abdissa Lencho

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ABBREVIATIONS

AHRI	Armauer Hansen Research Institute
ALERT	All African Leprosy Rehabilitation and Training Center
API	Analytical Profile Index
APSGN	Acute Poststreptococcal Glomerulonephritis
ARF	Acute Rheumatic Fever
ASO	Anti- streptolysin O
ATCC	American Type Culture Collection
BHI	Brain Heart Infusion
CDC	Centres for Disease Control and Prevention (USA)
COBA	Colistin-Oxolinic Acid-Blood Agar
CSA	Central Statistics Authority
ELISA	Enzyme Linked Immunosorbent Assay
EMA	Ethiopian Mapping Agency
GAS	Group A streptococci
Mga	Multigene regulator of GAS
mga	Gene Encoding Mga
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
PMN	Polymorphnuclear cells
RHD	Rheumatic Heart Disease
SLS	Streptolysin O
SLS	Streptolysin S

ABSTRACT

Post-streptococcal complications such as acute rheumatic fever (ARF) are reported to be common among Ethiopian children. Little is known, however, about the epidemiology of β-hemolytic streptococci, and Group A streptococci (GAS) in Ethiopia. A total of 937 randomly selected healthy schoolchildren residing in Addis Ababa (n=491), Gondar (n=265) and Dire-Dawa (n=181) were studied during a period November 2004 and January 2005. Throat specimens were collected and β-hemolytic streptococci were isolated on Columbia blood agar supplemented with Colistin-oxolonic acid. Gram reaction, catalase test Bacitracin susceptibility test and serological test were used to differentiate the organisms. In addition biochemical testing (API) was used to identify the species of isolates that could not be serogrouped.

GAS were the predominant β-hemolytic streptococci with a proportion of 55% in Addis Ababa, 61% in Gondar, and 47% in Dire-Dawa of the serogroups. The carrier rate of GAS in healthy schoolchildren was 10.8% in Addis Ababa, 8.7% in Gondar and 9.4% in Dire-Dawa. Seven isolates that were nongroupable confirmed to be Streptococcus constellatus with API biochemical testing. Molecular (emm gene) typing was performed on 82 GAS isolates and 44 different emm types were detected. Among the 82 isolates 18 strains belonged to 13 new, previously undescribed emm subtypes. Known rheumatogenic strains accounted for 56% of isolates in Addis Ababa, 35% in Gondar and 0% in Dire-Dawa.

Of the emm types prevalent in the study communities, 60% were not included in the 26 valent vaccine that was formulated for western countries. The distribution of emm types described in this study was compared with the study conducted on isolates collected 13 years ago in Addis Ababa, only 3/20 emm types identified in earlier study had been represented in the present study. Moreover, the type, which was predominant in the previous study, was not detected in the current one and the type that was predominant in our study was not detected in the previous one.

In conclusion, Group A was the most frequent serogroup among β-hemolytic streptococci isolated from throat swabs of healthy schoolchildren in the selected three sites of Ethiopia and the carriage rate of GAS ranged from 8.7 to 10.8%. Tremendous diversity of emm types was observed in this first large scale emm type survey in Africa, and a significant proportion of them are known to be rheumatogenic. The association of the prevalent emm types not previously linked to rheumatic fever needs to be explored particularly in Dire-Dawa.

Key Words: Beta-hemolytic streptococci, Group A Streptococci, *Streptococcus pyogenes,* M protein, *emm* typing

CHAPTER I INTRODUCTION

1.1. General Overview

Streptococcus pyogenes, also known as Group A Streptococci (GAS) are human pathogenic bacteria capable of causing a wide range of diseases. The clinical presentation varies from localized infections, such as pharyngitis and pyoderma, to severe sequelae, such as rheumatic fever and post streptococcal glomerulonephritis (Bisno, 1991). Infection with GAS can also lead to the development of highly invasive disease such as sepsis, necrotizing fasciitis and toxic shock-like syndrome (Bronze and Dale, 1996; Stevens, 1992).

There has been a steady decline in the incidence of serious streptococcal disease in the developed world since the beginning of the last century. However, in the 1980's an increase in serious, life threatening infection due to GAS and a re-emergence of acute rheumatic fever (ARF) (Bisno, 1991) has been observed in the western world and this has heightened interest in streptococci and streptococcal infections. In developing countries, the epidemiology of streptococci is poorly investigated. However, the available data indicate the high public health importance of streptococcal infection in these countries (Carapetis, 2003; Kaplan, 1993).

In Ethiopia, rheumatic heart disease (RHD) has an overall prevalence rate ranging from 4.6 (Oli *et al.*, 1992) to 7.1 per l000 children, depending on the socioeconomic status. This is among the highest in the world (Oli and Porteous, 1999). Moreover, up to 60% of admissions of cardiac cases in the children's hospital were reported to have a rheumatogenic origin (Daniel and Abegaz, 1993).

This clearly indicates a need for studies to understand the epidemiology of GAS in Ethiopia for subsequent implementation of more effective preventive and control activities. The purpose of this study is to survey the genetic diversity of GAS in Ethiopia by using M protein (*emm*) gene sequence analysis.

1.2. Review of the Literature

1.2.1. Historical Background

The first description of disease caused by *Streptococcus pyogenes* in the European medical literature was epidemic pharyngitis, with or without symptoms of scarlatina, reported from the early 16th century (Osterlund, 1997). However, further landmarks that contributed to current understanding of the bacterium were made in the 19th century as presented in Table 1.1.

Table 1.1. Milestones in the understanding of streptococci: Adapted from (Bisno and Stevens, 2000)

Year	**Development**	Person Introduced
1874	Demonstration of streptococci in wound infection	Billroth
1879	Demonstration of streptococci in blood from a patient with sepsis	Pasteur
1833	Isolation of streptococci from erysipelas lesions and demonstrated the organism as causative agent	Fehieisen
1903	Classification of streptococci based on their haemolytic characteristics	Schötmuller
1933	Classification of β-hemolytic streptococci into distinct serogroups	Lancefield
1935	The role of M protein as virulence factor and the type specific Nature of protective immunity to GAS were established	Lancefield
1949	Description of the method for titration of anti-streptolysin O (ASO)in serum	Todd
1996	Introduction of *emm* gene sequence based GAS typing	Beall

1.2.2. Classification of Genus Streptococci

Streptococci are spherical or ovoid bacteria that grow in pairs or chains of varied lengths. Most are facultative anaerobic, although some are obligate anaerobes. Streptococci are gram-positive, non-spore forming, catalase negative and ordinarily non-motile and have complex but variable nutritional requirements. Taxonomically, these organisms belong to the genus *Streptococcus*, of which there are over 30 identified species (Bisno and Stevens, 2000).

No single system of classification suffices to differentiate this heterogenous group of organisms. Instead, classification depends on a number of features. Colonies of certain strains on blood agar are surrounded by clear colorless zones within which the red cells in the medium have been completely lysed. This pattern is designated beta-hemolysis and is of considerable importance since it is exhibited by *Streptococcus pyogenes* and many of the other streptococci pathogenic to humans. A second group of organisms produces partial hemolysis, or alpha-hemolysis. Finally, the term gamma-hemolysis has been used to designate strains producing no hemolysis, although the term non-hemolytic streptococci is to be preferred (Bisno and Stevens, 2000; Facklam, 2002).

Most precise identification of the beta-hemolytic streptococci was accomplished by Lancefield, who succeeded in differentiating these organisms into serogroups by means of antigenic differences in cell wall carbohydrates (Table 1.2). To date, serogroups A through H and K through V have been designated. Groups A, B, C, D, and G are those most commonly found in humans; groups E, L, P, U, and V are isolated from humans rarely if at all (Facklam, 2002; Petts, 1999).

Table 1.2. Characteristics of clinically important β-hemolytic streptococci (Facklam, 2002)

Species	Lancefield Group	Bacitracin Sensitivity	Origin
S. pyogenes	A	+	Human
S. agalactae	B	-	Human, bovine
S. dysgalactiae Subsp. e*quismilis*	A, C, G, L	-	Human, animals
S.equi Subsp. z*ooepidemicus*	C	-	Animals, human
S. canis	G	-	Dog, human
S. anginosus (group)	A, C, G, F, None	-	Human
S. constellatus Subsp. *pharyngis*	C	-	Human
S. porcinus	E, P, U, V, None	-	Swine, human

Streptococcus pyogenes or Lancefield group A streptococcus (GAS) is the most pathogenic species in the genus. Group A streptococci are spheric or ovoid cells 0.6-1.0 micrometer in diameter and occur as pairs or as short to moderate sized chains in clinical specimens. GAS are facultative anaerobic, nutritionally fastidious and are cultivated in complex media, often supplemented with blood or serum (Bisno and Stevens, 2000; Facklam, 2002). When cultured on blood agar plates, GAS appear as white to grey colonies 1-2 mm in diameter surrounded by zones of complete (β) hemolysis (Bisno and Stevens, 2000).

1.2.3. Typing of Group A Streptococci (*S. pyogenes*)

Historically, streptococcal M protein, which extends from the cell membrane of GAS, has been used to classify *S. pyogenes* into serotypes. The M-typing system, a typical serologic system based on antigen-antibody reactions, is dependent on the preparation of type-specific antisera and extraction of M protein (Facklam *et al.*, 1999). The antisera against the M-protein antigens are produced with whole-cell streptococcal vaccines used to immunize rabbits (Facklam *et al.*, 1999). The N-terminal region of the M protein has been demonstrated to contain the type specific moiety and is recognized by specific typing sera in a precipitin test (Fischetti, 1989). There were several difficulties with M serotyping, including ambiguities in the results, discovery of new M types, difficulty in obtaining high-titered antisera against many strains, and the availability and high cost of preparing high-titered antisera for all known serotypes (Cunningham, 2000).

Thus, an alternative to the preparation of M-typing antisera has been developed. Approximately half of GAS produce opacity factor, a lipoproteinase that causes various types of mammalian serum to increase in opacity. Antibodies against the opacity factor are type specific and correlate with the M type. By using an opacity factor inhibition test, the M type of a GAS can be determined by determining the type of opacity factor (Johnson and Kaplan, 1993).

In addition, the T protein antigen is present at the surface of the GAS along with the M protein antigens (Schneewind *et al.*, 1990). In the laboratory, the T typing assay is performed as an agglutination test. The T typing of GAS has been important in the investigation of epidemiology of GAS infections and has identified strains associated with outbreaks when the M type was not identifiable. Because certain M types are associated with certain T types, the testing for M types can be shortened by knowledge of the T type (Beall *et al.*, 1996,1997).

As has been described earlier, producing type specific M typing antisera is difficult and specialized; no attempt has ever been made to produce them commercially, and only a few reference laboratories prepare them (Facklam *et al.*, 1999). Thus, characterization and serotyping of GAS has mostly been done on isolates from the western world. Moreover, with the available M-typing antisera, the success of M-typing is often poor among strains from tropical and subtropical countries (Kaplan *et al.*, 1992; Relf *et al.*, 1992). For example more than 80% of GAS isolated from the upper respiratory tract of patients in Thailand could not be characterized by M protein or opacity factor antisera, while more than 80% of the USA isolates could be identified (Kaplan *et al.*, 1992). The genetic analysis has shown that all non-typable GAS possess the gene for M protein. The M-protein expressed by these non-typeable strains may therefore represent new, previously unrecognized serotypes (Relf *et al.*, 1992).

Several reports confirm that there is a high proportion of T-untypable GAS isolates from developing countries. For example, 61% of Thailand isolates could not be T-typed whereas the typability rate among USA isolates was 93% (Kaplan *et al.*, 1992). In addition, a considerable proportion (43.6%) of group A streptococcal isolates from Ethiopia were not recognized by T typing antisera from Prague (Tewodros *et al.*, 1992).

These findings emphasize the need for comprehensive typing method for GAS using the recently developed molecular techniques that can also type isolates from the tropics where streptococcal infection and sequelae are of significant public health importance (Beall *et al.*, 1996, 1997). In response, many molecular methods have been developed; including the use of labeled oligonucleotide probes targeting a specific area of the gene encoding the M protein, the *emm* gene. These include oligonucleotide typing or dot blotting (Efstratiou, 2000) and the PCR *emm* gene ELISA. (Efstratiou, 2000; Saunders *et al.*, 1997). While these technologies are useful, they rely on oligonucleotides specific to known *emm* alleles, do not allow identification of new *emm* genes, and would be tedious for the identification of rarely occurring *emm* genes (Beall *et al.*, 1996).

The current "gold standard" molecular methodology is based upon *emm* gene sequence typing, which has overcome the limitations inherent in other typing techniques (Beall *et al.*, 1996; Facklam *et al.*, 1999). DNA sequencing is much more discriminating in that it allows for the rapid direct deduction of the sequence of up to 500 bases of the $5'$ end of *emm* genes. This is much more specific and reliable than serologic M typing. Moreover, nearly 100% of GAS isolates could be genotyped by the *emm* typing system (Facklam *et al.*, 1999).

Two isolates were regarded as sharing the same *emm* sequence type, if they were $\geq$ 95% identical over their $5'$ end 160 nucleotides (includes approximately 50 base pairs of the moderately conserved leader peptide-coding region), allowing for one frame shift or in-frame insertion/deletion of no more than seven codons (Beall *et al.*, 1996).

Recently, a modification has been made to this type definition. New types will now be identified on the basis of sharing less than 92% sequence identity over the first 90 bases encoding the deduced processed M protein of the type reference strain, using the SSEARCH program in the Wisconsin package version 10.3 and bases 1-90 of *emm* reference strain sequences to compare to the full length 150 base subtype-determining region of the query sequence. As before, a single interruption of the reference sequence-reading frame (through frameshift, in frame deletion or insertion) by no more than 7 codons is tolerated and not quantitated for mismatches. However, for each codon involved in such interruptions, a penalty of 0.5% is subtracted from the over all % identity score. However subtypes will continue to be assigned according to exact 150 base sequences encoding the N terminal 50 residues of the mature M protein (Beall, 2004a).

Ever since its development, several investigators have employed the *emm* typing technique and confirmed its value in differentiating GAS types. Moreover, the typing system is predictive of the M serotype. This potential is very crucial since it appears to relate fairly accurately to past and current findings for individual clones (Dicuonzo *et al.*, 2001).

1.2.4. Virulence Factors

Molecules produced by bacteria, which contribute to the degree of their disease causing capacity, are known as virulence factors. The outcome of an infection depends on the interaction between the virulence factors of the bacterium and the host immune system (Figure1.1). GAS has evolved an impressive range of strategies to evade the non-specific and the specific immune defense mechanisms of its human host.

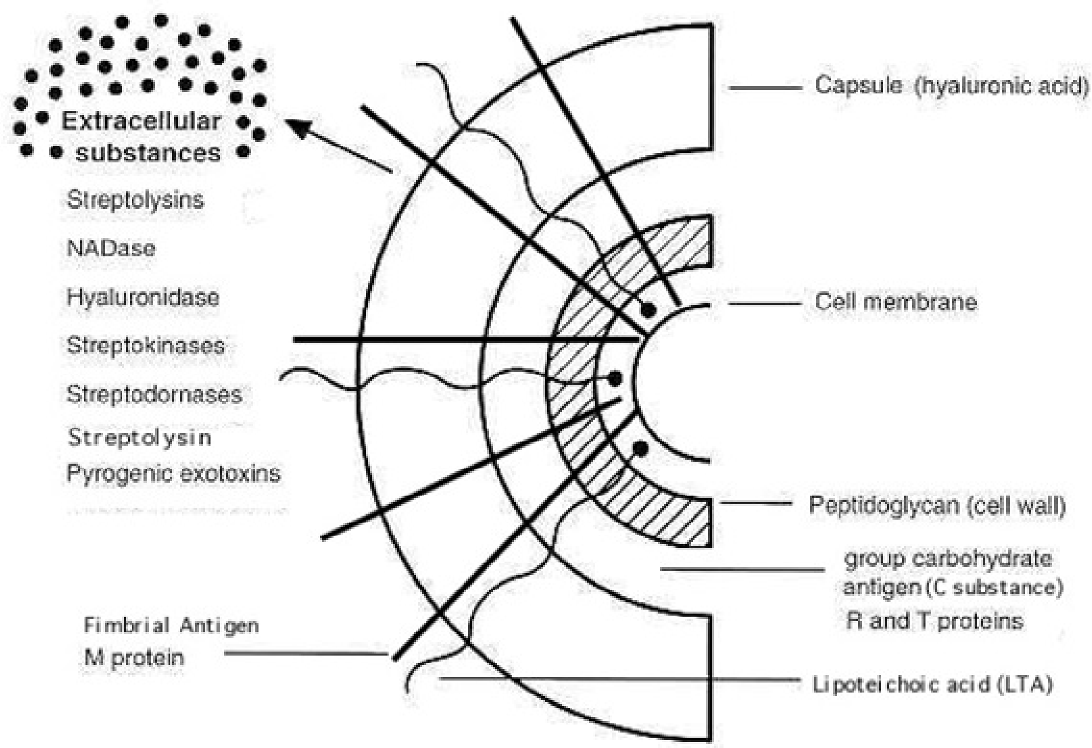

Figure 1.1 Cell surface structure of GAS and secreted products involved in Virulence (Todar, 2002)

a) M-protein

The M proteins were discovered by Lancefield and constitute the structural basis for typing of GAS. They are fibrillar proteins projecting about 60 nm out from the streptococcal cell wall with a molecular weight of 35, 000 KDa. Two alpha-helical proteins form a so-called coil-coiled structure which was quite similar to the alpha-helical coiled-coil structure in host tissue proteins such as tropomyosin and the keratin-desmin-vimentin and keratin-myosin-epidermin-fibrinogen-families of molecule (Fischetti, 1989).

In electron microscopy, they appear as fine hair-like projections radiating from the streptococcal surface. The M protein is anchored by its COOH-terminal end in the streptococcal cell wall. This region is highly conserved whereas the amino terminal half consists in its proximal part of repetitive structures but is variable particularly at the N-terminal end. The N-terminal tip of only about 11 amino acids is highly variable and form the basis for serological M typing of GAS (Fischetti, 1989).

Functionally, the M-protein inhibits phagocytosis, which is a primary virulence mechanism for survival in tissue. Absence of the *emm* gene allowed rapid phagocytosis of the streptococcus. Introduction of the *emm* gene into a M-negative strain converted it to M-positive strain and restored resistance to phagocytosis (Moses *et al.*, 1997). The antiphagocytic activity of M-protein is due to its binding of complement regulatory protein factor H (Perez-Casal *et al.*, 1992), and fibrinogen (Moses *et al.*, 1997).

Furthermore, M proteins mediate adhesion to and invasion of host respiratory and skin epithelial cells, and studies indicate that M proteins of different serotypes may differ in these functions, possibly of importance for differences in tissue tropism between serotypes (Berkower *et al.*, 1999; Courtney *et al.*, 1994; Ji *et al.*, 1998).

The genes encoding the M proteins and several other surface factors have been shown to be located together in a large, highly complex, gene locus designated the Mga operon. Cloning of an M6 gene (*emm* 6) was accomplished (Hollingshead *et al.*, 1986) and subsequently a gene regulating M protein expression was identified (Caparon and Scott, 1987). This transacting regulatory protein was designated Mga for Multi Gene Regulator of Group A streptococci. In the Mga operon is located the gene encoding Mga (*mga*), followed by one of the three genes encoding the M (*emm*) and M-like proteins, and a gene encoding streptococcal C5a peptidase (*scp A*) (Podbielski *et al.*, 1995).

The age of molecular biology brought cloning technology to the study of streptococcal antigens, and the *emm* genes from some of the serotype were cloned and their nucleotide sequences were deduced. The cloning and sequencing of the *emm* genes revealed repeating sequence motifs within I) the N-terminal region, II) the mid-molecule region and pepsin-sensitive region, and III) the conserved carboxyl-terminal region (Figure1.2). The N-terminal region, called the A repeat region, confers serotype specificity on the GAS and was found to be highly variable among M-protein serotypes. The midregion was also variable and was called the B repeat region. The carboxyl-terminal region also contained amino acid sequence repeats, which extend throughout the carboxyl-terminal one-third of the molecule (Fischetti, 1989). The highly conserved carboxyl-terminal region of M-protein contained sequence homology shared among most of the M-proteins (Hollingshead *et al.*, 1987).

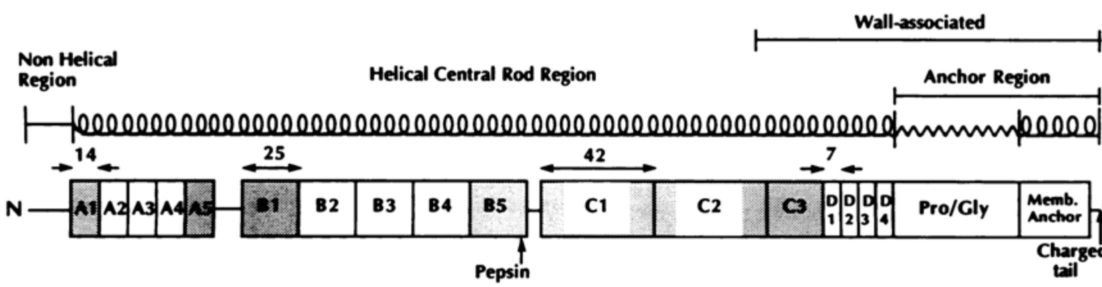

Figure 1.2 Basic structure of M protein (Fischetti, 1989).

The length of M-protein is dependent on the number of repeating units in the A and B repeat regions (Fischetti, 1989). Such changes in size may provide the bacterium with a selective advantage, as it would change antigenically and not be recognized by host antibody (Cunningham, 2000).

Apart from the classical M proteins a large group of proteins with homology to the M proteins and sharing the overall architecture are collectively referred to as the M-like proteins. Strains of GAS express one, two, or three M-like proteins (Kehoe *et al.*, 1996) which may be required for optimal resistance of phagocytosis (Thern *et al.*, 1998). The genes coding for the M-like proteins, the *emm* gene family, are flanked by *mga*, coding for Mga, a universal regulator of GAS (Hollingshead *et al.*, 1993).

b) Hyaluronic Acid Capsule

The GAS capsule is composed of a polymer of hyaluronic acid containing repeating units of glucuronic acid and N-acetylglucosamine (Cunningham, 2000). Hyaluronic acid capsule is required for resistance to phagocytosis (Wessels and Bronze, 1994). In addition, the capsule may be an important adherence factor in the pharynx, since it binds CD44 on epithelial cells (Schrager *et al.*, 1998). Streptococcal isolates vary in the amount of hyaluronic acid capsule that they produce (Moses *et al.*, 1997). Epidemiologic evidence linking highly mucoid strains with rheumatic fever and severe invasive streptococcal disease suggests that the capsule could play an important role in invasive infections in humans (Johnson *et al.*, 1992).

c) Other Virulence Factors

GAS has several cell wall constituents, including C5a peptidase that play critical role in pathogenesis. The cell bound C5a peptidase cleaves the C5a component of complement at its PMN-binding site (Cleary *et al.*, 1992b). This event then inhibits the recruitment of phagocytic cells to the site of infection (Ji *et al.*, 1997, 1998).

Moreover, numerous extracellular products are elaborated by this bacterium. Streptococcal pyrogenic exotoxins, formerly known as erythrogenic toxins, are well known for their pyrogenicity, enhancement of endotoxic shock, and superantigenic effects on the immune system. The pyrogenic exotoxins appear to be responsible for many of the manifestations of scarlet fever and toxic streptococcal syndrome (Cunningham, 2000).

GAS produce two hemolysins, namely streptolysin O (SLO) and streptolysin S (SLS). SLO is a well-characterized oxygen labile bacterial exotoxin. Early studies of purified SLO and SLS demonstrated that these hemolysins were toxic to the variety of human cells *in vivo* and *in vitro*. Recently, cytotoxic effects of SLO and SLS have been described to protect GAS from phagocytic killings and enhance bacterial virulence (Sierig *et al.*, 2003)

Another secreted protein, streptokinase, is of particular interest. Streptokinase can form a complex with plasminogen, the key molecule of the fibinolytic system, which subsequently is converted to its enzymatically active form. Such unphysiological activation may enhance bacterial invasion or movement through normal tissue barriers (Lottenberg *et al.*, 1994) Streptokinase has been associated with the pathogenesis of acute post streptococcal glomerulonephritis (Ohkuni *et al.*, 1991)

1.2.5 Epidemiology

GAS is by far the most common bacterial cause of acute pharyngitis, accounting for approximately 15-30 percent of cases in children and 5-10 percent of cases in adults (Bisno, 2001). However, strains of other serogroups especially groups C and G, may occasionally be involved (Bisno and Stevens, 2000).

The disease occurs primarily among children 5 to 15 years of age, with the peak incidence occurring during the first few years of school. All ages are susceptible, and there is no sex predilection. However, GAS pharyngitis is uncommon under two years of age. The disease is ordinarily spread by person-to-person contact, most likely via droplets of saliva or nasal secretions. Overcrowding, such as occurs in schools or barracks, favors interpersonal spread of the organism, and may also enhance its virulence by processes of natural selection (Bisno and Stevens, 2000).

Although environmental factors such as crowding certainly contribute to the risk of acquisition of GAS and the development of subsequent suppurative or nonsuppurative complications, they are clearly not the sole determinants. For instance, outbreaks of acute rheumatic fever (ARF) in the United States during the late 1980s occurred in middle-class populations with ready access to medical care. These outbreaks were attributed to the concomitant appearance of mucoid strains of GAS in the upper respiratory tracts of children in the community (Veasy *et al.*, 1994).

GAS and other β-hemolytic streptococci frequently colonize the throats of healthy children (see section 1.2.7). Most studies from the temperate regions showed a predominance of Lancefield group A streptococci (Quinn, 1989). On the other hand, in the subtropical and tropical climate group C and G streptococci were more frequently isolated from healthy schoolchildren (el-Kholy *et al.*, 1973; Lawal *et al.*, 1990).

Pyoderma occurs most frequently among economically disadvantaged children dwelling in tropical and subtropical climates. The peak incidence of pyoderma is in children aged 2-5 years. It is estimated that over 111 million cases are prevalent worldwide (Carapetis, 2003). The mode of spread of streptococcal pyoderma is poorly understood. Possible means of transmission include direct contact, environmental contamination, and arthropod vectors such as the Hippelates fly, and is often secondary to scabies (Bisno and Stevens, 2000).

ARF is most frequent among children in the 6-15 year-old age group. Indeed, its relative rarity in infant and pre-school-aged children has led some observers to question whether repeated "primary" infections might be required for the development of this disease. Both initial and recurrent episodes also occur in adults. The antecedent GAS pharyngitis is required to cause ARF. More recently, studies indicate that pyoderma can also initiate ARF (Carapetis, 2003).

1.2.6 Clinical Features

a) Pharyngitis

GAS are by far the most common bacterial cause of acute pharyngitis, accounting for approximately 15-30 % of cases in children and 5-10 % of cases in adults. Group C and G can also cause food-borne and water-borne outbreaks or sporadic cases of pharyngitis (Bisno, 2001). Certain M protein serotypes, such as M types 1, 3, 5, 6, 14, 18, 19, and 24 of GAS, are found associated with throat infections and rheumatic fever (Stollerman, 1997). More recently, types M1 and M3 were also epidemiologically associated with some severe forms of GAS infections, such as toxic shock syndrome (Kiska *et al.*, 1997). Although usually associated with streptococcal throat infection, scarlet fever may occur due to infections at other sites such as wound infections. The GAS strain producing scarlet fever does so because it carries the genes for one or more of the streptococcal pyrogenic exotoxins (Cunningham, 2000). Although they are not considered normal flora, pharyngeal carriage of GAS can occur without clinical symptoms of disease.

b) Pyoderma

Pyoderma or impetigo is the term used collectively to denote localized purulent streptococcal infections. The infection is limited to the epidermis, usually on the face or extremities. GAS, which invade the skin and cause impetigo are different M protein serotypes from those that cause pharyngitis. In addition, some of the skin strains are associated with production of acute poststreptococcal glomerulonephritis. M serotypes such as 2, 49, 57, 59, 60, and 61 are associated with pyoderma (Bisno and Stevens, 1996).

c) Non-suppurative Sequelae

i. Acute Rheumatic Fever and Rheumatic Heart Disease

In addition to suppurative infections, GAS are also able to cause acute rheumatic fever (ARF) and subsequent rheumatic heart disease (RHD) after infection in humans (Bisno, 1991). ARF occurs one to four weeks after a streptococcal infection and manifests itself as an inflammation of the joints (arthritis), heart (carditis), centeral nervous system (chorea), skin (erythema marginatum), and/or subcutaneous nodules. The carditis leads to the formation of Aschoff's bodies, a localized area with fibrinoid material and cellular infiltration (Stollerman, 1997, 2001). In this classic form, the disorder is acute, febrile, and largely self-limited. However, damage to heart valves may occur, which is referred to as rheumatic heart disease, total disability and, not infrequently, death may occur many years after the acute attack (Bisno and Stevens, 2000).

Epidemiological data show that some GAS serotypes have been clearly associated with epidemics of rheumatic fever. M5 is the most common serotype, and M1, 3, 5, 6, 14, 18, 19, 24, and a few others are well represented. Such prevalent pharyngeal types as M2, 4, and 12 are not associated with ARF (Stollerman, 1997). However, there is still much confusion about the notion of the rheumatogenic potential of specific M serotypes (Stollerman, 2001). Quite recently, investigators are also questioning the rheumatogenic potential of group C and G in areas where carriage of these organisms are common (Haidan *et al.*, 2000).

The pathogenic mechanism involved in the development of ARF/RHD remains unclear; however it is evident that an abnormal humeral and cellular immune response occurs. Antigenic mimicry between streptococcal antigens, mainly M protein epitope, and heart component has been proposed as the triggering factor leading to ARF (Brandt *et al.*, 2000).

It is believed that there is genetic susceptibility to rheumatic fever. Several genetic markers of susceptibility were studied and no consistent association was found. However, associations with different MHC class II antigens have been observed, since MHC class II antigens play an important role in the antigen presentation to the T-cell receptors (Guilherme *et al.*, 2000).

ii. Acute Poststreptococcal Glomerulonephritis (APSGN)

APSGN arises after a delay of one to four weeks, after a skin or throat infection with GAS. The clinical findings are dominated by malaise, slight fever, and nephritic syndrome. APSGN is triggered by antigen-antibody complexes either generated at the basement membrane or deposited there after at the site of infection. Data also suggest that streptokinase is involved in the pathogenesis (Ohkuni *et al.*, 1991). The strains belonging to the M serotypes 2, 4, 12, 49, 55, 57, and 60 have been claimed to induce APSGN (Cunningham, 2000).

1.2.7 The Carrier State

GAS have a potential to invade human epithelial cells at frequencies, equal to or greater than classic intracellular bacterial pathogens such as *Listeria* and *Salmonella* species. The potential to be internalized is suggested to play a role in the carriage and persistence of infection (Cunningham, 2000). The reason for invasion of host cells is not entirely clear, although the streptococci may find the intracellular environment to be a good place to avoid host defense mechanisms. Therefore, internalization of streptococci may lead to carriage and persistence. Studies showed that throat carriers served the purpose of maintaining and disseminating the organism in nature. For example, it was confirmed that GAS strains associated with invasive disease resided principally in a throat reservoir within the human population (Fiorentino *et al.*, 1997).

Furthermore, it has been demonstrated that re-infection of patients recovering from acute pharyngotonsillitis by new clones originated from healthy carriers (Nguyen *et al.*, 1997) In addition, in a study on a single clone of GAS that was responsible for an outbreak of invasive disease in Minnesota, the identical clone was found in association with asymptomatic throat carriage among healthy children within the same community (Cockerill *et al.*, 1997).

Epidemiological data indicate that being in the same school and classroom or living in an orphanage increases the prevalence of carriers, and the streptococcal populations circulating in schoolchildren tend to be highly related (Durmaz *et al.*, 2003). Studies on throat carriage of GAS in healthy children showed that carrier states among healthy children varied: Nigeria 2-6% (Lawal *et al.*, 1990), Iran 11% (Fazeli *et al.*, 2003), Turkey 14% (Durmaz *et al.*, 2003), Egypt 13-24% (el-Kholy *et al.*, 1973). In Ethiopia, one report on carrier rate of GAS reported 7.5% in October and 39% in March among health schoolchildren (Tewodros *et al.*, 1992).

Examples of changes in prevalent strains have been reported. A study with weekly or biweekly cultures reported the gradual introduction and increase in prevalence and retention of specific serotypes in skin and respiratory tract in a prospective study of children in a rural population (Kaplan *et al.*, 2001). In that report, the sudden appearance and rapid disappearance of a single serotype was noted. In another study, 15 different serotypes were found among more than 200 isolates cultured monthly over 4 years from 64 schoolchildren. Almost 90% of the M1 isolates during the entire 4 years were recovered during a single year (Kaplan *et al.*, 2001)

A longitudinal study followed schoolchildren (124) for two years and indicated that, of the children, 36 (29%) never become streptococcal carriers ever once during the period of examination and 88 (71%) became streptococcal carriers at least once during the 29 examinations; among these carriers, 29 children (23%) showed positive results in more than 50% of the examinations (Maekawa *et al.*, 1981). A study showed that children who cleared the carrier state spontaneously demonstrated that they were likely to become carriers again. In this study, ten of 11 children who were GAS carriers in the first year and were monitored over time remained carriers. This phenomenon may suggest a GAS carrier phenotype (Martin *et al.*, 2004).

It was observed that children who are carriers of GAS often acquire infection with new *emm* types. This has clinical implications for children who are known to be carriers. Although the conventional teaching has been that children who are carriers of GAS are not at risk to develop ARF, children may be at risk when they acquire new *emm* types (Martin *et al.*, 2004).

1.2.8 Laboratory Diagnosis of GAS Infection

a) Culture of Throat Specimen

Culture of a throat swab on a sheep-blood agar plate remains the gold standard for the confirmation of the clinical diagnosis of acute streptococcal pharyngitis and carriers. If done correctly, culture of a single swab on a blood agar plate has a sensitivity of 90-95% for the detection of the presence of GAS in the pharynx (Bisno, 2001).

Several variables affect the accuracy of throat culture results. For example, the manner in which the swab is obtained has an important impact on the yield of streptococci from the culture. Throat swab specimens should be obtained from the surface of both tonsils (on tonsillar fossae) and the posterior pharyngeal wall. Other areas of the oral pharynx and mouth are not acceptable sites. These sites should not be touched with the swab before or after the appropriate areas have been sampled (Bisno *et al.*, 2002).

The isolation of streptococci is often complicated by the presence of other bacterial flora, which may either overgrow or restrict the growth of the streptococci. A number of media selective for streptococci have been described. Columbia 5% sheep blood agar supplemented with Colistin and Oxolinic acid (COBA) is one of the best selective media. This medium is selective for streptococci of medical and veterinary importance, and is inhibitory for Gram-negative organisms, staphylococci, bacillus, and coryneforms. Thus, it is valuable for the isolation of streptococci in pure culture from mixed flora such as in throat specimens (Petts, 1984).

In addition, it has been reported that the use of anaerobic incubation may increase the proportion of positive culture results (Kellogg, 1990). Others recommend incubation at 5-10% CO_2 (Bisno *et al.*, 2002; Bourbeau, 2003). In general, data are conflicting with regard to the impact of atmosphere.

A test for bacitracin susceptibility and/or PYR (pyrrolidonyl aminopeptidase) are helpful for presumptive identification of GAS among β-hemolytic streptococci, which then can be confirmed by group specific antibodies (Bisno and Stevens, 2000).

b) Rapid Antigen Tests

The major disadvantage of culturing a throat specimen on blood agar plates is the delay (overnight or longer) in obtaining the result. Rapid antigen detection tests have been developed for the identification of GAS directly from throat swabs. Although these rapid tests are more expensive than blood agar culture, they provide results faster. Almost all such rapid tests involve an acid extraction step to solubilize GAS cell wall carbohydrate and to identify the presence by an immunologic reaction. The great majority of the rapid antigen detection tests currently available have an excellent specificity of ≥ 95%, compared with blood agar plate culture (Bourbeau, 2003; Gerber and Shulman, 2004). Neither conventional throat culture nor rapid antigen detection test accurately differentiate acutely infected persons from asymptomatic *Streptococcus* carriers with intercurrent viral pharyngitis. Nevertheless, they allow clinicians to withhold antibiotics from the great majority of patients with sore throats for whom results of culture and antigen detection tests are negative (Bisno *et al.*, 2002).

c) Serological Diagnosis

The host responds immunologically to streptococcal infections with a plethora of antibodies against many cellular and extracellular components. Serological diagnosis of GAS infection is based on immune responses against the extracellular products streptolysin O, DNase B, hyaluronidase, NADase, and streptokinse, which induce strong immune responses in the infected hosts. Anti-streptolysin O (ASO) is the antibody response most often examined in serological tests to confirm antecedent streptococcal infection, and helps in the diagnosis of rheumatic fever (Cunningham, 2000).

1.2.9. Treatment

Despite 50 years of extensive use, penicillin remains the treatment of choice for GAS infections. (Brook, 2001). However, reports of the failure of penicillin to eradicate GAS from oropharynx are now causing concern. Possibly, they are a result of the presence of beta-lactamase producing oral micro flora, and/or absence in tonsils of normal flora that are capable of interfering with the growth of GAS (Brook and Gober, 1999),.

Macrolides are also an alternative choice. In contrast to penicillin, erythromycin resistance in GAS has emerged and an increasing number of countries are reporting it (Bassetti *et al.*, 2000; Cha *et al.*, 2001; Palavecino *et al.*, 2001; Stamos *et al.*, 2001; Syrogiannopoulos *et al.*, 2001). It is therefore advisable to avoid the routine use of macrolides for GAS pharyngo-tonsilits and save these agents for those patients who are truly penicillin or cephalosporin allergic (Brook, 2001).

1.2.10. Prevention and Control

Reducing overall exposure to GAS is the mechanism by which many GAS diseases have been relatively uncommon in more developed countries over the past century. The importance of crowding in transmission of GAS has been well documented. Some strategies within this category may, theoretically, be feasible-e.g., reducing class room sizes in schools-but for the most part these changes are intrinsically linked with economic development (WHO, 1995).

Besides, there is currently no strategy for primary prevention of GAS disease that has a proven cost-effective benefit at the population level. Of the existing primary prevention interventions, antibiotic treatment of symptomatic GAS pharyngitis has been the most intensively studied, advocated and practiced. The efficacy of a large-scale primary prophylaxis program in reducing transmission of GAS and incidence of GAS pharyngitis has been demonstrated, although this strategy is unlikely to be cost-effective (Carapetis, 2003).

Of the available control strategies, secondary prophylaxis (the delivery of regular doses of penicillin to ARF/RHD) is the only one that has been definitively shown to be both effective and cost-effective at the community level. In populations, with high prevalence of RHD, delivery of secondary prophylaxis should be the major priority for control of GAS disease (Carapetis, 2003). Tertiary prevention of RHD (the provision of medical and surgical treatment to patients with RHD and heart failure) is largely expensive and palliative (Carapetis, 2003).

The development of a safe and efficacious vaccine against GAS has received a major impetus to move forward with the re-emergence of invasive disease in the developed countries and increased recognition of the devastating public health impact on developing countries. One of the major vaccine candidates is the surface M protein, which is a primary virulence determinant (Carapetis, 2003). Currently a 26 valent M protein based GAS vaccine has been formulated. The selection of M protein serotypes for inclusion in the vaccine was based on current epidemiologic studies of invasive disease, pharyngitis and historical information linking certain serotypes to the pathogenesis of ARF. In addition, certain serotypes, such as M19 and M24 that have historically been identified as rheumatogenic are also included even though they are not present in the population today. This vaccine was checked for its immunogencity and demonstrated to elict broadly protective opsonic antibodies (Hu *et al.*, 2002).

The highly conserved C5a peptidase that is expressed on the surface of GAS (see section 1.2.4 c) and other streptococcal species, associated with human infections, is another prime vaccine candidate under investigation (Cleary *et al.*, 2004).

1.2.11. Rationale of the study

The epidemiology and clinical patterns of streptococcal infections in the developed countries are well studied. In contrast, information from Africa is scanty. The high prevalence rate of rheumatic heart disease reported from a few African countries, however, has indicated the importance of streptococci as pathogens in this part of the world. In Ethiopia about 60% of all cases of heart morbidity among children are of rheumatic origin (Daniel and Abegaz, 1993). An average of about 100 cases of post-streptococcal glomerulonephritis per year are seen annually at the Tikur Anbessa Hospital, Pediatrics Department (Tewodros *et al.*, 1992).

These figures may not appropriately describe the burden of disease caused by GAS, since the studies are 13 years old and limited in scope. Diseases caused by GAS are denied attention and may be among the most neglected diseases. Therefore, intense control and prevention activities need to be launched. To design an efficient strategy, it is imperative that we understand the epidemiology of GAS in the country.

Moreover, a 26-valent M protein based vaccine by recombinant technology has recently been formulated for North America and Western Europe (Hu *et al.*, 2002), that requires a prior survey of *emm* types before application in other parts of the world.

This cross-sectional study aims at typing GAS strains circulating among healthy schoolchildren in Ethiopia, in order to provide knowledge of GAS types prevailing in the country and to describe the nature and proportion of highly virulent strains. In addition, the usefulness of the vaccine under development can be evaluated in the background of the findings. This first large-scale *emm* type survey in Ethiopia and Africa too may provide data that can be extrapolated for the entire country.

1.3. Hypothesis

Our hypothesis was that each pharyngeal GAS isolate from Ethiopian schoolchildren shared the same *emm*-sequence with one of the published *emm* sequence type or subtype from other parts of the world.

1.4. Objectives

1. To investigate the carriage rate of GAS and other β-hemolytic streptococci among healthy schoolchildren in Ethiopia.

2. To determine the relative prevalence of different *emm* types of Group A streptococci among healthy schoolchildren in Ethiopia.

3. To assess the proportion of prevalent *emm* types among Ethiopian children that are included in the 26 valent M protein based vaccine

CHAPTER II. MATERIALS AND METHODS

2.1 Study Design and Period

The study was a cross-sectional study conducted in Addis Ababa, Gondar, and Dire-Dawa. during the period of October 2004 to January 2005

2.2 Study Participants

The study participants were healthy schoolchildren between 6- 14 years of age, and attending primary schools. A total of 937 children participated in the study. Each study participant had a general physical examination conducted by a Paediatrician before being admitted to the study (Appendix IV). Children with any signs or symptoms of upper respiratory tract infection were excluded. A pre-tested questionnaire was administered to the study participants to document socio-demographic characteristics such as socioeconomic status, history of throat infection (Appendix III).

a) Sample Size

The sample size was calculated based on the reported carrier rate in the literature selecting the relatively higher rate of 35% (Fazeli *et al.*, 2003),

$$N = (Z/\alpha)^2 P (1- P)$$

N= Total sample size Z= 1.96

Z= 95% confidence interval α= 0.03 P= Proportion of carriers

$$N= (1.96/0.03)^2 \, 0.35X0.65$$

Total sample size = **971**

b) Selection of Participants

I) Eligibility Criteria

1. Children 6-14 years of age, who reside in the study area and attend the selected school.
2. Freely given informed consent by parent/guardian and assent by study participants

II) Exclusion Criteria

1. Children who have taken a course of antibiotic within the last four weeks
2. Children with any nasopharyngeal infections such as pharyngitis and tonsillitis.

2.3 Study Area

The study sites were Addis-Ababa, Gondar, and Dire-Dawa. Addis Ababa and Gondar were selected due to their high prevalence of rheumatic heart disease (RHD) cases (Ephrem *et al.*, 1990; Melka, 1996). The prevalence of RHD in Dire-Dawa is not known. Dire-Dawa has a lower altitude than both Addis and Gondar.

a) Addis Ababa

Addis Ababa is the capital city of the Federal Democratic Republic of Ethiopia and the major urban centre in the country. The city is setup with 10 Kifle Ketema and 328 kebeles. The projected population for the year 2004 was 3, 021,766 with a male to female ratio of 1:1.06 (CSA., 1995c). Addis Ababa is located at an altitude of 2,400 m above sea level (EMA, 1988). In the 2004/2005 Academic year a total of 384, 675 children were attending primary education (1-8 Grade level) in the city. Among these, 51% (196,819) were found in public schools, according to the data collected from Addis Ababa Administration Education Bureau.

b)Gondar

Gondar is the capital town of Gondar administrative zone. It is located 750 Kilometers north of Addis Ababa, and has an altitude of 2,200 m above sea level (EMA, 1988). The projected population for the year 2004 was 281, 000 with male to female ratio of 1:1.04 (CSA, 1995a). In the 2004/2005 academic year a total of 33,865 children were registered for primary education, of which 80% attended in public schools, according to data colleted from Gondar Wereda Education Desk.

C. Dire-Dawa

Dire-Dawa is the capital town of Dire-Dawa administrative council. It is located 535 kilometers east of Addis Ababa, and has an altitude of 1,300 m above sea level (EMA, 1988). The projected population size for year 2004 was 256,000 with male to female ratio of 1:1.006 (CSA, 1995b). In the academic year 2004/2005 30, 405 children were registered for primary education in the town; among these 74% (22,626) attended public schools, according to the data collected from Dire-Dawa Administrative Council Education Bureau.

Three schools were selected from Addis Ababa and two each from Gondar and Dire-Dawa for the investigation (Table 2.1). Only public schools were involved in the study, since children attending such schools represent the vast majority of schoolchildren in both urban and rural settings. The schools were selected owing to their relative overcrowding (estimated from the population size of schools) and convenience for the sample collection activity. The total sample size was allocated to the study sites based on proportional sampling. The list of all students in the age ranged of 6 to 14 years was the sampling frame. Computer generated random numbers from the list of schoolchildren were used to select the study participants regardless of their sex and grade level. The classrooms floor area and volume were measured to assess the crowding situation.

Table 2.1 Study Area and Study subjects

Study area	***Number of public schools in the city/town***	***Children attending primary education in public schools***	***Schools involved in the study***	***Target population (schoolchildren age range 6-14)***	***Number of selected participants***
Addis Ababa	80	196,000	1.Misrak Gohe 2. Meskerem 2 3.Yekatit 23 **Total**	1,800 1,300 5,000 **8,100**	98 91 302 **491**
Gondar	14	32,000	1. Abiyot Firae 2. Meseret **Total**	1,600 1,850 **3,450**	120 145 **265**
Dire-Dawa	14	22,000	1. Sabian 2. Legeharae **Total**	1,320 1,240 **2,560**	91 90 **181** **Grand Total = 937**

2.4 Obtaining Approval, Community Sensitization and Informed Consent

Permission to conduct the study was obtained from Addis Ababa Education Bureau and subsequently from Kifle Ketema Education Desks, Amhara Education Bureau, Bahir-Dar, Semen Gondar Education Bureau, Gondar Wereda Education Desk, Dire-Dawa Education Bureau. Eventually schools were selected and final permissions given by schools to conduct the study. The institutional and National ethical clearance committees had approved the protocol before the activities were initiated.

After securing approval from the respective education authorities and schools, sensitization of the selected schools' community was carried out. In Addis Ababa, advantage was taken of parents gathering arranged by schools and Kebele officials (for their own purpose) to sensitize parents. Gatherings within schools for the daily singing of the national anthem were used as the occasions to address the school community and create awareness about the study. Moreover, there was a regular broadcast program in all schools by "mini-media club" (group of interested students in journalism led by a teacher), before the day's classes begin, at break time, and after the day's classes end. Mini-media clubs of schools were used to disseminate information about the study and maximize the level of awareness. Notices describing the study were also posted in schools.

In Addis Ababa, after sensitizing the school communities, an information sheet (Appendix I) and consent form (Appendix II) were given to the randomly selected schoolchildren to deliver to their parents, get the signature of consent, and finally to return the consent form the next day. However, this approach was not good enough to get sufficient participants. Possibly, parents did not get enough information to decide favorably. Misinformation was propagated in schools from unknown sources that terrified the selected students and their parents, such as the tonsils of students will be excised, and so on… A lesson taken from the experience and amendment was made in the approach of obtaining consent in other sites.

In Dire-Dawa and Gondar basically the same approach was employed in sensitizing school communities. However, the technique of informing parents was changed. Invitation memo were sent to parents of the randomly selected schoolchildren, for them to attend a meeting in schools. All relevant information including the study purpose and procedures were explained to the parents at the meetings and clarification given to questions raised by the parents. In addition, the information sheet was distributed and read to the illiterate parents/guardians. This approach ensured that parents received adequate information before giving consent. Consent forms were then distributed to the parents so that they can return them after they gave their consent, confirmed by their signatures.

2.5 Ethical Considerations

Permission to conduct the study was given by all education authorities and schools. In addition, approval was given by the Health Bureaus of Addis Ababa City Administration, Amhara Region and Dire-Dawa Administrative Council for the respective study sites.

The project proposal was reviewed and approved by ethical review committees of AHRI/ALERT, and Addis Ababa University, Faculty of Medicine and finally by National Ethical Review Committee of Ethiopia. Informed consent was obtained from each child's parents or guardian. Verbal assent was obtained from all children. Children with clinical findings were treated. Medications were given free of charges according to prescription given by the examining study Paediatricians. Some children were referred to the nearby health institution for further investigation and management. Parents in Dire-Dawa and Gondar received compensation for the time they spent to come and attend the meeting, and their transport cost was reimbursed. A first aid kit was donated to each school to strengthen the schools Red Cross clubs.

2.6 Collection, Transportation, and Handling of Specimens

Tongue was depressed with a clean tongue depressor, while the tonsillar fauces and the posterior pharyngeal wall behind the uvula were swabbed (swab supplied with the transport media was used) vigorously. Swabbing the checks, tongues, lips or other areas was avoided. A Paediatrician took all swabs at each study site. The swab was immediately inserted into a tube containing Amies transport media (Copan, Italy). The collected throat specimens were transported and processed within 8 hours of collection.

The ALERT Bacteriology Laboratory was used for the processing of the specimens collected from Addis Ababa. The Microbiology Laboratory of Gondar University and the Harari Regional Laboratory were used for the processing of the specimens collected from Gondar and Dire-Dawa, respectively.

GAS and other β-hemolytic streptococci isolates were transported from Dire-Dawa and Gondar to Addis Ababa using Brain Heart Infusion broth (BHI) (Oxoid, UK) at +4 ^{o}C. All β- hemolytic isolates were stored in duplicate in BHI broth with 15 % glycerol at -70 ^{o}C until further processed.

2.7 Isolation and Identification of β-hemolytic streptococci

2.7.1. Isolation

Columbia blood agar (Oxoid, UK) supplemented with colistin-oxolonic acid (Oxoid, UK) (see Appendix V) was the growing medium used to isolate the organisms (Petts, 1984). Throat specimens were rolled firmly over one-sixth of the plate to deposit the specimen, then a loop was used to streak the inoculum over the surface of the plate and plates were incubated at 35 ^{o}C over night. CO_2 incubator was used in Addis Ababa, whereas in Dire-Dawa and Gondar candle jar was used to provide an atmosphere of 5% CO_2.

After 24 hours of incubation, each plate was checked for colonies with β-hemolytic characteristics. Culture plates negative for β-hemolytic colonies were incubated for additional

24 hours to allow for the recovery and detection of slow growers. Isolated β- hemolytic colonies were selected and subcultured onto Blood agar plates (with out supplement) to obtain a pure growth. After overnight incubation, the pure colonies were tested for their Gram reaction, catalase.

All β-hemolytic and catalase negative colonies were tested for Bacitracin susceptibility. For this purpose, the colonies were inoculated into BHI broth and incubated at 35 ^{o}C in 5% CO_2 overnight in order to obtain a bacterial suspension. The bacterial suspension was evenly spread onto blood agar plate using swab, and 0.05 U Bacitracin disc (Oxoid, UK) was placed on the inoculated surface and incubated for 18-24 hour at 35 ^{o}C in 5% CO_2. Any zone of inhibition surrounding the disc was indicative of a presumptive GAS (see Appendix V). All β-hemolytic colonies were further tested with the dry spot streptococcal grouping kit (Oxoid, UK) to determine their serogroup (Petts, 1999).

2.7.2. Serogrouping

Colonies to be tested were subjected to antigen extraction; for this purpose 2-5 colonies were mixed with 0.4 ml of extraction enzyme and incubated for 10 minutes at 37 ^{o}C in water bath. The result was an antigen extract ready for serogrouping. The reagent card sticks were placed on the card with the spots touching the antigen suspension. The card was rocked gently for one minute and checked for agglutination that indicated positive result for the respective tested antigen (see Appendix V).

2.7.3. API Testing

Non-groupable isolates and strains that could not be amplified were tested by API biochemical testing (BioMeurex, France) to determine their species. A turbidity standard of McFarland 4 was prepared and the suspension was distributed in the first half of the strip. The rest of the suspension was transferred into an ampoule of a medium supplied with the kit. Then, the new suspension was distributed into the second half of the strip; mineral oil was filled and incubated at 37 ^{o}C overnight. The next day reagents were added to the strip and incubated for 10 minutes at ambient temperature and then the reaction was read with reference to the reading table (see Appendix V).

2.7.4. Reference Strains

All batches of freshly prepared media were tested for sterility by incubating representative un-inoculated plates or tubes, and control organisms inoculated on the media and incubated parallel to the specimens. For the COBA plates, *Streptococcus pyogene* (ATCC 19615) was used as a positive control and *Proteus mirabilis* (ATCC 43071) was used as a negative control. For Bacitracin discs *Streptococcus pyogenes* (ATCC 19615) and *Streptococcus agalactae* (ATCC 12386) were used as a positive and negative control respectively. For the dry spot streptococcal grouping kit, control sticks consists of dried antigen extract of A, B, C, D, F and G streptococci were used. In addition control organisms including *Streptococcus pyogene* (ATCC 19615) and *Streptococcus agalactae* (ATCC 12386) were used to check the correct working of the latex reagent.

2.8. Molecular Typing

Frozen isolates were transported to Karolinska Institute, Stockholm (Sweden) on dry ice where molecular typing was conducted

2.8.1. DNA Extraction

DNA templates were prepared by boiling bacterial suspensions in distilled water (personal communication). A loop-full of colonies were picked from an overnight growth on blood agar and suspended in 100 µl distilled water. The suspension was then heated at 94 ^{o}C for 2-3 minutes and used for PCR immediately or stored at -20 ^{o}C until used.

2.8.2. Primers

Primers 1 and 2 as described by CDC (Table 2.2) were used for PCR. Primer 1 was also used for sequencing. For a few isolates, amplification was unsuccessful with primers 1 and 2, and thus the alternative primer set MF2/MR1 was used as recommended by CDC (Table .2.2).

Table 2.2 List of primers used for PCR and sequencing

Primer Name	Primers sequence	Reference	Synthesized by
Primer 1 (Forward)	5' TATTCGCTTAGAAAATTAA 3'	(Beall, 2005)	Cybergene, Sweden
Primer 2 (Reverse)	5' GCAAGTTCTTCAGCTTGTTT 3'	(Beall, 2005)	Cybergene, Sweden
MF2 (Forward)	5' GGATCCATAAGGAGCATAAAAATGGCTA3'	(Beall, 2005)	Cybergene, Sweden
MR1 (Reverse)	5' TGATAGCTTAGTTTTCTTCTTTGCGTTTT 3'	(Beall, 2005)	Cybergene, Sweden

2.8.3. PCR

PCR amplification of the *emm* gene was performed in a total volume of 50 µl reaction mixture contained 2 µl of the bacterial lysates, 5 µl 10X buffer PE GOLD, 5 µl $MgCl_2$ (25 mM) 2.5 µl of each primer 1 and 2 (10 pmole/ µl), 1 µl of dNTP(1 mMole of each)and 0.25 µl AmpliTaq Gold, Applied Biosystems. Amplification was done on a PCR Thermal cycler, GeneAmp, PCR system 9700, Applied Biosystems using the cycle parameters as described by CDC; 94 ^{o}C for 10 s followed by 10 cycles of 94 ^{o}C for 15 s, 46.6 ^{o}C for 30 s, 72 ^{o}C for 1 min 15 s followed by 20 cycles of 94 ^{o}C for 15 s, 46.5 ^{o}C for 30 s, 72 ^{o}C for 1 min 15 s with 10 s increment for each of the subsequent 19 cycles followed by 72 ^{o}C for 10 min then 4 ^{o}C storage (Beall, 2005); (Appendix V).

Amplification products were purified with JetQuick spin column technique (Germond, Germnay) according to the manufacturer's instructions. PCR products were then run at 100 Volt, 400 mA for 40-45 minutes on 1% agarose gel to ensure that there was a single band and to estimate the concentration of the products for the sequencing reaction.

2.8.4. Sequencing and BLAST Analysis

Approximately a volume of 4µl of PCR product was sequenced by using primer 1 (see Table 2.2) with the dye terminator mix version 3.1(Applied Biosystems, Foster City, CA, USA). The sequencing products were purified using ethanol sodium acetate precipitation (see Annex V). The purified sequencing products were subjected to automated sequence analysis after adding 10 µl of Formamide (Sigma, Germany) on a 3100 model autosequencer (Applied

Biosystems) as per manufacturer's instructions. The cycling parameters were 96 ^{o}C for 1 min for initial denaturation, followed by 25 cycles of 96^{o}C for 10 s, 50 ^{o}C for 5 s, 60 ^{o}C for 4 min and a holding temperature of 4 ^{o}C.

The sequencher^{TM} version 3.0 genetic software program was used to edit the sequences, if in case discrepancies occur between peaks in the electrophorogram and assigned nucleotide. The 5 ' end of the sequences were compared to sequences in the database available at the CDC website (Beall, 2004b). Pair wise comparison of the nucleotide homology for the first 150 bases of the hypervariable region was conducted. Strains which showed 100% homology with a reference strain, were designated the particular parental *emm* type. Sequences that showed one or more base pair discrepancies with reference sequences were sent to Dr Bernard Beall, CDC, Atlanta, for verification and subsequent designation of *emm* subtype.

Moreover, two of the newly described sequences were submitted to the European Molecular Biology Laboratory-European Bioinformatics Institute (EMBL-EBI) (Gene bank) for registration, and accession numbers (AJ890457 and AJ891032) were obtained. The remaining eleven will be submitted in the future.

2.9. Data Entry, Management, and Analysis

Data were submitted to the data management unit of AHRI. Information obtained from the questionnaire, physical examination and the laboratory result were double entered into Excel and Epi Info version, 6.04b, by a statistician and a data entry clerk, were verified and validated. In analyzing the data, both Epi Info and statistical package STATA software version 7 were used. $P < 0.05$ was used to determine statistical significance.

CHAPTER III. RESULTS

In this cross-sectional study we present the results of a survey on the prevalence of β-hemolytic streptococci and GAS among Ethiopian healthy schoolchildren in Addis Ababa, Gondar, and Dire-Dawa. The survey was carried out in November 2004 in Addis Ababa and it was conducted in December 2004 and January 2005 in Dire-Dawa and Gondar respectively. The total number of study participants screened was 988; out of this, complete data were available for 937 participants. Of the 937 children, 491(52.4 %) were from Addis Ababa, 181(19.3 %) from Dire-Dawa, and 265 (28.3 %) from Gondar. Parent refusal rate was 50% in Addis Ababa, and 2% in Dire-Dawa and Gondar. The median and the mean age of the study participants were 11 (ranged 6-14) and 10.5 (± 2 SD), respectively (Figure 3.1). Girls constituted 52% (486/937) of the study participants.

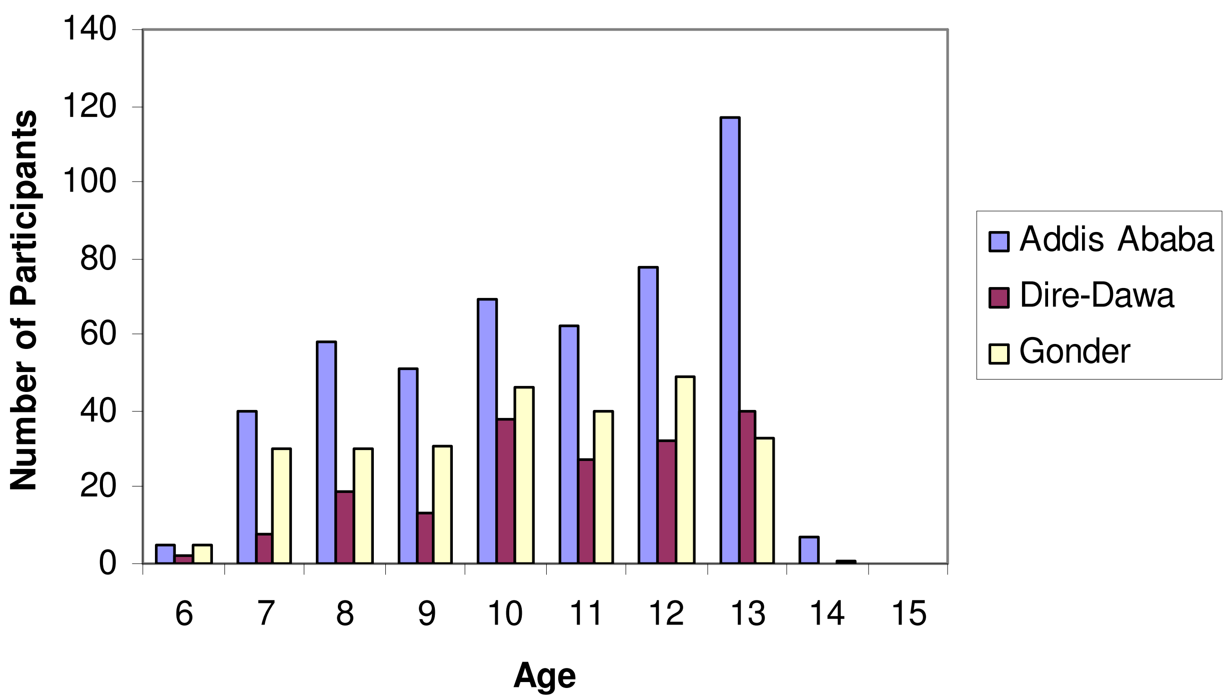

Figure 3.1 Age distributions of study participants in the three study sites.

3.1. Crowding

The bedroom of 34% (317/937) of participants was not separate from their kitchen and living room that might signify their poor economic status and 52% (488/937) of the students shared their bedroom with 4 or more people. In the classrooms, the floor area per pupil ranged from 0.24- 0.7 m^2 and the volume of classrooms per pupil ranged between 2.1-2.5 m^3, these parameters are much below the standards 1.4 m^2 (floor area/pupil) and 5.7 m^3 (volume/pupil) (Hailu *et al.*, 2002).

3.2. Clinical Findings

In Gondar 139 study participants were assessed for the status of uvula by the paediatrician. It was found that 45% (63/139) of them had uvulectomy. In addition, upon physical examination, 22% (205/932) of study participants were found to have tonsillar hypertrophy. Tonsillar hypertrophy found to have no association with GAS carriage P=0.124.

Among the study participants, 29 % (276/931) reported that they had experienced pharyngotonsillitis within the last month and among them 45% complained of recurrent pharyngotonsillitis (3 or more attack per year). This characteristic has no association with GAS carriage (P=0.23). The majority of participants who reported infection within the previous month did not go to a clinic. They rather used traditional treatment such as application of ginger, lemon, and salt to relieve their symptoms.

The result of physical examination revealed that 1.3 % (13/937) of the study participants had cardiac findings including murmur and/or dysrhythemia and they were referred to the nearby hospital for further management. Ten of them were from Addis Ababa. Children with cardiac finding did not report any family history of cardiac illness. No study participant was taking monthly benzanthin penicillin injections, in any of the three study sites.

3.3. Serogroup Prevalence

GAS accounted for 54.5% of all β-hemolytic streptococci. Group G, Group C streptococci accounted for 17.9% (30/167) and 12.6% (21/167), respectively of all streptococci isolated (see Fig. 3.2). A significant number of Group F streptococci were also collected, especially in Addis Ababa, 10/95 (10.5%). The colony characteristic of group F was quite different from others in that they were pinpoint sized and quite difficult to pick up from the plates, with loops. The frequency of GAS carriage in all age groups was found to be similar (P=0.678).

The carrier rate of GAS among the study participants was 10.8 % (52/491) in Addis Ababa, 8.7% (23/264) in Gondar, and 9.4% (17/181) in Dire-Dawa. The carriage rate did not show difference among study sites (P=0.590).

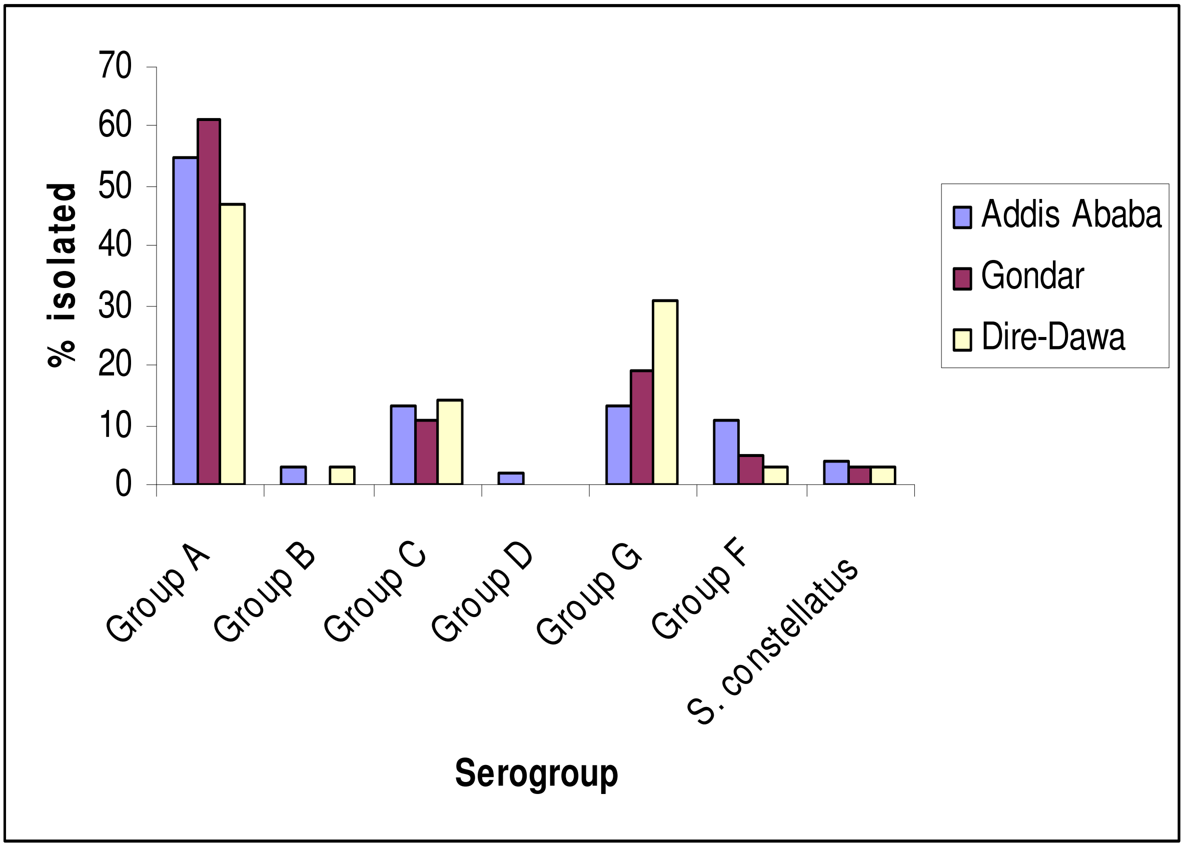

Figure 3.2 Proportion of Lancefield serogroups of β-hemolytic streptococci isolated from the throats of healthy schoolchildren in three cities in Ethiopia, 2005.

Table 3.1. Distribution of β-hemolytic streptococci among healthy schoolchildren in urban centres

Study site	Group A	Group B	Group C	Group D	Group F	Group G	*S.constellatus*	Total
Addis Ababa	52(54.7%)	3(3.1%)	12(12.6%)	2(2.1%)	10(10.5%)	12(12.6%)	4(4.2%)	**95 (100%)**
Gondar	22(61.1%)	0 (0%)	4(11.1%)	0(0%)	2 (5.5%)	7(19.4%)	1(2.7%)	**36 (100%)**
Dire-Dawa	17 (47.2%)	1(2.7%)	5 (13.8%)	0(0%)	1(2.7%)	11(30.5%)	1(2.7%)	**36 (100%)**
Total	**91(54.5%)**	**4(2.4%)**	**21(12.6%)**	**2 (1.1%)**	**13(7.7%)**	**30(17.9%)**	**6(3.5%)**	**167 (100%)**

All β-hemolytic streptococci were tested for their sensitivity to Bacitracin (0.05 U) and all GAS isolates demonstrated susceptibility (significant zone of inhibition), and all non group A streptococci were found to be resistant (no zone of inhibition).

Six of the isolated β-hemolytic streptococcal strains could not be grouped using the carbohydrate grouping technique. The API biochemical testing was used to identify their species. According to the result of API testing all six non-groupable isolates turned out to be *Streptococcus constellatus*. Four of the isolates obtained from Addis Ababa and 1 each from Gondar and Dire-Dawa. Group B and D were relatively less frequent. Three strains of Group B were detected from Addis, 1 from Dire-Dawa and none from Gondar. Group D streptococci were detected in two cases from Addis Ababa only (Table 3.1).

Among the 90 GAS isolates, only 82 were typed, the remaining were not typed. One of the un-typed isolates failed to be recovered in the laboratory where the molecular typing was carried out and the other seven isolates could not be amplified. Among them, agreement was lacking between serogrouping and biochemical testing for three of the isolates. The serogrouping technique (tested with a product of two different companies) identified these isolates as group A streptococci. Conversely, the biochemical testing detected them as *S. constellatus*, *S. agalactia*, and *S. suis II*. All these three isolates were collected from Addis Ababa. The other four isolates were *Streptococcus pyogenes*; however, their *emm* gene could not be amplified even with alternative set of primers that were recommended by CDC (Table 3.2).

Table 3.2. Serogrouping and API biochemical results of isolates which failed to be amplified

S.No	Geographical origin of the Isolate	Serogrouping result I (Oxoid, UK)*	API Result (Biomeurex, France)*	Serogrouping result II (Phadabect,Italy)*
1	Addis Ababa	GAS	*Streptococcus constellatus*	GAS
2	Addis Ababa	GAS	*Streptococcus agalactia*	GAS
3	Addis Ababa	GAS	*Streptococcus pyogenes*	GAS
4	Addis Ababa	GAS	*Streptococcus pyogenes*	GAS
5	Addis Ababa	GAS	*Streptococcus pyogenes*	GAS
6	Addis Ababa	GAS	*Streptococcus suis II*	GAS
7	Gondar	GAS	*Streptococcus pyogenes*	GAS

* indicates the company in which the products were manufactured.

3.4. emm Type Distribution

Ninety isolates were subjected to molecular typing and 82 of them were successfully typed. The PCR product of *emm* gene run on gel compared with 1.5 Kb DNA ladder, they ranged between 0.8 – 1.3 Kilobase (Figure 3.3). Gel visualization helped to estimate the concentration of the PCR product and to decide the amount of template to be added in the preparation of sequencing reaction.

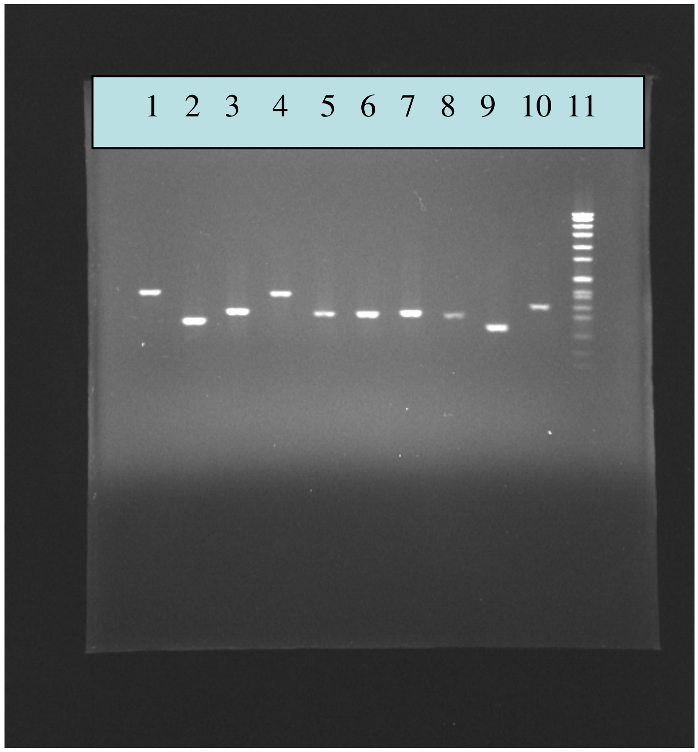

Figure 3.3 *emm* gene PCR products of the isolates run on 1% agarose gel.

Lane 1 *emm* 3.19, Lane 2 *emm* 89.0, Lane3 *emm* 30.2, Lane 4 *emm* 12,
Lane 5 *emm* 93, Lane 6, *emm* 80.0, Lane 7 *emm* 30.2, Lane 8 *emm* 8.1,
Lane 9 *emm* 85.0 Lane10 *emm* 14.8, Lane 11 1.5 Kb DNA ladder

The *emm* gene was sequenced from each of 82 isolates, and the *emm* type with 100% homology to the sequences in the CDC website and new *emm* types discovered in this study are listed in Table 3.3. The 82 isolates examined represent 44 different sequence types. Among them18 GAS isolates belong to 13 new *emm* subtypes.

Sequences of 18 strains demonstrated one or more base discrepancies with the database of CDC and thus the raw data were submitted to CDC for verification and obtaining designations for the new subtypes. For example a sequence of one strain had 99% (149/150) identity with reference sequence of *emm*119.1. The discrepancy was at the 143rd base, which was “A” in the query sequence while it was “G” in the reference sequence (Figure 3.4). This strain got new designation, *emm*119.3. Similarly, the 18 strains belonged to 13 new subtypes and designations were given for all of them.

```
Query: 36  GATCAGCCTAATCACCCTAGATATACCGATGCTAACAATGCAGTACGAAATGGGCTCTCT 95
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 1   GATCAGCCTAATCACCCTAGATATACCGATGCTAACAATGCAGTACGAAATGGGCTCTCT 60

Query: 96  CCATGGGATCGGGCTGTGTTAGCTGAAATAGACAAAAATGACAAACTTAGATTGGAAAAT 155
           ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 61  CCATGGGATCGGGCTGTGTTAGCTGAAATAGACAAAAATGACAAACTTAGATTGGAAAAT 120

Query: 156 GAAAAGCTTAAGGCTGGTTTACAGGAAAAG 185
           |||||||||||||||||||||| |||||||
Sbjct: 121 GAAAAGCTTAAGGCTGGTTTACGGGAAAAG 150
```

Figure 3.4. Comparison of a query sequence with the reference strain sequence.

Six different types, listed in a descending order of frequency, *emm* 3.19, *emm* 5.48, st62.0, *emm* 29.2, st 463.0, and st3757.0 made up 35.0% of the isolates (see Table 3.3). The most frequent type *emm* 3.19 comprised 14.6% of the total isolates. *emm* 3 is among the most prevalent type in the temperate regions. It is also important to note that the previously undescribed *emm* 5.48, one of the most common types in our collection was isolated only from Gondar.

Table 3.3 Distribution of Group A Streptococcal *emm* types isolated from the throats of healthy Ethiopian schoolchildren.

emm type	Geographical origin			Total No of isolates
	Addis Ababa	Dire-Dawa	Gondar	
emm 1.0	2	0	0	2
emm 2	2	0	0	2
emm 3.19	11	0	1	12
**emm* 5.47	2	0	0	2
**emm* 5.48	0	0	4	4
**emm* 6.40	1	0	0	1
emm 8	1	0	0	1
emm 12.0	0	1	0	1
**emm* 14.8	0	0	1	1
emm 18.0	1	0	0	1
emm 28.0	1	0	1	2
**emm* 28.5	1	0	0	1
emm 29.2	3	0	0	3
**emm* 29.5	1	0	0	1
**emm* 30.2	0	2	0	2
**emm* 38.1	1	0	1	2
**emm* 39.2	1	0	0	1
**emm* 42.5	1	0	0	1
emm 43.7	0	0	1	1
emm 44/61.0	2	0	0	2
**emm* 53.4	2	0	0	2
emm 56.0	1	0	0	1
emm 68.0	0	0	1	1
**emm* 68.5	1	0	0	1
emm 74.0	1	1	0	2
emm 75.0	1	0	0	1
emm 80.0	0	1	0	1
emm 85.0	0	1	0	1
emm 86.2	0	0	1	1
emm 89.0	0	1	0	1
emm 90.3	1	0	0	1
emm 92.0	1	0	0	1
emm 93	0	1	0	1
emm 95.0	1	0	0	1
emm 102.4	0	0	1	1
emm 103.0	1	0	0	1
emm 106.2	1	0	1	2
emm 109.1	0	0	1	1

**emm* 119.3	1	0	0	1
ST62.0	0	0	4	4
ST212.0	1	0	0	1
ST430	0	1	1	2
ST463.0	2	0	1	3
ST854.1	0	0	1	1
ST1731.1	1	1	0	2
ST3757.0	0	2	1	3
ST6735.0	0	0	1	1
Total	**47**	**12**	**23**	**82**

Note: * indicates an *emm* type that has previously not been described anywhere else.

The result showed that there was no significant overlap in distribution of GAS types in the three study sites. Only seven *emm* types were isolated from two study sites. Addis Ababa and Gondar shared many of the overlapping GAS types, specifically *emm* 3.19, *emm* 28, *emm* 38.1, *emm* 106.2, and st 463.0. In Addis Ababa and Dire-Dawa *emm* 74.0 and st1731.1 were overlapping, whereas Gondar and Dire-Dawa had only st3757.0 in common. However, not a single isolate occurred in all the three study sites (Table 3.3).

Epidemiological observations indicated that certain GAS types are associated with acute rheumatic fever and the prevalent types in study sites that are known to trigger ARF are indicated (see Table 3.4). Among the isolates, previously described rheumatogenic strains constituted 56% (17/30) for Addis Ababa and 35% (6/17) for Gondar. No known rheumatogenic strain was isolated from Dire-Dawa, (Table 3.4).

Table 3.4 Proportion of known rheumatogenic strains in three cities

Known Rheumatogenic strains	Number of isolates per site			
	Addis Ababa	Dire-Dawa	Gondar	Total
emm 1.0	2	0	0	2
emm 3.19	11	0	1	12
emm 5.47	2	0	0	2
emm 5.48	0	0	4	4
emm 6.40	1	0	0	1
emm 14.8	0	0	1	1
emm 18.0	1	0	0	1
Total number of known rheumatogenic strains	17	0	6	23
Unknown types	30	12	17	59
Proportion of known rheumatogenic strains	56%	0%	35 %	39%

The result here showed that quite large portion (12/26) of our collection was not in the vaccine that was formulated for USA and Western Europe. On the average 60% isolates were not represented in the vaccine (Table 3.5).

Table 3.5 *emm* types included in the 26 multivalent vaccine composed of 26 *emm* types (Hu *et al.*, 2002)

emm type	Represented	Addis Ababa	Gondar	Dire-Dawa	Total number
emm 1	yes	2	0	0	2
emm 1.2	No				
emm 2	yes	2	0	0	2
emm 3	yes	11	1	0	12
emm 5	yes	2	4	0	6
emm 6	yes	1	0	0	2
emm 11	No				
emm 12	yes	0		1	1
emm 13	No				
emm 14	yes	0	1	0	1
emm 18	yes	1	0	0	1
emm 19	No				
emm 22	No				
emm 24	No				
emm 28	yes	2	1	0	3
emm 29	yes	4	0	0	4
emm 33	No				
emm 43	yes	0	1	0	
emm 59	No				
emm 75	yes	1	0		1
emm 76	No				
emm 77	No				
emm 89	yes	0	0	1	1
emm 92	yes	1	0	0	1
emm 101	No				
emm 114	No				
Total no of *emm* types represented		10	5	2	17
Total no of prevailing *emm* types		26	17	10	43
Percentage of un represented *emm* types		62%	71%	80%	60 %

CHAPTER IV DISCUSSION

It is now well established that GAS disease constitutes a major public health problem in developing countries, most of which are located in subtropical and tropical climate. The epidemiology of GAS is poorly described and the pathogen not well characterized in these countries (Carapetis *et al.*, 1999). In the present study we presented survey on the prevalence of β-hemolytic streptococci and *emm* type distribution of GAS among Ethiopian healthy schoolchildren in Addis Ababa, Gondar, and Dire-Dawa. A total of 937 study participants were involved in the study of which females accounted for 52%. The age range of the participants was 6-14, and the median age was 11.

Our findings show that the proportion of GAS among β-hemolytic streptococcal isolates from throat swabs of healthy schoolchildren was 55% (52/95) in Addis Ababa, 61% (22/35) in Gondar and 47% (17/36) in Dire-Dawa. Thus, GAS appeared to be the predominant streptococci serogroup. Most studies from temperate regions also showed a predominance of GAS (Quinn, 1989). However, longitudinal studies from other parts of Africa revealed that serogroups other than GAS, mainly C and G were more frequent (el-Kholy *et al.*, 1973; Lawal *et al.*, 1990). A 3- year study conducted a decade after the first report from Nigeria had shown a continued predominance of streptococci of Group C and G. Based on these observations it was suggested that these serogroups were important pathogens of the throat in the tropics (Lawal *et al.*, 1990). In a study conducted in 1992 the frequency of GAS was higher (43%) when compared with Group C (30%) and G (7%) streptococci isolated from healthy schoolchildren in Addis Ababa (Tewodros *et al.*, 1992). The current finding and previous reports may demonstrate the consistent predominance and importance of GAS in Ethiopia.

Susceptibility to bacitracin is one of the preliminary laboratory tests employed in the presumptive differentiation of GAS from other β-hemolytic streptococci (Bourbeau, 2003). Pertinently in the present study, all GAS isolates were susceptible to bacitracin (0.05 U) in contrast to other β-hemolytic streptococci. Recently, bacitracin-resistant isolates were recovered from tonsillo-pharyngitis patients in Belgium (Malhotra-Kumar *et al.*, 2003), and it raised questions about the continued reliability of bacitracin sensitivity testing for GAS identification. In this study, however none was detected, and therefore we may keep using bacitracin sensitivity testing (for routine use) for identification of GAS in our country owing to its significant economic implication.

The serogrouping technique could not classify 4 % (6/166) of the isolated streptococci. Thus, API biochemical testing was employed to determine the species of these non-groupable isolates which all turned out to be *Streptococcus constellatus.* Similarly, it has been described that some strains of *S. constellatus* were nongroupable, but frequently they possess Lancefield group F antigens (Facklam, 2002). *S. constellatus* strains are often isolated from respiratory and many other sources, and are commonly associated with suppurative and deep-seated infections, particularly at thoracic sites (Chiang *et al.*, 2004).

In response to the repeated failure to amplify the *emm* gene of seven GAS isolates, API testing was conducted and four of them were confirmed to be *S. pyogenes.* Three of the GAS isolates, however, were not *Streptococcus pyogenes* as have been confirmed by API biochemical testing. They were *S. constellatus, S. agalactiae, and S.suisII.* The serogrouping result was re-checked by a product of a different company and a similar result was obtained. This might indicates that *S. pyogenes* was not the only *Streptococcus* species that might possess the group A antigen (Facklam, 2002; Lawrence *et al.*, 1985).

The carrier rate of GAS among schoolchildren was 10.8 % (52/491) in Addis Ababa, 8.7 % (23/264) in Gondar, and 9.4 % (17/181) in Dire-Dawa. The average carrier rate of GAS in the three sites was 10%. This result was markedly higher when compared to the isolation rate, 1.9%, from a similar survey in Lagos (Lawal *et al.*, 1990) However, it was lower than those reported from United Arab Emirates 35%(345/1000) (Ameen *et al.*, 1997) Korea 17% (98/581) (Kim and Lee, 2004), Turkey 14%, and comparable to Iran 11% (175/1588) (Fazeli *et al.*, 2003). The carrier rate described in this study was in agreement with a similar survey conducted before a decade in Addis Ababa which had also indicated that carrier rate of GAS in schoolchildren varied between 7.5% in October to 39% in March (Tewodros *et al.*, 1992). In the present study GAS carriage was found to be similar in all age groups as reported elsewhere (Durmaz *et al.*, 2003)

As we can infer from housing conditions of the study participants, their socioeconomic status was very low. The schoolchildren attended classes a day or half day long in very crowded classrooms. The carrier rate was, however, below that described for affluent children in more developed countries (Ameen *et al.*, 1997). This clearly showed that socioeconomic status and crowding conditions were not the only determinant factor for the epidemiology of GAS. Seasonal variation may play a major role in distribution of GAS (Tewodros *et al.*, 1992; Veasy *et al.*, 1994).

Studies indicated that a high frequency of GAS carriage existed among patients with hypertrophic tonsils and recurrent pharyngotonsillitis (Stjernquist-Desatnik *et al.*, 1991). However, the results here indicated that both groups of study participants (children with hypertrophic tonsil and recurrent pharyngotonsillitis) had no statistically significant different in their carrier status than others.

In Gondar, 139 children were investigated for the status of uvula during physical examination and surprisingly 45% were found to have their uvula excised. This practice should strictly be discouraged, especially in this era of Human Immunodeficiency Virus (HIV) (Miles and Ololo, 2003).

One of the major objectives of this study was to determine the prevalence of various GAS *emm* types. Homology to existing *emm* types was defined by examining the amino terminal region of *emm* genes, because this region imparts serotypic identity to each GAS strain. Since the introduction of the sequence typing method of GAS based on the heterogeneity of the the 5' end of the *emm* gene (Beall *et al.*, 1996), several previously undescribed new types have been detected. The number of distinct *emm* types has more than doubled in the last few years and currently the CDC database contains over 225 distinct *emm/*st types.

Allelic variations in the M proteins of our isolates, compared to reference sequences, were predominantly due to single base substitutions, with several new alleles. Thus, 18 isolates belonged to 13 previously unrecognized sequence types. The detection rate of unrecognized types varies from country to country. In general more new types were reported from Malaysia (Jamal *et al.*, 1999), Thailand (Pruksakorn *et al.*, 2000), Brazil (Teixeira *et al.*, 2001), USA (Beall *et al.*, 1997) and Australia (Bessen *et al.*, 2000) compared to that reported from European countries. It is interesting to note that no new *emm* type was detected among 133 isolates which were non typeable serotypes in Israel (Moses *et al.*, 2003). The number of newly discovered types among the Ethiopian isolates is comparable to that reported from Malaysia, Brazil and Thailand (Jamal *et al.*, 1999; Pruksakorn *et al.*, 2000; Teixeira *et al.*, 2001).

In this study, 6 strains (*emm 3.19*, *emm* 5.48, st62.0, *emm*29.2, st463.0, and st3757.0) accounted for 30.5% of isolates. The earlier study conducted in Addis Ababa detected 78 distinct *emm*/st types out of 217 isolates collected from symptomatic and asymptomatic participants (personal communication). The predominance of GAS isolates in both studies is therefore less than that seen in other studies. In Japan only 29 *emm*/st types were detected among 906 clinical isolates (Tanaka *et al.*, 2002). In Mexico, only 31 *emm* types were detected among 423 isolates (Espinosa *et al.*, 2003). Six serotypes accounted for 60% of all isolates recovered from patients with uncomplicated pharyngitis collected in the United States (Johnson *et al.*, 1992).

Few reports examining *emm* type diversity outside of western nations are available; however most support the presence of predominant types. A community study in Korea examining community-wide GAS strain diversity found that *emm* 78 and *emm* 23 accounted for 69% of GAS isolates in one region, whereas, in another region 4 types accounted for 52% of isolates (Kim *et al.*, 1996). By contrast, studies with isolates collected in Australia and India revealed much greater diversity of GAS strains (Dey *et al.*, 2005; Gardiner and Sriprakash, 1996). The factors that influence the epidemiological distribution of particular *emm* types have yet to be determined.

The epidemiological pattern is inconsistent among the study sites. For example, our data showed that in Addis Ababa*,* *emm* 3.19 is a major type among the isolates. However, only one such strain was identified in Gondar and none in Dire-Dawa. In Gondar, *emm* 5.48 and st62.0 were the most predominant and accounted for (4/23) 14.7% each. The result clearly showed that there was no significant overlap in distribution of GAS types in the three study sites, except for seven isolates. Five out of the seven duplications (*emm* 3.19, *emm* 28, *emm*38.1, *emm* 106.2, and st 463.0) occurred in Addis Ababa and Gondar. This may show that the two sites may have a sort of resemblance in GAS epidemiology than each of them do have with Dire-Dawa. This might be attributable to the climatic difference.

As mentioned earlier, this study found that the type *emm* 3 was the most prevalent in pharyngeal isolates of healthy schoolchildren in Addis Ababa. Type M3 was disproprtionaly represented in invasive diseases, in various reports (Chelsom *et al.*, 1994; Cleary *et al.*, 1992a; Colman *et al.*, 1993; Johnson *et al.*, 1992; Musser *et al.*, 1995). It has been showed that M3 was equally prevalent in pharyngeal isolates from throats of schoolchildren (Kiska *et al.*,

1997) Furthermore, the M3 isolates causing invasive infections had PFGE patterns that were identical to those of concurrent pharyngeal isolates (Ho *et al.*, 2003; (Kiska *et al.*, 1997). In this study involving a comparison of a large number of invasive isolates with control strains, there was again no evidence of association between a particular clone and invasive infection (Johnson *et al.*, 1992). Despite the prevalence of *emm* 3 type GAS carriage in Addis Ababa severe invasive disease due to GAS, such as streptococcal toxic shock like syndrome and necrotizing fasciitis have not been reported to the best of our knowledge. Therefore, clinicians should be on the alert to detect such cases.

Based on decades of epidemiological observations GAS serotypes M1, 3, 5, 6, 14, 18, 19, and 24 are known to be associated with rheumatic fever (Stollerman, 2001). Of these rheumatogenic types *emm* 1, 3, 5, 6, 14, and 18 were represented among our collection (Table 3.4). In Addis Ababa 56%, and in Gondar 35% of strains were rheumatogenic. In Dire-Dawa however, no rheumatogenic strains were detected; nevertheless, this does not mean that prevailing types would not trigger rheumatic fever. In a previous study conducted in Addis Ababa, *emm* 74 and st62 were detected from the throats of ARF cases, although it was not possible to discriminate whether these isolates were in the carrier state or whether they were actually responsible for triggering rheumatic fever (Personal Communication). In the present study both types were detected.

Epidemiological surveys from Europe, USA and the Caribbean islands have associated M serotypes 2, 4, 12, 15, 25, 49, 55, 56, 59, 60, and 61 with acute poststreptococcal glomerulonephritis (APSGN) (Bisno, 1991; (Cunningham, 2000). Of these, only *emm* 2, *emm* 56, and *emm* 12 were represented among our collection, the former two were collected from Addis Ababa where as the latter was isolated in Dire-Dawa. However, it was indicated that types other than what have been reported elsewhere as nephritogenic are more important in causing acute poststreptococcal glomerulonephritis (APSGN) in Ethiopia (Personal Communication).

The concept of distinct throat and skin *emm* types has been widely accepted. It has been described that two *emm* types with three isolates each, *emm* 80 and st212, were associated with skin origin only (Personal Communication). However both of them were isolated from throats of healthy schoolchildren in this study.

The highly diversified nature of the GAS population in Ethiopia challenges the application of a multivalent vaccine composed of the N terminal region of the most common *emm* types described earlier. A multivalent vaccine composed of 26 M protein N terminal region was anticipated to protect about 90% invasive GAS infections in USA (Hu *et al.*, 2002). Out of these 26 *emm* types included in this multivalent vaccine only 38% of isolates in Addis Ababa, 29% in Gondar, and 20% in Dire-Dawa were detected among the GAS collection (Table 3.5). In addition, ten out of the seventeen (59%) most frequently isolated *emm* in the present study were not included in the vaccine. Theoretically it would prevent only 40% of the infection, if applied in the study area.

Thus, the 26 *emm* multivalent vaccine, which is basically designed for the USA population, may prove ineffective in preventing GAS infection in the Ethiopian population. Even if all the currently circulating *emm* types are covered, it is still possible that new strains not covered by the vaccine could be introduced into the vaccinated population and that naïve hosts could become colonized. Therefore, this particular strategy may appear to have limited effect on GAS infection and only serve to alter the circulating GAS *emm* types (Kaplan *et al.*, 2001).

A similar *emm* type survey had been conducted on stored isolates from healthy schoolchildren (Addis Ababa) collected in 1990 and showed that *emm* 12 was the predominant sequence type accounting for 23% (9/39) of all isolates (Personal Communication), whereas in our study *emm* 3.19 was the most common type with 23% (11/47) prevalence in Addis Ababa. In addition, out of 20 *emm* types demonstrated in 1990 only 3 (15%) sequence types (*emm* 1, *emm* 28, *and emm106.2*) were represented in our study. Examples of changes in the prevalent strains have been reported elsewhere. A two year intensive study with weekly or biweekly cultures revealed the gradual introduction and increase in prevalence and retention of specific serotypes in a study of children. In that report, the sudden appearance and rapid disappearance of a single serotype was noted (Kaplan *et al.*, 2001). Likewise observation in this study demonstrated possible replacement of *emm* types in Addis Ababa; however the rate of replacement with new *emm* types is not known and warrants further study.

The observed displacement of prevalent *emm* type with new types in Addis Ababa further complicates the design of M protein amino-terminal vaccines for specific geographical locations, especially in areas of high transmission. As more and more GAS isolates are being collected from different locations, particularly from the developing countries where the disease burden is still high, it is important to continuously monitor the emerging *emm* types (Dey *et al.*, 2005).

CONCLUSIONS

- Group A streptococci are the most common β- hemolytic streptococci isolated from the throats of healthy schoolchildren carriers in Ethiopia

- There is a high diversity of Group A streptococcal *emm* types among healthy Ethiopian schoolchildren

- Considerable proportion of GAS types were known rheumatogenic strains

- Significant proportion of *emm* types in Addis Ababa are strongly associated with invasive diseases elsewhere

- GAS *emm* types circulating at a certain time may be replaced by others over the years.

- Theoretically, 26 valent GAS vaccine under development may not provide significant protection against GAS disease and its subsequent complications to Ethiopian schoolchildren, because it does not include more than half of the prevalent *emm* types circulating in schoolchildren.

RECOMMENDATIONS

- This finding indicated that there were significant proportions of GAS *emm* types that might be able to trigger rheumatic fever among schoolchildren; therefore clinicians should consider all sorts of throat infections seriously, in order to combat the sequelae.

- Parents/guardian should not underestimate sore throat even if it is self-limiting, thus they have to visit clinics in good time for proper management and prevent the severe complications.

- The most predominant strain represented in this study is strongly associated with severe invasive diseases in industrialized countries thus; clinicians should be alert to detect invasive diseases due to GAS.

- Health education should be given to the community against the traditional practice of uvulectomy.

- The high diversity of *emm* types encountered in this study and the observed replacement of *emm* types over time suggested that any M protein based multivalent vaccine would have to be specifically tailored for this region in addition, investigation on another vaccine candidate, highly conserved C5a peptidase should be intensified to address the burden of disease in this region

FUTURE RESEARCH DIRECTION

This work can serve as a baseline study for further complementary investigations to get fairly clear picture of GAS epidemiology in Ethiopia, thus the following research areas are suggested to be investigated.

- ☞ Studies should identify the *emm* types responsible for causing pharyngitis, pyoderma, rheumatic fever and acute post-streptococcal glomerulonephritis in Ethiopian children.

- ☞ The typing of streptococcal isolates from cases of tonsillopharyngitis and rheumatic fever or rheumatic heart disease in Dire-Dawa is particularly important, since the prevalent *emm* types there have previously not been recognized as rheumatogenic.

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Personal Communication

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APPENDIX I Information sheet

የዋና ተመራማሪ ስም፡ ዓለምሰገድ አብዲሣ
የድርጅቱ ስም፡ አዲስ አበባ ዩኒቨርሲቲ ሕክምና ፋካልቲ የማይክሮባዮሎጂ ኢሚዮኖሎጂ እና ፖራሲቶሎጂ ትምህርት ክፍል
የስፖንሰሩ /ደጋፊው/ ስም አርማወር ሀንሰን የምርምር ኢንሲቲትዮት/አህሪ/

ከአዲስ አበባ ፣ ከጎንደር እና ከድሬዳዋ ከተሞች ከተመረጡ አንደኛ ደረጃ ትምህርት ቤቶች ውስጥ ትምህርታቸውን ከሚከታተሉ ተማሪዎች መካከል ግሩፕ ኤ ስትሪኘቶኮክሳይ በተሰኘው ባክቴሪያ ላይ በሚደረገው ጥናት ውስጥ ተሳታፊ ለሚሆኑ ተማሪዎችና ወላጆቻቸው የተዘጋጀ የማብራሪያ ፅሁፍ፡፡

የዚህ ጥናት ዋና ተመራማሪ ከላይ በተጠቀሰው የትምህርት ክፍል ውስጥ የድህረ ምረቃ ትምህርታቸውን በማጠናቀቅ ላይ የሚገኙ ሲሆን ይህንን ማብራሪያ ያዘጋጀነው ዋና ተማራማሪውን ጨምሮ ጥናቱን ለማካሄድ የምርምር ቡድን ያቋቋምን ተመራማሪዎች እና ሀኪሞች ነን፡፡

<u>**የጥናቱ ዓላማ**</u>

የዚህ ጥናት ዓለማ "ግሩፕ ኤ ስትሪኘቶኮክሳይ" በመባል በሚታወቀው እና ለጉሮሮ በሽታ፣ ለልብ ህመም እንዲሁም ለሌሎች የጤና እክሎች መንስኤ የሆነውን ባክቴሪያ በአገራችን ያለውን አጠቃላይ ስርጭት ለመረዳት እንዲሁም በብዛት ተሳፍፍተው የሚገኙትን የዝርያ ዓይነቶች ለይቶ ማወቅ ነው፡፡

<u>**ጥናቱ የሚያስገኘው ጥቅም**</u>

ይህ ጥናት የልብ ህመምን ጨምሮ ሌሎች የከፋ የጤና ችግሮች የሚያስከትሉ የባክቴሪያው ዝርያዎች ያላቸውን የስርጭት መጠን በማወቅ የመከላከል እርምጃ ለመውሰድ ከፍተኛ አስተዋፅኦ ያደርጋል፡፡ በተጨማሪም ለባክቴሪያው የተዘጋጁ እና በዝግጅት ላይ ያሉ ክትባቶችን በአገራችን ውስጥ ጥቅም ላይ ለማዋል የሚያስፈልጉትን ቅድመ ሁኔታዎች ያመቻቻል፡፡

<u>**ከጥናቱ ተሳታፊዎች ምን ይጠበቃል**</u>?

የጥናቱ ተሳታፊዎች ከጉሮሮአቸው አካባቢ በጥጥ አማካኝነት ናሙና እንዲወሰድ ፍቃደኛ መሆን እና ናሙና ለመውሰድ ለሚደረገው ጥረት ተባባሪ መሆን ይጠበቅባቸዋል፡፡

<u>**ስጋትና ጉዳት**</u>

ናሙና የመውሰዱ ጥረት በጥናቱ ተሳታፊዎች ምናልባትም በጣም በጥቅት ልጆች ላይ ወዲያውኑ የሚጠፋ የማቅለሽለሽ ስሜት ሊያስከትልባቸው ይችላል፡፡

ጥቅሞች

ልጅዎ በዚህ ምርምር ውስጥ ቢሳተፍ/ብትሳተፍ የሚቀጥሉትን ጥቅሞች ያገኛል/ታገኛለች ብለን እንገምታለን

- ናሙናው የሚሰበሰበው ልምድ ባለው ባለሙያ ነው፡፡
- የሕክምና ምርመራ በሕፃናት ሐኪም ይደረግለታል/ላታል፡
- የሕክምና ምርመራ በሚደረግበት ወቅት ሕመም ቢገኝበት/ባት ነፃ ሕክምና የማግኘት መብት አለው/አላት፡

ምስጢራዊነት

ከዚህ ምርምር የምንሰበስበው መረጃ በምስጢር ይያዛል፡፡ ከልጅዎ እና ከርስዎ የሚገኘው መረጃ የሚከማችበት መዝገብ ውስጥ ከልጅዎ ስም ጋር የተያያዘ ፋይል እንዲኖር አይደረግም፡፡ በምስጢር ቁጥር ማለትም በኮድ መልክ ይመዘገባል፡፡ የቱ ኮድ ወይንም ቁጥር የየትኛው ተሳታፊ ተማሪ እንደሆነ የሚታወቅበት ሰነድ በተቆለፈ ቦታ ይቀመጣል፡፡ ይህም ምስጢር ምርምሩን ለሚያቀናጁት እስፖንሰሮች ለጭብጥ ታማኝነትና ደህንነት ቦርድና ለሀኪሙ /ይህም እጅግ አስፈላጊ የሆነ ጊዜ ብቻ/ ካልሆነ በስተቀር ለሌላ ለማንኛውም ሰው አይሰጥም፡፡

ያለመቀበል ወይንም ጥሎ የመውጣት መብት

ፍቃደኛ ካልሆኑ ልጅዎ በዚህ ጥናት እንዲሳተፍ/እንድትሳተፍ አይገደድም/አትገደድም፡፡ ላለማሳተፍ መወሰንዎ በልጅዎ የትምህርት ክትትል ላይ እክል አይፈጥርም፡፡ ልጅዎ በትምህርት ቤት ውስጥም ሆነ በጤና ድርጅቶች የሚገባውን/ባትን ማናቸውንም ጥቅሞች ሌሎች ተማሪዎች በሚያገኙት መልኩ በሙሉ ያገኛል/ታገኛለች፡፡

ልጅዎ በጥናቱ መሳተፍ ከጀመረ/ች በኃላ በማናቸውም ሰዓት ከጥናቱ ጥሎ/ላ እንዲወጣ/እንድትወጣ ሊያደርጉ ይችላሉ፡፡ ይህንን በማድረግዎ የልጅዎ የተማሪነት መብት አይሸራረፍም፡፡

የበለጠ መረጃ ከፈለጉ

ይህ የጥናት እቅድ በአዲስ አበባ ዩኒቨርሲቲ የሕክምና ፋካሊቲ እና በአህሪ አለርት የስነምግባር ቅኝት ኮሚቴዎች ተገምግሞ ፀድቋል፡፡ ስለነዚህ ኮሚቴዎች የበለጠ ለማወቅ ከፈለጉ የኮሚቴዎቹን ሊቀመንበሮች ዶ/ር ለገሠ ዘሪሁን /ስ.ቁ 51 12 11 አዲስ አበባ እና ዶ/ር ሀዋርድ ኤንገርስ /ስ.ቁ 21 13 34 አዲስ አበባ ሊያነጋግሩ ይችላሉ፡፡

ይኸው የጥናት እቅድ በኢትዮጵያ ሳይንስና ቴክኖሎጁ ኮሚሽን በተቋቋመው ብሔራዊ የስነምግባር ቅኝት ኮሚቴ ተገምግሞ እንዲካሄድ ፈቃድ አግኝቷል፡፡ ስለዚህ ኮሚቴ መረጃ ከፈለጉ የኢትዮጵያ ሳይንስና ቴክኖሎጂ ኮሚሽን የጤና ቢሮ ኃላፊና የብሔራዊ የጤና ሳይንስ

ቴክኖሎጂ ምክር ቤት ፀሐፊ ዶ/ር የማነ ተክላይን በስልክ ቁጥር 53 49 45 ማነጋገር ይችላሉ፡፡ ጥያቄ ካለዎት በማንኛውም ወቅት ጥያቄዎችን ሊያቀርቡ እና ማብራሪያ ሊያገኙ ብት ይችላሉ፡፡

ዶ/ር አብርሃም አሰፋ	**01-21 13 34**	**09-24 75 25**
ዶ/ር ዳንኤል አስራት	**01-52 87 26**	**09-22 30 19**
አቶ ዓለምሰገድ አብዲሣ	**01-40 44 31**	**09-40 90 74**

APPENDIX II Consent Form

የስምምነት ቅፅ

የ የተሳታፊው ልዩ መለያ ቁጥር ________________

የተሳታፊ ተማሪ ሙሉ ስም ______________________

የወላጅ ወይም የአሳዳጊ ሙሉ ስም

____

እኔ ______________________ ስሙ/ስሟ ከላይ የተጠቀሰው ተማሪ ወላጅ/አሳዳጊ መሆኔን እያረጋገጥኩ የጎሮሮ እና ሌሎች ህመሞች እንዲሁም የልብ ችግር መንስኤ የሆነውን ግሩፕ ኤ ስትሪፕቶኮክሳይ "Group A Streptococci" በመባል የሚታወቀውን ባክቴርያ ላይ ሊደረግ ስለታሰበው ጥናት መረጃ አግኝቻለሁ። ለዚሁም ይረዳ ዘንድ ከልጄ/ከማሳድገው/ጋት ልጅ ጉሮሮ አካባቢ በጥጥ አማካይነት ናሙና መውሰድ እንደሚፈለግ ተረድቻለሁ። ስለጥናቱ ዓላማ እንዲሁም ናሙና የመውሰዱ ጥረት በልጄ/በማሳድገው/ጋት ልጅ ላይ ምናልባትም መጠነኛ የማቅለሽለሽ ስሜት ለሰማዉ/ትእንደሚችል ተገንዝቤያለሁ።

በተጨማሪም በመጠይቁ ውስጥ በተካተቱት ጥያቄዎች መሰረት የምሰጣቸው መረጃዎች በጠ ቅላላ በሚስጥር እንደሚጠበቁ ተገልፆልኛል። እንዲሁም ልጄን/የማሳድገውን ልጅ በተመለከተ የምጠየቀውን መረጃ ያለመስጠትና በጥናቱ ያለመተባበር ከጥናቱ በማናቸውም ወቅት ልጄን/የማሳድገውን/ጋትን ልጅ የማግለል መብቴ የተጠበቀ መሆኑ የተገለጸልኝ ሲሆን ይህንንም ማድረጌ በልጄ/በማሳድገው/ጋት ልጅ አጠቃላይ የትምህርት ክትትልና የትምህርት ቤት እንቅስቃሴ ላይ ምንም አይነት እክል የማይፈጥርበት/ባት መሆኑን በሚገባ ተረድቻለሁ። ከዚህ በላይ በልጄ/በማሳድገው/ጋት ልጅ ላይ የህክምና ምርመራ በሚደረግበት ወቅት ህመም ቢገኝበት/ባት ህክምና የማግኘት መብት እንዳለው/ላት ተገንዝቤያለሁ።

ስለዚህ የስምምነት ቃሌን የሰጠሁት በአጠቃላይ ሁኔታውን በመረዳትና በፍፁም ፈቃደኝነት ነው። ከልጄ/ከማሳድገው/ጋት ልጅ የሚወሰደው ናሙና ለወደፊት በመጠበቅ ለአዲስ ግኝትና ለምርምር ዓላማ ይውሉ ዘንድ ተስማምቻለሁ።

የወላጅ ወይም የአሳዳጊ ፊርማ　　　　　　　　　　የዋናዉ ተመራማሪ ፊርማ

___________________________　　　　　___________________________

APPENDIX III Questionnaire

Questionnaire number ______________________________

City/Town _______________________________________

School Name _____________________________________

Grade and Section _________________________________

Part I Socioeconomic Data

101. Identification

101.1. Full Name of the Participant ____

101.2. Subject Initials ____

101.3. Subject Unique Identity Number ____________________________________

102. Age (Completed year) ____________________

103. Sex 1. Male 2. Female

104. Ethnicity
1. Amhara
2. Oromo
3. Somalia
4. Tigray
5. Gurage
6. Others (Specify)_________________

105. Monthly Income of the family (in Birr) ____________
1. 0 - 500
2. 501 - 1000
3. > 1000

106. Family Size _____________
1. < 5
2. 5 - 8
3. > 8

107. Is your bedroom separated from the kitchen?

1. Yes 2. No

108. Is your bedroom separated from the living room?

1. Yes   2. No

109. With how many people do you share the bedroom? __________

1. < 4
2. 4 - 6
3. > 6

Part II Class room Profile

201. Average volume of the classroom in the school (m^3) _____________

202. Average class size in the school ________________________________

203. Percentage of window area per the floor area ___________________

203.1 Average area of the floor (m^3) _____________________________

203.2. Average area of the window (m^3) _________________________

Part III History of sore Throat

301. Have you ever had sore throat in the last one-year?

1. Yes   2. No

301.1. If yes for Q. No301, how many times in the year? ________

1. < 3
2. 3 - 5
3. >5

302. When did you experience the last episode?
1. A week ago
2. Two weeks ago
3. A month ago
4. Three months ago
5. Don't know

302.1. Was the last episode of sore throat accompanied by skin rash?

1. Yes   2. No   3. Don't know

302.2 Had measure been taken for treatment?

1. Yes   2. No

302.2.1. If yes, what measure had been taken?

1. Visit a clinic
2. Traditional medicine (Specify)__________

302.2.1. If you visited a clinic, did you receive penicillin injection?

1. Yes 2. No 3. Don't know

303. Have you been subjected to tonsillectomy?

1. Yes 2. No 3. Don't know

Part IV History of Cardiac Illness

401. Do you have shortness of breath when exercising, than your friends?

1. Yes 2. No 3. Don't know

402. Do you feel strong palpitations when you exercise?

1. Yes 2. No 3. Don't know

403. Do you need many pillows to sleep comfortably?

1. Yes 2. No 3. Don't know

404. Do you wake up at night with shortness of breath?

1. Yes 2. No 3. Don't know

405. For Q No 401 – 404, if the answer for two or more questions is yes, When these symptoms start to occur? ______________

1. A month ago
2. Three months ago
3. Six months ago
4. A year ago

406. Do you take monthly injection of antibiotic, this time?

1. Yes 2. No 3. Don't know

406. Do you have family history of cardiac illness?

1. Yes 2. No 3. Don't know

APPENDIX IV Physical Examination

Date _________________

Subject Full Name ________________________

Subject unique Id No_______________

1.General appearance __________________

1. Vital signs

Pulse _____ /min Respiratory Rate ______/min

Temperature ______ °C

3. Anthropometry

Weight ________Kg Height _______ M Head Circumference _______cm

4. HEENT

4.1. Tonsils enlarged 1. Yes 2. No

4.2. Tonsils inflamed 1. Yes 2. No

4.3. Pharynx inflamed 1. Yes 2. No

4.4. Tonsillopharyngeal exudates 1. Yes 2. No

4.5. Ear discharge 1. Yes 2. No

4.6. Otoscopic examination 1. Abnormal 2. Normal

4.7. Runny nose 1. Yes 2. No

4.8. Conjunctivae 1. Injected 2. Not injected

5. Lymphograndular system

5.1. Cervical lymphadenopathy 1. Not enlarged 2. Enlarged

3. Tender enlarged 4. Non-tender enlarged

5.2. Submandibular lymphadenopathy 1. Present 2. Absent

5.3. Others ______________________

6. Chest

6.1. Signs of respiratory distress 1. Present 2. Absent

If present, describe ______________________

7. Cardiovascular system

7.1. Cardiac murmur 1. Yes 2. No

If yes, characterize __________________________

7.2. Dysrhythemia 1. Yes 2. No

If yes, characterize __________________________

7.3. Other cardiac finding _________________________

8. Abdomen

8.1. Hepatomegally 1. Yes 2. No

8.2. Splenomegally 1. Yes 2. No

9. Genitourinary system

9.1. Costovertebral angle tenderness 1. Yes 2. No

10. Musculoskeletal

10.1. Joint tenderness or swelling 1. Yes 2. No

If yes, characterize ______________________

10.2. Leg oedema 1. Yes 2. No

11. Integumentary

11.1. Skin rash 1. Yes 2. No

If yes, characterize _______________________

11.2. Subcutaneous nodule 1. Yes 2. No

If yes, characterize ________________________

12. Centeral nervous system

12.1. Abnormal body movement 1. Yes 2. No

If yes, characterize ________________________

13 . Conclusion 1. Eligible for the study

2. Excluded from the study

14. Treatment given, if any. ____
____

15. Referred to: ____

Clinician Name Signature

____________________________ _________________________

APPENDIX V Laboratory Procedures

1. Preparation of Colistin-Oxolinic Acid Blood Agar (COBA) Plates

1. Columbia blood agar base (19.5 g) (Oxoid, UK) was suspended in 500 ml of distilled water and boiled.

2. The suspension was autoclaved at 121 °C for 15 minutes, and then cooled to 50 °C.
3. Five hundred ml of the medium and 25 ml of sheep blood were mixed homogenously
4. One vial of colistin-oxolonic acid was reconstituted in 2 ml of sterile distilled water.
5. The content of the vial was added aseptically to 500 ml of sterile Columbia blood agar.
6. The medium was mixed gently and poured into sterile plates.

2. Inoculation of Plates and Incubation

1. Prior to inoculation, the media were brought to room temperature
2. Throat swabs were rolled firmly over one-sixth of the plate to deposit the specimen.
3. A sterile loop was used to carefully streak the inoculum over the surface of the plate and the plate incubated at 35 °C.
4. CO_2 incubator was used in Addis, whereas in Dire-Dawa and Gondar candle jar was used to provide an atmosphere of 5% CO_2.

3. Catalase Test

1. Using a Pasteur pipette 2-3 drops of hydrogen peroxide (3%) solution was placed on a microscopic slide.
2. Using a loop, suspected colonies were picked without touching the plate and immersed in the hydrogen peroxide solution.
3. The suspension was checked for immediate bubbling. Active bubbling indicated a positive test.

4. Bacitracin Sensitivity Testing

1. Catalase negative isolate was inoculated into Brain Heart Infusion (BHI) broth and incubated at 35 °C in 5% CO_2 overnight in order to obtain a bacterial suspension.
2. The bacterial suspension was evenly spread onto blood agar plate using sterile swab.
3. Bacitracin disc (0.05 U) was aseptically placed on the inoculated surface and incubated

for 18-24 hour at 35 °C in 5% CO_2.

4. The plate was examined for the presence of a zone of inhibition around the disc.

5. Any zone of inhibition surrounding the disc was indicative of a presumptive GAS.

5. Serogrouping Technique

A. Extraction procedure

1. Extraction enzyme (0.4 ml) was dispensed into test tubes
2. β-hemolytic colonies (2-5) were selected with an inoculating loop and added to the tube
3. The tubes were incubated for 10 minutes at 37 °C in a water bath. After 5 minutes Of incubation the tubes were shaken vigorously for 2-3 seconds, then the incubation continued at 37 °C for 5 minutes. The result was an antigen extract ready for serogrouping.

B. Test Method

1. One drop of antigen extract was added to each of three small circles in the test oval for group A, C, and G. (This procedure was repeated for group B, D, and F if isolate was found to be negative for the group A, C, and G).
2. Test reagent card sticks were removed from the pouch, and then placed on the card with the spots touching the antigen suspension.
3. The card was gently rocked up to 1 minute in a circular motion
4. Each circle was checked for agglutination.

6. API 20 Strep Test Procedure

A. Preparation of the strip

1. Five ml of distilled water was distributed on an incubation box (tray and lid) to create a humid atmosphere.

2. Strip was removed from its individual package and placed in the incubation box.

B. Preparation of the inoculum

1. Using a swab, all the culture from a fresh growth was harvested
2. A suspension with a turbidity standard of McFarland 4 was prepared in a tube containing 2ml of distilled water.

C. Inoculation of the strip

1. In the first half of the strip, 100 µl of the suspension was distributed, (avoiding formation of bubbles)
2. An ampoule of API GP medium was opened and the rest of the suspension was transferred into it and mixed well.
3. In the second half of the strip 180 µl of the new suspension was distributed
4. Cupules in the second half of the strips were filled with mineral oil to obtain a convex meniscus, and the lid was placed on the strip.
5. Incubated at 37 ^{o}C overnight

D. Reading and Interpretation

1. Reagents were added to the cupules and incubated for 10 minutes at ambient temperature.
2. The reaction was read with reference to the reading table.

6. Molecular Typing

A.PCR

1. Fifty µl of reaction mixture was prepared:

   - 5 µl 10X buffer, with out $MgCl_2$

   - 5 µl $MgCl_2$ (25 mM)

- 2.5 μl of each primer 1 and 2 (10 pmole/ μl)
- 0.25 μl AmpliTaq Gold polymerase, Applied Biosystems
- 33.75 μl of distilled water

2. Two μl of bacterial lysate was added

7. Placed in PCR Thermal cycler, GeneAmp, PCR system 9700, Applied Biosystems using the cycle parameters as described by CDC (Beall, 2005). The cycling conditions were: 94 °C for 10s followed by 10 cycles of 94 °C for 15s, 46.6 °C for 30s, 72 °C for 1 min 15s followed by 20 cycles of 94 °C for 15s, 46.5 °C for 30s, 72 °C for 1 min 15s with 10s increment for each of the subsequent 19 cycles followed by 72 °C for 10 min and subsequent 4 °C storage

8. Amplification products were purified with JetQuick spin column technique (Germond, Germany) according to the manufacturer's instructions.

9. PCR products were then run at 100 Volt, 400 mA for 40-45 minutes on 1% agarose gel to ensure that there was a single band and to estimate the concentration of the products for the sequencing reaction.

B. Preparation of Sequencing Reaction

1. One μl of Big dye terminator V3.1 (Applied Biosystems, Foster City, CA, USA) was added to a tube

2. Dilution buffer (1.5 μl) supplied with the kit was added to the Big dye terminator containing tube

3. Distilled water (1.5 µl) poured into the mixture
4. Two µl of diluted Primer 1 (see Table 4) diluted as (1:16) was added
5. Finally, approximately 4 µl of PCR product was added to the mixture
6. The sequencing products were purified using ethanol sodium acetate precipitation technique as follows.

C. Precipitation of sequencing products

1. One µl of $NaOCH_3C_2OH$ (3M, pH 4.8) (Karolinska Hospital, Sweden) and 25 µl of 95% ethanol (Sigma, Germany) were mixed in a tube and the entire sample from the sequencing reaction was added.
2. The solution was vortexed and left on bench for 30 minutes.
3. Centrifuged for 20 minutes, the tubes were marked to ensure which side is up in the centrifuge all the time.
4. The supernatant was removed with vacuum sucker (care had been taken not to lose the precipitated DNA which may usually not be seen).
5. Washed with 100 µl of 70% Ethanol, and vortexed.
6. Spun for 5 minutes, and supernatant removed using vacuum suction.
7. The product was dried in an incubator (37 ^oC) kept overnight, with the lids removed.

The purified sequencing products were subjected to automated sequence analysis after adding 10 µl of Formamide (Sigma, Germany) on a 3100 model autosequencer (Applied Biosystems) as per manufacturer's instructions. The cycling parameters were 96 ^oC for 1 min for initial denaturation, followed by 25 cycles of 96^oC for 10s, 50 ^oC for 5s, 60 ^oC for 4 min and a holding temperature of 4 ^oC.

APPENDIX VI Laboratory data

Subject unique No ________________ Subject initials ____________________

Site ______________________________ School ____________________________

Date of specimen collection ___________________

1. **Culture result**

1. Growth of beta-hemolytic colonies   1. Yes   2. No

Gram reaction	Positive		Negative	
Shape	Cocci		Bacilli	
Arrangement	In chain		In cluster	
Catalase	Positive		Negative	
Bacitracin	Sensitive		Resistant	
Group A	Positive			
Group C	Positive			
Group G	Positive			
Group B	Positive			
Group D	Positive			
Group F	Positive			

2.*emm* – sequence

3. *emm*-type of the isolate ___________

Signature ------------------------

DECLARATION

I, the undersigned, declare that this M.Sc thesis is my original work, has not been presented for a degree in any other University and that all sources of materials used for the thesis have been duly acknowledged.

Alemseged Abdissa ______________________________

M.Sc candidate Signature

Date of submission

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