Throat Carriage Rate and Antibiotic Susceptibility Pattern of *Beta*-Hemolytic Streptococci and gram negative oral commensals in Children with Rheumatic Heart Disease on Secondary Antibiotic Prophylaxis in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia

By

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Abbreviations and Acronyms

AAU	Addis Ababa University
AHRI	Armauer Hansen Research Institute
ALERT	All African Leprosy-TB Rehabilitation and Training Center
ARF	Acute Rheumatic Fever
ASO	Anti-Streptolysin O
CDC	Communicable Disease Control
GAβHS	Group A *Beta*-Hemolytic Streptococci
GAS	Group A Streptococcus
GCS	Group C streptococcus
GGS	Group G Streptococcus
GGT	Gamma-Glutamyl Transferase
MALDI TOF- MS	Matrix Assisted Laser Desorption Ionization/ Time of Flight Mass Spectrometry
RF	Rheumatic Fever
RHD	Rheumatic Heart Disease
SOP	Standard Operational Procedures
STGG	Skim milk, Trypton Soya broth, Glucose, Glycerol
TASH	Tikur Anbessa Specialized Hospital
WHO	World Health Organization

Abstract

Background: Rheumatic fever and rheumatic heart disease remain significant causes of cardiovascular diseases in Ethiopian children.

Objectives: This study was conducted to determine the throat carriage rate and antibiotic susceptibility pattern of *beta*-hemolytic Streptococci and gram negative oral commensal bacteria, such as Neisseria species in order to have a better understanding of their composition and dynamics in children having rheumatic heart disease who received on-going monthly Penicillin G prophylaxis.

Methods: Throat swab from 234 children who had rheumatic heart disease and received on-going secondary prophylaxis was collected and inoculated onto Sheep blood and Modified Thayer-Martin agar plates. The bacterial strains were analyzed by conventional methods as well as advanced mass spectrometry and molecular methods. Antimicrobial susceptibility testing of streptococci was performed by Kirby Bauer disc diffusion method. ASO titer determination was also done for infection or colonization differentiation.

Results: Out of 234 participants, 38.03% were referred for possible surgical intervention but only three participants received this treatment. Throat carriage rate of *beta*-hemolytic streptococci was 23.93 % (56/234). Of these, 4 were *S. pyogenes*. *Streptococcus dysgalactaie subsp. equisimilis* possessing Lancefield group A, C and G were isolated. Six different *emm* gene types with one newly discovered subtype (*stGrobn.1*) were identified. The Streptococcal glycosyltransferase in strain *emm* 68.2 (multidrug resistant strain) had N-linked glycosylation carrying a unique HexNAc-deoxyhexose; a novel post-translational modification not previously recognized or studied which indicates it is not only M protein responsible for rheumatic fever disease. All *beta*- hemolytic Streptococci were susceptible to penicillin except *S. agalactaie*. Erythromycin and tetracycline resistant *S. pyogenes* were isolated. Among 234 participants, 29.49% (69) was positive to ASO. Of the positives, 66.67 % (46 / 69) showed increased ASO titers (ASO > 200 units/µL) and the rest 33.3% (23/69) was ASO =200 units/ µL. Children who received antibiotic prophylaxis within two weeks showed significantly lower *beta*- hemolytic streptococcal throat carriage than 4 weeks of injection (p=0. 004). The isolation rate of gram negative oropharyngeal commensals was 55.1% (129/234). Throat carrier rate of *N. meningitidis, N. lactamica, M. catarrhalis, K. denitrificans* and *K. kingae* were 4.27%, 4.27%, 19.66 %, 15.81% and 2.56 % respectively.

Conclusion: Characterization of carrier strains and increased ASO titer in rheumatic heart disease is likely to elucidate the significance and mechanisms for carriage and drug resistance during on-going penicillin G monthly prophylaxis. The throat carriage rate of gram negative commensals seems affected by on-going penicillin G prophylaxis.

Recommendation: The current schedule benzathine penicillin prophylaxis injection should be revised (changing schedule of benzathine penicillin G prophylaxis from four to two weeks). Treatment intervention has to be established to provide services for those who need repair or replacement of their damaged heart valves. Penicillin failure to clear pathogenic streptococci needs further investigations.

Key words: Group A streptococcus, *beta*-hemolytic streptococci, rheumatic heart disease, rheumatic fever, antibiotic prophylaxis, susceptibility pattern, *emm* type, protein profile, gram negative oral commensal, Ethiopia

Chapter I: Introduction

1.1.General Introduction

Streptococcus pyogenes [group A Streptococcus (GAS)] is the main human pathogen associated with local or systemic invasive and post immunologic disorders (Facklam, 2002; Bisno, 1991). Rheumatic fever (RF) is a non-suppurative, auto-inflammatory multi-system response following infection by group A, ß-hemolytic streptococcus (GAβHS) also known as *S. pyogenes* (Lee *et al.,* 2009). *Streptococcus dysgalactiae subsp. equisimilis* (SDSE) possessing Lancefield group A, C, or G are phylogenetically related to *S. pyogenes.* It has recently emerged pathogen and causes human infections similar to *S. pyogenes* including rheumatic fever (Bramhachari *et al*., 2010; Liao *et al*., 2008; Rantala *et al.,* 2008; Cohen-Poradosu *et al*., 2004; Brandt *et al*., 1999; Schnitzler et al., 1995).

Rheumatic heart disease (RHD) is the most dreaded complication of rheumatic fever. The term rheumatic heart disease refers to the chronic heart valve damage that can occur after a person has had an episode of acute rheumatic fever. This valve damage can eventually lead to heart failure (Marijon *et al*., 2012; Lee *et al*., 2009; Carapetis *et al.,* 2005a).

The incidence of rheumatic fever has declined remarkably in the industrialized countries of the world, where the disease has become rare. However, in many developing countries, which account for almost two thirds of the world's population, streptococcal infections, rheumatic fever and rheumatic heart disease remain a very significant public health problem (Seckeler and Hoke, 2011; Lee *et al*., 2009). It appears that a threshold level where higher socioeconomic status is associated with reduced prevalence of rheumatic heart disease is not reached in developing countries (Steer *et al*., 2002).

Although penicillin is effective in the prevention of the disease, treatment of advanced stages uses up a vast amount of resources, which makes disease management, especially challenging in developing nations (Marijon *et al*., 2012; WHO, 2004). Other Studies have observed various GAS illness rates during secondary prophylaxis regimens (Kaplan *et al*., 2006; Kaplan and Johnson, 2001) and the current protective effects of benzanthine penicillin G are unclear or diminished (Broderick *et al., 2012;* Kaplan *et al*., 2006; Lily *et al*., 2006; Kaplan and Johnson, 2001).

Rheumatic heart disease is the number one cardiac problem in Ethiopian children (Habte *et al*., 2010; Oli and Asmera, 2004; Oli and Porteous, 1999 a; Oli *et al*., 1992). There is a lack of good quality prevalence surveys of rheumatic heart disease in developing countries. Many regions need well designed studies of rheumatic heart disease that incorporate assessment of environmental factors as well as the study of the microbiological epidemiology of rheumatic heart disease and GAS (Steer *et al*., 2002).

The present study was conducted to determine the throat carriage rate of *beta*-hemolytic Streptococci and gram negative oral commensal bacteria, such as Neisseria species in order to have a better understanding on their composition and dynamics in children having rheumatic heart disease who received ongoing monthly Penicillin G prophylaxis during the period of July 2013 to June 2014 to contribute for prevention of aggravation of RHD.

1.2.Historical Perspective

Acute rheumatic fever is post infectious, non-suppurative sequelae of pharyngeal infection with *S. pyogenes*. About 100 years back RF/RHD was believed to be a disease of "temperate climate" (Kumar and Tandon, 2013). At present incidence and prevalence of Acute Rheumatic Fever (ARF) and RHD have been decreasing in developed nations though they continue to be major causes of morbidity and mortality among young people in developing nations. More recent data using echocardiography to screen for RHD in developing nations have led to a marked increase in the recognized prevalence in these regions (Seckeler and Hoke 2011; Bhaya *et al*., 2010).

1.3.Microbiology of Group A Streptococcus/S. pyogenes

The group A streptococci is gram positive spherical bacteria that characteristically form pairs or chains during growth. Most streptococci that contain the group A antigen are *S. pyogenes.* It is a prototypical human pathogen and the main human pathogen associated with local or systemic invasion and post streptococcal immunologic disorders (Lee *et al.,* 2009; Bisno and Stevens, 2000; Bisno, 1991)

Streptococcus pyogenes typically produces large (1 cm in diameter) zones of hemolysis around colonies greater than 0.5 mm in diameter. They are PYR positive (hydrolysis of L-pyrrolidonyl-2-naphthylamide) and usually are susceptible to bacitracin. The *S. pyogenes* cell wall contains proteins

(M, T, R antigens), carbohydrates (group-specific), and peptidoglycans. A hair like pili project through the capsule of GAS which consists partly of M protein and is covered with lipoteichoic acid is important in the attachment of streptococci to epithelial cells (Bisno and Stevens, 2000).
The carbohydrate is contained in the cell wall of many streptococci and forms the basis of serologic grouping into Lancefield groups A–H and K–U. The serologic specificity of the group specific carbohydrate is determined by an amino sugar (Facklam, 2002; Petts, 1999; Bisno, 1996).

Group A Streptococci are classified into many *emm*-types based on the sequence of the amino terminal hyper variable region of the M surface protein (Beal *et al*., 1997). Currently there are above 93 validated M serotypes and >200 *emm* genotypes of Lancefield group A streptococcus (Beall *et al*., 1996). M Protein is a major virulence factor of group A *S. pyogenes*. M protein appears as hairlike projections on the streptococcal cell wall. When M protein is present, the streptococci are virulent, and in the absence of M type-specific antibodies, they are able to resist phagocytosis by polymorphonuclear leukocytes. *Streptococcus pyogenes* that lack M protein are not virulent (Bradit *et al*., 2000).

Immunity to infection with GAS is related to the presence of type specific antibodies to M protein. Because there are many types of M protein, a person can have repeated infections with group A *S. pyogenes* of different M types. Both group C and group G streptococci have genes homologous to the genes for M protein of group A, and M protein has been found on group G & C streptococci. The M protein molecule has a rod like coiled structure that separates functional domains. The structure allows for a large number of sequence changes while maintaining function, and the M protein immune determinants, therefore, can readily change. Studies showed that the overall genetic diversity of the M proteins is high. There are two major structural classes of M protein, classes I and II. (Smeesters *et al*., 2008; Brandt *et al*., 2001; Bradit *et al*., 2000). The majority of Class I strains (with reactive M- protein) are implicated in RF. Of the M-protein types identified, M types such as 1, 3, 5, 6, 14, 18, 19 and 24 have been associated with rheumatic fever (WHO, 2004).

1.4. Epidemiology of Group A Streptococci Infection, Rheumatic Fever and Rheumatic Heart Disease

The epidemiology of ARF is similar to GAS upper respiratory tract infections. Acute rheumatic fever mostly occurs in children age range between 5 and 15 years. Epidemiologic risk factors

classically associated with individual attacks and especially with outbreaks of ARF include lower standards of living, especially crowding. The disease has been more common among socially and economically disadvantaged populations. However, the organism itself as well as the degree of host/herd immunity to the prevalent M-types in an affected community are equally important risk factors (Seckeler and Hoke 2011; Carapetis *et al*., 2005b).

The incidence of pharyngeal *beta*-hemolytic streptococcal infections can vary between countries and within the same country, depending upon season, age group, socioeconomic conditions, environmental factors and the quality of health care (WHO, 1988). The need for a better understanding of the epidemiology of streptococci is underscored by a report that one GAS serotype can be rapidly and completely replaced by another serotype in a stable population with adequate access to health care (Rubio-López *et al*. 2012; WHO, 2004). This serotype change still has not been adequately explained and it raises questions about the efficacy of any type specific streptococcal vaccine that is synthesized by combining M protein sequences from virulent streptococcal serotypes. Furthermore, the ability of streptococci to infect the host after a prior infection by a different M serotype strain, suggests there is no broad, non-type specific immunity directed against conserved M-protein epitopes or their extracellular products, which complicates the development of an RF vaccine aimed at conserved M-protein sequences (WHO, 2004).
The epidemiological association between GAβHS infections and the subsequent development of ARF has been well established (WHO, 2004). The streptococcal M protein (a major virulence factor) seems to play an important role in the pathogenesis of rheumatic fever (Guilherme et al., 2006).

The epidemiological data are scarce for ARF/RHD in Africa. Twenty million peoples are affected in the developing world with highest prevalence in sub-Saharan Africa. Two million children are affected worldwide from which 1 million are found in sub-Saharan Africa. Determinants of persistence of RF and RHD in Africa are poverty; overcrowding; malnutrition; cultural aspects/low level of disease awareness; shortage of resources; inadequate healthcare expertise; more virulent GAβHS/genetic susceptibility that leads to increased transmission of streptococci infections, inadequate diagnosis/treatment and poor adherence to secondary measures (Marijon *et al*., 2012; Brink and Aalbers, 2009;; Gerber *et al*., 2009; Nkomo, 2007).

In Ethiopia Rheumatic heart disease is an important cause of mortality and morbidity. Analysis of medical admissions to different hospitals showed cardiovascular diseases to be responsible for up to 35% all admissions, of which RHD was the leading cause (Habte *et al*., 2010; Mehadi *et al*., 2006; Oli and Asmera, 2004).

A Study done in Ethiopian school children showed the RHD prevalence of 4.6 per 1000 (Oli *et al*., 1992). Some studies showed a high diversity of GAS isolates recovered from Ethiopian children (Abdissa *et al*., 2006; Tewodros and Kronvall, 2005). A study conducted to determine the carriage rate of GAS among Ethiopian school children showed that 9.7% was noted to have GAS with high diversity in which, among 82 isolates 43 denote dissimilar *emm* types and of the *emm* types prevalent in the study communities, 46% were not included in the 26-valent GAS vaccine (Abdissa *et al*., 2006).

1.5.Pathogenesis of Rheumatic Fever and Rheumatic Heart Disease

Rheumatic fever (RF) is a non-suppurative, auto-inflammatory multi-system response (joints, skin, brain, subcutaneous tissue and heart) following infection by GAβHS (Lee *et al.,* 2009), and the clinical manifestation of the response and its severity in an individual is determined by host genetic susceptibility; the virulence of the infecting organism; and a conducive environment (Marijon *et al*., 2012; Lily *et al*., 2006; WHO, 2004).

Initial streptococcal infection in a genetically predisposed host in a susceptible environment leads to the activation of T-cell and B-cell lymphocytes by streptococcal antigens and super antigens (SAgs), which results in the production of cytokines and antibodies directed against streptococcal carbohydrate and myosin (Cunningham, 2012; WHO, 2004). Super antigens from GAS are potent T cell mitogens, and have been suggested to play a role in severe streptococcal disease (Guilherme *et al*., 2004; Roberts *et al*., 2001; WHO, 2004).

It appears that M protein and perhaps other streptococcal cell wall antigens have an important role in the pathogenesis of RF. Purified streptococcal cell wall membranes induce antibodies that react with human cardiac sarcolemma. The characteristics of the cross reactive antigens are not clear. A component of the cell wall of selected M types induces antibodies that react with cardiac muscle tissue. Conserved antigenic domains on the class I-M protein are similar with human cardiac muscle,

and the class I-M protein may be a virulence determinant for RF (Cunningham, 2012; Brandt *et al*., 2000).

It has been proposed that injury to the valvular endothelium by the anti-carbohydrate antibodies leads to an up regulation of VCAM1 and other adhesion molecules (Roberts *et al*., 2001). VCAM1 expression is a hallmark of inflammation and it heralds cellular infiltration. VCAM1 interacts with VLA4 on activated lymphocytes and leads to an influx of activated CD4+ and CD8+ T-cells. A break in the endothelial continuity of a heart valve would expose sub endothelial structures (vimentin, laminin and valvular interstitial cells) and lead to a chain reaction of valvular destruction. Once valve leaflets are inflamed through the valvular surface endothelium and new vascularization occurs, the newly formed microvasculature allows T-cells infiltrate and perpetuate the cycle of valvular damage. The presence of T-cell infiltration, even in old mineralized lesions, is indicative of persistent and progressive disease in the valves. Valvular interstitial cells and other valvular constituents under the influence of inflammatory cytokines perpetuate aberrant repair (Cunningham, 2012; WHO, 2004).

1.6.Clinical Features of Rheumatic Fever and Rheumatic Heart Disease

Acute rheumatic fever, the precursor to RHD, can affect different organs and lead to irreversible valve damage and heart failure. Patients usually present with an acute febrile like illness 2-3 weeks after streptococcal throat infection (Marijon *et al*., 2012; Dajani *et al*., 1992; Jones, 1944).

1.6.1. Carditis (Rheumatic Heart Disease)

The carditis of ARF affects all parts of the heart involving the pericardium, myocardium, and endocardium. Most literatures showed evidence of carditis which is characterized by sinus tachycardia, the murmur of mitral regurgitation, a sound3 (S3) gallop, a pericardial friction rub, and cardiomegaly. The introduction of echocardiography has assisted in the identification of subtle abnormalities of the mitral valve, has revealed a higher RHD burden than previously thought. A prolonged PR interval and evidence of heart failure may be present as well, but these are nonspecific and may be found in a number of other diseases (Reményi *et al., 2012*; Essop *et al*., 1993; Edwards *et al*., 1993).

Healing of the rheumatic valvulitis may cause fibrous thickening and adhesion, resulting in the most serious complication of RF, i.e., valvular stenosis and/or regurgitation. The mitral valve is involved most frequently, followed by the aortic valve. However, the isolated aortic valve disease as a consequence of ARF is quite rare. In patients with aortic valve disease due to RF, the mitral valve is almost always simultaneously affected. Minor degrees of rheumatic valvular involvement can lead to susceptibilities to infective endocarditis. Although rheumatic pericarditis can cause a serious effusion, fibrin deposits, and pericardial calcification, it does not lead to constrictive pericarditis (Marijon *et al*., 2012; WHO, 2004).

1.6.2. Migratory polyarthritis

A migratory polyarthritis is present in as many as 75% of cases, most often affecting the ankles, wrists, knees, and elbows over a period of days. It usually does not affect the small joints of the hands or feet and seldom involves the hip joints (Sanyal *et al*., 1982; Jones, 1944).

1.6.3. Sydenham's chorea

Sydenham's chorea occurs in <10% of patients with RF. It is characterized by emotional liability, uncoordinated movements, and muscular weakness. The inability to maintain a voluntary contraction (i.e. Motor impersistence) as is seen during manual grip (milkmaid grip) tests or tongue protrusion, is a characteristic feature of chorea and results in the dropping of objects and clumsiness. Muscle stretch reflexes are often hung-up and pendular. In severely affected patients, a peculiar dancelike gait may be noted (Aron *et al*., 1965; Jones, 1944).

1.6.4. Subcutaneous nodules and erythema marginatum

Subcutaneous nodules and erythema marginatum are rare major manifestations, usually present in <10% of cases. Subcutaneous nodules are found over the extensor surfaces of joints, are seen most often in patients with long standing RHD, and are extremely rare in patients experiencing an initial attack. Erythema marginatum is an uncommon manifestation. It is an evanescent macular eruption with rounded borders usually concentrated on the trunk (Bisno, 1991; Massell *et al*., 1958).

1.7. Diagnosis of Rheumatic Fever and Rheumatic Heart Disease

There are no specific laboratory tests that can establish a diagnosis of RF. The diagnosis, therefore, is a clinical one, but requires supporting evidence from the clinical microbiology and clinical immunology laboratories (WHO, 2004; Dajani *et al*., 1992; WHO, 1988).

1.7.1. Jones major and minor criteria

Because of the variety of signs and symptoms associated with the RF syndrome, in 1944 Jones first proposed criteria to assist the clinician in standardizing the diagnosis of RF (Jones, 1944). The modification of the Jones criteria (Updated Jones Criteria) was published in 1992 by a Special Writing Group of the American Heart Association. A recent consideration of the Jones criteria by the American Heart Association and WHO has basically not resulted in change (WHO, 2004; Dajani *et al*., 1992).

1.7.1.1.Major Jones criteria

There are five criteria termed major because they are most commonly found in patients with RF. These are carditis, migratory polyarthritis, Sydenham's chorea, subcutaneous nodules, and erythema marginatum (WHO, 2004; Dajani *et al*., 1992; Jones, 1944).

1.7.1.2.Minor Jones criteria

The minor criteria are nonspecific. These are fever, arthralgia, elevated acute phase reactants and prolonged PR wave interval which may be present in many clinical conditions (Jones, 1944).
For the diagnosis of a primary episode of RF, it is recommended that the major and minor clinical manifestations of RF, the laboratory diagnosis, and evidence of a preceding streptococcal infection should all continue according to the 2002-2003 WHO recommendations. In the context of a preceding streptococcal infection, two major manifestations, or a combination of one major and two minor manifestations, provide reasonable evidence for a diagnosis of RF. World Health Organization (WHO) has continued to maintain that a diagnosis of a recurrence of RF in a patient with established RHD should be permitted on the basis of minor manifestations plus evidence of a recent streptococcal infection (WHO, 2004; Dajani *et al*., 1992; Jones, 1944).

Physicians should use their clinical judgment to diagnose carditis in an episode of RF, especially during a recurrence of RF, and should use the above recommendations as guidelines for the diagnosis. Such recommendations are in keeping with the original intent of the Jones criteria, which were established as a universal standard for the diagnosis of RF (WHO, 2004; Dajani *et al*., 1992; Jones, 1944).

Clinical examination remains the basis of a diagnosis of RF and carditis, and the role of echocardiography should be considered supportive. However, an echo-Doppler examination should be performed if the facilities are available. The other invasive and noninvasive diagnostic modalities for RF, such as endomyocardial biopsy and radionuclide imaging, should be considered as research tools (Reményi *et al* 2012; WHO, 2004).

Hospital admission may be helpful for confirming a diagnosis of rheumatic Fever (RF), for instituting treatment and for educating the patients and family. Initial tests should include a throat culture (or in some circumstances rapid streptococcal antigen detection test), a measurement of streptococcal antibody titers (e.g. ASO or anti DNase B), an assessment of acute phase reactants (e.g. ESR or CRP), a chest X-ray, an electrocardiogram, and an echocardiogram. A blood culture may help to exclude infective endocarditis (Thenmozhi *et al*., 2010; Carapetis *et al*., 2005a; WHO, 2004).

1.8.Management of Rheumatic Fever and Rheumatic Heart Disease

1.8.1. Antimicrobial therapy

Eradication of the pharyngeal streptococcal infection is mandatory to avoid chronic repetitive exposure to streptococcal antigens (Broderick *et al., 2012;* Gerber *et al*., 2009). The eradication of pharyngeal streptococci should be followed by long term secondary prophylaxis to guard against recurrent pharyngeal streptococcal infections (Armstrong C, 2010; WHO, 2004).

1.8.2. Management of heart failure

Heart failure in RF generally responds to bed rest and steroids, but in patients with severe symptoms, diuretics, angiotensin converting enzyme inhibitors, and digoxin may be used (Carapetis *et al*., 2005a; WHO, 2004).

1.8.3. Management of chorea

The signs and symptoms of chorea generally do not respond well to anti-inflammatory agents. Neuroleptics, benzodiazepines and antiepileptics are indicated, in combination with supportive measures such as rest in a quiet room. Haloperidol, diazepam, carbamazepine have all been reported to be effective in the treatment of chorea. There is no convincing evidence in the literature that steroids are beneficial in the therapy of the chorea associated with rheumatic fever (Carapetis *et al*., 2005a; WHO, 2004).

1.8.4. Surgery for rheumatic heart disease

Surgery is usually indicated for chronic rheumatic valve disease. It is rarely required during ARF and the necessity for surgical treatment is determined by the severity of the patient's symptoms and/or evidence that cardiac function is significantly impaired (Carapetis *et al*., 2005a; WHO, 2004).

1.9.Prevention of Rheumatic Fever and Rheumatic Heart Disease

1.9.1. Primary prevention of rheumatic fever

Rheumatic fever is quite a preventable disease. The following are important ways to prevent ARF and RHD. The most important determinant of RF is socioeconomic rather than medical. Crowding is by far the most important risk factor. One has to improve crowding both at household and school level to reduce the incidence of the disease. This is obviously achieved over a long period at the national level through improved economy. Other more important and practical measures include improved environmental and personal hygiene; Avoidance of contact with individuals having a streptococcal throat infection; early recognition and treatment of streptococcal upper respiratory tract infection (Gerber *et al*., 2009; WHO, 1999).

The primary prevention of RF is defined as the adequate antibiotic therapy (Pencillin G, Penicillin V, Amoxacillin and for individuals allergic to penicillin: Narrow-spectrum cephalosporin, Clindamycin, Azithromycin, Clarithromycin, Erythromycin) of GAS Upper Respiratory Tract (URT) infections to prevent an initial attack of acute RF. Primary prevention is administered only when there is group A streptococcal URT infection. The therapy is therefore intermittent, in contrast to the therapy used for the secondary prevention of RF, where antibiotics are administered continuously (Armstrong, 2010; Gerber *et al*., 2009; WHO, 1999).

1.9.2. Secondary prevention of rheumatic fever

Secondary prevention of RF is defined as the continuous administration of specific antibiotics to patients with a previous attack of RF, or a well-documented RHD. The purpose is to prevent colonization or infection of the URT with GAβHS and the development of recurrent attacks of RF. Secondary prophylaxis is mandatory for all patients who have had an attack of RF, whether or not they have residual rheumatic valvular heart disease (Armstrong C, 2010; Gerber *et al*., 2009; WHO, 1999).

As it is depicted above recurrence is the most important and frequent determinant of disease progression in patients with prior history of ARF/RHD. Hence, monthly benzathtine penicillin injection is universally recommended for those with documented history of ARF/RHD and is considered as the single most effective measure (Gerber *et al*., 2009; WHO, 1999).

1.10. Statement of the problem

Rheumatic heart disease is the number one cardiac problem in Ethiopian children (4.6-7.1 per 1000) (Oli and Porteous, 1999 a; Oli *et al*., 1992). Hospital based studies showed 32.8% (256) (Habte *et al*., 2010) and 39.2% (127) (Mehadi *et al*., 2006) were RHD patients among all heart cases. Of 457 cardiovascular deaths including cerebrovascular accidents (CVA) from January 1995 to December 2001; 26.5 % (121) deaths were due to RHD (Oli and Asmera, 2004). Günther also reported 125.3 per 1000 person -year mortality rate during about 7 years follow up (Günther *et al*., 2006).

The economic effects of the disability and premature death caused by these diseases are felt at both the individual and national levels through higher direct and indirect health care costs (WHO, 2004). Implementation of secondary antibiotic prevention was challenged by impaired awareness of RHD; poor quality of service delivery and inadequate cooperation with family physicians (Shrestha *et al*., 2012; Petricca *et al*., 2009; Günther *et al*., 2006). In addition, low rate of regular prophylaxis and drug discontinuation are our problems (Günther *et al*., 2006; Oli and Porteous, 1999 b, Melka, 1996).

Studies have observed various GAS illness rates during secondary prophylaxis regimens (Kaplan *et al*., 2006; Kaplan and Johnson, 2001) and the current protective effects of benzathine penicillin G are unclear or diminished (Broderick *et al., 2012;* Kaplan *et al*., 2006; Lily *et al*., 2006; Kaplan and Johnson, 2001). Two weekly and 3 weekly intramuscular penicillin injections are better in reducing recurrence of rheumatic fever and streptococcal throat infections compared to 4-weekly intramuscular penicillin injection (Kassem *et al*., 1996; Lue *et al*., 1996). Reemergence of different virulent M serotype strains of GAS are also challenges (Abdissa *et al*., 2006; WHO, 2004).

In Ethiopia the current secondary rheumatic fever antibiotic prophylaxis regimen is 4 weekly intramuscular penicillin injections. For those allergic to penicillin the alternative is daily Erythromycin. There is limited published data on GAS and other *beta*-hemolytic throat carriage rate of children having rheumatic heart disease who are on secondary rheumatic fever antibiotic prophylaxis (Tewodrosl *et al*., 1992). Therefore the present study was carried out to determine the effectiveness of secondary antibiotic prophylaxis in children with rheumatic heart disease to GAS and other *beta*-hemolytic carriage rate, which could be important to the care of the children to prevent aggravation of RHD. In this study, the carriage rate of gram negative oral commensal bacteria, such as Neisseria species was also determined in order to have a better understanding in their composition

and dynamics in those patients with RHD and on secondary prophylaxis. These gram negative commensals could be potential pathogens and can cause endocarditis, meningitis and septicemia when entered to sterile body sites and aggravate the existing medical problem.

1.11. Hypothesis

1. Children who are on secondary prophylaxis for RF/RHD are not colonized or infected by GAS in their throat.

2. Oral gram negative commensal carriage rate is not affected by monthly penicillin G prophylaxis in children having rheumatic heart disease.

Chapter II. Objectives of the Study

General objective

- This study was conducted to determine the throat carriage rate of *beta*-hemolytic Streptococci and gram negative oral commensal bacteria, such as Neisseria species in order to have a better understanding of their composition and dynamics in children having rheumatic heart disease who received ongoing monthly Penicillin G prophylaxis in Tikur Anbessa Specialized Teaching Hospital (TASH), Addis Ababa, Ethiopia during the period of July 2013 to June 2014.

Specific objectives

- To isolate group A streptococci (GAS) and other *beta*-hemolytic streptococci from throat swabs of children with rheumatic heart disease
- To determine the *emm* gene type of the GAS and a large colony forming *S. dysgalactaie subsp. equisimilis*
- To determine protein profile of selected strains
- To determine ASO titer level in children with rheumatic heart disease
- To perform drug susceptibility pattern of GAS and other *beta*-hemolytic streptococci isolates to selected antimicrobial agents
- To isolate oral gram negative commensals such as *Neisseria* and *Moraxella* species from throat swabs of children with rheumatic heart disease

Chapter III: Materials and Methods

3.1. Study Area and Period

The study was conducted at pediatric cardiac clinic of Tikur Anbessa specialized referral teaching hospital located in Addis Ababa from July 2013 to June 2014 G.C. The hospital provides health service to patients' referred from different parts of the country. It also provides research and training services in the country.

3.2. Study Design

Cross sectional study design was employed during the study period.

3.3. Source Population and Study Participants

I. Source Population

All children who have had rheumatic fever/ rheumatic heart disease (RF/RHD) and attending pediatric cardiac clinics of TASH during the study period were source population.

II. Study participants

All children who have had RHD and attending pediatric cardiac clinics of TASH during the study period and those fulfilling the inclusion criteria were study participants.

3.4 Eligibility Criteria

I. Inclusion criteria

Children whose age range from 5- 15 years with confirmed RF/RHD; children whose parents/guardians gave written informed consent and children who gave verbal assent were included in the study.

II. Exclusion criteria

Children with RF/RHD who were visiting the follow up clinic for the first time and those not received secondary antibiotic prophylaxis were excluded from the study.

3.5. Sample Size Determination

The sample size was determined by using single proportion sample size determination.

$$n = \frac{(Z\text{-}\alpha/2)^2 P (1-P)}{d^2}$$

Where,

n = Minimum sample size of population

d=margin of error=0. 05

P= Proportion of the isolates that estimate maximum sample size =18. 7% (Kassem *et al.,* 1996; Feinstein *et al.,* 1959)

Z-α/2 =Confidence certainty used as 95%=1.96

$$n = \frac{(1.96)^2 \text{ X } 0.187\text{x } (1-0.187)}{(0.05)^2}$$

n=234

3.6. Sampling Method

Convenient sampling technique was used to select 234 children with RHD fulfilling the inclusion criteria and attending TASH during the study period.

3.7. Ethical Considerations

The M Sc research project was ethically cleared by the Department Research and Ethical Review Committee (DREC) and approved by the Department of Microbiology, Immunology and Parasitology, School of Medicine, Addis Ababa University, Addis Ababa, Ethiopia. The proposal was also approved by the ethical committee of Armauer Hansen Research Institute/All African Leprosy Rehabilitation and Training Centre (AHRI/ALERT). Official permission was obtained from the Department of Paediatric and Child Health, School of Medicine Addis Ababa University. Written informed consent was obtained from each child's parent or guardian and assent from study participants older than 12 years. Parents/Guardians were requested for permission to send bacteria isolates for molecular typing abroad and sample export permission was obtained from the Ethiopian Institute of Biodiversity. The laboratory findings were communicated for further treatment and management of the study participants.

3.8. Sample Collection, Handling and Transport

A. Throat swab

Tongue was depressed with a clean tongue depressor to swab the tonsillar fauces and the posterior pharyngeal wall behind the uvula using the sterile swab applicator (Thermo Fisher Scientific, USA) by trained nurses. The swab was immersed to a test tube containing medium, STGG (skim milk-tryptone-glucose-glycerin) (Thermo Fisher Scientific, USA). The samples were transported in cold chain with eight hours to the AHRI bacteriology laboratory.

B. Blood specimen

Two ml of blood specimen was collected from each study participants by trained nurses. It was collected using 25G needle and 10ml syringe. The collected blood was immediately transferred to a glass tube without anticoagulant. It was transported to AHRI and then serum was separated at 3000 RPM for 10 minutes and stored at -80°C for ASO titer determination.

3.9. Culture and Identification of GAS and other ß-hemolytic streptococci

The throat swab was rolled on a portion of 7% defibrinated sheep blood agar plate (Becton, Dickinson, USA) and it was stroked evenly to get a single colony at the end. The rest swab transferred aseptically to sterile cryo-tubes and had been stored at -80 °C. The plates were incubated in a CO2 incubator containing 5% CO_2 at 37°C for 24-48 hrs. The grown bacteria were characterized by the presence of *beta*-hemolytic colonies and were sub cultured on blood agar for 24-48 hrs in CO_2 at 37°C to obtain pure colonies for further characterizations. All *beta*-hemolytic colonies were then subjected to the gram stain, catalase test and bacitracin susceptibility testing (see Annex IV). All gram positive cocci, catalase negative, positive or negative for bacitracin test were stored in STGG media at -80°C for further characterizations.

3.10. Serogrouping

All *beta*-hemolytic streptococci were serogrouped by streptococcal grouping latex kit (Pro-Lab Diagnostics, USA) according to the manufacturer's instructions (See Annex IV).

3.11. Species Identification by Mass spectrometry

Conventional matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) Mass Spectrometry (MS) analyses was performed by the direct colony method by using the Microflex mass spectrometer (Bruker Daltonics, Germany) for species differentiation. First Streptococcal and gram negative commensal strains isolated from throat swabs were cultivated on 5% human blood agar media. A moderate quantity of the bacteria was obtained from a fresh culture using a loop, deposited on the target, and smeared. The film was overlaid first with 1 µL formic acid (100%) and then with 1µL matrix α-cyano-4 hydroxy-Cinnamic acid (HCCA). Two spots were prepared for each bacterium. The plate was inserted into the Microflex mass spectrometer and the spectra generated were analyzed by the MALDI Biotyper 3.0 software (Bruker Daltonics, Germany) and matched to the Biotyper 3.0 database.

3.12. *emm* typing of GAS and S. dysgalactiae subsp. equisimilis

The following procedures were conducted for *emm* typing of GAS, GCS and GGS (large colony forming) isolate.

A. Bacterial cell lysate preparation (DNA extraction)

Cell lysate preparation was adapted from CDC (Beall, 2005) (www.cdc.gov/streplab/protocols.html). Half of a loop full fresh growth from *S. pyogenes* and *S. dysgalactiae subsp. equisimilis* were resuspended in 300 µL normal saline (0.85% NaCl) in Eppendorf tubes. It was heated at 70°C for 15 minutes. The samples were span down full speed for 2 minutes in microfuge and the supernatant was discarded. The pellet was re-suspended in 50 µL TE (10mM Tris, 1 mM EDTA), 10 µL mutanolysin (3000units/mL, M9901 Sigma), 2µL hyaluronidase (30mg/mL, H3506 Sigma). It was then incubated at 37°C for 30 minutes and then it was heated at 100 °C for 10 minutes. Finally, it was spinning down full sped for 2 min and the supernatant was stored at -20 °C for later use. The supernatant was used as DNA template for PCR amplification**.**

B. PCR

PCR amplification was optimized first with 20µL PCR reaction mixtures. After we got the optimization it was done at 50 µL PCR reaction mixtures contained 0.5µL cell lysate, 5µL 10x NH4 reaction buffer, 1µL 10uM forward primer (Table 3.1), 1µL 10uM reverse primer (Table 3.1), 2µL

50xmM MgCl2, 2µL 4x5 mM dNTPs, 0.2µL Bio-Taq polymerase (5 units/µL) and 38.3 µL Milli-Q water.

The samples were placed in thermo cycler (ESCO, Swift MaxPro) using the following programs (CDC) (Beall, 2005). Initial denaturation 94°C for 1 minute, 10x (94°C: 15s, 46.5°C: 30s, 72C: 1 minutes 15s and the next 20x: (94°C: 15s, 46.5°C: 30s, 72°C: 1 min 15s with a 10 Sec increment for each of the subsequent 19 cycles.), 72°C for 10 min and then 4°C storage. The PCR product was stored at -20°C until used.

C. DNA Purification by Illustra GFX PCR DNA and Gel Band Purification

PCR products were purified using illustra GFX PCR DNA and gel band purification kits (GE Healthcare, UK) according to the manufacturer's instruction (See Annex IV).

D. Sequencing and BLAST Analysis

After DNA was purified the concentration was measured using nano drop (Thermo Scientific Nanodrop 2000 C Spectrophotometer) at 260nm. Ten microliter [10µl (5µl 20-80ng/µl DNA, 5µl 5uM *emmseq2* primer (Table 3.1)] was used for sequencing. After doing some calculations to determine the high and low range values the DNA-sequence primer mixture was sent in duplicates to Germany for sequencing**.** The results were downloaded online from GATC Biotech. com. *emm* typing was performed according to Beall *et al.* (1996, 1997) recommendation. The sequence was subjected to a homology search (Streptococci Group A Subtyping Request Form, BLAST 2.0 server (Beal, 2004b) (http://www2a.cdc.gov/ncidod/biotech/strepblast.asp). The first 240 bases of the 5'end of the *emm* gene sequences were compared with those in the CDC *S. pyogenes emm* sequence database. An *emm* type showing 100% homology with a CDC reference strain was identified as that particular *emm* type (Beal, 2004a) (http://www.cdc.gov/streplab/assigning.html). One sequence was sent to CDC (bbeall@cdc.gov.) for new *emm* subtype verification.

Table 3. 1. Primers used for PCR and sequencing

	Primers used for PCR amplification and sequencing	Ref
S. pyogenes	F: TATTCGCTTAGAAAATTAA R: GCAAGTTCTTCAGCTTGTTT	(Beal, 2005).
S. dysgalactiae	F: TATTCGCTTAGAAAATTAA R: GCAAGTTCTTCAGCTTGTTT	
emmseq2 primer (Sequencing primer)	TATTCGCTTAGAAAATTAAAAACAGG	

3.13. Protein Profiling of some Selected Strains by Electrospray

Next-generation high end MS was performed by the new electrospray-based high resolution Q-Exactive OrbiTrap mass spectrometer (Thermo-Fischer, Germany), after trypsin shaving of fresh streptococcal cells to enrich surface proteins. Thereby high resolution strain characterization was achieved. Modified proteins in streptococcal cell lysates were investigated using liquid chromatography–OrbiTrap mass spectrometry approaches (Q-Exactive) and Bioinformatics analyses (Proteome Discoverer 1.4) (Taylor *et al*., 2006) in addition to manual inspection.

Proteins of some selected strains (strains were selected based on the presence of *emm* gene) were compared with databases. Samples were prepared using in-gel trypsin digestion (samples were reduced and alkylated prior to trypsin digestion). Samples were run on the Q-executive mass spectrometer coupled to EASY 1000 NLC using a data dependent MS^2 method with TopN=8. Gradient used for peptide separation (C_{18} column) by NLC was a 105 minute gradient, with solvent A being 0.1% formic acid and solvent B being acetonitrile with 0.1% formic acid. With the following steps: from 0 to 35% B in 90 minutes and then up to 90% B from 90 to 105 minutes. MaxQuant software was used to search the data from the Q-exhaustive analysis. Two databases were used, STRP6 and STREQ.

STRP6:

Proteome ID	Organism	Last modified	Protein Count
UP000001167	Streptococcus pyogenes serotype M6 (strain ATCC BAA-946 / MGAS10394)	2014-07-04	1879

STREQ:

Proteome ID	Organism	Last modified	Protein Count
UP000016889	Streptococcus dysgalactiae subsp. equisimilis 167	2014-07-15	2202

3.14. Antibiotic Susceptibility Testing

All *beta*-hemolytic streptococci were tested for drug susceptibility. The drug susceptibility test was done by a disk diffusion method using Muller Hinton Agar (MHA) (Thermo Fisher Scientific, USA) supplemented with 5% defibrinated sheep's blood. Colony suspension was made using normal saline (0.85% NaCl) equivalent to 0.5% McFarland standard from grown overnight colonies (18-24 hours) on sheep blood agar plate. The suspension was inoculated to MHA plate with 5% sheep's blood using a culture swab and incubated at 5% CO_2 18 to 24 hours. A maximum of 8 antibiotic disks on a 150 mm plate and four antibiotic disks on 100 mm plate were placed. The diameter of zones of inhibition including the diameter of the disk was measured. Interpretation was done according to the Clinical Laboratory Standards Institute (CLSI) (Cavalieri *et al.,* 2005). Twelve antimicrobial agents (Table 3.2) (Thermo Fisher Scientific, USA) were tested against all *beta*-hemolytic streptococci.

Table 3. 2. Antimicrobial agents tested against beta-hemolytic streptococci

Groups	Antimicrobial agents	Concentration
Penicillins	Penicillin (P)	10 units
	Oxacillin (OX)	1µg
Cephalosporin	Ceftriaxone (CRO)	30µg
Glycopeptides	Vancomycin (VA)	30µg
Macrolides	Erythromycin (E)	15µg
Tetracyclines	Tetracycline (TE)	30µg
Fluroquinolones	Ofloxacin (OFX)	5µg
Phenols	Chloramphenicol (C)	30µg
Lincosamides	Clindamycin (DA)	2µg
Streptogramines	Quinopristin-Dalfopristin (QD)	15µg
Oxalolidinones	Linezolid (LZD)	30µg
Sulfonamides	Trimethoprim-sulfamethoxazole (SXT)	25 µg

3.15. Determination of Anti Streptolysin O (ASO) Titer

Anti-Streptolysin O- latex reagents (LiNEAR Chemicals. s. L, Spain) were used to determine ASO titer. All test reagents and serum samples were brought to room temperature before the test was done. Normal saline (0.85 % NaCl) was used for dilution purpose. Fifty micro liter of serum and one drop of the ASLO-Latex antigen were mixed using disposable stirrer on a circle on the card and rotated on the mechanical rotator (100 RPM) for 2 minutes. Both positive and negative controls were run in parallel. It was observed under visible light source for any degree of agglutination. When the test becomes reactive, 50 µL of the serum diluted with 50, 100, 150,200 µL of saline etc. until the result became non-reactive. Then antibody titer was determined from tested sera. An increased titer of ASO indicates recent infection of GAS, Group C Streptococcus (GCS) and Group G Streptococcus (GGS).

3.16. Culture and Identification of Gram Negative Commensals

Throat swab specimens were inoculated onto Modified Thayer Martin agar supplemented with [Vancomycin (300 μg), Colistin (750 μg), Nystatin (1250 units), Trimethoprim (500 μg) (VCNT)] and isovitalex (Thermo Fisher Scientific, USA). The plates were incubated at 37°C in 5 % CO_2 for 24-72 hrs. For those plates that showed bacterial growth, oxidase and gram stain tests were done. All gram negative diplococci and positive for oxidase test were identified as Nessieria/Moraxella species, then subjected to biochemical tests for species differentiation using *Gamma*-Glutamyl Transferase (GGT), *Ortho*-Nitrophenyl-β-galactoside (ONPG) and Tributrine tests (see annex IV). Those strains that were positive for GGT, ONPG and Tributrine were presumptively identified as *Nessieria meningitidis*, *Nessieria lactamica* and *Moraxella catarrhalis*, respectively. In addition to conventional methods, species differentiation was performed by MALDI-TOF MS and 7 out of 10 serogroup of *Nessieria meningitidis* were rechecked by PCR.

3.17. Reference Strains

Streptococcus pyogenes (ATCC 19615), *Neisseria meningitidis* A (13077), *Neisseria lactamia* (23970) were used as a quality control for culture media, culture and identification of GAS and Neisseria.

3.18. Variables

A. Dependent Variables

- Group A Streptococci and other *beta*-hemolytic streptococci carriage rate among children on secondary RF prophylaxis
- *emm* types
- Protein profiles
- Drug susceptibility pattern of *beta*-hemolytic streptococci
- Gram negative oral commensal carriage rate among children on secondary RF prophylaxis

B. Independent Variables

Demographic parameters

- Age
- Sex
- Resident (urban, rural)
- Living conditions
- Family size

Clinical parameters

- Compliance
- Frequency of secondary prophylaxis given
- Types of secondary RF prophylaxis given

3.19. Data Quality Control Measures

Pre analytical

- Standard Operational Procedures were strictly followed during collection, preservation and transportation of throat swab and blood specimens.
- All samples were correctly labeled with patient identifiers.
- Sufficient quantity and quality of swab sample was collected.
- Two ml of venous blood was collected aseptically and properly.
- The swabs and bacterial samples were transported appropriately to the Armauer Hansen Research Institute (AHRI) bacteriology laboratory and Oslo University Rikshospitalet, Norway respectively according to SOP.

Analytical

- Standard Operational Procedures were strictly followed during: staining, culturing, serogrouping, susceptibility testing, mass spectrometry (both MALDI TOF and Electrospray), PCR, sequencing and ASO titer determination. Cross-contamination was looked for on a continuous basis

- Daily positive control samples were run in parallel with sample cultures to verify that the media are functioning as expected.

Post analytical

- The results of throat culture, drug susceptibility, staining, biochemical tests, serogrouping, mass spectrometry (both MALDI TOF and Electrospray), PCR, sequencing and ASO titer determination were recorded timely and properly.
- Experts were involved in interpreting the results.

3.20. Operational Definitions

- Rheumatic fever is a non-suppurative, auto-inflammatory multi-system response following infection by *S. pyogenes* [group A streptococcus (GAS)].
- Rheumatic heart disease refers to the chronic heart valve damage that can occur after a person has had repeated episode of acute rheumatic fever
- Streptococcal colonization: those with positive throat swab culture alone.
- Streptococcal infection: those associated with positive throat swab culture and increased in ASO titer from the cut of point (ASO titer > 200 units/µL).
- Primary prevention of rheumatic fever is defined as the adequate antibiotic therapy of Group A Streptococcus upper respiratory tract infections to prevent an initial attack of acute rheumatic fever
- Secondary prevention of rheumatic fever is the continuous administration of specific antibiotics to patients with a previous attack of rheumatic fever or a well-documented rheumatic heart disease.

3.21. Data Entry, Management and Analysis

Data was entered and summarized using SPSS version 20 software (USA) and analyzed using STATA software. Comparisons were made using the Chi - square test. A p-value of ≤ 0.05 was considered indicative of a statistically significant difference.

Chapter IV: Results

4.1. Socio-Demographic Characteristics of Study Participants

Two hundred and thirty four children participated in this study. All of the children already developed rheumatic heart disease and they were on secondary rheumatic fever prophylaxis. All of the participants took monthly penicillin G prophylaxis except one who took Erythromycin daily. Among 231 participants chart review, 30.74 % (71) had previous history of rheumatic fever recurrence and 92.31 % (216) had mitral regurgitation, 18.80 % (44) had mitral stenosis, 54.27 % (127) had aortic regurgitation, 2 had aortic stenosis, 67.09% (157) had tricuspid regurgitation and 8.55% (20) had pulmonary regurgitation. Out of 234 participants, 38.03% were referred for possible surgical intervention in which only three participants received surgical intervention. A delay in surgical intervention for more than one year was 46.5 % (40/86).

The participants' age ranges 5-15 years. The majority of participants were aged beteween10-15 years (75.88%), ethnic Oromo (48.29%) and females (59.2%) as shown in (Table 4.1). H

Table 4. 1. Socio-demographic characteristics of children with rheumatic heart disease investigated for beta-hemolytic streptococci in TASH (July 2013-June 2014)

Characteristics		**No.**	**(%)**
Age (years) (n=228)	5-9	55	24.12
	10-15	173	75.88
Sex (n=233)	Male	95	40.8
	Female	138	59.2
Ethnicity (n=234)	Amhara	54	23.08
	Oromo	113	48.29
	Guragie	30	13.04
	Tigre	8	3.42
	Silte	6	2.56
	Others	23	9.83
Residence (n=234)	Urban	122	52.1
	Rural	112	47.9

4.2. Isolation Rate of *Beta*-hemolytic Streptococci + Serogrouping + Species differentiation

A total of 58 *beta*-hemolytic streptococci were isolated with two multiple carriages. The throat carriage rate of *beta*-hemolytic streptococci was 23.93 % (56/234). Group A Streptococcus proportion and carriage rate were 10.34 % (6/58) and 2.56% (6/234), respectively. Most of *beta*-hemolytic streptococci were serogroup F (43.1%) followed by serogroup G streptococci (34.48%) as shown in (Table 4.2). In addition to conventional characterization all streptococcal strains were identified to the species level by MALDI-TOF mass spectrometry using the Bruker Daltronics Microflex equipment (Table 4.3).

Species differentiation of 58 *beta*-hemolytic Streptococci showed 6.9% *S. pyogenes*, 6.9% *S. dysgalactaie subsp. equisimilis*, 36.21% *S. anginosus*, 43.10% *S. constellatus*, 1.72% *S. agalactaie* and 5.17% unclassified.

Among 6 group A streptococci, four of them were bacitracin susceptible *S. pyogenes* and the other 2 were bacitracin resistant *S. dysgalactaie subsp. equisimilis.* In this study *S. dysgalactaie subsp. equisimilis* possessing Lancefield group A, C and G were isolated. Almost all group F and G are *S. constellatus* and *S. anginosus* respectively (Table 4.3).

Forty one *alpha*-hemolytic streptococci colonies were also characterized to species level that were difficult to differentiate them either *alpha* or *βeta* hemolytic on sheep blood agar plates. Species differentiation of these *alpha*-hemolytic streptococci / *beta*-hemolytic like colonies showed that 31.71% *S. parasanguinis*, 31.71% *S. mitis*, 7.32% *S. oralis,* 4.88% *S. pneumoniae*, 2.44 % *S. perioris*, 2.44% *S. constellatus* and 19.5% unclassified streptococci (Table 4.4). Bacitracin susceptibility test result of *alpha*-hemolytic streptococci showed 84.6% of *S. parasanguinis*, 54.5% of *S. mitis* and 100% of others (unclassified) were susceptible to bacitracin. *S. parasanguinis* possessing group B, C, G, and F were detected. One *alpha*-hemolytic *S. constellatus* possessing Lancefield group C was also detected.

Table 4. 2. Serogrouping result of 58 beta-hemolytic streptococci isolated from 234 children with rheumatic heart disease on secondary prophylaxis in TASH (July 2013-June 2014)

Serogroup	**No**	**%**
A	6	10.34
B	1	1.72
C	2	3.45
F	25	43.10
G	20	34.48
G & F	2	3.45
Non typeable	2	3.45
Total	58	100

Table 4. 3. Species differentiation of 58 beta-hemolytic streptococci isolated from 234 children with rheumatic heart disease on secondary prophylaxis in TASH (July 2013-June 2014)

Species	Serogroup	No	%
S. pyogenes	A	4	6.9
S. dysgalactaie subsp. equisimilis	A, C, G	4	6.9
S. agalactie	B	1	1.72
S. anginosus	G, G & F, Non typable	21	36.21
S. constellatus	F, Non typable	25	43.10
Not differentiated	F, G	3	5.17
Total		58	100

Table 4. 4. Species differentiation of 41 alph-hemolytic like streptococci isolated from 234 children with rheumatic heart disease on secondary prophylaxis in TASH (July 2013-June 2014)

Species	**No**	**%**
S. constellatus	1	2.44
S. parasanguinis	13	31.71
S. mitis	13	31.71
S. orali	3	7.32
S. perioris	1	2.44
S. pneumoniae	2	4.88
Not differentiated	8	19.5
Total	41	100

4.3. *emm* sequence typing

Selected *beta*-hemolytic streptococcal strains (*S. pyogenes* and *S. dysgalactiae subsp. equisimilis)* were also subjected to molecular characterization using PCR-sequencing of the *emm* gene. All *S. pyogenes* (4) and *S.dysgalactaie subsp. equisimilis* (4) *emm* gene was amplified by the primer 1 and primer 2 (Table 3.1) as recommended by CDC (Beal, 2005) which is originally designed for *emm* typing of group A streptococci. Six different subtypes were found in this study (Table 4.5.). *StGrobn.1* subtype was *S. dysgalactaie subsp. equisimilis* possessing Lancefield group A which was the newly discovered subtype in this study. PCR products of the selected Streptococci are shown in Fig. 4.1.

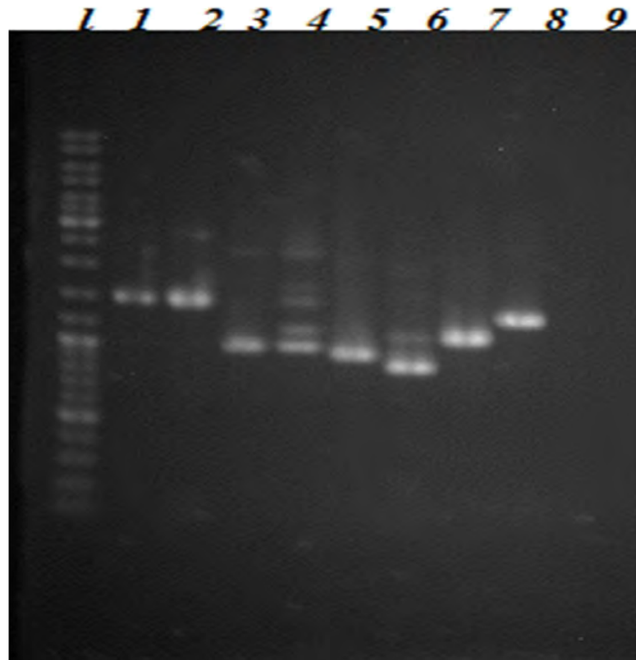

Figure 4. 1. PCR products of emm genes of S. pyogenes and S. dysgalactaie subsp. equisimilis isolated from children with RHD on secondary antibiotic prophylaxis in TASH (July 2013- June 2014)

Lane L=DNA ladder (100 to 1000 with 100 base difference, then 1200, 1500 up to 6000 with 500 base difference, and finally 8000 and 10000 bases), lane1=*stGrobn*.1, lane 2 *stGrobn.1*, lane 3=*emm* 70.0, lane 4=*emm* 70.0, lane 5=*emm* 68.2, lane 6 =*emm*77.0, lane7= *stG4831.0*, lane 8= *stC839.0*, lane 9=Milli Q water.

Table 4. 5. emm typing result of S. pyogenes and S. dysgalactaie subsp. equisimilis streptococci isolated from 234 children with rheumatic heart disease on secondary antibiotic prophylaxis in TASH (July 2013-June 2014)

emm type	Group	Species	No	Comment
emm 68.2 *emm*68.2 subtype	A	*S. pyogenes*	1	
*emm*70. 0 *emm*70. 0 subtype	A	*S. pyogenes*	2	
*emm*77. 0 *emm*77. 0 subtype	A	*S. pyogenes*	1	
STGROBN.1 stGrobn.1 subtype	A	*S. dysgalactaie subsp. equisimilis*	2	New subtype
STG4831.0 stG4831.0 subtype	G	*S. dysgalactaie subsp. equisimilis*	1	
STC839.0 stc839. 0 subtype	C	*S. dysgalactaie subsp. equisimilis*	1	

4.4. Protein Profile of some Selected Strains

In addition to molecular *emm* typing, selected *beta*-hemolytic streptococcal strains (*S. pyogenes* and *S. dysgalactiae*) were also subjected to advanced molecular protein profiling based on next-generation OrbiTrap-based mass spectrometry. This electrospray-based analysis yielded high-resolution characteristics of the streptococcal strains, including the identification of >1800 streptococcal proteins. The in-gel digestion and OrbiTrap analysis also yielded the identification of novel post-translational modifications (PTMs) not previously recognized or studied. For example, the streptococcal glycosyltransferase in *S. pyogenes* strain *emm* 68.2 had N-linked glycosylation carrying a unique N-acetylhexoseamine (HexNAc)-deoxyhexose.

Additionally, we compared proteins shared between group A *S. pyogenes* and Group A *S. dysgalactaie subsp. equisismilis* with reference strains (strain 105- group A *S. pyogenes*, strain 15 and 5- group A *S. dysgalactaie subsp. equisismilis*). Combined from both database searches and for all

three samples (strain 105, 15 and 5) a total number of 2049 proteins were identified. In total, from the database STRP6 1070 proteins were identified and from the database STREQ 1186 proteins, where 207 protein identifications overlapped between the two databases. Strain 105 had a higher number of protein match from STRP6 database, while strain 15 and 5 had the highest number of protein match from STREQ database. This result showed both group A *S. pyogenes* and Group A *S. dysgalactaie subsp. equisimilis* share many proteins (Fig 4.2, Table 4.6).

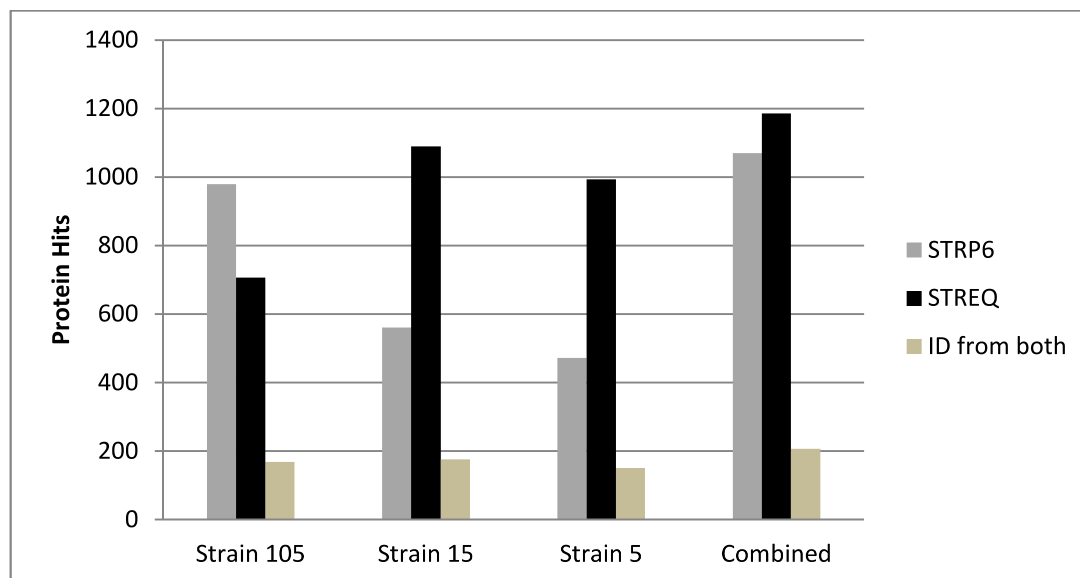

Figure 4. 2. Protein identification from search against the database STRP6 and STREQ.

Table 4. 6. Protein identification from search against the database STRP6 and STREQ

	Strain 105	Strain 15	Strain 5	Combined
STRP6	979	561	472	1070
STREQ	706	1089	993	1186
ID from both	168	176	151	207

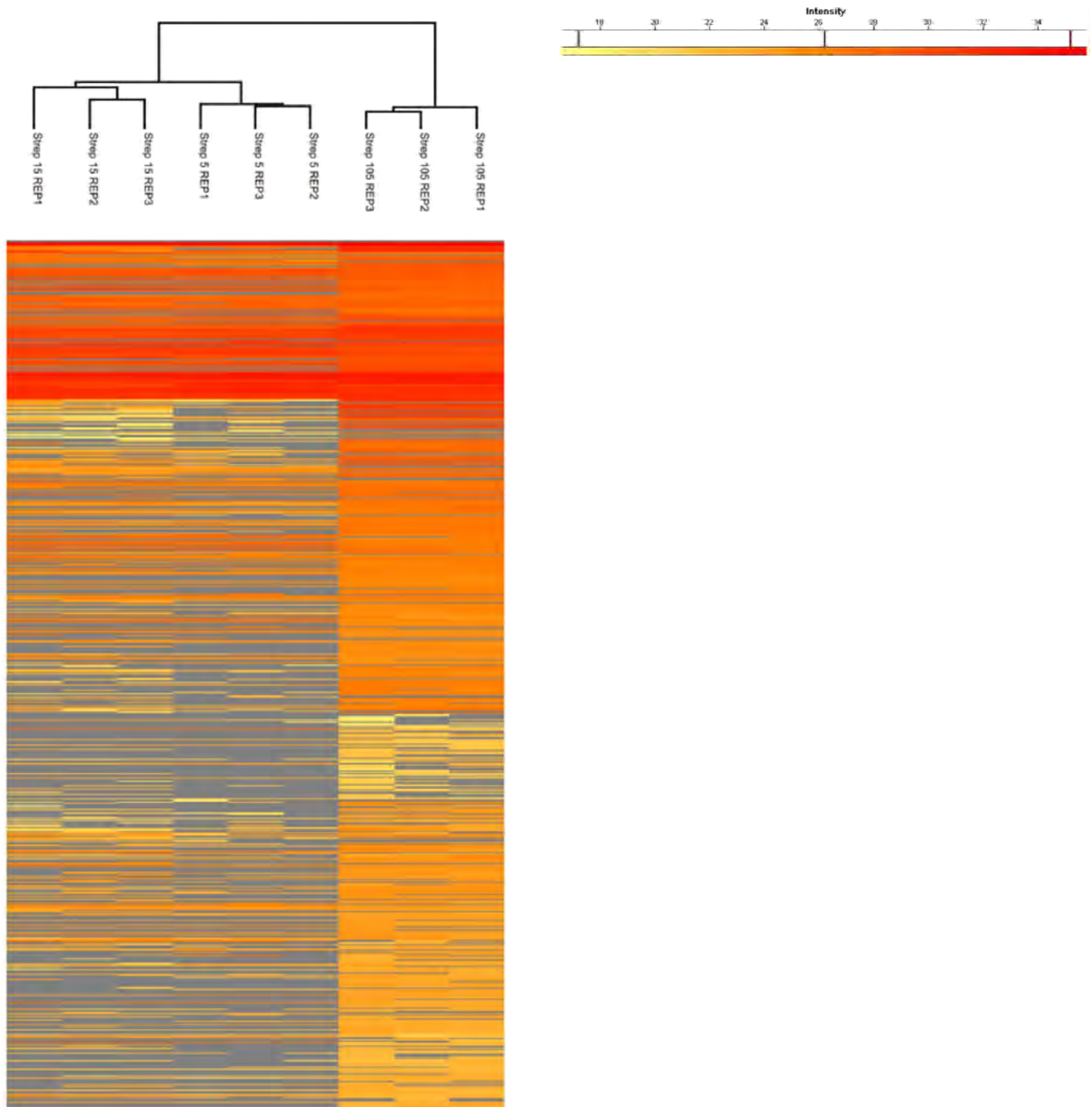

Figure 4. 3. Heatmap of STRP6 database search. Color scheme shown in the upper right corner, yellow (low abundance protein) -red (high abundant protein). The color scheme is based on log2 scale of label free quantitative (LFQ) protein intensity measurements calcula

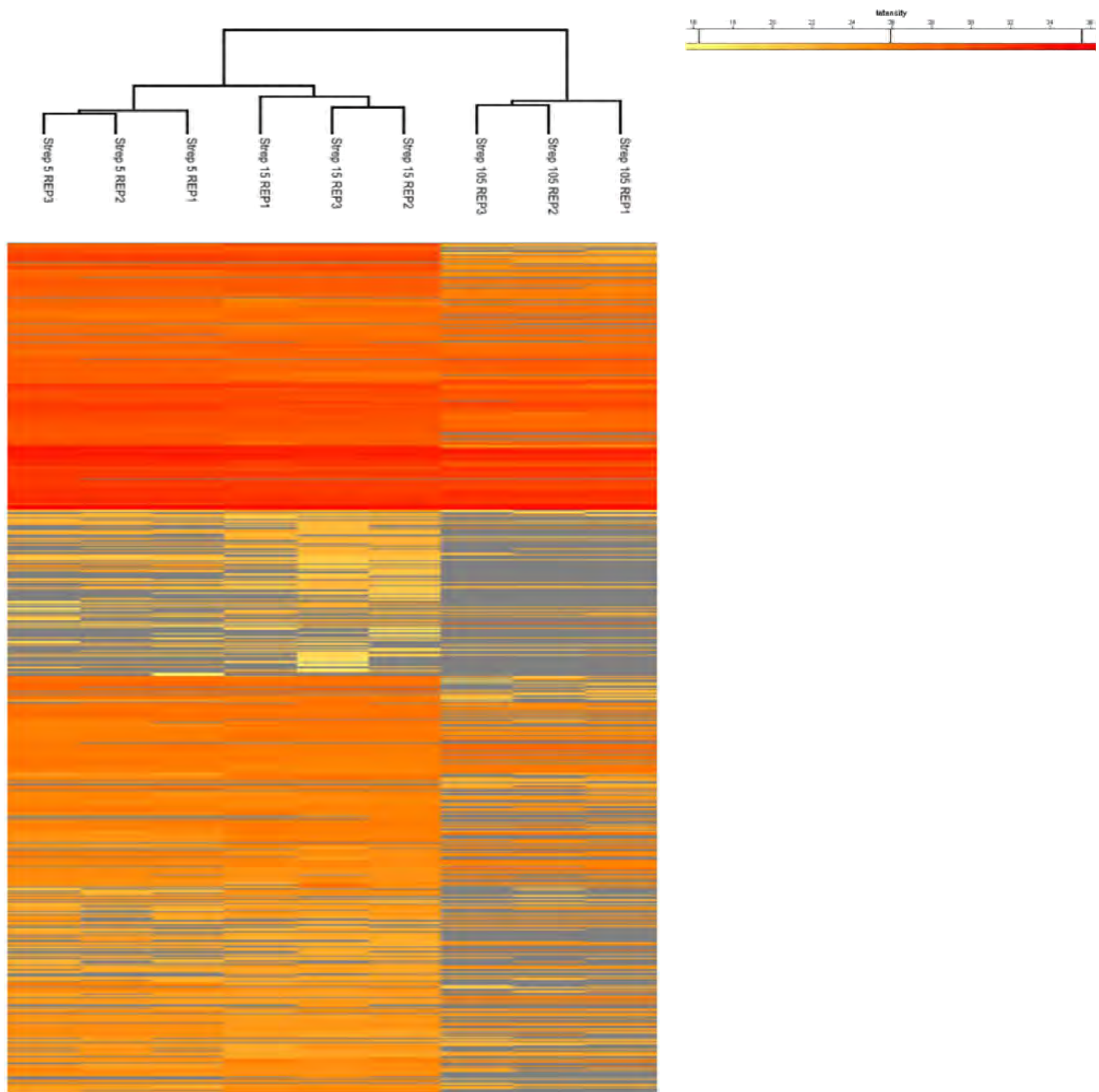

Figure 4. 4. Heatmap of STRP6 database search. Color scheme shown in the upper right corner, yellow (low abundance protein) -red (high abundant protein). The color scheme is based on log2 scale of label free quantitative (LFQ) protein intensity measurements calcula

4.5. Antibiotic Susceptibility Data

A. GAS (*S. pyogenes*)

[Resistance (R) / intermediate susceptibility (I) (R /I)] to penicillin (0/0), Oxacillin (2/ 0) Erythromycin (1/1), Ceftriaxone (2 /0), Trimethoprim-sulfamethoxazole (3/0), Vancomycin (0/0), Tetracycline (2/1), Ofloxacin (1/0), Chloramphenicol (1/0), Clindamycin (0/2) Quinopristin-Dalfopristin (0/1) and Linezolid (1/0) were observed for GAS isolates.

All *S. pyogenes* were susceptible to penicillin and Vancomycin but resistant to Sulphamethoxazole-trimethoprim. One intermediate and one resistant *S. pyogenes* to Erythromycin were also found. Two *S.pyogenes* showed multi drug resistance pattern. One of them was resistant to Ofloxacin, Oxacillin, Ceftriaxone, Trimethoprim-sulfamethoxazole, Linezolid, Chloramphenicol, and Tetracycline. It was also intermediately resistant to Erythromycin, Clindamycin and Quinopristin-Dalfopristin. The other one was resistant to Erythromycin, Ceftriaxine, Tetracycline, Trimethoprim-sulfamethoxazole and intermediately resistant to Clindamycin (Table 4.7).

B. Other *beta*-hemolytic streptococci

[Resistance (R %) / intermediate susceptibility (I %) (R % /I %)] to penicillin (1.96/0), Oxacillin (76.47/0), Erythromycin (5.77/3.84), Ceftriaxone (45.1/0), Trimethoprim-sulfamethoxazole (9.8/1.96), Vancomycin (3.92/1.96), Tetracycline (4.17/2.08), Ofloxacin (0/1.96), Chloramphenicol (1.96/7.84), Clindamycin (0/0), Quinopristin-Dalfopristin (0/9.8) and Linezolid (1.96/0) were observed.

All other *beta*-hemolytic streptococci were susceptible to Clindamycin. They were also susceptible to Penicillin except *S. agaglactaie. Streptococcus agalactaie* was resistant to multi drugs (Table 4.7).

C. *Alpha*-hemolytic streptococci

[Resistance (R %) / intermediate susceptibility (I %) (R % /I %)] to penicillin (33.3/0), Oxacillin (82.1, / 0) Erythromycin (23.1/15.4), Ceftriaxone (64.1 / 0), Trimethoprim-sulfamethoxazole (46.2/17.9), Vancomycin (10.3/0), Tetracycline (27/18.9), Ofloxacin (0/2.6), Chloramphenicol (2.6/7.7), Clindamycin (2.6/0), Quinopristin-Dalfopristin (0/25.6) and Linezolid (2.3/0) were observed (Table 4.8.).

Table 4. 7. Antibiotic susceptibility pattern of beta-hemolytic streptococci isolated from 234 children with rheumatic heart disease on secondary antibiotic prophylaxis in TASH (July 2013-June 2014)

		Antimicrobial agents no. (%)											
***Beta*-hemolytic streptococci**		P	OX	E	CRO	SXT	VA	TE	OFX	C	DA	QD	LZD
S. pyogenes (n=4)	S	4	2	2	1	-	3	-	2	2	1	2	2
	I	-	-	1	-	-	-	1	-	-	2	1	-
	R	-	2	1	2	3	-	2	1	1	-	-	1
S. dysgalactaie subsp. equisimilis (n=4)	S	4	4	3	4	-	2	3	4	4	4	4	4
	I	-	-	1	-	1	1	-	-	-	-	-	-
	R	-	-	-	-	3	1	-	-	-	-	-	-
S. agalactaie (n=1)	S	-	-	-	-	1	-	-	1	-	1	-	-
	I	-	-	1	-	-	-	-	-	1	-	1	-
	R	1	1	-	1	-	1	1	-	-	-	-	1
S. anginosus (n=20)	S	20(100)	5(25)	18(90)	8(40)	19(95)	20(100)	18(94.7)	20(100)	18(90)	20(100)	16(80)	20(100)
	I	-	-	-	-	-	-	-	-	1(5)	-	4(20)	-
	R	-	15(75)	2(10)	12(60)	1(5)	-	1(5.3)	-	1(5)	-	-	-
S. constellatus (n=26)	S	26(100)	3(11.5)	25(96.2)	16(61.5)	25(96.2)	26(100)	24(96)	25(96.2)	24(92.3)	26(100)	26(100)	26(100)
	I	-	-	-	-	-	-	1(4)	1(3.8)	2(7.7)	-	-	-
	R	-	23(88.5)	1(3.8)	10(38.5)	1(3.8)	-	-		-	-	-	-
Total (n=55)	S	54(98.2)	14(25.5)	48(87.3)	29(53.7)	45(83.3)	51(94.4)	45(88.2)	52(96)	48(87.3)	52(96)	48(88.9)	52(96)
	I	-	-	3(5.5)	-	1(1.9)	1(1.9)	2(3.90)	1(2)	4(7.4)	2(4)	6(11.1)	-
	R	1(1.8)	41(74.5)	4(7.3)	25(46.3)	8(14.8)	2(3.7)	4(7.8)	1(2)	2(3.7)	-	-	2(4)

Key- P-Penicillin, OX-Oxacillin, E-Erythromycin, CRO-Ceftriaxone, SXT-Trimethoprim-sulfamethoxazole, VA-Vancomycin, TE-Tetracycline, OFX-Ofloxacin, C-Chloramphenicol, DA- Clindamycin, QD- Quinopristin-Dalfopristin, LZD-Linezolid

Table 4. 8. Antibiotic susceptibility pattern of αlpha hemolytic streptococci isolated from 234 children with rheumatic heart disease on secondary antibiotic prophylaxis in TASH (July 2013-June 2014)

		Antimicrobial agents no. (%)											
***alpha* hemolytic streptococci**		P	OX	E	CRO	SXT	VA	TE	OFX	C	DA	QD	LZD
S. parasanguinis (n=26)	S	7(53.8)	2(15.4)	7(53.8)	5(38.5)	4(30.8)	11(84.6)	6(46.2)	13(100)	11(84.6)	13(100)	11(84.6)	12(92.3)
	I	-	-	1(7.7)	-	3(23)	-	3(23)	-	1(7.7)	-	2(15.4)	-
	R	6(46.2)	11(84.6)	5(38.5)	8(61.5)	6(46.2)	2(15.4)	4(30.8)	-	1(7.7)	-	-	1(7.7)
S. mitis (n=13)	S	7(63.6)	-	6(54.5)	4(36.4)	3(27.3)	11(100)	6(54.5)	10(90.1)	10(90.1)	10(90.1)	6(54.5)	11(100)
	I	-	-	2(18.2)	-	-	-	1(9.1)	1(9.9)	1(9.9)	-	5(45.5)	-
	R	4(36.4)	11(100)	3(27.3)	7(63.6)	8(72.7)	-	4(36.4)	-		1(9.9)	-	-
S .oralis (n=3)	S	2	1	2	-	1	2	-	3	3	3	2	3
	I	-	-	1	-	2	-	-	-	-	-	1	-
	R	1	2	-	3	-	1	2	-	-	-	-	-
S. pneumoniae(n=2)	S	2	1	2	1	-	2	2	2	2	2	2	2
	I	-	-	-	-	-	-	-	-	-	-	-	-
	R	-	1	-	1	2	-	-	-	-	-	-	-
S. perioris (n=1)	S	-	-	1	1	1	1	1	1	1	1	1	1
	I	-	-	-	-	-	-	-	-	-	-	-	-
	R	1	1	-	-	-	-	-	-	-	-	-	-
Unclassified (n=9)	S	8	3	6	3	5	8	5	9	8	9	7	9
	I	-	-	2	-	2	-	3	-	1	-	2	-
	R	1	6	1	6	2	1	1	-	-	-	-	-
Total (n=39)	S	26(66.7)	7(17.9)	24(61.5)	14(35.9)	14(35.9)	35(89.7)	20(54.1)	38(97.4)	35(89.7)	38(97.4)	29(74.4)	38(97.7)
	I	-	-	6(15.4)	-	17(17.9)	-	7(18.9)	1(2.6)	3(7.7)	-	10(25.6)-	-
	R	13(33.3)	32(82.1	9(23.1)	25(64.1)	18(46.2)	4(10.3)	10(27)	-	1(2.6)	1(2.6)	-	1(2.3)

4.6. ASO Titer Result

Among 234 participants, 29.49% (69) and 70.51% (165) were positive and negative to ASO respectively. Of the positives, 66.67 % (46 / 69) showed increased ASO titers (ASO > 200 units/µL) and the rest 33.3% (23/69) was ASO =200 units/ µL. Anti-Streptolysin O titer > 200 units/µL indicates recent infection. But ASO =200 units/ µL is on the borderline and difficult to interpret whether it is infection or not at this time.

In this study positive for throat culture and ASO =200 units/ µL was found and may be considered as infection with group A, C, or G streptococci. Among 8 *S. pyogenes* and *S. dysgalactaie,* six showed increased ASO titer/infection and the other two were colonization.

Table 4. 9. Association of ASO titer result with *beta*-hemolytic streptococci infection in 234 children with RHD on secondary antibiotic prophylaxis in TASH (July 2013-June 2014)

		ASO			
		Positive	Negative	Total	P-value
***Beta* - hemolytic Streptococci**	Culture positive	22	33	55	0.05
	Culture negative	47	132	179	
	Total	69	165	234	

4.7. Isolation Rate and Species Differentiation of Gram Negative Commensals

The isolation rate of gram negative, oxidase positive oropharyngeal commensals was 55.1% (129/234). Throat carrier rate of *N. meningitidis, N. lactamica, M. catarrhalis, K. denitrificans, K. kingae, N. elongate* and *Capnocytophaga sputigena* were 4.27% (10/234), 4.27% (10/234), 19.66 % (46/234), 15.81% (37/234), 2.56% (6/234), 0.85% (2/234) and 1.28% respectively.

Species differentiation showed that proportion of *M. catarhalis* (35.66%), *K. denitrificans* (28.68%), *N. meningitidis* (7.75%), *N. lactamica* (7.75%), *K. kingae* (4.65%), *Capnocytophaga sputigena* (2.32 %), *N. elongata* (1.55 %), and others (Table 4.10). The Serogroup of seven *N. meningitidis* strains out of 10 was rechecked by PCR. Identification of the serogroup of *N. meningitidis* by MALDI-TOF MS was not consistent. One strain which was serogroup W/A/no serogroup became W135 by PCR. Another strain which was W/Y became non groupable by PCR. Five strains were serogroup Y in

both methods. One non-groupable and 2 serogroup A by MALDI-TOF MS but not rechecked by PCR were also identified. Among the two serogoup A *N. meningitidis* one was GGT negative, which is not common.

Table 4. 10. Species differentiation of 129 gram negative commensals isolated from 234 children with rheumatic heart disease on secondary prophylaxis in TASH (July 2013 to June 2014)

Species	**No**	**%**
N. meningitidis	10	7.75
N. lactamica	10	7.75
M.catarrhalis	46	35.66
K. denitrificans	37	28.68
K. kingae	6	4.65
Capnocytophaga sputigena	3	2.32
N. elongate	2	1.55
Brevibacillus borstelensis	2	1.55
Chrysobacterium indologenes	1	0.77
Aeromonas caviae	1	0.77
M .oblonga	1	0.77
Not differentiated	10	7.75
Total	129	100

4.8. Risk Factors

Those participants missing the prophylaxis showed statistically significant association to have *beta*-hemolytic streptococci in their throat (p=0. 0003) (Table 4.11). Those children who received antibiotic prophylaxis between 22- 28 days had 1.6 times risk to have *beta*-hemolytic streptococci than those ≤16 days (p=0. 021, OR=2. 6, CI =1. 16-5.85) (Table 4.12). The longer timing of receiving of antibiotic prophylaxis is an important risk factor for colonization with *beta*-hemolytic streptococci, for example, those received antibiotic prophylaxis ≥ 29 days is 2.61 times riskier to have *beta*-hemolytic streptococci colonization (p= 0.003, OR=3. 61, CI = 1.5-8.46) (Table 4.12).

Among three participants who had *S. pyogenes*, two of them were above 16 days duration after injection and one less than 16 days. In the same way among 3 participants who had *S. dysgalactaie subsp. equisimilis* two of them were above 16 days duration after injection and one less than 16 days.

Table 4. 11. Risk factors associated with beta-hemolytic streptococci colonization in children with RHD on secondary antibiotic prophylaxis in TASH (July 2013-June 2014).

		***Beta*-hemolytic streptococci**		**P-value**
Risk factors		**Culture positive**	**Culture negative**	
Age	5-9 (n=55)	13	42	0.9372
	10-15 (n=173)	40	133	
Sex	Male (n= 95)	26	69	0.262
	Female (n =138)	29	109	
Residence	Urban (n=122)	27	95	0.6052
	Rural (n=112)	28	84	
Family size	<6 (n=140)	32	108	0.776
	≥7 (n=94)	23	71	
Missing prophylaxis	Missing (n=33)	16	17	0.0003
	Not missing (n=201)	39	162	
Hx of Family pharyngitis	Yes (n=25)	5	20	0.7321
	No (n=204)	47	157	
Family educational status	Illiterate (n=68)	18	50	0.458
	Literate (n=164)	36	128	
Uvelectomy	Yes (n=57)	16	41	0.3435
	No (n=160)	35	125	

Table 4. 12. Timing of antibiotic prophylaxis injection as risk factor for colonization rate of beta-hemolytic streptococci

		***Beta*-hemolytic streptococci**			
Risk factor		Culture positive	Culture negative	OR (95% CI)	P-value
Timing of antibiotic prophylaxis	≤16 days (n=98)	15	83	1	
	17-21 days (n=25)	5	20	1.4(0.5-4.3)	0.57
	22-28 days (n=50)	16	34	2.6(1.2-5.9)	0.02
	≥29 days (n=38)	15	23	3.6(1.5-8.5)	0.003

CI-confidence interval

OR-odds ratio

Chapter V: Discussion

The global morbidity of RHD is estimated to be 15.6-19.6 million people (2.4 million children aged 5-14 years) with a mortality rate of 233 364-294 398 deaths per year (annual mortality rate of 1.5%). Though the incidence of rheumatic heart disease is decreasing in developed countries, it is still a major challenge in developing nations. The highest prevalence of RHD is in sub-Saharan Africa with a prevalence of 5.7 per 1000, compared with 1.8 per 1000 in North Africa, and 0.3 per 1000 in economically developed countries (Carapetis *et al*., 2005b, WHO, 2005). Both population and hospital based studies showed rheumatic heart disease is the number one cardiac problem in Ethiopian children (Habte *et al*., 2010; Günther *et al*., 2006; Mehadi *et al*., 2006; Oli and Asmera, 2004; Oli and Porteous, 1999 a; Oli *et al*., 1992). Group A streptococci is the cause for RF/ RHD (WHO, 2005) but the latest studies reporting another large colony forming serogroup C and serogroup G streptococci are also emerging (Sitkiewicz and Hryniewicz, 2010; Bramhachari *et al*., 2010). The present study was carried out to determine the effectiveness of secondary antibiotic prophylaxis in children with rheumatic heart disease to GAS and other *beta*-hemolytic carriage rate, which could be important to the care of the children to prevent aggravation of RHD.

Two hundred and thirty four children participated in this study. They have had rheumatic heart disease and they were on secondary rheumatic fever prophylaxis. In this study, all children already developed rheumatic heart disease and there was no patient with acute rheumatic fever without carditis encountered during the study period. This may indicate poor prevention of rheumatic heart disease which is in agreement with a study done somewhere in Africa (Bassill *et al*., 2000). Out of 234 participants, 38.03% were referred for possible surgical intervention, of which only 3 had got the chance for surgery. A delay in surgical intervention for more than one year was 46.5%. This was a problem in Egypt also (Bassill *et al*., 2000). The majority of the participants were females (59.2%). Rheumatic heart disease is higher in females (59.2%) which are in agreement with population and hospital based studies conducted in Ethiopia and elsewhere (Bhardwaj *et al*., 2012; Tewodros *et al*., 1992). The reseason why higher in females is not known.

In this study among 231 participants chart review, 30.34 % had previous history of rheumatic fever recurrence which is comparable to a study conducted in a pacific island population (38.2% of 158

participants) (p=0. 09) (Seckeler *et al*., 2010). The recurrence could be due to schedule of injection, noncomplaince, and development scape mechanism of the pathogen for penicillin.

Throat carrier rate and distribution of *beta*-hemolytic streptococci varies from place to place and based on seasonal changes (Bramhachari *et al*., 2010; Abdissa *et al*., 2006; Tewodros *et al*., 1992). In the current study, though the children received ongoing monthly penicillin G prophylaxis their *beta*-hemolytic carrier rate (23.93%) was significantly higher than Abdissa and his colleagues study (17.72%) (P=0. 03) (Abdissa *et al*., 2006) but it was similar to a study conducted on chronic rheumatic heart disease patients about 25 years ago (20.45%) (p=0. 62) (Tewodros *et al*., 1992). It is different from Abdissa *et al* study, it could be because of they did on healthy school children but it is similar with Tewodros *et al* which was similar methodology and participants. The reason for lower group A atreptococci could be because of on-going monthly prophylaxis.

Serogroup distribution of streptococci species is different among parts of the world. Group C and G streptococcus disease burden was higher than group A streptococcus among Mumbai school children (Bramhachari *et al*., 2010) which is similar to the present study for group G and group A streptococci but the distribution of group A streptococcus was higher in Ethiopian healthy school children (Abdissa *et al*., 2006). In the present study, serogroup G and serogroup F were predominant streptococci which are comparable with other studies conducted elsewhere (Devi et al., 2011; Bramhachari *et al*., 2010).

Species differentiation of the genus streptococcus is dynamic. In addition to traditional methods (*beta*-hemolytic characteristics, catalase test, gram stains and serogouping), species differentiation was also performed by MALDI-TOF MS (Bruker Daltonics, Germany). Although *S. pyogenes* is the main human pathogen, large colony forming *S. dysgalactiae subsp. equisimilis* is becoming emerging pathogen (Sitkiewicz and Hryniewicz, 2010; Bramhachari *et al*., 2010).
Of 6 group A streptococci, four of them were *S.pyogenes*. The other 2 were bacitracin resistant *S. dysgalactaie subsp. equisimilis.* In the current study, *S. dysgalactaie subsp. equisimilis* possessing Lancefield group A, C and G (GAS, GCS, and GGS) were isolated. There are reports for *S.dysgalactaie subsp. equisimilis* possessing Lancefield group A increment (Katsukawa *et al*., 2002; Brandt *et al.,* 1999).

In the present study carrier rate of *S. pyogenes* is 1.7%, which seems a little bit lower than another study (4.5%) conducted in 44 chronic rheumatic heart disease patients (Tewodros *et al*., 1992).

Even though the type prophylaxis and the methodology is different, the present finding showed a lower carriage rate as compared with other study with a carrier rate of 15.4 % GAS among 26 patients on once weekly azithromycin (AZT) but higher than 22 patients taking oral penicillin (0%) (Gopal *et al., 2009*).

Some *alpha*-hemolytic streptococci create confusion to differentiate whether they are *alpha* or *beta*-hemolytic colonies, mainly when incubation time was extended to 48 hours. Many of them were bacitracin susceptible which adds confusion for group A streptococcus presumptive identification (84.6% of *S. parasanguinis*, 54.5% of *S. mitis* and 8 others (unclassified) were susceptible to bacitracin). In other studies, bacitracin susceptibility was reported in some GCS and as high as 67% among GGS (Vartian *et al*., 1985) and 12.2% non group A *beta*-hemolytic streptococci (Gunn, 1976). In this study and in another study GAS isolates were resistant to trimethoprim-sulfamethoxazole which is considered as presumptive identification of GAS (Gunn, 1976) but it contrasts with Abdissa *et al*. (2011) findings, where all GAS isolates were susceptible to trimethoprim-sulfamethoxazole.

For *beta*-hemolytic characterization sheep blood agar was better than a human blood agar plate. In this study, it was observed that some *beta*-hemolytic colonies became either *alpha* or non-hemolytic on human blood agar plates. Other studies reported that human blood agar plate is not recommended for *beta*-hemolytic appreciation and drug susceptibility testing (Satzke *et al*., 2010; Russell *et al*., 2006). It was also observed that small *alpha*-hemolytic streptococcus colonies and very small oxidase positive, fastidious, gram negative, spreading like colonies were not correctly identified by MALDI- TOF MS speciation (very small score value and inconsistent results). A study conducted by Seng *et al*. (2009) found that some strains were not correctly identified by this method and they recommend improving this method for correct identification of microorganisms. In this study, two microorganisms which are not yet present in the database were found, in which another literature also recommend the database to contain all microorganisms to make MALDI-TOF MS the better diagnostic tool (Carbonnelle *et al*., 2011)

Selected streptococcal strains (*S. pyogenes* and *S. dysgalactaie subsp. equisimilis)* were subjected for *emm* typing. *Streptococcus pyogenes* (group A) and *S. dysgalactaie spp. equisimilis* (group A, C, G) are evolutionary related, share virulence associated genes and same tissue niche in humans. They can also exchange genetic materials and cause a similar spectrum of disease. Both are also grouped under pyogenic streptococci (Bramhachari *et al*., 2010; Sitkiewicz and Hryniewicz, 2010; Brandt and Spellerberg, 2009). There are reports that *S. dysgalactaie subsp. equisimilis* can cause rheumatic fever, similar to *S. pyogenes* (Brandt and Spellerberg, 2009; Mathur *et al*., 2004). In this study, among eight strains (4 *S. pyogenes* and 4 *S. dysgalactaie subsp. equisimilis*), a total of six different *emm* gene types was identified, in both cases *emm* gene was amplified by the same primers originally designed for *S. pyogenes* only. Of six, *emm* 70.0 gene type has been reported in a previous study conducted in Ethiopia (Tewodros and Kronvall, 2005), but there was no such *emm* subtype observed in another study conducted in Ethiopia (Abdissa *et al*., 2006).

emm 77.0 was included in 26 multivalent vaccine trials (Hu *et al*., 2002). From the reports of Tewodros *et al*. (2005) 42.3% and Abdissa *et al.* (2006) about 46% GAS collections were not included in the 26 multivalent vaccine. Theoretically, this proves the 26 multivalent vaccine could not be a beneficiary in protecting GAS infection for Ethiopian population. In this study, the newly discovered subtype was *S. dysgalactaie subsp. equisimilis* possessing Lancefield group A in which the sequence was reviewed and assigned designation "*stGrobn.1*" by CDC and deposited at both CDC (ftp://ftp.cdc.gov/pub/infectious_diseases/biotech/tsemm/stGrobn.1.sds) and NCBI with the accession number of GenBank KM524257.

Those selected *beta*-hemolytic streptococcal strains (*S. pyogenes* and *S. dysgalactiae*) in addition to molecular *emm* typing they were also subjected to advanced molecular protein profiling based on next-generation OrbiTrap-based mass spectrometry. This electrospray based analysis yielded high resolution characteristics of the streptococcal strains, including the identification of >1800 streptococcal proteins. The in-gel digestion and OrbiTrap analysis also yielded the identification of novel post-translational modifications (PTMs) not previously recognized or studied. For example, the streptococcal glycosyltransferase in *S. pyogenes* strain *emm* 68.2 had N-linked glycosylation carrying a unique HexNAc-deoxyhexose. The rheumatogenicity of some streptococcus strains has been considered belonging to specific M serotypes (M types such as 1, 3, 5, 6, 14, 18, 19 and 24) (WHO,

2004). However, the data showed that rheumatogenic M serotypes were infrequently identified in communities with high burdens of acute rheumatic fever and rheumatic heart disease. These results question the potential importance of other disease causing serotypes (Marijon *et al*., 2012).

Pathogenic mechanisms of cross-reactive auto antibodies which target the valve in rheumatic heart disease and the neuronal cell in Sydenham chorea share a common streptococcal epitope N-acetylglucoseamine (GlcNAc) and target intracellular biomarkers of disease, including cardiac myosin in the myocardium and tubulin, a protein abundant in the brain (Cunningham, 2012). In the current study, *emm* 68.2 subtype found to have HexNAc-deoxyhexose which has pathogenic potential in rheumatic fever disease. This result showed it is not only M protein responsible for rheumatic fever disease. The strains of *S. pyogenes* and *S. dysgalactiae* assessed here had different PTM patterns, enabling distinction of subgroups of these closely strains with pathogenic potential in rheumatic fever disease. Therefore, findings from this study generated novel knowledge on *beta*-hemolytic streptococci in rheumatic fever disease.

Studies support antimicrobial drug resistant strains among the β-hemolytic streptococci is emerging (Devi *et al*., 2011; Traub and Leonhard, 1997). In the present study, all GAS isolates were susceptible to penicillin, which is comparable with other studies conducted in Ethiopia and elsewhere (Traub and Leonhard, 1997; Abdissa *et al*., 2011). Up to date penicillin resistant group A streptococci is not yet reported (Yang *et al*., 2013; Devi *et al*., 2011; Traub and Leonhard, 1997) but Erythromycin and tetracycline resistant GAS are increasing from time to time, 93.7% were resistant to tetracycline (Yang *et al*., 2013) and 38 % (Syrogiannopoulos *et al*., 2001) and 96.1% (Yang *et al*., 2013) were resistant to erythromycin. In this study, two resistant and one intermediately susceptible *S. pyogenes* to tetracycline and one resistant and one intermediately susceptible to erythromycin were found. There is a report macrolide treatment failure in streptococcal pharyngitis resulting in acute rheumatic fever because of macrolide antibiotics used as second choice for the treatment of bacterial pharyngitis (Logan *et al*., 2012).

Penicillin resistant group B streptococcus and susceptible group G and F streptococci were reported in the present study, which is in contrast with studies conducted elsewhere (Devi *et al*., 2011; Traub and Leonhard, 1997).

Among 8 strains of *S. pyogenes* and *S.dysgalactaie, emm* 68.2 and *emm* 77.0 subtypes were resistant to multiple drugs. Especially *emm* 68.2 suptype was resistant to 7 drugs and intermediately susceptible to 3 drugs out of 12 antibiotics tested, even though some studies reported no association between multi drug resistance and GAS *emm* types (Dhanda *et al*., 2013).

Penicillin and Oxacillin susceptible results showed discordant in this study. Many of the strains were penicillin susceptible, but resistant to oxacillin. Jetté and Sinave (1999) reported that absence of zone of inhibition around the 1-µg oxacillin disk be regarded as an indicator of no susceptibility to penicillin and ceftriaxone, pending the results of minimum inhibitory concentration (MIC) quantitation method otherwise MIC has to be done for penicillin to report as resistance to penicillin when zone size of Oxacillin 1µg is $\leq$19 (Clinical and Laboratory Standards Institute, 2012). The document says when zone size of oxacillin 1µg is $\leq$19 the microorganism could be susceptible, intermediate or no susceptible to penicillin.

Anti-streptolysin O titer is one of the antibodies which confirm a recent streptococcal suppurative infection and/or non-suppurative streptococcal complications (Hahn *et al*., 2005). In the current study, 29.49 % of the participants were ASO positive out of which 66.67 % showed recent streptococcal infections. There was a statistically significant association between the throat carrier rate of *beta*-hemolytic streptococci and positive for ASO (P=0. 05).

The oropharynx and the nasopharynx are frequently colonized by both commensals and pathogenic bacteria (Chi *et al*., 2003). The microbiota diversity could be affected by infection (Allen *et al*., 2014), season (Bogaert *et al*., 2011), and vaccine or antibiotic pressure (Biesbroek *et al*., 2014; Pettigrew *et al*., 2012). These commensals could be potential pathogens and can cause endocarditis, meningitis and septicemia when entered to sterile body sites and aggravate the existing medical problem. This study also aimed to get a better understanding of the composition and dynamics of the oropharyngeal gram negative, oxidase positive microbiome during on-going monthly penicillin G challenge on children having rheumatic heart disease.

Rheumatic heart disease increases the risk of bacterial attachments to the heart valves and causes endocarditis (Mitchell *et al*., 2007). Rheumatic heart disease is responsible for 63% of all cases of infective endocarditis in developing countries (WHO, 2005). In the present study, gram negative

commensals were isolated and composed of *Nessieria*, *Moraxella* and *Kingella* and other species. The most common illness caused by *Neisseria elongate subsp. nitroreducens* is endocarditis, for which one of the risk factor is rheumatic heart disease (Wong and Janda, 1992). Kingella species have also been recovered from endocarditis patients more frequently (WHO, 2004) which was the second highest gram negative commensal isolated in the present study.

The isolation rate of gram negative oropharyngeal commensals was 55.1% in the present study. Throat carriage rate of *N. meningitidis* found 4.27% (10/234) in the current study, which is lower than the MenAfriCar survey (10%) in which about 1% was serogroup A outside epidemic periods (MenAfriCar Consortium, 2013). Two non-groupable *N. meningitidis* were found in the current study. A review study reported that the capsule deficient state of meningococcal strains in the pharynx may aid evasion of the human immune defense and hence be selected to survive nasopharyngeal colonization (Yazdankhah and Caugant, 2004).

Throat carriage rate of *N. lactamica* were found 4.27% in this study, *Neisseria lactamica*, a harmless human commensal found predominantly in the upper respiratory tracts of infants, is closely related to *Neisseria meningitidis*, a pathogen of global significance. Colonization with *N. lactamica* may be responsible for the increase in immunity to meningococcal disease that occurs during childhood (Bennett *et al*., 2005).

The carrier rate of *M. catarrhalis* was 19.66 % in the current study, which is again less than healthy carriers without antibiotic pressure (38.5%) (Sehgal and al Shaimy, 1994). *M. catarrhalis* isolated from patients with antibiotic challenge was higher (85.7%) in producing *beta*-lactamase than healthy subjects (41.9%) (Sehgal and al Shaimy, 1994). In general, when we compare the present finding with other literatures, the throat carriage rate of gram negative commensals is lower than healthy persons without antibiotic pressure.

The monthly benzathine penicillin G secondary prophylaxis seems not 100% effective against *beta*-hemolytic streptococci throat carriage. ASO titration indicates 19.66% of participants had recent group A, C, or, G streptococcal infection and culture of throat swab reveals 23.93% *beta*- hemolytic streptococcal throat carriage. Recurrence of rheumatic fever and reduced ability of penicillin to eradicate *beta*-hemolytic streptococci are reported continuously from parts of the world, indicating

failure to benzathine penicillin G prophylaxis (Seckeler *et al*., 2010; Kaplan *et al.,* 2006; Kaplan and Johnson, 2001).

Why the prevalent *beta* hemolytic streptococci carriage is not explained by penicillin resistance in the present study. It could be explained by other factors. In this study, commensal *alpha*-hemolytic streptococci found resistance to penicillin (33.3%) and if the mechanism for resistance is through producing *beta*-lactamase, it can disrupt penicillin G before it actually clears the pathogenic *beta*-hemolytic streptococci. In addition brands of penicillin prescribed may raise doubts of effectiveness as explained by a study (Hannan *et al*., 2010). Biofilm formation and ability of *S. pyogenes* to enter into epithelial cells may not be undermined mechanisms to escape from penicillin and erythromycin, though they are susceptible (Baldassarri *et al*., 2006).

The other reasons could be the timing of antibiotic prophylaxis injection and compliance. Children who received antibiotic prophylaxis within two weeks showed significantly lower *beta*- hemolytic streptococcal throat carriage than 4 weeks of injection (p=0. 004). Studies after 1990 showed decrease serum penicillin G below minimum-protective concentration for three weeks and four weeks with known and unknown reasons. Some specific populations showed faster penicillin G degradation in the serum (Broderick *et al*., 2012; Kaplan and Johnson, 2001). This is aggravated when the schedule of injection is three weeks or four week interval (Manyemba *et al*., 2002; Kassem *et al*., 1996; Lue *et al*., 1996) and there is no agreement on the best effective ways of giving penicillin (Manyemba and Mayosi, 2002).

Regarding compliance the children were not taking the injections as prescribed by the physician. To start with, only 26.6% (62) participants were taking the injections at exactly at 28 days. The rest approximates and taking the injections every 30 days (data not shown). Missing of injections in which most of the reasons were personal showed significantly higher *beta*-hemolytic throat carriage rate (p=0.0003). And 8.63 % (17) were taking 5 or less injections for a six month survey of the number of injections.

Other studies reported that implementation of secondary antibiotic prevention was challenged by impaired awareness of RHD; poor quality of service delivery and inadequate cooperation with family physicians (Shrestha *et al*., 2012; Petricca *et al*., 2009; Günther *et al*., 2006). In addition, low rate of

regular prophylaxis and drug discontinuation have been observed as contributing problems in Ethiopia (Günther *et al*., 2006; Oli and Porteous, 1999 b; Melka, 1996).

Other associated factors assesed here do not show any significant association to have beta hemolytic streptococci.

Conclusions

Relatively high prevalence of *beta*-hemolytic streptococci was detected among rheumatic heart disease patients who were under secondary antibiotic prophylaxis. Eight important strains (4 *S. pyogenes* and 4 *S. dysgalactaie subsp. equisimilis*) were detected in this study. Among 6 group A streptococci, the 2 were *S. dysgalcataie subsp. equisimilis* strain, indicating all group A streptococci are not belonging to *S. pyogenes*. *emm* typing of these 2 strains also showed they are new and not found in the database before and they are also bacitracin resistant strains. In *emm* 68.2 strain N-acetylhexoseamine was found in the streptococcal glycosyltransferase enzyme which was not found previously, indicating it is not only M protein responsible for rheumatic fever disease. This strain was either intermediate or resistant to all drugs except penicillin and vancomycin. All *beta*-hemolytic streptococci except *S. agalactaie* were susceptible to penicillin. The throat carriage rate of gram negative commensals was also observed and it seems affected by on-going penicillin G prophylaxis. In general characterization of carrier strains in rheumatic heart disease is likely to elucidate the significance and mechanisms for carriage and drug resistance during on-going penicillin G monthly prophylaxis.

Recommendations

Based on the present study findings, the following recommendations are made: -

- The current schedule benzathine penicillin prophylaxis injection should be revised (changing schedule of benzathine penicillin G prophylaxis from four to two weeks)
- Penicillin bioavailability in the blood after an injection in Ethiopian children has to be determined.
- Continuous health education to caretakers and children regarding the importance of taking the benzathine penicillin prophylaxis on time as prescribed is recommended.
- Treatment intervention has to be established to provide services for those who need repair or replacement of their damaged valves.
- Primary prevention has to be given attention to prevent the first attack of rheumatic fever.
- More study is needed for *S. dysgalactaie subsp. equisimilis* as a causative agent for rheumatic fever and to find out the best mechanism of pathogenesis.
- Penicillin failure to clear pathogenic streptococci needs further investigations.

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Annex I: Information Sheet English and Amharic Version

Information Sheet for Parents/Guardians

Principal investigator: Nigus Zegeye

Organization: DMIP School of Medicine Addis Ababa University and Armauer Hansen Research Institute

Sponsors: Armauer Hansen Research Institute and AAU-post graduate studies

We would like to conduct a medical research entitled "Throat carriage rate and antibiotic susceptibility pattern of group A streptococci (GAS) in children with rheumatic fever on secondary antibiotic prophylaxis in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia"

We are requesting you to allow your child to voluntarily participate in this study. We are going to inform you about the purpose, responsibility of investigators or data collectors to keep confidentiality and how we are going to use the data.

Description and purpose of the study

Rheumatic fever is a multi-system response following infection by Group A Streptococci. Rheumatic heart disease is the most serious complication of rheumatic fever. RHD is the number one cardiac problem in Ethiopian children. Group A Streptococci profile among children on secondary Rheumatic Fever prophylaxis follow up has not been studied well in Ethiopia. This study is important in the care of the children to prevent aggravation of RHD.

Procedures

If you agree your child to participate in this study and you sign the consent form the following will be done:

- ✓ Your child's medical history will be reviewed
- ✓ You will have interview with us for some time about risk factors of the disease
- ✓ We will take throat swab from your child. We will take blood sample (2mL or half of one teaspoonful) of blood.

- ✓ If Group A streptococcus is found from the swabs , the bacteria will be stored for further molecular typing abroad in the future

Risks and discomforts

During all sample collections we will follow standard operational procedures. Your child may experience some pain during the collection of blood; nausea during throat swab collection. Appropriate medical care will be provided to your child.

Benefits

This study may not benefit your child directly. But if it is found that your child has group A streptococci, it will be communicated with your child's physician. The findings from this study will also be useful for the care of children with this condition.

Compensation

Your child will not be offered payment for being in this study.

Costs

We expect that there will be no extra cost to your child because of participation in the research but if there is any, this will be compensated for by the project so that your child does not have to pay for the research.

Confidentiality

We respect your child's privacy and confidentiality. Your child's study records will be identified by a subject identification number and not by your child's name. Any information that identifies your child will not be shared with anyone else. If a research article or publication comes from this study, anything that identifies your child should not go for publication. The information we collect from your child as part of the study will be kept in a locked file cabinet in a locked building. We will be entering some of your child's information into a computer file. This will be protected by a password only accessible to personnel involved in the study.

Voluntary Participation and Withdrawal from the Study

Your child's participation is completely voluntary and you have the right to refuse your child's participation in this study. You can stop your child to participate in the study at any time after giving your consent. If you withdraw your child from the study, the samples will be withdrawn from analysis. This decision will not affect in any way your child's current or future medical care or any other benefits to which your child is otherwise entitled. The study doctor/investigator may stop your child from taking part in this study at any time if they decide it is in your child's best interest.

If you have any questions about this study you can contact the following investigators and the ethics committee for further information.

Nigus Zegeye, Phone: 0912907050,E-mail: kingster2002@gmail.com, Dr. Etsegenet Gedlu, Phone: 0911608705,E-mail: etsegedlu@gmail.com , Dr. Daniel Asrat, Phone:0911223019 E-mail: asratdan@gmail.com; Dr.Yimtubezinash Woldeamanuel Phone:0911225832 E-mail: yimtuwa@gmail.com; Dr. Abebe Habte, Phone: 0913677377, E-mail: abebehabte@gmail.com; Dr. Abraham Aseffa, Phone:0911247525, E-mail: aseffa@gmail.com.

Information Sheet for Parents/Gurdians Amharic Version

ቅጥያ III የወላጆች/የአሳዳጊዎች የመረጃ ቅጽ

የዋና ተመራማሪ ስም፤ ንጉስ ዘገየ

የድርጅቱ ስም፤ በአዲስ አበባ ዩኒቨርሲቲ የህክምና ትምህርት ቤት ማይክሮባዮሎጂ፣ኢሚኖሎጂ እና ፓራሲቶሎጂ ትምህርት ክፍል እና አርማውር ሃንሰን የምርምር ተቋም

ድጋፍ አድራጊ (ስፖንሰር) ፤ አርማውር ሃንሰን የምርምር ተቋም እና አዲስ አበባ የኒቨርሲቲ የድህረ ምረቃ ትምህርት ቤት

ከላይኛው የመተንፈሻ አካላት ከባክቴሪያ ጋር ተያይዞ ሊከሰት የሚችል ህመም ባለባቸው የሁለተኛ መከላከያ መድሃኒት በጀመሩ ልጆች ላይ ባክቴሪያው መኖር ወይም አለመኖሩን እና በመድሃኒት መጠቃቱን መፈተሽ በሚለው ጥናት ውስጥ ተሳታፊ ለሚሆኑ ህመምተኛ ልጆች ወላጆቻቸው/አሳዳጊዎቻቸው የተዘጋጀ የማብራሪያ ጽሁፍ፡፡

ከላይ በተጠቀሰው ርዕስ ላይ በጥቁር አንበሳ ስፔሻላይዝድ ሆስፒታል ጥናት ለማካሄድ አስበናል፡፡ በከፍተኛ አክብሮት እና ትህትና ልጅዎ/የሚያሳድጉት/ጓት ልጅ በዚህ ጥናት ውስጥ እንዲሳተፍ/እንድትሳተፍ ይጠየቃሉ፡፡ ይህ ፎርም በዚህ ጥናት ውስጥ ልጅዎ/የሚያሳድጉት/ጓት ልጅ እንዲሳተፍ/እንድትሳተፍ ወይም እንዳይሳተፍ/እንዳትሳተፍ ከመዎሰንዎ በፊት ሊያውቋቸው የሚገቡ ጠቃሚ መረጃዎችን በሙሉ አካቶ የያዘ በመሆኑ በጥሞና እንድያነቡ እና እንዲመረምሩ ወይም ሲነበብለዎት በጥሞና እንዲያዳምጡ እንምከራለን፡፡

ስለ ጥናቱ በጥቂቱ

ከላይኛው የመተንፈሻ አካላት ጋር ተያይዞ ሊከሰት የሚችል ህመም በባክቴሪያ የሚመጣ በሽታ ነው፡፡ ይህ በሽታ እየተደጋገመ ሲመጣ የልብ ጡንቻዎችን በመጉዳት የልብ ህመም ያስከትላል፡፡ ይህም የሚሆነው የዚህ ባክቴሪያ እንግዳ አካል ከልብ ጡንቻዎች ጋር ስለሚመሳሰል ለባክቴሪያው የተመረተው የሰውነታችን መከላከያ የራሳችንን የልብ ጡንቻዎች ስለሚጎዳ ነው፡፡ የልብ ህመም ያለባቸው ሰዎች ለሁለተኛ ጊዜ ይህ ባክቴሪያ ከያዛቸው በሽታውን በማባባስ ልጆችን ለከፍተኛ ጉዳት እና ለህልፈተ ህይወት ይዳርጋቸዋል፡፡ በዚህም ምክንያት የልብ ህመም ያለባቸው ሰዎች ባክቴሪያው ሁለተኛ እንዳይዛቸው በእድሜያቸው ዘመን ሙሉ የመከላከያ መድሃኒት እንዲወስዱ ይደረጋል፡፡ የዚህ ጥናት አላማም የሁለተኛ የመከላከያ መድሃኒት በሚወስዱ የልብ ህመምተኛ ልጆች ላይ የዚህ ረቂቅ ተህዎሲያን መኖር ወይም አለመኖሩን ለመፈተሽ ነው፡ በዚህ ጥናት ውስጥ ይህ ረቂቅ ተህዋሲያን ከተገኘ የማስተካከያ እርምጃዎች እንዲዎሰዱ ይደረጋል፡፡

የጥናቱ ሂደት ዝርዝር

በዚህ ጥናት ውስጥ ልጅዎ/የሚያሳድጉት/ጓት ልጅ እንዲሳተፍ/እንድትሳተፍ ከተስማሙ የሚከተሉትን ነገሮች እናደርጋለን፡፡

1. ከልጅዎ/ከሚያሳድጉት/ጓት የህክምና መዝገብ እንዲሁም ከራስዎ አንደበት በቃለ መጠይቅ የልጅዎን/የሚያሳድጉትን/ጓትን ልጅ የህክምና መረጃ ይሰበሰባል አካላዊ ምርመራም ይደረጋል፡፡
2. ግማሽ የሻይ ማንኪያ (2 ሚሊ ሊትር) ያህል የልጅዎ/የሚያሳድጉት/ጓት ደም ስር ላይ እጅግ በጣም አነስተኛ በሆነ ንጽህናው በተረጋገጠ ሲሪንጅ ና መርፌ የደም ናሙና ይወሰዳል፡፡
3. ከ ልጅዎ/ከሚያሳድጉት/ጓት ጉሮሮ እንጥል አካበቢ ጠረግ ጠረግ ተደርጎ ናሙና ይወሰዳል፡፡
4. ልጅዎ/የሚያሳድጉት/ጓት ልጅ የቆዳ ቁስል ከተገኘበት/ባት ቁስሉ አካባቢ ጠረግ ጠረግ ተደርጎ ናሙና ይወሰዳል፡፡
5. ከልጅዎ/ከሚያሳድጉት/ጓት ልጅ የተወሰደው ናሙና ላይ ባክቴሪያው ከተገኘ ይህ ረቂቅ ተህዋሲያን ከተጠበቀ በኋላ ወደ ውጭ አገር ተልኮ ተጨማሪ ምርምር ይደረግበታል፡፡

ስጋትና ጉዳት

በአጠቃላይ ከላይ የተጠቀሱት ናሙናዎች ሲወሰዱ ህክምናው የሚያስገድደውን የአሰራር ሂደት ስለምንከተል ሊያገጥሙ የሚችሉ ጉዳቶች በጣም አነስተኛ ናቸው፡፡ ቢሆንም እጅግ በጣም አነስተኛ በሆኑ አጋጣሚዎች በክንድ የደም ስር በመቅዳት እና በቆዳ ቁስል ላይ ናሙና ሲወሰድ ሊያጋጥሙ የሚችሉ ችግሮች ልክ እንደ ሕመም፣ መቅላት፣ መቆጥቆጥ ወይም የመሳሰሉ ችግሮች ይኖራሉ፡፡ጉሮሮ አካባቢ ናሙና ሲዎሰድም ቶሎ የሚጠፋ የማቅለሽለሽ ስሜት ሊኖር ይችላል፡፡ እነዚህ ያልተለመዱ ችግሮች ልጅዎን/የሚያሳድጉትን/ጓትን ልጅ ካጋጠመው/ማት ተገቢ የሆነ የህክምና እርዳታ እንዲያገኝ/እንድታገኝ ይደረጋል፡፡

ሊያገኟቸው የሚችሉ ጥቅሞች

ልጃችሁ/የሚያሳድጉት/ጓት ልጅ ከሚገኙት መረጃዎች በቀጥታ ተጠቃሚ ባይሆንም/ባትሆንም ረቂቅ ተህዋሲያኑ ከተገኘበት/ባት ከልጁ/ቷ ሃኪም ጋር የመረጃ ልውውጥ እናደርጋለን፡፡ በተጨማሪም የዚህ ጥናት ውጤት ለሌሎች ተመሳሳይ በሽታ ላለባቸው ልጆች ጥንቃቄ ለማድረግ ይጠቅማል፡፡

የካሳ ክፍያ

ልጅዎ/የሚያሳድጉት/ጓት ልጅ በዚህ ጥናት ውስጥ በመሳተፉ/ፏ ከላይ ከተጠቀሰው ጥቅም በስተቀር በጥሬ ገንዘብ የሚደረግ የካሳ ክፍያ አይኖርም፡፡ ይህንን ጥናት በገንዘብ የሚደጉሙት ድርጅቶች ከዋና በሽታው ጋር የተያያዙ የህክምና ችግሮች ክፍያ ወይንም የተለየ የህክምና እርዳታ አያደርጉም፡፡

የሚየወጡት ትርፍ ወጪ

ልጅዎ/የሚያሳድጉት/ጓት ልጅ በዚህ ጥናት ውስጥ በመሳተፉ/ፏ ለጥናቱ ተብሎ የሚየወጡት ትርፍ ወጪ ይኖራል ብለን አናስብም፡፡ቢሆንም ለጥናቱ ተብሎ የሚያዎጡት ትርፍ ወጪ ካጋጠመ በፕሮጀግቱ የሚሸፈን ይሆናል፡፡

የጥናቱ ሚስጥራዊነት

ይህ ጥናት የህክምና እና የሳይንሳዊ ጥናቶች ስነምግባርን በመከተሉ ሰብአዊ እንዲሁም የልጅዎን/የሚያሳድጉትን/ጓትን ልጅ ግለሰባዊ መብትን በማክበር የተነሳ ሁሉንም እሱን/ሷን የሚመለከት የጤና መረጃዎች በሚስጥር እንጠብቃለን፡፡ የልጅዎን/የሚያሳድጉትን/ጓትን ልጅ ማንነት በሚያጋልጥ መልኩ የተዘጋጀን መረጃ ይፋ አናደርግም፡፡የጥናቱ መረጃዎች በሙሉ የሚቀመጡት

ከልጁ/ጅቷ ስም ጋር ሳይሆን ለጥናቱ ተብሎ በሚሰጣቸው ስውር ቁጥር ሲሆን ጥናቱን ከሚያካሂዱት ባለሞያዎች በስተቀር ማንም ሊያውቅ አይችልም፡፡ ይህ ጥናት ሳይንሳዊ መረጃ እንደመሆኑ መጠን በወረቀት ታትሞ ቢዎጣ ወይንም በሚዲያ ቢነገር የልጅዎ/የሚያሳድጉት/ጓት ልጅ ስም በምንም መልኩ አይጠቀስም፡፡ ስለ ልጅዎ/የሚያሳድጉት/ጓት ልጅ የምንሰበስበው ማንኛውንም መረጃ በተቆለፈ ቢሮ እና በተቆለፈ ቁምሳጥን ውስጥ ይቀመጣል፡፡ከተሰበሰቡት መረጃዎች የተወሰኑትን ወደ ኮምፒውተር በማስገባት የምንጠቀምባቸው ሲሆን መረጃው የተቀመጠበት መዝገብ በስውር ኮድ ይታሰራል፡፡ይህን ስውር ኮድ ከአጥኚዎች በስተቀር ማንም እንዳያውቅ ይደረጋል፡፡

ያለመቀበል ወይንም ጥሎ የመውጣት መብት

ልጅዎ/የሚያሳድጉት/ጓት ልጅ በዚህ ጥናት ውስጥ የሚኖረው/ራት ተሳትፎ ሙሉ በሙሉ በፈቃደኝነት የተመሰረተ ይሆናል፡፡ልጅዎ/የሚያሳድጉት/ጓት ልጅ በዚህ ጥናት ውስጥ የመሳተፍ መብቱ/ቷ ሙሉ በሙሉ የተጠበቀ ነው፡፡ ልጅዎ/የሚያሳድጉት/ጓት ልጅ በዚህ ጥናት እንዲሳተፍ/እንዲትሳተፍ ፈቃድ ቢሰጡም በማንኛውም ጊዜ ከጥናቱ የማስዎጣት አማራጭ መውሰድ ይችላሉ፡፡ልጅዎን/የሚያሳድጉትን/ጓትን ልጅ ከጥናቱ የሚያሶጡት/የሚያሶጧት ከሆነ የተዎሰዱት ናሙናዎች ምርመር አይደረግባቸውም፡፡ በጥናቱ ባለመሳተፉ/ፏ ወይም ከጥናቱ በመገለሉ/ሏ ምክንያት በአሁኑ ወይም የወደፊት የህክምና እርዳታ ለይ ተጽዕኖ አይፈጥርም፡፡ ከዚህ በፊት ሲያገኘው/ስታገኘው ከነበሩት ጥቅሞች አንዷ እንኳ አይጎድልበትም/ባትም፡፡ ጥናቱን የሚያከናውነው አካል ወይም ድጋፍ አድራጊ አካል ለልጁ/ቷ ጥቅም ሲባል በጥናቱ እንዳይሳተፍ/እንዳትሳተፍ ሊከለከል/ልትከለከል ይችላል/ትችላለች፡፡

ጥያቄ አለዎት?

- ✓ ስለዚህ ጥናት ወይም እርስዎ በዚህ ጥናት ውስጥ ስለሚኖርዎ ድርሻ
- ✓ በዚህ ጥናት አማካይነት በልጅዎ/በሚያሳድጉት/ጓት ልጅ ላይ ደረሰበት/ባት ስለሚሉት ጉዳት
- ✓ ስለ ጥናቱ ማንኛውንም ጥያቄ፣ አሳሳቢ ጉዳት፣ ወይንም ቅሬታ ካለዎት የሚከተሉትን ስልኮች በመጠቀም የጥናቱን ባለቤቶች ማነጋገር ይችላሉ፡፡

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Information Sheet for Children between 12-15 Years

Principal investigator: Nigus Zegeye

Organization: DMIP School of Medicine Addis Ababa University and Armauer Hansen Research Institute

Sponsors: Armauer Hansen Research Institute and AAU-post graduate studies

We would like to conduct a medical research entitled "Throat carriage rate and antibiotic susceptibility pattern of group A streptococci (GAS) in children with rheumatic fever on secondary antibiotic prophylaxis in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia"

We are requesting you to voluntarily participate in this study. We are going to inform you about the purpose, responsibility of investigators or data collectors to keep confidentiality and how we are going to use the data.

Description and purpose of the study

Rheumatic fever is a multi-system response following infection by Group A Streptococci. Rheumatic heart disease is the most serious complication of rheumatic fever. RHD is the number one cardiac problem in Ethiopian children. Group A Streptococci profile among children on secondary Rheumatic Fever prophylaxis follow up has not been studied well in Ethiopia. This study is important in the care of the children to prevent aggravation of RHD.

Procedures

If you agree to participate in this study the following will be done:

- ✓ Your medical history will be reviewed
- ✓ We will take throat swab from your throat.
- ✓ We will take blood sample (2mL or half of one teaspoonful) of blood.
- ✓ If Group A streptococcus is found from the swabs , the bacteria will be stored for further molecular typing abroad in the future

Risks and discomforts

During all sample collections we will follow standard operational procedures. You may experience some pain during the collection of blood; nausea during throat swab collection. Appropriate medical care will be provided to you if these symptoms occur.

Benefits

This study may not benefit you directly. But if it is found that you have group A streptococci, it will be communicated with your physician. The findings from this study will also be useful for the care of children with this condition.

Compensation

You will not be offered payment for being in this study.

Costs

We expect that there will be no extra cost to you because of participation in the research but if there is any, this will be compensated for by the project so that you do not have to pay for the research.

Confidentiality

We respect your privacy and confidentiality. Your study records will be identified by a subject identification number and not by your name. Any information that identifies you will not be shared with anyone else. If a research article or publication comes from this study, anything that identifies you should not go for publication. The information we collect from you as part of the study will be kept in a locked file cabinet in a locked building. We will be entering some of your information into a computer file. This will be protected by a password only accessible to personnel involved in the study.

Voluntary Participation and Withdrawal from the Study

Your participation is completely voluntary and you have the right to refuse your participation in this study. You can stop to participate in the study at any time after giving your assent. If you withdraw from the study, the samples will be withdrawn from analysis. This decision will not affect in any way your current or future medical care or any other benefits to which you otherwise entitled. The study doctor/investigator may stop you from taking part in this study at any time if they decide it is in your best interest.

If you have any questions about this study you can contact the following investigators and the ethics committee for further information.

Nigus Zegeye, Phone: 0912907050,E-mail: kingster2002@gmail.com, Dr. Etsegenet Gedlu, Phone: 0911608705,E-mail: etsegedlu@gmail.com , Dr. Daniel Asrat, Phone:0911223019 E-mail: asratdan@gmail.com; Dr.Yimtubezinash Woldeamanuel Phone:0911225832 E-mail: yimtuwa@gmail.com; Dr. Abebe Habte, Phone: 0913677377, E-mail: abebehabte@gmail.com; Dr. Abraham Aseffa, Phone:0911247525, E-mail: aseffa@gmail.com.

Information Sheet for Children between 12-15 Years Amharic Version

ቅጥያ III የልጆች የመረጃ ቅጽ

የዋና ተመራማሪ ስም፤ ንጉስ ዘገየ

የድርጅቱ ስም፤ በአዲስ አበባ ዩኒቨርሲቲ የህክምና ትምህርት ቤት ማይክሮባዮሎጂ፣ኢሚዮሎጂ እና ፓራሲቶሎጂ ትምህርት ክፍል እና አርማውር ሃንሰን የምርምር ተቋም

ድጋፍ አድራጊ (ስፖንሰር) ፤ አርማውር ሃንሰን የምርምር ተቋም እና አዲስ አበባ ዩኒቨርሲቲ የድህረ ምረቃ ትምህርት ቤት

ከላይኛው የመተንፈሻ አካላት ከባክቴሪያ ጋር ተያይዞ ሊከሰት የሚችል ህመም ባለባቸው የሁለተኛ መከላከያ መድሃኒት በጀመሩ ልጆች ላይ ባክቴሪያው መኖር ወይም አለመኖሩን እና በመድሃኒት መጠቃቱን መፈተሽ በሚለው ጥናት ውስጥ ተሳታፊ ለሚሆኑ ህመምተኛ ልጆች የተዘጋጀ የማብራሪያ ጽሁፍ፡፡

ከላይ በተጠቀሰው ርዕስ ላይ በጥቁር አንበሳ ስፔሻላይዝድ ሆስፒታል ጥናት ለማካሄድ አስበናል፡፡ በከፍተኛ አክብሮት እና ትህትና አንተ/አንቺ በዚህ ጥናት ውስጥ እንድትሳተፍ/እንድትሳተፊ ትጠየቃያለህ/ትጠየቂያለሽ፡፡ ይህ ፎርም በዚህ ጥናት ውስጥ ለመሳተፍ ከመዎሰንህ/ሽ በፊት ልታውቀው/ቂው የሚገቡ ጠቃሚ መረጃዎችን በሙሉ አካቶ የያዘ በመሆኑ በጥሞና እንድታነብ/ነቢ እና እንዲትመረምር/ሪ ወይም ሲነበብልህ/ሽ በጥሞና እንድታዳምጥ/እንድታዳምጪ እንምክራለን፡፡

ስለ ጥናቱ በጥቂቱ

ከላይኛው የመተንፈሻ አካላት ጋር ተያይዞ ሊከሰት የሚችል ህመም በባክቴሪያ የሚመጣ በሽታ ነው፡፡ ይህ በሽታ እየተደጋገመ ሲመጣ የልብ ጡንቻዎችን በመጉዳት የልብ ህመም ያስከትላል፡፡ ይህም የሚሆነው የዚህ ባክቴሪያ እንግዳ አካል ከልብ ጡንቻዎች ጋር ስለሚመሳሰል ለባክቴሪያው የተመረተው የሰውነታችን መከላከያ የራሳችንን የልብ ጡንቻዎች ስለሚጎዳ ነው፡፡ የልብ ህመም ያለባቸው ሰዎች ለሁለተኛ ጊዜ ይህ ባክቴሪያ ከያዛቸው በሽታውን በማባባስ ልጆችን ለከፍተኛ ጉዳት እና ለህልፈተ ህይወት ይዳርጋቸዋል፡፡ በዚህም ምክንያት የልብ ህመም ያለባቸው ሰዎች ባክቴሪያው ሁለተኛ እንዳይዛቸው በእድሜያቸው ዘመን ሙሉ የመከላከያ መድሃኒት እንዲወስዱ ይደረጋል፡፡ የዚህ ጥናት አላማም የሁለተኛ የመከላከያ መድሃኒት በሚወስዱ የልብ ህመምተኛ ልጆች ላይ የዚህ ረቂቅ ተህዎሲያን መኖር ወይም አለመኖሩን ለመፈተሽ ነው፡ በዚህ ጥናት ውስጥ ይህ ረቂቅ ተህዋሲያን ከተገኘ የማስተካከያ እርምጃዎች እንዲዎሰዱ ይደረጋል፡፡

የጥናቱ ሂደት ዝርዝር

በዚህ ጥናት ውስጥ ለመሳተፍ ከተስማማህ/ሽ የሚከተሉትን ነገሮች እናደርጋለን፡፡

1. የህክምና መዝገብህ/ሽ እንዲሁም ከራስህ/ሽ አንደበት በአጭር ቃለ መጠይቅ የህክምና መረጃ ይሰበሰባል አካላዊ ምርመራም ይደረጋል፡፡

2. ግማሽ የሻይ ማንኪያ (2 ሚሊ ሊትር) ያህል የደም ስርህ/ሽ ላይ እጅግ በጣም አነስተኛ በሆነ ንጽህናው በተረጋገጠ ሲሪንጅ ና መርፌ የደም ናሙና ይወሰዳል፡፡
3. ጉሮሮህ/ሽ እንጥል አካበቢ ጠረግ ጠረግ ተደርጎ ናሙና ይወሰዳል፡፡
4. የቆዳ ቁስል ከተገኘብህ/ሽ ቁስሉ አካባቢ ጠረግ ጠረግ ተደርጎ ናሙና ይወሰዳል፡፡
5. የተወሰደው ናሙና ላይ ባክቴሪያው ከተገኘ ይህ ረቂቅ ተህዋሲያን ከተጠበቀ በኋላ ወደ ውጭ አገር ተልኮ ተጨማሪ ምርምር ይደረግበታል፡፡

ስጋትና ጉዳት

በአጠቃላይ ከላይ የተጠቀሱት ናሙናዎች ሲወሰዱ ህክምናው የሚያስገድደውን የአሰራር ሂደት ስለምንከተል ሊያገጥሙ የሚችሉ ጉዳቶች በጣም አነስተኛ ናቸው፡፡ ቢሆንም እጅግ በጣም አነስተኛ በሆኑ አጋጣሚዎች በክንድ የደም ስር በመቅዳት እና በቆዳ ቁስል ላይ ናሙና ሲወሰድ ሊያጋጥሙ የሚችሉ ቸግሮች ልክ እንደ ሕመም፣ መቅላት፣ መቆጥቆጥ ወይም የመሳሰሉ ችግሮች ይኖራሉ፡፡ጉሮሮ አካባቢ ናሙና ሲዎሰድም ቶሎ የሚጠፋ የማቅለሽለሽ ስሜት ሊኖር ይችላል፡፡ እነዚህ ያልተለመዱ ችግሮች ካጋጠመህ/ሽ ተገቢ የሆነ የህክምና እርዳታ እንድታገኝ/ኚ ይደረጋል፡፡

የምታገኘው/ኚው ጥቅም

ከጥናቱ ከሚገኙት መረጃዎች በቀጥታ ተጠቃሚ ባትሆንም/ባትሆኚም ረቂቅ ተህዋሲያኑ ከተገኘብህ/ሽ ከሃኪምህ/ሽ ጋር የመረጃ ልውውጥ እናደርጋለን፡፡ በተጨማሪም የዚህ ጥናት ውጤት ለሌሎች ተመሳሳይ በሽታ ላለባቸው ልጆች ጥንቃቄ ለማድረግ ይጠቅማል፡፡

የካሳ ክፍያ

አንተ/አንቺ በዚህ ጥናት ውስጥ በመሳተፍህ/ሽ ከላይ ከተጠቀሰው ጥቅም በስተቀር በጥሬ ገንዘብ የሚደረግ የካሳ ክፍያ አይኖርም፡፡ ይህንን ጥናት በገንዘብ የሚደጉሙት ድርጅቶች ከዋና በሽታው ጋር የተያያዙ የህክምና ችግሮች ክፍያ ወይንም የተለየ የህክምና እርዳታ አያደርጉም፡፡

የሚየወጡት ትርፍ ወጪ

አንተ/አንቺ በዚህ ጥናት ውስጥ በመሳተፍህ/ሽ ለጥናቱ ተብሎ የምታወጣው/ጪው ትርፍ ወጪ ይኖራል ብለን አናስብም፡፡ቢሆንም ለጥናቱ ተብሎ የምታወጣው/ጪው ትርፍ ወጪ ካጋጠመ በፕሮጀግቱ የሚሸፈን ይሆናል፡፡

የጥናቱ ሚስጥራዊነት

ይህ ጥናት የህክምና እና የሳይንሳዊ ጥናቶች ስነምግባርን በመከተሉ ሰብአዊ እንዲሁም የአንተን/የአንችን ግለሰባዊ መብትን በማክበር የተነሳ ሁሉንም የሚመለከት የጤና መረጃዎች በሚስጥር እንጠብቃለን፡፡ የአንተን/የአንችን ማንነት በሚያጋልጥ መልኩ የተዘጋጀን መረጃ ይፋ አናደርግም፡፡የጥናቱ መረጃዎች በሙሉ የሚቀመጡት ከአንተ/ከአንቺ ስም ጋር ሳይሆን ለጥናቱ ተብሎ በሚሰጥህ/ሽ ስውር ቁጥር ሲሆን ጥናቱን ከሚያካሂዱት ባለሞያዎች በስተቀር ማንም ሊያውቅ አይችልም፡፡ ይህ ጥናት ሳይንሳዊ መረጃ እንደመሆኑ መጠን በወረቀት ታትሞ ቢዎጣ ወይንም በሚዲያ ቢነገር የአንተ/የአንቺ ስም በምንም መልኩ አይጠቀስም፡፡ ስለ አንተ/አንቺ የምንሰበስበው ማንኛውንም መረጃ በተቆለፈ ቢሮ እና በተቆለፈ ቁምሳጥን ውስጥ ይቀመጣል፡፡ከተሰበሰቡት መረጃዎች የተወሰኑትን ወደ ኮምፒውተር በማስገባት የምንጠቀምባቸው ሲሆን መረጃው የተቀመጠበት መዝገብ በስውር ኮድ ይታሰራል፡፡ይህን ስውር ኮድ ከአጥኚዎች በስተቀር ማንም እንዳያውቅ ይደረጋል፡፡

ያለመቀበል ወይንም ጥሎ የመውጣት መብት

አንተ/አንቺ በዚህ ጥናት ውስጥ የሚኖርህ/ሽ ተሳትፎ ሙሉ በሙሉ በፈቃደኝነት የተመሰረተ ይሆናል፡፡በዚህ ጥናት ውስጥ የመሳተፍ መብትህ/ሽ ሙሉ በሙሉ የተጠበቀ ነው፡፡ በዚህ ጥናት እንዲትሳተፍ/ተፊ ፈቃድ ብትሰጥም/ጪም በማንኛውም ጊዜ ከጥናቱ የመውጣት አማራጭ መውሰድ ትችላለህ/ትችያለሽ፡፡ ጥናቱ ከተጀመረ በኋላ ጥናቱን የምታቋርጥ/ጪ ከሆነ የተዎሰዱት ናሙናዎች ምርመር አይደረግባቸውም፡፡ በጥናቱ ባለመሳተፍህ/ሽ ወይም ከጥናቱ በመገለልህ/ሽ ምክንያት በአሁኑ ወይም የወደፊት የህክምና እርዳታህ/ሽ ለይ ተጽዕኖ አይፈጥርም፡፡ ከዚህ በፊት ስታገኘው/ኘው ከነበሩት ጥቅሞች አንዱ እንኳ አይጎድልብህም/ሽም፡፡ ጥናቱን የሚያከናውነው አካል ወይም ድጋፍ አድራጊ አካል ለአንተ/አንቺ ጥቅም ሲባል በጥናቱ እንዳትሳተፍ/ፊ ልትከለከል/ልትከለከይ ትችላላህ/ተችያለሽ፡፡

ጥያቄ አለህ/ሽ?

- ✓ ስለዚህ ጥናት ወይም አንተ/አንቺ በዚህ ጥናት ውስጥ ስለሚኖርህ/ሽ ድርሻ
- ✓ በዚህ ጥናት አማካይነት ደረሰብኝ ስለምትለው/ይው ጉዳት
- ✓ ስለ ጥናቱ ማንኛውንም ጥያቄ፣ አሳሳቢ ጉዳት፣ ወይንም ቅሬታ ካለህ/ሽ የሚከተሉትን ስልኮች በመጠቀም የጥናቱን ባለቤቶች ማነጋገር ትችላለህ/ትችያለሽ፡፡

ንጉስ ዘገየ፣ ስልክ ቁጥር፤ 0912907050፣ ኢ-ሜይል፤ kingster2002@gmail.com, ዶ/ር ዕፀገነት ገድሉ፣ ስልክ ቁጥር፤ 0911608705፤ ኢ-ሜይል፤ etsegedlu@gmail.com, ዶ/ር ዳኒኤል አስራት፣ ስልክ ቁጥር፣ 0911223019፣ ኢ-ሜይል፤ asratdan@gmail.com, ዶ/ር ይምጡበዝናሽ ወ/አማኑኤል፣ ስልክ ቁጥር፣ 0911225832፣ ኢ-ሜይል፤ yimtuwa@gmail.com, ዶ/ር አበበ ሃብቴ፣ ስልክ ቁጥር፤ 0913677377፣ ኢ-ሜይል፤ abebehabte@gmail.com, ዶ/ር አብርሃም አሰፋ፣ ስልክ ቁጥር፣ 0911247525፣ ኢ-ሜይል፤ aseffa@gmail.com.

Annex II: Consent and Assent form English and Amharic Version

Parental/Guardian Consent for Age bellow 12 Years

Participant Full Name____

- I confirm that I am the parent/guardian of the above mentioned child.
- I understood the purpose of the study entitled "Throat and antibiotic susceptibility pattern of group A streptococci (GAS) in children with rheumatic fever on secondary antibiotic prophylaxis in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia."
- I agree to collect samples from my child.
- I understand that the information will be confidential.
- I understand that if I refuse/stop my child to participate in the study, it will not affect him/her current and future medical services.
- I agree that there is no direct benefit.
- I agree that the samples taken from my son/daughter will be stored for future molecular characterization abroad.

Agree ☐ Do not agree ☐

Therefore I give my consent freely for my child to participate in this study.

Participant parent/Gardians Name________________________Signature _________Date____

Interviewer's Name_______________________ Signature _________Date___________

Witness'

1. Name ________________________________Signature__________Date______________
2. Name_________________________________Signature__________Date______________

Parental/Guardian Consent for Age bellow 12 Years Amharic Version

ከ12 አመት በታች ለሆኑ ልጆች የወላጆች/የአሳዳጊዎች የፈቃደኝነት መግለጫ ቅጽ

የተሳታፊው/ዋ ሙሉ ስም___________________

- ✓ እኔ_________________________________ስሙ/ስሟ ከለይ የተጠቀሰው ልጅ ወላጅ/አሳዳጊ መሆኔን አረጋግጣለሁ፡፡
- ✓ የልብ ህመም፣ የጉሮሮ እና የሌሎች ህመሞች መንስኤ የሆነውን ባክቴሪያ ላይ ሊደረግ ስለታሰበው ጥናት አላማ መረጃ አግኝቻለሁ፡፡
- ✓ ከልጄ/ከማሳድገው/ጋት ጉሮሮ አካባቢ በጥጥ አማካኝነት ናሙና እንዲወሰድ፤ ከክርን አካባቢ የደም ናሙና በመርፌ እንዲወሰድ እና ቆዳው/ዋ አካባቢ ቁስል ከተገኘ በጥጥ አማካኝነት ናሙና እንዲወሰድ ተስማምቻለሁ፡፡
- ✓ ስለ ጥናቱ አላማ እንዲሁም ናሙናዎቹ በሚወሰዱበት ወቅት በልጄ/በማሳድገው/ጋት ልጅ ላይ ምናልባትም መጠነኛ የሆነ የማቅለሽለሽ ስሜትና የህመም ስሜት ሊሰማው/ት እንደሚችልም ተገንዝቤያለሁ፡፡
- ✓ በልጄ/በማሳድገው/ጋት ልጅ ላይ ናሙናዎቹ በሚወሰዱበት ምክንያት የህመም ስሜት ቢሰማው/ት አስቸኳይ የህክምና እርዳታ እንደሚደረግለት/ላት ተገንዝቤያለሁ፡፡
- ✓ በከልጄ/ከማሳድገው/ጋት የሚሰበሰቡት መረጃዎች በጠቅላላ በሚስጥር እንደሚያዙ ተረድቻለሁ፡፡
- ✓ ልጄን/የማሳድገወን/ጋትን ልጅ በተመለከተ የምጠየቀውን መረጃ ያለመስጠት እና በጥናቱ አለመተባበር የምቸል መሆኑ የተገለጸልኝ ሲሆን ይህንንም ማድረጌ በልጄ/በማሳድገው/ጋት የህክምና ምርመራ እና ክትትል ላይ ምንም አይነት እክል የማይፈጠርበት/ባት መሆኑን በሚገባ ተረድቻለሁ፡፡
- ✓ በማንኛውም ወቅት ልጄን/የማሳድገውን/ጋትን ልጅ ከጥናቱ የማግለል መብቴ የተጠበቀ መሆኑን ተረድቻለሁ፡፡
- ✓ ልጄ/የማሳድገው/ጋት ልጅ በጥናቱ በመሳተፉ/ፏ ምክንያት ቀጥተኛ ጥቅም ባለመኖሩ ተስማምቻለሁ፡፡
- ✓ ከልጄ/ከማሳድገው/ጋት ከሚወሰዱት ናሙናዎች ላይ ባክትሪያው ከተገኘ ለወደፊት በመጠበቅ ወደ ውጭ አገር ተልኮ ለአዲስ ግኝትና ለምርምር አላማ ይውል ዘንድ ተስማምቻለሁ፡፡

ተስማምቻለሁ ☐ አልተስማማሁም ☐

ስለዚህ የምርምር ቃሌን የሰጠሁት በአጠቃላይ ሁኔታውን በመረዳት በፍጹም ፈቃደኝነት ነው፡፡

*የወላጅ/አሳዳጊ ስም*_______________________________ ፊርማ____________ቀን_

*የውይይቱን ያካሄደው ሰው ስም*__________________ ፊርማ____________ቀን______

ምስክሮች፤

1. *ስም* _______________________________________ *ፊርማ* ____________*ቀን*_____
2. *ስም* ____________________________________*ፊርማ*________________*ቀን*______

Parental/Guardian Consent for Age between 12-15 Years

Participant Full Name____

- I confirm that I am the parent/guardian of the above mentioned child.
- I understood the purpose of the study entitled "Throat and antibiotic susceptibility pattern of group A streptococci (GAS) in children with rheumatic fever on secondary antibiotic prophylaxis in Tikur Anbessa Specialized Hospital, Addis Ababa, Ethiopia."
- I agree to collect samples from my child.
- I understand that the information will be confidential.
- I understand that my son/daughter can refuse to participate in the study
- I understand that my son/daughter can stop the study at any time.
- I understand that if my son/daughter does not want to take part, it will not affect him/her current and future medical services.
- I understand that if I refuse/stop my child to participate in the study, it will not affect him/her current and future medical services.
- I agree that there is no direct benefit.
- I agree that the samples taken from my son/daughter will be stored for future molecular characterization abroad.

Agree ☐ Do not agree ☐

Therefore I give my consent freely for my child to participate in this study providing that my son/daughter is voluntary to participate.

Participant parent/Gardians Name________________________Signature __________Date____

Intreviewer's Name_______________________ Signature _________Date___________

Witness'

1. Name ________________________________Signature__________Date______________
2. Name_________________________________Signature___________Date______________

Parental/Guardian Consent for Age between 12-15 Years Amharic Version
ከ12-15 አመት ላሉ ልጆች የወላጆች የፈቃደኝነት መግለጫ ቅጽ

የተሳታፊው/ዋ ሙሉ ስም______________________

- ✓ እኔ_______________________________________ስሙ/ስሟ ከላይ የተጠቀሰው ልጅ ወላጅ/አሳዳጊ መሆኔን አረጋግጣለሁ፡፡
- ✓ የልብ ህመም፣ የጉሮሮ እና የሌሎች ህመሞች መንስኤ የሆነውን ባክቴሪያ ላይ ሊደረግ ስለታሰበው ጥናት አላማ ተረድቻለሁ፡፡
- ✓ ከልጄ/ከማሳድገው/ጋት ጉሮሮ አካባቢ በጥጥ አማካኝነት ናሙና እንዲወሰድ፤ ከክርን አካባቢ የደም ናሙና በመርፌ እንዲወሰድ እና ቆዳው/ዋ አካባቢ ቁስል ከተገኘ በጥጥ አማካኝነት ናሙና እንዲወሰድ ተስማምቻለሁ፡፡
- ✓ በከልጄ/ከማሳድገው/ጋት የሚሰበሰቡት መረጃዎች በጠቅላላ በሚስጥር እንደሚያዙ ተረድቻለሁ፡፡
- ✓ ልጄ/የማሳድገው/ጋት ልጅ በጥናቱ ያለመሳተፍ ወይም ጥሎ የመውጣት መብት እንዳላት ተረድቻለሁ፡፡
- ✓ ልጄ ከጥናቱ ባለሳተፉ/ፏ ምንም አይነት የህክምና ጉድለት እንደማይደርስበት ተረድቻለሁ፡፡
- ✓ ልጄ/የማሳድገው/ጋት ልጅ በጥናቱ በመሳተፉ/ፏ ምክንያት ቀጥተኛ ጥቅም ባለመኖሩ ተስማምቻለሁ፡፡
- ✓ ስለ ጥናቱ አላማ እንዲሁም ናሙናዎቹ በሚወሰዱበት ወቅት በልጄ/በማሳድገው/ጋት ልጅ ላይ ምናልባትም መጠነኛ የሆነ የማቅለሽለሽ ስሜትና የህመም ስሜት ሊሰማው/ት እንደሚችልም ተገንዝቤያለሁ፡፡
- ✓ በልጄ/በማሳድገው/ጋት ልጅ ላይ ናሙናዎቹ በሚወሰዱበት ምክንያት የህመም ስሜት ቢሰማው/ት አስቸኳይ የህክምና እርዳታ እንደሚደረግለት/ላት ተገንዝቤያለሁ፡፡
- ✓ ልጄን/የማሳድገወን/ጋትን ልጅ በተመለከተ የምጠየቀውን መረጃ ያለመስጠት እና በጥናቱ አለመተባበር የምቸል መሆኑ የተገለጸልኝ ሲሆን ይህንንም ማድረጌ በልጄ/በማሳድገው/ጋት የህክምና ምርመራ እና ክትትል ላይ ምንም አይነት እክል የማይፈጠርበት/ባት መሆኑን በሚገባ ተረድቻለሁ፡፡
- ✓ በማንኛውም ወቅት ልጄን/የማሳድገውን/ጋትን ልጅ ከጥናቱ የማግለል መብቴ የተጠበቀ መሆኑን ተረድቻለሁ፡፡
- ✓ ከልጄ/ከማሳድገው/ጋት ከሚወሰዱት ናሙናዎች ላይ ባክትሪያው ከተገኘ ለወደፊት በመጠበቅ ወደ ውጭ አገር ተልኮ ለአዲስ ግኝትና ለምርምር አላማ ይውል ዘንድ ተስማምቻለሁ፡፡

ተስማምቻለሁ ☐ አልተስማማሁም ☐

ስለዚህ የምርምር ቃሌን በፍጹም ፈቃደኝነት የሰጠሁት በአጠቃላይ ሁኔታውን በመረዳት እና ልጄ/የማሳድገው/ጋት ልጅ በጥናቱ ለመሳተፍ ፈቃደኛ የሚሆን/የምትሆን ከሆነ ነው፡፡

*የወላጅ/አሳዳጊ ስም*_______________________________ *ፊርማ*________________ *ቀን*_

*የውይይቱን ያካሄደው ሰው ስም*______________________ *ፊርማ*________________ *ቀን*______

ምስክሮች፤

1.ስም _______________________________________ *ፊርማ* ______________ *ቀን*_____

2.ስም _________________________________ *ፊርማ*____________________ *ቀን*_______

Children Assent for Age between 12-15 Years

- I, ________________________________ understand that my parents (mom/dad)/guardian have/has given permission (said it's okay) for me to take part in a project entitled as "Throat carriage rate and antibiotic susceptibility pattern of group A streptococci in children with rheumatic fever on secondary antibiotic prophylaxis."
- I agree to collect samples from me.
- I understand that the information will be confidential.
- I understand that I can stop the study at any time.
- I understand that if I don't want to take part, it will not affect my current and future medical services.
- I agree that there is no direct benefit.
- I agree that the samples taken from myself will be stored for future molecular characterization abroad.

Agree ☐ Do not agree ☐

Therefore I give my Assent freely to participate in this study.

Signature___________________________________ Date____________________

Signature of the interviewer ________________ Date ________________

Children Assent for Age between 12-15 Years Amharic Version

ከ12-15 አመት ለሆናቸው ልጆች የፈቃደኝነት መግለጫ ቅጽ

እኔ---የተባልኩ ልጅ ወላጆቼ (እናቴ/አባቴ)/አሳዳጊዎቼ ከላይኛው የመተንፈሻ አካላት ከባክቴሪያ ጋር ተያይዞ ሊከሰት የሚችል ህመም ባለባቸው የሁለተኛ መከላከያ መድሃኒት በጀመሩ ልጆች ላይ ባክቴሪያው መኖር ወይም አለመኖሩን እና በመድሃኒት መጠቃቱን መፈተሽ በሚለው ጥናት እንድሳተፍ መስማማታቸውን ተረድቻለሁ፡፡

- ✓ እኔ መስማማት/አለመስማማቴን ተጠይቄ መስማማቴን ገልጫለሁ፡፡
- ✓ ናሙናዎቹ ከ እኔ እንዲወሰዱ ተስማምቻለሁ፡፡
- ✓ ከእኔ የሚሰበሰቡት መረጃዎች በሚስጥር እንደሚያዙ ተረድቻለሁ፡፡
- ✓ በማንኛውም ጊዜ ጥናቱን ማቋረጥ እንደምችል ተረድቻለሁ፡፡
- ✓ በጥናቱ ባለመሳተፌ/በማቋረጤ በአሁኑ/የወደፊቱ የህክምና ግልጋሎቴ ላይ ምንም አይነት እክል እንደማይፈጥርብኝ ተረዷቻለሁ፡፡
- ✓ እኔ በጥናቱ በመሳተፌ ምክንያት ቀጥተኛ ጥቅም ባለመኖሩ ተስማምቻለሁ፡፡
- ✓ እኔ ላይ ከተወሰዱት ናሙናዎች ባክትሪያው ከተገኘ ለወደፊት በመጠበቅ ወደ ውጭ አገር ተልኮ ተጨማሪ ምርምር እንዲደረግበት ተስማምቻለሁ፡፡

ተስማምቻለሁ ☐ አልተስማማሁም ☐

ስለዚህ በአጠቃላይ ሁኔታውን በመረዳት በዚህ ጥናት ውስጥ ለመሳተፍ ፈቃደኛ ነኝ፡፡

ፊርማ--------------------ቀን----------------

ውይይቱን ያከናወነው ሰው ስም---

ፊርማ--ቀን_______________

Annex III: Questionnaire English and Amharic Version

English version

Code number__________________

Interview

We thank gratefully for your agreement your child to participate in this study. Now we are going to undertake interview with you for sometime. The interview is about general socio demographic characteristics and risk factors for the spread of Group A Streptococci. The information you give us is very essential for this study.Therefore we politely ask you to give us the right response.

Socio Demographic Data

1. Age (completed year)____________________________

2. Sex:

A. Male

B. Female

3. Residence

A. Urban

B. Rural

4. Ethnicity

A. Amara

B. Oromo

C. Tigre

D. Somalia

E. Gurage

F.Others(specify)______

Factors facilitating aggravation of RHD and some symptoms

1. When was your child diagnosed to have RF/RHD?__________________(In years)

2. How frequently is your child taking injections?

A. 21 days

B. 28 days

C. 30 days

D. Daily

3. Has your child ever missed the injections as prescribed by your child's physician?

A. Yes B. No

4. If your answer is yes for question #3 what was the reason?

A. Personal problems __________________(specify)

B. Health facility was not accessible

C. Others (specify) __________________________________

5. Did you have other children or family member who had pharyngitis within one year?

A. Yes C. Don't remember

B. No

6. Did your child have pharyngitis/ sore throat after starting the injections?

A. Yes

B. No

C. Don't remember

7. How many family members live in your house?___________________________

8. How many people share the same bedroom with your child? ________________

9. Is your child a student?

A. Yes B. No

10. If your answer is yes for question # 9 how many students learn in one class?_______________

11. Is your child's bedroom separated from the kitchen?

A. Yes B. No

12. Is your child's bedroom separated from the living room?

A. Yes B. No

13. Is your child's bedroom separated from the living room?

A. Yes B. No

14. Did your child subjected to tonsillectomy?

A. Yes

B. No

C. Don't remember

15. Does your child have skin lesions?

A. Yes B. No

16. What is your educational status (the care giver)__________________________

A. I am illiterate B. I am literate

17. If your answer is "I am literate in question #16

A. Grade______________________

B. Degree(s)____________________

18. Do you know the importance of the secondary antibiotic prophylaxis given to your child?

A. Yes B. No

19. Do you know the type of the drug given to your child?

A. Yes B. No

20. Do you know the interval the drug should be administered?

A. Yes B. No

21. How many injections did your child take with in the last 6 months? ___________

22. Monthly Income of the family (in Birr) ___________

For normal flora

23. Did your child have either of these symptoms now or in the last week?

A. Runny nose 1. Yes 2. No

B. Sore throat 1. Yes 2. No

C. Cough 1. Yes 2. No

24. Did your child have been injected with vaccine against meningitis in the past 6 months

A. Yes C. I don't remember

B. No

Name of the interviewer___________________________

Sign________________

Date _____________________

Amharic Version

መጠይቃዊ ቅጽ

መለያ ቁጥር___________________

ቃለ ምልልስ

ልጅዎ በጥናታችን እንዲሳተፍ/እንዲትሳተፍ በመፍቀድዎ እያመሰገንን ቃለ ምልልስ ከእኛ ጋር ያደርጋሉ፡፡ ቃለ ምልልሱ የሚያተኩረው የልብ በሽታውን የሚያባብሱ ነገሮች ላይ እና አንዳንድ የበሽታው ምልክቶች ላይ ነው፡፡ አሁን የሚሰጡን መረጃ ለጥናቱ በጣም አስፈላጊ በመሆኑ ሳይሰለቹ ጥያቄዎቹን በጥሞና ካዳመጡ በኋላ ትክክለኛ ምላሽ እንዲሰጡን በትህትና እንጠይቃለን::

አጠቃላይ ሁኔታ

1. ዕድሜ____________________

2. ጾታ:

ሀ. ወንድ | ለ.ሴት

3. የሚኖሩበት ቦታ

ሀ. ከተማ ነው | ለ. ገጠር ነው

4.ብሄር

ሀ. አማራ | መ. ሶማሊያ

ለ. ኦሮሞ | ሠ. ጉራጌ

ሐ. ትግሬ | ረ. ሌላ (ይጠቀስ)

የልብ ህመሙን የሚያባብሱ ነገሮች እና አንዳንድ ምልክቶች

1. ልጅዎ/የሚያሳድጉት/ጓት ልጅ በልብ ህመም መያዙ/መያዟ የታወቀው መቼ ነው?__________(በአመት)

2. ልጅዎ/የሚያሳድጉት/ጓት ልጅ መርፌውን የሚወስዱት በየትኛው የጊዜ መደብ ውስጥ ነው?

ሀ. በየ ሃያ አንድ ቀን | ሐ. በየ ሰላሳ ቀን

ለ. በየ ሃያ ስምንት ቀን | መ.በየቀኑ

3.ልጅዎ/የሚያሳድጉት/ጓት ልጅ ሃኪሙ መርፌውን ባዘዘው መሰረት ሳይወስድ/ሳትወስድ ቀርታ የውቃል/ታውቃለች ወይ?

ሀ.አዎ | ለ. የለም

4.ለተራ ቁጥር 3 መልስዎ አዎ ከሆነ ምክንያቱ ምን ነበር?

ሀ. የግል ችግር (ይግለጹት)--------

ለ. የአቅርቦት ችግር

ሐ. ሌላ (ይግለጹት)------

5. ከቤተሰብዎ መካከል በዚህ አንድ አመት ውስጥ ጉሮሮውን የታመመ ሰው ነበረ ወይ?

ሀ. አዎ

ለ. የለም

ሐ. አላስታውስም

6. ልጅዎ/የሚያሳድጉት/ጓት ልጅ መርፌውን ከጀመረ/ች ወዲህ የጉሮሮ ቁስለት አጋጥሞት/ሚት ያውቃል ወይ?

ሀ. አዎ

ለ. የለም

ሐ. አላስታውስም

7. በአንድ ቤት ስንት የቤተሰብ ብዛት አላችሁ______________?

8. ከልጅዎ/ከሚያሳድጉት/ጓት ልጅ ጋር አብረው የሚተኙት ሰዎች ብዛት ስንት ነው___________?

9. ልጅዎ/የሚያሳድጉት/ጓት ልጅ ተማሪ ነው/ናት?

ሀ. አዎ

ለ. አይደለም/አይደለችም

10. ለተራ ቁጥር 9 መልስዎ አዎ ከሆነ በአንድ ክፍል ውስጥ ስንት ተማሪ ይኖራል____________?

11. የልጅዎ/የሚያሳድጉት/የሚሳድጓት ልጅ የምኝታ ክፍል ከኩሽና ቤቱ የተለየ ነው ወይ?

ሀ. አንድ ላይ ነው

ለ. የተለየ ነው

12. የልጅዎ/የሚያሳድጉት/ጓት ልጅ የምኝታ ክፍል ከ ሳሎን ክፍሉ ጋር የተለየ ነው ወይ?

ሀ. የተለየ ነው

ለ. አንድ ላይ ነው

13. የልጅዎ የምኝታ ክፍል ከ ሳሎን ቤቱ ጋ የተለየ ነው ወይ?

ሀ. የተለየ ነው ለ. የተለየ አይደለም

14. ልጅዎ እንጥሉን/እንጥሏን ተቆርጦ ወይም ተቆርጣ ታቃለች ወይ?

ሀ. አዎ ለ. አልተቆረጠም/አልተቆረጠችም ሐ. አላስታውስም

15. ልጅዎ የቆዳ ቁስል አለበት/አለባት?

ሀ. አለበት/ባት　　ለ. የለበትም/ባትም

16. የእርስዎ የትምህርት ደረጃ ምንዲን ነው?

ሀ. ተምሪያለሁ　　ለ. ምንም አልተማረኩም

17. ለ ጥያቄ ቁጥር 16 ምልስዎ ተምሪያለሁ ከሆነ እስከ ምን ድረስ ተምረዋል?

ሀ. እስከ ስንተኛ ክፍል----------------　　ለ. ድግሪ-------------------

18. ለልጅዎ የሚሰጠው መከላከያ መድሀኒት ምን እንደሆነ ያውቃሉ?

ሀ. አዎ　　ለ. አላቅም

19. ለልጅዎ የሚሰጠው መከላከያ መድሀኒት ከምን እነደሚመደብ ያውቃሉ?

ሀ. አዎ　　ለ. አላቅም

20. ለልጅዎ የሚሰጠው መከላከያ መድሀኒት በየ ስነት ቀኑ እንደሚሰጥ ያውቃሉ?

ሀ. አዎ　　ለ. አላቅም

21. ባለፈው ስድስት ወር ውስጥ ልጅዎ ስንት የመከላከያ መድሃኒት ወስዳል/ች?-----

22. የቤተሰቡ የወር ገቢ በብር ስንት ነው ?----------------?

23. ከባለፈው ሳምንት ወዲህ ልጅዎ የሚከተሉትን የበሽታ ምልክት አሳይቶ/ታ ያቃል/ታቃለች ወይ

1. የንፍጥ መዝረብረብ

ሀ. አዎ

ለ. የለም

የጉሮሮ እብጠት	**ሳል**
ሀ. አዎ	ሀ. አዎ
ለ. የለም	ለ.የለም

24. ባለፈው ስድስት ወር ውስጥ ልጅዎ የማግራት ገትር በሽታ መከላከያ ወስዶ/ዳ ያቃል/ታቃለች ወይ

ሀ. አዎ

ለ.አልዎሰደችም

ሐ. አላስታውስም

Laboratory and clinical data records of the participant

Participant unique identity number______________________

Site _________________________

1. Year of the diagnosis______________________

2. Status of the participant

A. rheumatic fever   B. rheumatic heart disease

3. What is the initial presentation of the participant ____________________

____

____

____

____

4. What is the past history of rheumatic fever recurrence? ______________________

____

5. Past history of pharyngitis/ tonsillitis prior to rheumatic fever or rheumatic heart

disease____

____

6. Types of diagnostic tests performed_______________________________________

____

____

____

7. Types of rheumatic heart disease

A. Mitral regurgitation

B. Mitral regurgitation

C. Aortic regurgitation

D. tricuspid valve regurgitation

E. pulmonary regurgitation

8. Types of the drug the participant is taking

A. Benzathine Penicillin G

B. Erythromycin

C. Others (Specify)_________________________

9. The date on which the child was assessed a candidate for surgery? ________________

10. Date of heart valve surgery if performed________________________

Name of the collector_____________________________

Sign__________________

Date _______________________

Annex IV: Laboratory Procedures

1. **Preparation of Blood Agar Plates**

1. Blood agar base (20 g) (Oxoid, UK) was suspended in 500 ml of distilled water and boiled.
2. The suspension was autoclaved at 121 ^oC for 15 minutes, and then cooled to 50 ^oC.
3. Five hundred ml of the medium and 35 ml of sheep blood was mixed homogenously
4. The medium was mixed gently and poured into sterile plates.

2. Inoculation of Plates and Incubation

1. Prior to inoculation, the media was brought to room temperature
2. Throat swabs swab was 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 ^oC.
4. CO_2 incubator was used to provide an atmosphere of 5% CO_2.

3. Gram Staining

1. One or fair amount of colonies were placed to a microscope slide. Spread the culture over 1/3 to ½ to the total area of the slide.
2. Allow the smear to air dry. This may take up to 1 hour depending on the temperature and humidity of the room.
3. Fast flamed 2-3 times
4. Cover the bacterial smear with crystal violet stain and allow standing 1 minute. Gently is the stain off with cool tap water and drain water from slide.
5. Cover the smear with grams iodine and allow standing 1 minute. Gently wash the iodine off with water and drain the water from the slide.
6. Rinse the bacterial smear with decolorizer solution for 10 seconds; decolonization is complete when the solution runs clear from the slide. Gently rinse with water and drain the slide.
7. Cover the bacterial smear with safranin stain, and allow standing for 1 minute, and then gently washing the stain from the slide.
8. Blot the slide dry with absorbent paper and examine the slide under oil immersion lens.

4. 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.

5. Bacitracin Sensitivity Testing

1. Catalase negative isolates evenly spread on blood agar plate

2. Bacitracin disc (0.04 U) was aseptically placed on the inoculated surface and incubated for 18-24 hour at 35 oC in 5% CO_2.

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

4. Any zone of inhibition surrounding the disk was indicative of a presumptive GAS.

6. Drug susceptibility

1. using a sterile wire loop touch 3-5 well-isolated colonies of similar appearance to the test organism and emulsify in 500µL normal saline.

2. In a good light match the turbidity of the suspension to the turbidity standard (mix the standard immediately before use). (0.5% McFarland)

3. using a sterile swab, inoculate a plate of Mueller Hinton agar. Remove excess fluid by pressing and rotating the swab against the side of the tube above the level of the suspension

4. Streak the swab evenly over the surface of the medium in three directions, rotating the plate approximately 60 to ensure even distribution

5. With the petri dish lid in place, allow 3-5 minutes (no longer than 15 minutes) for the surface of the agar to dry.

6. Using a multidisc dispenser, place the appropriate antimicrobial discs, evenly distributed on the inoculated plate should be applied (90 mm dish).

7. Within 30 minutes of applying the discs, invert the plate and incubate it aerobically at 35C for 16-18 h

8. After overnight incubation, examine the control and test plates to ensure the growth is confluent or near confluent. Using a ruler on the underside of the plate measure the diameter of each zone of inhibition in mm. The endpoint of inhibition is where growth starts.

9. Interpretation of zone sizes using the Interpretative Chart, interpret the zones sizes of each antimicrobial, reporting the organism as Resistant, Intermediate/Moderately susceptible, Susceptible.

7. **ASO titer determination by latex agglutination test**

1. Bring the test reagents and samples to room temperature.
2. Resuspend the antigen vial gently.
3. 0.85 % saline was used for dilution purpose. 50 μL of serum and one drop of the ASLO-Latex antigen were mixed using disposable stirrer on a circle on the card and rotated on the mechanical rotator (100 rpm) for 2 minutes.
4. Both positive and negative controls were run side by side. It was observed under visible light source for any degree of agglutination. When it was found reactive 50 μL of the serum each is diluted with 50, 100, 150,200 μL of saline etc. until it became non-reactive.

Interpretation

The titer of the specimen is reported as the highest dilution that shows reactivity multiplied by 200 IU/Ml.

8. Serogrouping

Test procedure

All components should be at room temperature prior to use.

1. Re-suspend the test latex reagents by gently inverting the dropper bottle several times. Examine the dropper bottles to ensure that the latex particles are properly suspended before use. Do not use if the latex fails to re-suspend.
2. Label one test tube for each isolate to be tested.
3. Add 1 drop of Extraction Reagent 1 to each tube.
4. Select 1-4 *beta*-haemolytic colonies using a disposable loop or needle and suspend them in the Extraction Reagent 1. If the colonies are small, pick several well isolated colonies to be tested such that the Extraction Reagent 1 solution becomes turbid. In all cases the streptococcal colonies should be picked from an area which will afford the lowest probability of contamination with another organism.
5. Add 1 drop of Extraction Reagent 2 to each tube.
6. Mix the reaction by gently tapping the tube with a finger for 5-10 seconds.

7. Add 5 drops of Extraction Reagent 3 to each tube and mix by gently tapping the tube with a finger for 5-10 seconds.

8. Dispense one drop of each group latex reagent onto separate circles on separate test cards labelled for each isolate being tested.

9. Using a Pasteur pipette, for each test place one drop of extract beside each drop of latex reagent.

10. Mix the latex and the extract with the sticks provided, using the complete area of the circles. A new stick should be used with each test circle.

11. Gently rock the cards allowing the mixture to flow slowly over the entire test ring area.

12. Observe for agglutination for up to one minute.

9. Protocol for MALDI-TOF species identification (Bruker Daltonics MALDI Biotyper, Bruker Daltonics Flex control, MALDI Biotyper RTC)

1. MALDI target plates were inoculated with small amount of overnight grown colonies on human blood agar plate.
2. Two target plates were used for a single microorganism.
3. The film was overlaid with 100% 1ul formic acid (0.5µl for each) and let it air dry.
4. Then it was overlaid with 1ul matrix α-cyano-4 hydroxy-Cinnamic acid (HCCA) and allowed air dry at room temperature.
5. The plate was inserted in to the MALDI TOF and the results were printed out.

10. Protocol for *emm* typing

A. Lysate preparation

1. With a loop pick up a fair amount of fresh growth (perhaps half of a standard loop-full). Resuspend in 300 ul 0.85% NaCl.
2. Heat at 70C for 15 minutes.
3. Spin down samples full speed for 2 min in microfuge and pipet out supernatant.
4. Resuspend pellet in 50 ul TE (10mM Tris, 1mM EDTA, pH8), 10 ul mutanolysin (3,000 units/ml), and 2 ul hyaluronidase (30 mg/ml, Sigma H-3506; 300-750 units/mg).).
5. Incubate 37C, 30 min
6. Heat at 100C for 10 min. Proceed immediately with PCR or store lysates at -20C until use.

B. PCR

Prepare master mix with this ratio of components:

50 µL PCR reaction mixtures contained, , , , and.

The samples were placed in thermo cycler (ESCO, Swift MaxPro) using the following programs (CDC). Initial denaturation 94°C for 1 minute, 10x (94°C: 15s, 46.5°C: 30s, 72C: 1 minute 15s and the next 20x: (94°C: 15s, 46.5°C: 30s, 72°C: 1 min 15s with a 10 sec increment for each of the subsequent 19 cycles.) 72°C for 10 min and then 4C storage. The PCR product was stored at -20°C until used.

5µL 10x NH4 reaction buffer

2µL 50xmM MgCl2,

2µL 4x5 mM dNTPs

1µL 10uM primer1 (TATTCGCTTAGAAAATTAA),

1µL 10µM primer2 (GCAAGTTCTTCAGCTTGTTT)

0.2µL Bio-Taq polymerase (5units/µL)

38.3 µL MQ water

Prepare 20 ul PCR reaction:

1. Spin down lysate full speed 1 min.
2. For 1 sample on ice aliquot 19.5 µl master mix.
3. Add 0.5 µl lysate supernatant.
4. Place in cycler using the following program. Put samples in thermocycler only after the initial sample temperature (94C) is reached.

94C 1 min

Do the following 10X:

94C: 15s.

46.5C: 30s.

72C: 1 min 15s.

followed by the following 20X:

94C: 15s.

46.5C: 30s.

72C: 1 min 15s with a 10 sec increment for each of the subsequent 19 cycles.

72C 10 min., then 4C storage.

Store PCR products at -20C until use

C. Protocol for purification of DNA

1. Add 500 µl Capture buffer type 3 to 45 µl sample.

2. Mix thoroughly. Capture buffer type 3- sample mix is yellow

3. For each purification that is to be performed, place one GFX MicroSpin column into one Collection tube.

4. Centrifuge Capture buffer type 3-sample mix briefly to collect the liquid at the bottom of the tube.

5. Load the Capture buffer type 3-sample mix onto the assembled GFX MicroSpin column and

Collection tube.

6. Spin the assembled column and Collection tube at 16 000 × g for 30 seconds.

7. Discard the flow through by emptying the Collection tube. Place the GFX MicroSpin column back inside the Collection tube.

8. Add 500 µl Wash buffer type 1 to the GFX MicroSpin column.

9. Spin the assembled column and Collection tube at 16 000 × g for 30 seconds.

10. Discard the Collection tube and transfer the GFX MicroSpin column to a fresh DNase-free 1.5 ml microcentrifuge tube.

11. Add 45 µl Elution buffer **type 4** to the center of the membrane in the assembled GFX MicroSpin column and sample Collection tube.

12. Incubate the assembled GFX MicroSpin column and sample Collection tube at room temperature for 1 minute.

13. Spin the assembled column and sample Collection tube at 16 000 × g for 1 minute to recover the purified DNA.

14. Proceed to measuring the concentration and sequencing procedures.

15. Store the purified DNA at -20°C.

D. PCR sequencing

10µl (5µl 20-80ng/µl DNA,) was needed

1. Measure 2.5µl 5uM *emm*seq2 primer (TATTCGCTTAGAAAATTAAAAACAGG)
2. Add low and high concentration of DNA
3. Add MQ water to make a final volume of 10µl
4. Label the tubes with the barcode and one lable for each tube on the lab note book with the correct labllings.
5. Sent the tubes to Germany for sequencing
6. Sequences were downloaded from GATC biotec.com
7. Sequence balsting was done using CDC *emm* database.

11. Gamma-Glutamyl-Aminopeptidase Test

1. Before starting of processing any new batch of samples, be sure to run a control test with *Neisseria meningitidis* and *Neisseria lactamica* known strains.
2. Mark GGT on a clean test tube, followed by the sample ID

3. Add 0.25 ml of saline solution to the tube
4. Remove a 10 µl loopful of bacteria from the BAP
5. Make a fine suspension of the bacteria in the saline solution
6. Put the tablet in the tube
7. Close the tube
8. Incubate at 35-37°C for 4 hours (It is also possible to incubate overnight)
9. Read the reaction:

Interpretation of Results

After four hours

Positive reaction-deep red

Quality Control

Use *N. meningitidi*s as the positive control and *N. lactamica* as the negative one.

12. Ortho-nitrophenyl β-D-galactopyranoside Test

1. Before starting of processing any new batch of samples, be sure to run a control test with *Neisseria meningitidis* and *Neisseria lactamica* known strains.
2. Mark a clean test tube with the sample ID
3. Add 0.25ml saline solution to the test tube
4. Remove a 10µl loop full of bacteria from the BAP
5. Make a fine suspension of the bacteria in the saline solution
6. Add 1 *Beta*-galactosidase (ONPG) tablet to the solution
7. Close the test tube
8. Incubate at 35-37^{0} C for 4h (It is also possible to incubate overnight)

Interpretation

Positive reaction-yellow

N. lactamica as the positive control and *N. meningitidi*s as the negative one

13. Tributyrin Test

1. Before processing any new batch of samples, be sure to run a control test with *Neisseria meningitidis* and *M. catarrhalis* known strains (see below). Mark a clean test tube Trib followed by the sample ID
2. Add 0.25 ml saline to the test tube
3. Remove 10ul loopful of bacteria from the BAP
4. Emulsify the bacteria in the saline solution
5. Add 1 tributyrin tablet to the solution
6. Close the test tube
7. Incubate at 35-37^0 C for 4 h (It is also possible to incubate overnight)
8. Read the colour reaction
9. Report the observed result on the Excel Report Form

Interpretation of Results

Positive reaction-yellow/yellow-orange

Negative reaction-red

Quality Control

Use *M. catarrhalis* as the positive control and *N. meningitidis* as the negative one.

Declaration

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

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Addis Ababa, Ethiopia