Rheumatic Fever and Rheumatic Heart Disease: Prevalence among Yemeni School Children and Studies of the Immunopathogenesis of the Disease

By

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Dedication

This thesis is dedicated to:

My father, Ali, who taught me the best knowledge to have, is that which is learned for its own sake.

My mother, Thuriya who taught me that even the largest task can be accomplished if it is done step by step.

My husband, Ahmed who has been a great source of inspiration, unwavering encouragement, motivational support, unending help and dynamic generous spirit that continues to enrich my life.

My daughters, Daliah, Fatima and Dalal for their unconditional love, support and a treasure beyond compare throughout the course of this thesis.

It is dedicated to all our journeys in learning to thrive

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Thesis Structure

Chapter one includes a general overview of the problem of Acute Rheumatic Fever (ARF) and Rheumatic Heart Diseae (RHD) following acute pharyngotonsillits with Group A β haemolyitc Streptococcal (GAS) infection. Chapter two describes the type of patients and the methodology including study design, sample size, paraclinical investigations and the statistical analysis used in the different phases of the research. The prevalence of RHD among a representative sample of 6000 Yemeni schoolchildren in Aden City is dealt with in Chapter three. Chapter four describes the prevalence of GAS infections among 730 patients with acute pharyngotonsillitis attending public and private primary health centres and school children. Chapter five gives the prevalence of Non Group A β haemolytic Streptococcal (SNA) pharyngotonsillits among patients and school children. The *emm* genotyping and the virulence factors including superantigen prophage exotoxin and *sof* genes of 21 GAS and 13 SNA throat isolates from patients with history of ARF and RHD is discussed in chapter six. Chapter seven includes the antimicrobial sensitivity and resistance patterns in 24 GAS and SNA pharyngotosnillitis throat isolates. The immunopathogenesis and genetic aspects of ARF and RHD including the study of 14 cytokines and chemokines in 49 patients with ARF and RRF is reviewed in chapter eight. The general discussion, conclusions and recommendations with study limitations are outlined in chapter nine. The final chapter ten includes all the references reviewed in this thesis.

Declaration of work done

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference has been made.

Some of the work in this study was shared with other individuals. My contribution to the reported work was as follows:

Activity	Responsibility
Recruitment of RHD subjects	Sole
Initial clinical evaluation of subjects	Sole
Evaluation of cardiac lesions	Shared
Recruitment of pharyngotonsillitis subjects	Sole
Collection of samples	Sole
Processing of throat swabs	Shared
EMM genotyping and virulence factor analysis	Shared
Antibiotic disc sensitivity tests	Shared
Cytokine and chemokine analysis	Shared
Analysis and interpretation of results	Sole

Abstract

Background: The epidemiological, genetic and host immunogenetic association between Group A β haemolytic Streptococcal (GAS) pharyngotonsillitis and the subsequent development of Acute Rheumatic Fever (ARF) and Rheumatic Heart Disease (RHD) is an area of major interest. RHD still remains an important contributor to cardiovascular disease in children and adults in Yemen.

Aims: The purpose of this study was to determine: (i) the prevalence of RHD among primary school children in Aden City, Yemen, (ii) the prevalence of GAS and Non Group A β haemolytic Streptococcal (SNA) pharyngotonsillitis among patients attending primary health care centres, (iii) the distribution of *emm* genotypes and selected superantigen prophage exotoxin and *sof* genes among GAS and SNA , (iv) the antimicrobial susceptibility pattern of GAS and SNA in patients with a history of ARF and RHD, (v) comparision of a profile of selected cytokines and chemokines between ARF and recurrent rheumatic fever (RRF) patients.

Methods: A cross-sectional case-finding survey of RHD was conducted in 6000 school children aged 5 – 16 years in Aden City to determine the prevalence of RHD. A cross-sectional descriptive survey was undertaken in 730 children aged 1- 16 years with acute pharyngotonsillitis to determine the prevalence of GAS and SNA infections. Thirty four throat culture isolates from patients with GAS and SNA pharyngotonsillitis with history of ARF and echo-proven cases of RHD were analyzed by a multiplex PCR method to determine the emm genotypes, presence of superantigen prophage-associated virulence genes and *sof* genes. Antibiotic sensitivity tests were conducted on 24 GAS and SNA throat culture isolates using the BSAC disc diffusison method. Fourteen serum cytokine and chemokine concentrations including interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-12p70 (IL-12p70), tumor necrosis factor (TNF-α), interferon gamma (IFN-γ), chemokines monocyte chemotactic protein-1(MCP-1), macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), human interferon inducable protein-10 (IP-10) and regulated upon activation, normal T-cell expressed and secreted (RANTES) protein levels from children with ARF and RRF were analyzed by the BD FACS Array Bioanalyzer using FCAP Array *Sof*tware.

Results: The prevalence of RHD was 36.5/1000 school children which is one of the highest reported among school echocardiography surveys in the world. RHD had a high preponderance in 10-16 years old students. 49.8% had mitral regurgitation (MR) lesions, 26.6% hadMR with mitral valve prolapse (MVP) and 17.8% with combined MR and aortic regurgitation (AR) lesions. RHD was diagnosed in more than one family member in 53 (24.2%) of the children. A high prevalence of GAS pharyngotonsillitis (41.5%) was noticed in children of 11 – 15 years of age. A red erythematous uvula and petechie on the soft palate were observed significantly more commoinlyin GAS pharyngotonsillitis. Group B (GBS), Group C (GCS) and Group G β haemolytic streptococci (GGS) were isolated from pharyngotonsillitis in 4.3% patients with history of ARF/RHD.

The most frequent GAS isolates among ARF and RHD patients with pharyngotonsillitis were *emm*87, *emm*12, *emm*28 and *emm*5. This is the first report of *emm*87 and *emm*28 genotypes to be potentially rheumatogenic. The 11 *emm*87 GAS isolates shared a common PFGE pattern and profile of five exotoxin prophage genes *spec, spdl, sdn, silC* and *silD* with the *sof* 87 sequence. *emm*12 and *emm*28 GAS strains were positive for gene *sof* , *spec* and *spdl.* This is the first report to describe the pattern of exotoxin prophage genes of *spec, spdl*, *sdn, silC* and *silD* among *emm*87, *emm*12, *emm*28 and *emm*5 GAS and SNA (GBS, GCS and GGS) isolates with history of ARF and RHD. The genotypic characteristics of GBS, GCS and GGS isolates confirmed seven new *emm* sequence types first detected among children with acute pharyngotonsillitis. GAS and SNA isolates were susceptible to the β-lactam antimicrobials, penicillin and amoxicillin. Erythromycin resistance was detected in *sof* positive *emm*12 and *emm*28 in 50% and 33% of isolates respectively.

Chemokine MCP-1 was significantly correlated with cytokines, IL-1β, IL-6, IL-10, IL-12p70, TNF-α, IFN-γ and RANTES in patients with RRF. This suggests that MCP-1 could serve as a potential inflammatory biomarker for patients with RRF having underlying RHD. MIP-1β had significant correlations with IL-8, IL-10, IL-12p70, IP-10, TNF-α and IFN-γ in patients with ARF. MIP-1β may serve as a potential inflammatory biomarker in patients with ARF without RHD.

Conclusions: The high prevalence of RHD is an alarming public health problem in Yemen. Urgent screening surveys and a preventive RHD prophylactic program with appropriate management of GAS pharyngotonsillitis are required. Future studies are needed to confirm the rheumatogenic GAS and SNA strains with their exotoxin prohage genes and the role of the chemokines and cytokines as biomarkers for ARF within the complex network of autoimmune reactions in RF/RHD. This study hopes to provide a further small step in elucidating the pathogenesis of this complex immunological disease.

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List of abbreviations

AOR	Adjusted Odds Ratio
AP	Alternative pathway
APR	Acute Phase Reactants
ARF	Acute Rheumatic Fever
AR	Aortic Regurgitation
AS	Aortic Stensois
ASD	Atrial Septal Defect
ASO	Anti Streptolysin Titer
CCF	Congestive Heart Failure
CD4	Cluster of Differentiation-4
CDC	Centers for Disease Control and Prevention
CFH	Complement factor H
CHD	Congenital Heart Disease
CRHD	Chronic Rheumatic Heart Disease
CI	Confidence Interval
CRP	C Reactive Protein
DALYs	Disability-adjusted life years
DNA	Deoxyribo-Nucleic Aacid
EDTA	Ethylenediaminetetra-acetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EMRO	East Mediterranean Regional Office
Factor H	FH
FHL-1	Factor H-like protein 1
SFB	Streptococcal Fibronectin-binding protein
GAS	Group A β haemolytic Streptococcal infection
GBS	Group B β haemolytic Streptococal infection
GCS	Group C β haemolytic Streptococal infection
GGS	Group G β haemolytic Streptococal infection
HLA	Human Leukocyte Antigen
IL	Interleukins
INF-γ	Interferon-gamma
IP-10	Human interferon inducible protein-10
IQR	Inter Quartile Range
MBL	Mannan-binding lectin
MCP-1	Monocyte Chemotactic Protein-1
MFI	Mean Flourescent Intensity
MHC	Major Histocompatibility Complex
MIP-1α	Macrophage Inflammatory Protein-1α
MIP-1β	Macrophage Inflammatory Protein-1β
MLST	Multilocus sequence typing
MR	Mitral Regurgitation
MS	Mitral Stenosis
MVP	Mitral Valve Prolapse
OR	Odds Ratio
PDA	Patent Ductus Arteriosus

PFGE	Pulse Field Gel Electrophoresis
PCR	Polymerase Chain Reaction
PHC	Primary Health Care
PI	Prinicpal Investigator
PS	Pulmonary Stenosis
RADT	Rapid Antigen Detection Test
RANTES	Regulated upon Activation, Normal T-cell Expressed and Secreted
ARF	Acute Rheumatic Fever
RHD	Rheumatic Heart Disease
RRF	Recurrent Rheumatic Fever
SD	Standard Deviation
SIL	Streptococcal Invasive Locus
SOF	Serum Opacity Factor
SNA	Non-group A β haemolytic streptococci
SPSS	Statistical Package for the Social Sciences
TA	Triscupid Atresia
Th	T helper
TNF-α	Tumor Necrosis Factor-alpha
TOF	Tetralogy of Fallot
TLRs	Toll-like receptors
VCAM-1	Vascular Cell Adhesion Molecule 1
VSD	Ventricular Septal Defect
WHO	World Health Organization
YR	Yemeni Riyals

Chapter 1

1 Introduction

1.1 General overview on GAS

Infection with Streptococcus pyogenes known as group A β haemolytic Streptococcus(GAS), an important Grampositive extra cellular bacterial pathogen, has remained among the most universal human bacterial pathogens with great diversity for centuries (Bisno, *et al* 2003, Dagnelie, *et al* 1998, Tart, *et al* 2007)

. GAS is responsible for a wide spectrum of human diseases, ranging from the relatively benign pharyngitis to potentially life threatening invasive disease and post-infectious sequelae such as acute rheumatic fever (ARF)(Carapetis 2008a, Cunningham 2000, Lazar R. 2004).

Humans are the only natural reservoir for GAS which has a world-wide distribution(Ahmed, *et al* 2003, Pichichero 1998). Millions of children each year develop GAS pharyngotonsillitis(Bessen, *et al* 2000, Sierig, *et al* 2003). The prevalence of GAS in throat accounts for approximately 20 to 30% of sore throats with an estimated seven sore throat episodes occurring per child per year (Armengol, *et al* 2004, Darrow and Buescher 2002). Occurrence of GAS tonsillopharyngitis is known to be highest in 5 – 18 years old school-aged children, the target population, irrespective of gender (Brook and Dohar 2006, Tart, *et al* 2007). It varies geographically and in temperate climates GAS tonsillopharyngitis is more common during the winter and early spring months (Bisno, *et al* 2003, Shulman, *et al* 2006).

Modern molecular biology techniques has taught us much about the GAS virulence and some types of its genomes (Bisno, *et al* 2003).The basic phenotypic typing methods of serotyping, biotyping and antibiotic resistance patterns have been supplemented with molecular methods such as random amplified polymorphic DNA analysis, 16S rRNA gene sequencing, restriction fragment length polymorphism, pulsed-field gel electrophoresis (PFGE) and PCR serotyping (Chiou, *et al* 2004, Desai, *et al* 1999, Seppala, *et al* 1994a, Stanley, *et al* 1995). Sequence-based typing methods such as

multilocus sequence typing and *emm* sequence typing have also been introduced (Bessen, *et al* 2008, Carriço, *et al* 2006, Maiden, *et al* 1998). These techniques have a wide application as they provide unambiguous data, form databases that are easily accessible through the internet and allow comparison with reports from different countries.

Multilocus sequence typing (MLST) provides a practical approach to study the molecular epidemiology and population structures of bacterial species such as GAS (Enright and Spratt 1999, Maiden, *et al* 1998). MLST can be used to identify clusters of isolates that have similar or closely related genotypes (Maiden, *et al* 1998, Urwin and Maiden 2003). The method is based on the nucleotide sequence variation in seven stable housekeeping genes (Brian G 1999, McGregor and Spratt 2005). A disadvantage of MLST for routine use is that the set-up procedures are slow and they are expensive as seven DNA sequences are needed for each isolate (Cooper and Feil 2004, McGregor, *et al* 2004b, Richardson, *et al* 2011).

The *emm* gene sequencing method has been shown to be an accurate and reliable substitute for M typing (Beall, *et al* 1996, Neal, *et al* 2007). Anti-M serum is produced in a limited number of central laboratories, is quite expensive and does not type all strains. The gene coding for GAS M protein, the most important virulence determinant of the organism, has a hypervariable 59 region with many single nucleotide polymorphisms. Detection of the variation in this region is the basis for GAS *emm* typing isolates (Bahnan, *et al* 2011, Beall, *et al* 1997, Pruksakorn, *et al* 2000). Despite the limitations of basing a typing system on a highly recombinant region subject to a high degree of selective pressure, *emm* gene sequencing is currently considered the molecular gold standard for typing GAS (Neal, *et al* 2007, Steer, *et al* 2009d).

Analysis of the clonal relationships between clinical throat isolates of GAS from patients by various typing methods is a practical approach to clarify the epidemiology of ARF. Some GAS strains which cause acute pharyngotonsillitis are clearly also associated with the non-suppurative sequelae of ARF and these strains have been found to have particular M types (Cunningham 2000, Kaplan 1991, Seckeler and Hoke 2011). The M protein is a cell-surface protein that is the major virulence and immunological factor of GAS responsible for rheumatogenicity (Bisno, *et al* 2003, Meisal, *et al* 2010,

Metzgar and Zampolli 2011). The M type is now determined by DNA sequencing of the *emm* gene and the method has detected several previously unknown GAS types (Bahnan, *et al* 2011, MA, *et al* 2009, Sakota, *et al* 2006). Recovery of GAS isolates from the infected throat of patients in areas where ARF is common, combined with *emm* sequence genotyping, could clarify the epidemiological associations of M types with sequelae in these countries (Bisno, *et al* 2003, Steer, *et al* 2009d, Teixeira, *et al* 2001).

Although previous epidemiological studies have shown specific GAS types responsible for rheumatogenecity, it remains necessary to study new strains in low resource settings in order to further clarify strain associations (Cunningham 2000, MA, *et al* 2009, Sakota, *et al* 2006, Seckeler and Hoke 2011). A better understanding of the disease epidemiology will help in the practical application of public health measures for improved protection against ARF (Seckeler and Hoke 2011, Smeesters, *et al* 2009) and will also guide the future development of an effective and safe vaccine with a combination of M protein serotypes and genotypes from different rheumatogenic strains in different areas of the world (Metzgar and Zampolli 2011, Shulman, *et al* 2009, Steer, *et al* 2009d).

To date, a number of phenotyping and genotyping schemes have been described for GAS but these have not yet been conducted in Yemen. This study will be the first to undertake research on GAS in Yemen (Chapter 4).

The epidemiological association between GAS infections and the subsequent development of acute rheumatic fever (ARF) has been well established (Fae, *et al* 2005, Gibofsky, *et al* 1998, Hahn, *et al* 2005). This delayed autoimmune systemic response with variable multiorgan involvement in an individual with GAS pharyngotonsillitis is determined by host genetic susceptibility,virulence of GAS and a favourable environment (Taranta and Markowitz 1989, WHO 2004).

Humans are exposed to GAS infections through the environment. Environmental factors and poor socioeconomic status facilitate the transmission and increase the frequency of GAS sore throats (Griffiths and Gersony 1990, Jaine, *et al* 2011). Poverty is an important risk factor and overcrowding promote GAS transmission and disease development (Carapetis, *et al* 2005a, Zaman, *et al* 1997).

GAS virulence is enhanced in favourable conditions such as overcrowding and close interpersonal contacts . This favours person-to-person transmission and contribute to the rapid spread and persistence of virulent GAS strains (Cunningham 2000, Steer, *et al* 2002, Thakur, *et al* 1996). Poor housing conditions are a further risk factor for ARF and RHD (Bryant, *et al* 2009, Gerber, *et al* 2009). Other factors such as decreased access to medical care, shortage of resources for providing quality health care, inadequate expertise of health-care providers, and a low level of awareness of the disease in the community can all affect the expression of ARF in a population (Carapetis, *et al* 2000, Seckeler and Hoke 2011)

Variations in the rheumatogenicity of prevalent GAS probably account for the marked temporal and geographic fluctuations in the incidence of ARF (Sakota, *et al* 2006, Shulman, *et al* 2006). The incidence of ARF varies by season, in time and by location. In many areas ARF has a high incidence in early autumn, late winter and early spring, and this coincides with variations in the frequency of GAS throat infections (Johnson 1992, Örün, *et al* 2012).

Several authors have found that GAS throat infections caused by particular *emm* strains are associated with high rates of ARF (Erdem, *et al* 2007, Smeesters, *et al* 2009, Steer, *et al* 2009d). GAS pharyngitis outbreaks caused by *emm* types 1, 3, 5, 6, 14, 18, 19, 24, 27and 29 were associated with ARF, but other common *emm* types 2, 4, 12, 22, and 28 were not associated with ARF even in highly susceptible individuals (Kaplan, *et al* 2001, Shulman, *et al* 2004, Steer, *et al* 2009d). Bisno et al reported multiple epidemics of ARF caused by *emm* 3, 5, 14 and 18 strains while *emm* 6,19,27 and 29 were correlated with a single epidemic (Bisno, *et al* 2003).

Different GAS strains are more likely than others to be rheumatogenic (Martin and Barbadora 2006). GAS isolates with high M protein content, a mucoid colonial morphology, encapsulation and lack of opacity factor are associated with increased virulence causing ARF (Bisno, *et al* 2003, Johnson, *et al* 2006). During the ARF resurgence in the intermountain area of the US, the high ARF incidence was associated with the occurrence of an increasing number of GAS mucoid strains in the community (Kaplan 1991). The marked decrease in the prevalence of ARF in some countries has been attributed to the decline of the proportion of children with rheumatogenic GAS

types, particularly the mucoid strains in throat infections (Abdul-Mohsen and Lardhi 2011, Veasy, *et al* 2004). An increase in extractable M protein has been reported from some GAS strains associated with outbreaks of pharyngitis and ARF, compared with GAS strains of the same serotypes isolated in communities where pharyngitis was common but ARF was rare (Maxted, *et al* 1973).

The distribution of GAS pharyngeal *emm* types and their degree of diversity exhibit geographical differences (Shulman, *et al* 2004, Teixeira, *et al* 2001). *emm* GAS distributions were documented to be similar in some countries, but unusual inter-site variation within season and intra-site variation from season to season has been reported (Shulman, *et al* 2009, Tewodros and Kronvall 2005). The increased levels of immunity to certain predominant types have also been associated with a decline in prevalence of some rheumatogenic GAS types in recent years and a decline in ARF (Carapetis, *et al* 2000, Cunningham 2000).

Changes in the circulating strains of GAS can explain some of the observed temporal and geographic fluctuations in ARF incidence (Erdem, *et al* 2007, Shulman, *et al* 2006). Other factors may also be important. For example, differences in the population prevalence in the same geographical area have been attributed to low socioeconomic groups and differences in the genetic predisposition to ARF (WHO 2001).

The global burden of disease caused by ARF currently falls disproportionately on children living in developing countries (Abella-Reloza 1998, Carapetis 2007). The overall annual incidence of the disease worldwide is between 100 and 200/100,000 school age children, whereas in industrialized countries it is only 0.5 to 2/100,000 (Olivier 2000, Omurzakova, *et al* 2009, Tibazarwa, *et al* 2008).

1.2 Acute Rheumatic fever

ARF is prevalent in the age of 5 – 14 years in different developed and developing countries (WHO 2001, Zaman, *et al* 1998a). ARF may cause death due to acute carditis or complications of RHD resulting from previous episodes of ARF (Carapetis, *et al* 2005b, Vázquez, *et al* 1991). But RHD is the major source of morbidity and mortality in ARF (Carapetis and Currie 1999, Seckeler, *et al* 2010).

The first episode of ARF usually occurs between 5 – 14 years (Jaine, *et al* 2011, Steer, *et al* 2002). It has rarely been reported in children less than age of 5 years and is very rare or almost unheard below 3 years (Carapetis, *et al* 2005a, Lennon and Craig 2009, Rosenthal, *et al* 1968). The few reported cases of ARF vary from 1 to 32 cases in children less than 5 years of age (Abdin and Eissa 1965, Tani, *et al* 2003) . The reported prevalence of ARF in children less than 5 years of age varies from lower values of 1% - 2% to higher values of 4.5% to 6.8% in different countries (Abdin and Eissa 1965, Jaine, *et al* 2008, Vázquez, *et al* 1991, Zaman, *et al* 1998a). There is lack of data on the clinical pattern of ARF in young children. It may due to the fact that some cases may be undiagnosed or there may be an underestimation of the disease in children younger than 5 years of age (Richmond and Harris 1998, Seckeler and Hoke 2011). There is also insufficient data on the age and cause specific mortality under 5 years of age for ARF and RHD (Bitar, *et al* 2000) . Mortality rates for RHD are reported to vary from 0 - 30% during childhood or early adulthood (Carapetis and Currie 1999, Seckeler and Hoke 2011) . Around 92% of cases occur under the age of 18 years (Carapetis, *et al* 2005a, Stollerman 2001a).

The main clinical manifestations of ARF are carditis and arthritis, less commonly there is chorea and rarely subcutaneous nodules and erythema marginatum (revised Duckett Jones criteria update 1992) (Ferrieri 2002). Most manifestations of the disease are transient and leave no residua except rheumatic carditis, the most severe complication of ARF, can lead to valvular scarring with permanent sequelae in 35 to 79% of cases (Padmavati 2001, Ravisha, *et al* 2003, Saxena 2002).

Certain reports in developed countries documented that protein energy malnutrition (PEM) was likely to be associated with ARF(Wahab 1986, Zaman, *et al* 1998b). Although PEM and ARF still remain high in low resource settings, there is insufficient data on this association. Children with PEM in early life are among the high risk group who develop ARF (Tahernia, *et al* 1971, Wilcox and Galloway 1954). This may be explained by the fact that PEM may modify the immunologic system and the immune response to GAS infection(Zaman, *et al* 1998b) . This possibly leads to a state of immune hypersensitiveness to a specific GAS cell-wall antigen (Zabriskie 1985). Children with PEM exposed to rheumatogenic strains of GAS particularly with a

genetic susceptibility could have a greater likelihood to develop an attack of ARF (Seckeler and Hoke 2011).

Protein and iron deficiencies during childhood are common and cause PEM in several underdeveloped and developing countries which is also documented in ARF (Steer, *et al* 2009d, Zaman, *et al* 1997). A few studies have reported that poor nutrition and low egg consumption and soybean oil may be related to ARF risk (Zaman, *et al* 1998b). It is suggested that it is not the protein content but the alcohol-soluble material, choline, found in abundance in the egg yolk functions in the synthesis of serum phospholipid, which is an active component of the natural serum inhibitor of GAS haemolysin, streptolysin (Coburn 1950). Both egg yolk and soybean oil contain an anti-inflammatory compound, A/-(2-hydroxyethyl) palmitamide that is assumed to suppress the hyper-responsiveness of susceptible subjects and maturation of the rheumatic process (Swift 1931).

A study was conducted on the effect of low thiamine, riboflavin, ascorbic acid and vitamins A and D with ARF which reported that low vitamin A consumption was associated with ARF. Another study did not support the association of alpha-tocopherol and beta-carotene with ARF (Zaman and Yoshiike 2000). Patients with ARF were reported to have low albumin stores compared to healthy subjects. Iron deficiency may predispose to repeated GAS infections which may favour ARF (Zaman, *et al* 1998c).In ARF, the lesion in affected organs contain T cells, and PEM favours T cell infiltration of damaged tissues mediating imbalance in the immunologic functions (Guilherme and Kalil 2010). Furthermore PEM leads to lymphopenia, thymic atrophy and altered cell-mediated and antibody responses that may predispose to ARF (Zaman and Yoshiike 2000). These findings are inconclusive and require further quantitative and qualitative specific dietary assessment studies.

Recently, the balance of cytokine production has been implicated as being a key coordinator in the development and progression of inflammatory disease activity in patients with ARF. In this study, the role of individual cytokines will be reviewed as an initial analytic step to evaluate the mechanisms that favour the development of autoimmune disease among rheumatic patients (Chapter7).

1.3 Rheumatic heart disease

Rheumatic heart disease (RHD) is the most serious complication of ARF which develops 4–8 weeks (or later) after GAS infection in 30–45% of individuals (Chen, *et al* 2003, Oli and Asmera 2004) . There is increasing evidence to indicate that patients with ARF may be genetically programmed to respond abnormally to GAS infections (Ralph, *et al* 2006, Roodpeyma, *et al* 2005, Smoot, *et al* 2002a).

ARF represents an autoimmune response that occurs in individuals who have untreated infection with rheumatogenic strains of GAS pharyngitis and who are genetically and environmentally predisposed (Guilherme and Kalil 2010, Kaplan 1985). Patients with ARF have an abnormal immunological response at both humoral and cellular level to GAS antigens which are cross reactive with human tissues (Guilherme and Kalil 2010, Kaplan 1980). It is this abnormal immune response on the part of the host that is genetically programmed (Bryant, *et al* 2009, Kurahara, *et al* 2002, Zabriskie 1985). Antigenic mimicry between GAS antigens, mainly M protein epitopes and heart proteins such as myosin and laminin, is proposed to be the triggering factor causing autoimmunity in patients with a genetic predisposition to ARF (Guilherme, *et al* 2006). It is also likely that the genetic background directs the immune response towards either a predominantly Th1 or Th2 pattern .

The aggregation of cases in families, similar disease patterns between siblings, identical twins and HLA correlation studies provide evidence for a genetic influence on ARF susceptibility (Chang 2011, Wilson and Schweitzer 1954, Zabriskie and Tabaqchali 1983). Twin studies, for example, demonstrate estimated heritability across studies of 60% (Engel, Stander et al. 2011).An association with different HLA class II antigen related loci (HLA-DR, DQ) as markers for risk of ARF and RHD but with different alleles has been demonstrated in several populations(Haydardedeo□lu, *et al* 2006). It is likely that the HLA-DR antigen may be responsible to mediation of this abnormal immunological response in ARF. Other new genes related to immune regulation are currently being investigated for understanding how they act in the complex network of autoimmune reactions that occur in ARF/RHD(Guilherme, *et al* 2011, Örün, *et al* 2012). Although there is controversy regarding an inheritance mode, several reports have

provided evidence that ARF occurs in genetically predisposed individuals (Engel, *et al* 2011, Lee, *et al* 2009)

Molecular antigenic mimicry between GAS antigens, mainly M protein epitopes and heart components has been proposed as the triggering factor leading to autoimmunity in susceptible individuals (Stanevicha, *et al* 2003). Known contributing factors include substandard living conditions with poverty, overcrowding, malnutrition, and low government health financial support. These effects are long-lasting producing a growing population of patients with chronic RHD, whose lifestyle and employment are severely affected (Chockalingam, *et al* 2003, Schaffer, *et al* 2003).

RHD is a major public health problem and is the leading cause of acquired cardiovascular morbidity and mortality in children and young adults in developing countries (Chen and Zhang 1981, Chockalingam, *et al* 2004, Supino, *et al* 2002). In low and low-middle income countries the prevalence of RHD ranges from 1.0 to 10 per 1000. The World Health Organisation estimates that approximately 12 million people are affected by RHD/RF globally per year, resulting in about 40 000 deaths annually (WHO 2004). ARF with RHD is the major cause of cardiac admissions, 40% to the medical centres, and the primary cardiac surgical indication in underdeveloped countries, in its acute, recurrent or chronic form. It still remains a real threat to the disadvantaged population as it did decades ago (Cilliers 2006).

Besides the enormous burden of medical and surgical costs, recurrent ARF and RHD cause severe hardship to patients and their families, with repeated hospitalisations, disability, and premature death (Clur 2006, Folomeeva, *et al* 2003, Steer, *et al* 2002).In Yemen RHD is a significant cause of acquired heart disease in children with a reported prevalence of 3.6/1000 school children in Sana'a (Al-Munibari, *et al* 2001). Symptomatic RHD patients are usually detected through the paediatric clinics and hospitals in different districts of the country. This will be the first study to assess the prevalence of RHD in primary school children in Aden city using echocardiography (Chapter 3).

Although some progress has been made in the understanding of the autoimmune pathogenesis in ARF and RHD, there is still much to be elucidated about the disease

process (Dinkla, *et al* 2003, Zhimin, *et al* 2006). Disease susceptibility factors, the major histocompatibility antigens (HLA) are under investigation as potential risk determinants for the disease. The genes of the HLA complex are attractive candidates likely to play a role in the genetic susceptibility to RHD and appear to be related to racial and ethnic origin (Stanevicha, *et al* 2003, Weisz, *et al* 2004).

The determination of a genetic pattern of susceptibility to ARF and RHD has been sought for more than a century and has often been debated (Wilson and Morton 1937). A familial incidence and a single recessive gene suggested that genetic factors play a role in ARF susceptibility (Wilson, *et al* 1943). Some have assumed an autosomal recessive model and a Mendelian inheritance (Davis 1970, Winter 1972). Recent studies have uncovered specific markers such as B-cell alloantigen D8/17 (Kaur, *et al* 1998, Rodriguez, *et al* 1990). Subsequent studies of HLA class II antigens have disclosed an association with different HLA-DR alleles according to the population analyzed (Ayoub, *et al* 1986). A molecular study of TNF-α documented its association with a gene having inflammatory function located in the MHC class II regions (Ramasawmy, *et al* 2007). A key role for TNF- α in immunopathogenesis of ARF/RHD has been proposed (Guilherme, *et al* 2007).

The association of HLA with ARF and RHD was to be explored in Yemeni children but it was not conducted due to the passing away of one my principal supervisors during the clinical laboratory analysis of this research and the limitations of funding.The association of toxins of GAS strains with pharyngotonsillitis and ARF as well as the genetic susceptibility in the causation of RHD is an area of worldwide research (Steer, *et al* 2009b). The purpose of this study is to determine the distribution of genes coding for toxins, relate their frequency to the M antigen protein genotypes inGAS and characterize an association between ARF/RHD and cytokines. The pathogenesis of ARF and RHD includes a network of events with a complex immunological, pathological and clinical process. It is ironic that an innocuous "sore throat" should extract such a high price from the host and their family (WHO 2004). This study hopes to provide a further small step in elucidating the pathogenesis of this complex immunological disease.

Objectives

Objective: 1

To determine the prevalence of rheumatic heart disease among primary school children in Aden

Objective: 2

To determine the prevalence of Group A β haemolytic Streptococcal infectionamong children with acute pharyngotonsillitis

Objective: 3

To determine the prevalence of Non-Group A β haemolytic Streptococcal infection among children with acute pharyngotonsillitis

Objective: 4

To determine the prevalence of emm serotypes, exotoxin gene and serum opacity factor gene among patients diagnosed with Group A β haemolytic Streptococcal and Non-Group A β haemolytic Streptococcal GAS and SNA pharyngotonsillitis

Objective: 5

To analyse the antimicrobial susceptibility pattern and genetic diversity among patients with Group A β haemolytic Streptococcal and Non-Group A β haemolytic Streptococcal pharyngotonsillitis

Objective: 6

To compare the concentrations of selected cytokines and chemokines in Yemeni children with acute rheumatic fever and recurrent rheumatic fever.

Chapter 2

2 Methodology

2.1 Introduction

This research was conducted in Aden City, Republic of Yemen, implemented in four phases. The methodology undertaken for each objective will be discussed separately within the respective section on patients and methods.

2.1.1 Study location

Yemen is located in the south western corner of the Arabian Peninsula occupying an area of over half a million square kilometres. It is bordered by the Kingdom of Saudi Arabia to the north, the Arabian Sea and Gulf of Aden to the south, the Sultanate of Oman in the east, and the Red Sea to the west. The Bab Al Mandab strait lies off the south-western tip of the Republic, and Mayoun, a Yemeni island in the middle of the strait, controls the passage into and out of the Red Sea. There are over 112 Yemeni Islands in the Red Sea and the Arabian Sea. Yemen can be divided geographically into five major areas: Mountainous area, Coastal area, Plateau area, Desert (Al-Ruba Al-Khali) area, and Yemeni islands.

Yemen has a population of 21,069,869 as of the year 2005 in more than 122,000 settlements and villages. Administratively, the country is divided into 22 governorates which are further divided into 333 districts, each of around 45,000 inhabitants. Yemeni society is labelled as traditional, with the agricultural sector absorbing about half of the total work force (MOPIC 2005).

The health care system in Yemen consists of a large public sector along with a sizable private sector. Public health care is organized in three levels: primary health care (PHC) supported by secondary and tertiary referral care. PHC focuses on preventive and preventive health programs (immunization, MCH and family planning, health education, etc) and provides first aid and curative care. It starts at the village level where PHC units are run by paramedical staff. The units are backed up by PHC centres, often managed by a physician and have laboratory and X-ray facilities.

The recommended course of the national Expanded Programme on Immunization [EPI] is described below.

Table 2. 1 Schedule for Childhood Routine Immunizations, Yemen

Antigen (or nutritional supplement)	Description	Schedule
BCG	Bacille Calmette-Guérin vaccine	Birth
DT	Tetanus and diphtheria toxoid childrens' dose	6, 10, 14 weeks
DTwPHibHep	Diphtheria and tetanus toxoid with whole cell pertussis, Hib and HepB vaccine	6, 10, 14 weeks
HepB	Hepatitis B vaccine	6, 10, 14 weeks
Measles	Measles vaccine	9, 18 months
OPV	Oral polio vaccine	birth; 6, 10, 14 weeks
Pneumo_conj	Pneumococcal conjugate vaccine	6, 10, 14 weeks
TT	Tetanus toxoid	1st contact; +1, +6 months; +1, +1 year
VitaminA	Vitamin A supplementation	9, 18 months

Source: Ministry of Public Health and Population of Yemen. Health Partners Reform *plus* WHO/Yemen, WHO Vaccine-preventable diseases: monitoring system 2011

Patients who cannot be properly cared for at the PHC level are referred to district or governorate hospitals (secondary care) for further diagnostic and curative treatment. Some of these hospitals also support national or regional immunization and disease control programs. Finally, tertiary hospitals provide specialized care and serve as teaching hospitals for the medical faculties of the country's universities.

The average number of persons per family is 7.1 and the average number of persons per dwelling is 6.9 persons, while the average number of persons per sleeping room is 4.0.The under-15 age group represents 46.3% of the population with a rapid annual growth of 3.5%. The total fertility rate is 6.2% birth/woman. Around 28 percent of children reaching their first birthday are fully immunized. The percent of low-weight births (less than 2,500 grams) is 19 percent. The crude birth rate is 40 per 1,000 populations and the crude death rate is 12.6 per 1,000 populations. The under five mortality rate is 105 per 1,000 live births. Mortality rate among infants less than a year is (74.8) infant per 1000 births. Infant mortality rates account to 37.3deaths/1,000 live births. The average life expectancy at birth is 62.9 years (MOPIC2006).

Yemen's health sector has been described as being in an early stage of epidemiological and demographic transition, with morbidity and mortality rates from communicable diseases dominating those from non-communicable diseases. Diarrhoeal diseases, malnutrition, acute respiratory infections, tuberculosis, malaria and pregnancy related complications are among the most prevalent health problems. Chronic diseases such as diabetes and hypertension have started to become more prevalent in Yemen.

About half of children under five suffer from malnutrition, 50 percent are stunted and 13 percent show signs of wasting. Among children, diarrhoeal diseases and acute respiratory infections are the major causes of morbidity and mortality. Furthermore, one-third of all deaths among the under-fives occur because of vaccine preventable diseases. An estimated 12% of children suffer from disabilities, and there are few specialized facilities for disabled children.

This study was based in Aden, the economical capital and second largest city of a United Yemen. Aden was a British territory from 1839; it became part of independent South Yemen in 1967 and was the capital of South Yemen until 1990. Mid-way between Europe and the Far East, Aden lies on amajor world trading route through the Suez Canal, located at the south west tip of Yemen and the Arab peninsula; situated 346 km south of the capital city Sana'a, and 160 km east of Strait of Bab Al-Mandab.

Geographically Aden is situated at latitude 12.47° north and at longitude 44.57° east. Aden has no defined climatic season except for two named weathers, a cool winter and a hot humid summer. The area of Aden Governorate is 8,321 square kilometres and the population is 590,000 inhabitants (MOPIC 2005). It is one of the largest natural harbours in the world with an area of about 70 km^2 of sheltered water surrounded by Shamsan Mountain, Khormakser, and the shore which extends to the hills of Little Aden. The port of Aden consists of the Inner Harbour, the Oil Harbour serving Aden Refinery and the anchorage and approach channels of the outer harbour.

In the health field, Aden has 8 districts provided with 5 central hospitals with a capacity of more than 1337 beds, 12 health complexes and 25 health facilities in which 799 doctors, 1127 nurses, 57 assistant doctors, 83 pharmacists and 138 dentists work. The private sector has proved to be efficient in providing the public with health services

through 414 health institutions, 27 opticians, 8 dental workshops, 8 X-ray centres, 78 laboratories, 88 general doctor offices, 180 specialized doctor offices, 9 medical centres, 13 clinics and 3 hospitals, with a capacity of 402 beds. The total capacity reached, therefore, 1739 beds. In Yemen, there is 1 physician for 4,650, 1 nurse for 2,913 and only 1 trained midwife for 14,465 populations. Together Aden and Sana'a lay claim to 50% of all physicians.

2.2 Objectives: 1 Prevalence of rheumatic heart disease

To determine the prevalence of rheumatic heart disease among primary school children in Aden city

2.2.1 Study site and design

Aden governorate has eight districts with 59 primary public schools from first to ninth primary class of whom 47 683 were males and 40 952 females. This was a descriptive cross-sectional case-finding survey of school children with RHD.

The target population were school children in established governmental primary schools of Aden city segregated by gender and selected by clusters through multistage sampling with probability proportionate to size (PPS). The schools were selected proportionately by stratified random sampling by school size and sex to include 6 schools from each of the centre and slum areas that give a total of 12 schools with an estimate number of 6000 students.

The inclusion criteria were age group 5 – 16 years, both sexes, Yemeni nationality. Each student in the selected school population had an equal independent chance of being recruited and selected in the study.

2.2.2 Sample size

Based on a study done by Al-Munibari et al(Al-Munibari, *et al* 2001)the prevalence of RHD in Yemeni school children was 3.6/1000, taking the CI of 95%, power 80%, and a precision of +10% then the number of students was calculated. Epi-Info 2002 (Statcalc) was used to estimate the required sample size of 6000 students with a maximum sampling error of ±1.3%.

Table 2. 2The Prevalence of RHD among school children was reviewed in different neighbouring countries

Country	Study Year	Prevalence RHD/1000	Sample size
Kanpur. India	2000	4.54	3963
Sanaur. Indi	1998	3.6	5000
Egypt	1998	9.8	4700
Nepal, India	1997	1.2	4736
Kinshasa, Zaire	1996	14.03	4848
Kenya	1996	2.7	1115
Saudi Arabia	1990	2.4	9418
Ethiopia	1990	4.6	3235
Sudan	1986	3.0	13322
Nigeria	1978	0.08	12755

Source: (WHO 2004)

2.2.3 Informed consent and confidentiality

Permission to conduct the study was obtained from the Ethics Review Committee of Aden. The importance and objectives of the screening program was discussed with the General Education Department, Aden governorate and the Primary School Health Director Officer. The District Education Officers (DEO) for government schools, and head teachers of each school were subsequently informed. It was then discussed with the health and social workers in the selected schools who discussed the study with the parent schools committees and relevant parents. Informed consent was obtained in writing from parents willing to allow their children to undergo the clinical screening, blood investigations and echocardiography if found to have an abnormal heart.Teachers or school administrators had no role in the selection of children.

2.2.4 Method of examination

The investigator performed the cardiac examination on all recruited students and those with organic murmurs were documented and given an appointment with a paediatric cardiologist in the central paediatric hospital for echocardiography. If an organic heart disorder was detected e.g. congenital heart disease or RHD they were followed up by the paediatric cardiology service. The features of an organic murmur are in Table.2.2.

The children were investigated with a complete blood cell count with antistreptolysin titres (ASO) and C-reactive protein (CRP) followed by the echocardiography

Table 2. 3Differentiating non-organic from organic/pathological heart murmurs

Characteristics	Non-organic murmur	Organic/pathological murmur
History and physical	Asymptomatic	Symptoms and signs of cardiac disease
Timing	Systolic ejection murmur, (except venous hum)	All diastolic, pansystolic or continuous
Grade	$\leq 3/6$	$> 3/6$ (palpable thrill)
Splitting	Physiologic S2	Fixed splitting or single S2
Extra sounds/clicks	None	Present
Change of position	Murmur varies	Unchanged

Modified from P12 – Pediatrics MCCQE 2002 Review Notes

2.2.5 Data collection

The students with echo proven RHD were interviewed through an open questionnaire by the investigator including information on demographic data, socio-economic factors, family history of ARF or RHD and previous history of ARF fulfilling the Duckett Jones criteria (Table3.1).

2.3 Objective 2: Prevalence of GAS infections in pharyngotonsillitis

Determine the prevalence of GAS infection among symptomatic Yemeni children with pharyngotonsillitis.

2.3.1 Study site, design and strategy

This cross-sectional descriptive survey was initially intended to be undertaken among children with acute pharyngotonsillitis in primary public health care polyclinics in Aden City. However, it became apparent that it was difficult to recruit the required number of

patients because a high proportion of patients attending the polyclinics were already receiving antibiotic treatment, which was an exclusion criterion.

It was thus decided that children could also be selected from schools if they had acute pharyngotonsillitis and before receiving antibiotic treatment at the polyclinics or pharmacies.

2.3.2 Participants

All Yemeni children with acute pharyngotonsillitis attending public polyclinics and primary elementary schools during the study period that fulfilled the inclusion criteria of age 1– 16years, both sexes, symptoms of sore throat with evidence of fever, tonsillar swelling and exudates and tender anterior cervical adenitis had an equal independent chance of being selected after obtaining a verbal consent from the parents through the health social worker at school.

Patients with coryza, cough, conjunctivitis and use of antibiotics in the two-week period prior to onset of current illness were excluded from the study.

2.3.3 Sampling

A sample size of 730 children was based on published prevalence of GAS from the neighbouring countries of 24.3% with similar climatic conditions (Table 2.4). Sample size was calculated through Epi-Info 2002 (statcalc) with a CI of 95%, power 80%, and a precision degree of ± 10% and a maximum sampling error of ±3.6%.

Table 2. 4 Presence of GAS in children with symptomatic pharyngotonsillitis

Year	City/country	Patients with pharyngotonsillitis	GAS positive (%)[a]
1997	Netherlands	558	33.0 and 75.0b
1995	Northern India	910	13.5
1995	Souise Tunisia	474	17.7
1993	Cairo, Egypt	451	24.0
1992	Zagreb, Croatia	629	44.7
1992	Creteil, France	307	36.8
1987	Havana, Cuba	480	25.0 and 34.5b
1980s	Rhode Island USA	8668	24.3

[a] GAS positive = patients positive for GAS[b] Patients with clinical features of GAS (fever, tonsillar exudates, anterior cervical lymhpadenopathy) Source: (WHO 2000)

2.3.4 Specific Methods

2.3.4.1 Collection of throat swab

Two sterile throat swabs were collected by rubbing sterile cotton tipped swabs over the tonsillar area on the eligible child without touching the tongue or lips.

2.3.4.2 First swab

The first swab was tested by Rapid Antigen Detection Test (RADT)using a Reveal Color Strept A Latex agglutination test (Murex Company), which detected GAS antigen in pharyngeal swabs. The swab was used for evaluation with Group A Strept Test kit performed according to the manufacturer instructions. The results of the test was obtained in 10 – 15 minutes and read as positive, negative or non-specific.

2.3.4.3 Second swab

The second swab was rolled on a Blood Agar plate containing 5% sheep blood. The blood plates were prepared from Trypticase Soy Agar with 5% sheep blood agar (Bellon et al 1991) that contained tryptone 15g, phytone/soytone 5g, sodium chloride 5g, agar 15g and distilled water 1 litre. These ingredients were exposed to heat to dissolve agar, then autoclaved for 15 min at 121°C and then cooled to 50°C. Then 50 ml of sheep blood was added to the melted agar, after which it was mixed and poured into sterile 15 x 100 mm petri dishes with a final pH base of 7.3 ± 0.2 (BAM Media M20: Blood Agar, January 2001). When the throat swab reached the laboratory, it was inoculated on the prepared sheep blood agar plate.

The inoculum was further distributed by streaking it with a loop to obtain isolated colonies. The plates were then incubated at 35°C under anaerobic conditions for 24 to 48 hours and checked for GAS. Potential GAS was checked by Gram stain, catalase (negative) and subcultures on to sheep blood agar for a bacitracin test. A bacitracin disc was added to a subculture of the initial isolate and cultures that gave a large zone around bacitracin disk > 15 mm inhibition in diameter were considered Lancefield

group A streptococci (GAS). The patients were treated with penicillin if positive for GAS infection with either RADTs or culture.

A glycerol nutrient broth was prepared to preserve GAS isolates in the cryobeads. Glycerol broth was prepared from a formula preparations of nutrient broth 84ml and glycerol 16ml that was mixed well and dispensed in 5ml amounts inscrew cap tubes (Cheesebrough M 1989). Those confirmed as having isolates of GAS by RADT, gram stain and culture had colonies stored in glycerol nutrient broth and cryobeads in freezers at –70°C and then later transported to Department of Microbiology at University of Liverpool. Pulsed field gel electrophoresis (PFGE) and Polymerase Chain Reaction (PCR) techniques were later undertaken which involved DNA isolation and PCR amplification of the toxin genes.

2.3.5 Statistical analysis

Data were entered into computer database SPSS Version 17 for windows (2008 SPSS Inc. Chicago, USA). Statistical analysis included quantitative descriptive analysis and summary statistics (mean, percentages, and standard deviations) for describing the prevalence of GAS. Quantitative analysis of the studied variables included 95% CI, Chi squares and rates stratified by gender, age and other variables. Univariate analysis based on "odds ratio" (prevalence ratio was denominated OR) and 95% CI and p values <0.05. Analyses was further based on chi-square tests, exact Fisher tests followed by logistic regression analysis for categorical variables and evaluation of the relationship between different symptoms and signs of GAS infections in the study population. Sensitivity and specificity was calculated for each cut off point and ROC drawn to find the optimal cut off point for highest sensitivity and specificity.

2.4 Objective: 3 Prevalence of SNA pharyngotonsillitis

Determine the prevalence of SNA among children with acute pharyngotonsillitis

2.4.1 Study site, design and strategy

This cross-sectional descriptive survey was undertaken among children with acute pharyngotonsillitis in Aden city similar to that described in objective 2.

2.4.2 Study subjects

The participants were the same patients described in objective 2.

2.4.3 Sampling

The numbers of patients included in this objective were recruited from the participants of objective 2

2.4.4 Collection of throat swab

The methodological process for the two throat swab collections followed the same procedures as described in objective 2. The positive culture sheep blood agar plates were subcultured on another plate with the addition of bacitracin. Bacitracin sensitive β-haemolytic streptococci that were isolated from patients with a negative RADT were subsequently tested for the Lancefield group by the latex agglutination test, the Oxoid streptococcal grouping kit, used for the identification of the streptococcal group (Diagnostic Reagents Streptococcal Grouping Kit Code: DR0585).

2.4.5 Diagnostic Reagents Streptococcal Grouping Kit Code: DR0585

The Oxoid Streptococcal Grouping Kit, (Oxoid Basinpotche, UK) a latex agglutination test was used for the identification of the streptococcal group and reagents were provided for groups A, B, C, D, F and G.Each latex reagent and the positive control reagent contained 0.1% sodium azide. After reconstitution the extraction enzyme solution contained 0.01% thiomersal.

Table 2. 5Components of the Oxoid Streptococcal Grouping Kit

Kit	Group
DR0586	Latex Group Reagent A
DR0587	Latex Group Reagent B
DR0588	Latex Group Reagent C
DR0589	Latex Group Reagent D
DR0590	Latex Group Reagent F
DR0591	Latex Group Reagent G
DR0592	Polyvalent Positive Control
DR0593	Extraction Enzyme
DR0500	Disposable reaction cards

2.4.6 Preparations of cultures samples for identification

The streptococci were recultured from the cryobeads and grown on a blood agar plate overnight at 37°C under anerobic condition. The haemolytic reaction of suspect colonies was noted. The bottle of Oxoid Streptococcus Extraction Enzyme (DR0593) was reconstituted with sterile distilled water to the amount shown on the label. The eppendorf tubes were labelled appropriately and 0.4 ml of enzyme was dispensed into each tube. Then 2-5 test colonies equivalent to 2-3 mm of growth were selected with a bacteriological loop and emulsified in the enzyme preparation.

2.4.7 Diagnostic reagent streptococcal kit test method

The latex reagents were brought to room temperature by warming the bottles by hand. The latex suspensions were mixed by vigorous shaking. Any latex from the dropper pipette was expelled for complete mixing. Then 1 drop from each latex reagent was dispensed into the circular rings on the reaction card (DR0500). Using a Pasteur pipette, 1 drop of extract was added to each of the 6 rings. With the mixing sticks provided, the mixture was spread over the entire area of the ring using a separate stick for each ring. Each card was gently rocked. The rings were examined for any agglutination reaction that would normally take place within 30 seconds. The positive control was used to check performance of latex reagents. Then the reaction card was safely disposed into a suitable disinfectant.

2.4.8 Interpretation of Results

The test was considered positive when agglutination occurred with one grouping reagent or when one grouping reagent gave a substantially stronger reaction than the other five. The test was considered negative when no agglutination occurred.

2.4.9 **Statistical analysis**

Data were entered into computer database SPSS Version 17 for windows (2008 SPSS Inc. Chicago, USA). Statistical analysis included quantitative descriptive analysis and summary statistics (mean, percentages, and standard deviations) for describing the prevalence of SNA. Univariate analysis based on "odds ratio" and p values <0.05. Analyses was further based on chi-square tests, exact Fisher tests followed by logistic

regression analysis for categorical variables and evaluation of the relationship between different symptoms and signs of SNA infections in the study population.

2.5 Objective 4: Prevalence of emm genotypes, exotoxin superantigen superantigen and sof genes

Determine the prevalence of *emm* genotypes, exotoxin genes and serum opacity factor genes among patients diagnosed with GAS and SNA pharyngotonsillitis in Aden, Yemen.

2.5.1 Sample size and sampling strategy

The required numbers of patients were recruited from objective 2 and objective 3.Objectives 2 and 3 aimed to establish prevalence of these pathogens in symptomatic children. In retrospect it would have been useful to include a group of asymptomatic children to document the carriage in apparently healthy children. (Nandi *et al*, 2002, Durmaz *et al,* 2003). This would allow estimation of prevalence in the general population and broaden interpretation of the findings. This is thus a limitation of the study. Selection of GAS and SNA isolates for genotyping was restricted by logistic constraints. The investigator only had resources to sequence 34 isolates. All SNA isolates were considered to be of special interest and were included. Available funding for genotyping the GAS samples, after inclusion of SNA isolates, allowed the selection of 21 samples at random.

2.5.2 Methods for Pulsed Field Gel Electrophoresis (PFGE) of GAS

Day 1 The organisms were plated onto blood agar and incubated at 37°C overnight.

Day 2 A single, well isolated colony was picked and suspended in 110µl cold, cell suspension buffer .The cell suspension buffer comprises: 10mM tris, 20mM sodium chloride, 50mM EDTA (pH 7.2) in a 1.5ml eppendorf tube, to a McFarland Standard of **"1"**. We used the small gel frame for the PFGE tank when running fewer than 10 samples. This needed 100mls of gel and a 10 slot comb. On this gel we would usually run the marker, a control strain if we had one, and 8 samples(9 samples for 100ml /10 comb gel). The large gel frame needed 150mls of gel and takes a 15 slot comb on which

we could run 13 samples. The marker we used for both was from Sigma: D2416 Pulsed Marker 50-1000kb.

Samples were then centrifuged at 13k for 1 minute. The supernatant was aspirated using a fine tipped pipette and discarded. The resulting pellet was re-suspended in a further 110µl of cold, cell suspension buffer. The tube was maintained at 60°C in a water bath for the next stage. 4µl lysozyme solution was added on the side wall of each tube. 10mls of 2% pulsed field grade agarose in single distilled water was prepared in a microwave and when melted, the agarose was placed in water bath to keep it molten. Keeping the samples at 60^0C, lysozyme droplet was shaken down into buffer of first tube, 110ul of molten agarose was added, and contents mixed thoroughly and quickly pipetted into 2 labeled plug moulds. Once all plugs were filled they were left at 4^0C for 15 minutes to set. The two plugs for each sample were placed into individual plastic universal bottles with 1ml Lysozyme buffer plus 40ul Lysozyme and incubated for 1 hour at 37^0C in a water bath. Lysozyme solution was aspirated and the plugs rinsed with 1.5ml of 1x washing buffer for 10 mins. The wash buffer was removed and replaced with 1ml of Proteinase K buffer containing 40ul of Proteinase K (25mg/ml). The samples were left to deproteinate at 50^0C in a water bath overnight.

Day 3 Next day Proteinase K solution was carefully aspirated and samples washed with gentle agitation, in 1.5ml of 1x washing buffer for 30-60 minutes at RT. The wash buffer was discarded and replaced with 1.0ml of 1x wash buffer containing 20ul of 1.7% PMSF. Samples were gently agitated for 30 minutes at RT. After carefully discarding PMSF solution, plugs were washed 3 times, for 30 min each, in 1ml of 1x wash buffer. After the last wash, one of the plugs was removed from 1x wash buffer, placed in 500µl of **0.1x** wash buffer in eppendorf tube and agitated at RT for 30-60 minutes to eliminate EDTA salts (the other was stored at 4°C). The wash buffer was removed and replaced with 500µl 1x *smaI* reaction buffer ("React2" 10x buffer diluted 1:10 in sdw). The samples were agitated for a further 30-60 minutes at RT before buffer was removed and replaced with 300µl of fresh 1x *smaI* reaction buffer containing 4 µl of*smaI* enzyme. The tubes were incubated at 37^0C overnight in a water bath (the tubes may be stored at 4^0C after digestion - for a maximum of 4 days).

Day 4 A 1% w/v pulse field grade agarose gel was prepared (1g of agarose to 100ml of 0.5x TBE buffer for 100 ml gel (10 well)). After cooling slightly the agarose was poured into a combed PFGE tray. The gel was left to solidify for 30 minutes at RT before removing the comb. Each plug was processed individually by tipping into a Petri dish and cut in half lengthways with a scalpel. One half plug was placed into each well; final well had a sliver of pulse field marker (50-1000kB). The wells were sealed with 1% agarose and allowed to set for 10 min. The remaining half plugs were stored at 4°C, in digestion solution, until results were obtained.The genomic DNA in the plugs was incubated with 24 U of *Sma*I (Promega Corporation, Madison, Wis.) for 24 h at 25°C in a water bath.

2.5.2.1 PFGE interpretation

PFGE of the agarose plug inserts was then performed on a CHEF-DR III system (Bio-Rad Laboratories, Hercules, CA, USA) on horizontal 1% agarose gel for 20h at 6v /120° angle, with a switch time of (initial & final) 3 to 30 s at 14 °C. The gel was stained with ethidium bromide (0.5µg ml^{-1} in water) for 45 – 60 mins and photographed on a UV transilluminator. Isolates differing in PFGE fragment patterns, by one or two bands, were regarded as closely related; minor mutational changes would result in such patterns according to Tenover criteria (Tenover, *et al* 1995) (Table 6.1).

Isolates are considered genetically indistinguishable if their restriction patterns have the same numbers of bands and the corresponding bands are of the same apparent size. These isolates are considered to represent the same strain as the common outbreak strain. If the PFGE pattern of the isolate differs from the outbreak pattern by changes consistent with a single genetic event, i.e., a point mutation or an insertion or deletion of DNA; with changes resulting in two to three band differences, then these are considered to be closely related to the outbreak strain. If the PGEF patterns differ from the outbreak pattern by changes consistent with two independent genetic events i.e., four to six band differences, they are considered possibly related. If an isolate differs from the outbreak pattern with three or more independent genetic events (generally seven or more band differences), then it is considered an unrelated strain.

The control strain was processed along with the unknown isolates tested to confirm that the process of cell lysis, washing, and endonuclease digestion steps was working, the

gel electrophoretic conditions was correct and the results reproducible from run to run within the laboratory were similar to those reported by others for the same strain. A molecular size standard must be run in at least one lane of the gel to provide size orientations of the fragments. Standards are needed to evaluate minor profile differences that may result from single genetic events such as deletions, insertions, or mutations. Preparations consisting of phage lambda concatamers, as a ''lambda ladder,'' were used as molecular size standards with an enhanced 48-kb band, helpful for size orientation in the gel. Thus the presence of one of these strains in each group of isolates to be tested provides both a procedure control and a molecular size standard.

Selected strains were typed by PFGE of chromosomal digests using *Sma*I. Isolates differing by only 1±6 bands from a common reference strain for each group were assigned a common type. More than six bands of difference from subtype 1 of each type were considered unrelated isolates and assigned a different PFGE type (Haukness, *et al* 2002, Tenover, *et al* 1995).

2.5.3 Isolation of chromosomal DNA

GAS chromosomal DNA was isolated using the PureGene DNA purification kit (Gentra Systems, Minneapolis, MN). The protocol supplied by the manufacturer was used with the following modifications: with addition of mutanolysin lysozyme (Sigma-Aldrich, St. Louis, MO),

2.5.3.1 Analysis of the *emm* gene

In the late twentieth century with advances in DNA-sequencing technology, a method was developed to determine the M GAS type from the sequence of the gene encoding M protein, the *emm* gene (Beall, *et al* 1997). The *emm* gene of GAS is the gene that encodes the M protein, which forms the basis of a serological typing scheme. A method has been developed to determine the M GAS type from the sequence of the gene encoding M protein, the *emm* gene (Beall, *et al* 1997). *emm* sequence typing is currently the most widely used method to define GAS strains through sequence analysis of PCR products of the N-terminal hypervariable region of the M protein gene and its excellent typeability and concordance values (Beall, *et al* 1996). It has become a powerful epidemiological tool to study GAS M-type with identification accessible in all

laboratories with DNA-sequencing capability. It has resulted in a dramatic increase in the number of identified M and emm types(Bisno, *et al* 2003, Cunningham 2000).

The *emm* gene was amplified by PCR using primers *emm*1b and *emm*2. PCR was performed in a reaction mixture volume of 25µl under the following conditions: initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min 45 s, and a final extension step at 72°C for 5 min. Size variation in *emm* PCR product was assessed by gel electrophoresis using 1.5% agarose gels. PCR products were purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA) according to manufacturer's protocol. The primers used were primer 1: TAT TCG CTT AGA AAA TTA A and primer 2 GCA AGT TCT TCA GCT TGT TT and Sequencing primer emm seq2 TAT TCG CTT AGA AAA TTA AAA ACA GG. Although primer 1 may also be used for sequencing and these came from the CDC website: www.cdc.gov/ncidod/biotech/strep/protocol_emm-type.htm, Sequencing was done by Macrogen/ Lark at the DNA level.

Purified PCR product was used as template for cycle sequencing with either *emm*1b or *emm*2 primer under the following conditions: 25 cycles of 96°C for 10 s, 50°C for 5s, and 60°C for 4 min. Sequence data were analyzed by BLAST search performed against the Centers for Disease Control and Prevention (CDC) *Streptococcus pyogenes emm* sequence database (http://www.cdc.gov/ncidod/biotech/strep/strepindex.htm). To be identified as a particular *emm* type, the *emm* sequences had to have greater than 98% identity with the *emm* sequence of CDC reference strain. Reference strain sequences are available from CDC at URL: http://www .cdc.gov/ncidod/biotech/strep/*emm*types.htm.

2.5.4 Exotoxin pyrogenic gene phage determination

All strains were cultured on blood agar plates Agar plates were stored at 4°C; DNA lysates were stored at -20°C and bacterial strains were stored at -80°C in the freezer at the Microbiology Laboratory Department, University of Liverpool.

Emm sequence typing was performed as described by Beall *etal* on the CDC web site http://www.cdc.gov/ncidod/biotech/strep/protocol_*emm*type.htm). DNA was extracted with a commercial chelex resin-based DNA extraction kit (InstaGene Matrix 1, Bio-

Rad, France). All *emm* sequences used in this study are available at http :www.cdc.gov.Ncidod biotech.strep.strepindex.html) and were independently obtained in the CDC streptococcal laboratory from CDC reference strains.PCR of GAS isolates was performed according to recommendations of Division of Bacterial and Mycotic Diseases, Centres for Disease Control and Prevention (CDC), *Streptococcus pyogenes emm* sequence database (http://www.cdc.gov/ncidod/biotech/ strep/doc.htm).

The primers used for amplification of GAS at the DNA wereprimer 1 (5' TATTCGCTTAGAAAATTAA 3') and primer 2 (5' GCAAGTTCTTCAGC TTG TTT 3'). PCR products were purified with Wizard columns (Promega, Madison, Wis.) as described by the manufacturer. Primer 1 (F) TATT^GCTTAGAAAATTAA 5' ends of *emmL* genes GCAAGTTCTTCAGCTTGTTT 3' ends of *emmL* genes. The sequence DNA analysis were subjected to homology searches against the bacterial DNA database with GCG software (Wisconsin package, version 8). Sequences for new *emm* gene GenBank entries were obtained from one strand of PCR fragments. PCR fragments using methods and the primers used were described in Table 2.5 (Page 22).

PCR detection of the *sil* locus was performed with two sets of primers: silC-Fand silC-R for the *sil*C while ORF and silD-F and silD-Rfor the *sil*D ORF (Table 2.5) All PCR mixes consisted of a 50-μl volume with 25 μl of 2× QIAGEN Multiple PCR Master Mix (QIAGEN, Courtaboeuf, France), 5 μl of 5x Q-solution, 1 μM each primer and 5 μl of bacterial DNA extract. PCR was performed with a Bio-Rad i-cycler thermal cycler as follows: DNA denaturation and polymerase activation for 15 min at 95°C; 30 cycles of 30 s at 94°C, 90 s at 55°C, and 90 s at 72°C; and a final extension step for 10 min at 72°C. The PCR detected the positive toxin gene profile for the GAS and SNA isolates.

The PCR was used for toxin-gene profiling (*speC*, *spd1* and sdn) using the primers aas shown in Table 2.5 as described by Schmitz *et al*(Schmitz, *et al* 2003). GAS colonies were resuspended in 20 mL of water and boiled for 5 min at 95°C and then centrifuged with brief high-speed. PCRs were setup in a total volume of 50 mL, with 1 mL of cell lysate as a template, 5 mL of 10x PCR buffer, 5 mL of 10xMgC12 (25mmol/L), 2 mL of dNTPs (10 mmol/L each), 1 mL of polymerase (3.5 U/mL; High Fidelity PCR System; Roche), and 1 mL of each oligo (100 pmol/L). PCR was performed by denaturing for 5

min at 96°C, 30 cycles for 50 s at 96°C, annealing for 65 s at 44°C, and elongation for 70 s at 72°C, followed by 5 min at 72°C. The PCR detects the positive toxin gene profile for the studied GAS and SNA isolates.

2.5.5 Serum opacity factor (*sof*) **determination** and sequencing

All strains were cultured on blood agar plates and stored at - 20°C until characterization. *SOF* gene sequence specific typing with PCR was performed using standard protocols Boehringer Mannheim Hi Fidelity system described by Beall et al. 2000 (Beall *et al.*, 1998; CDC web site) http://www.cdc.gov/ncidod/biotech/strep/protocol-*emm* type.htm).

DNA was extracted with a commercial chelex resin-based DNA extraction kit as recommended by the manufacturer (InstaGene Matrix 1, Bio-Rad, France). Conserved primer sets were based upon comparison of the *sof* gene with GenBank accession sequence encoding the mature protein plus 22 residues of signal sequence amplification PCR fragment using methods and primers described in Table 2.6(Beall, *et al* 2000).

2.5.6 Data analysis

The SPSS 17 statistical package was used for all analyses. Chi square or Fisher's exact test was used for statistical analysis. A P value of <0.05 was considered significant.

Table 2. 6Primer sequences used for GA S genotyping and exotoxin genes by PCR emm and streptococcus invasive locus (sil) analysis

Amplicon type	Gene	Primer1	Primer 2	Amplicon size (bp)
Prophage-associated virulence factor				
emm	Emm	GGGAATTCTATTSGCTTAGAAAATTAA	GCAAGTTCTTCAGCTTGTTT	Variable
mf2	spd1	CCCTTCAGGATTGCTGTCAT	ACTGTTGACGCAGCTAGGG	400
Prophage 315.6	Sdn	AACGTTCAACAGGCGCTTAC	ACCCCATCGGAAGATAAAGC	489
Prophage 370.1	Spec	TCTAGTCCCTTCATTTGGTG	GTAAATTTTTCAACGACACA	459
sil	silC	ATATCTCCACCAATCACTTTAAGTA	ACTATAAAGATAAGATACTCAACAGT	189
sil	silD	GATGAAGTTCGTCAAGCTGACT	TCGGCTATAGCGATACGTTTAAT	148
sof	Sof	GTAAAGGATGCTTCACGTTTGTCTCCAG	GAAG/CAAATTGACGAAGGTGCCGATGT	560 -700

2.6 Objective: 5 Analyze the antimicrobial susceptibility pattern

Analyse the antimicrobial susceptibility pattern and genetic diversity among patients with GAS and SNA pharyngotonsillitis

2.6.1 Patients and methods

The susceptibility pattern was determined on a total of 24 culture isolates from patients with acute pharyngotonsillitis of whom 11 GAS isolates were selected from objective 4 and all the13 SNA isolates were collected from objective 5.

2.6.2 Antibiotic disc sensitivity test procedure

Antibiotic disc sensitivity testing using penicillin G (1 unit), amoxicillin (10μg), erythromycin (5 μg), tetracycline (10 μg) and chloramphenicol (10 μg) was conducted on the 24 culture proven GAS and SNA isolates on the isosensitest agar supplemented with 5% defibrinated horse blood. The diameter of the zone of inhibited growth around the antibiotic disk, known as the diameter of zone of inhibition was measured to the nearest millimetre with a ruler and according to the standard Table by the BSAC system the bacteria were classified as sensitive and resistant. The range of concentrations test and interpretation of results was as per the BSAC guidelines on antimicrobial sensitivity testing (Andrews 2001b).

2.6.2.1 Reference for breakpoints

The breakpoint refers to the concentration of antibiotic dividing isolates of GAS as susceptible or resistant to a particular antibiotic. The interpretation of zone diameters in (mm) according to the BSAC standard for the six antibiotics of penicillin G, amoxicillin, erythromycin, chloramphenicol and tetracycline were sensitivity of ≥20mm and a resistance of ≤19mm (Andrews 2007) .

2.6.3 Data analysis

The data was then analyzed by SPSS17.0 software and the level of significance was determined as $p<0.05$.

2.7 Objectives: 6 Compare the levels of selected cytokines and chemokines

Compare the concentrations of selected cytokines and chemokines in Yemeni children with ARF and RRF.

2.7.1 Sample size

The sample size was based on a study on cytokines in ARF which included 27 patients (12 ARF and 15 RHD) (Yegin, *et al* 1997) and another study on 18 patients with RRF (WHO 2004). Cytokines and chemokines were analyzed from a subset of 50 patients analysis in the current study to compare the results from the two groups ARF (Group A), RRF (Group B). A total of 25 children fulfilling the diagnosis of ARF and 25 children fulfilling the diagnosis of RRF were recruited from the patients in the private polyclinics and those admitted in public and private hospitals.

2.7.2 Blood collection for Cytokines

Peripheral blood was taken into sterile 5ml containing tubes, immediately centrifuged at 600g (+4'C) for 10 min, serum collected in tubes and kept frozen at –70°C until transported to Microbiology Department at University of Liverpool for analysis. Commercially available BD™ Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit were used with FCAP Array *sof*tware (Cat. No. 641488)

2.7.3 FCAP Beads assay

Bead assays determined the concentrations for multiple analytes in the given sample In a bead assay, one or more beads with discrete and distinct fluorescence intensities were used to simultaneously detect multiple analytes in a small sample volume. The beads captured and quantified soluble analytes through a sandwich schema. A particular analyte in the sample was bound to a corresponding bead with given fluorescent characteristics. The bead was coated with capture antibodies specific for that analyte. A reporter antibody (different from the capture antibody) bound to the analyte and was conjugated with fluorescent molecules (different color from those used to distinguish beads). Unbound (excess) reporter antibodies and analytes were eliminated by washing. Samples are acquired with a flow cytometer and acquisition *sof*tware capable of saving data in a Flow Cytometry Standard (FCS) 2.0 file format. FCAP Array *sof*tware

analyzes the FCS 2.0 files. Bead populations were found by clustering, and assigning beads to clusters. Reporter antibody florescence intensity within a population measured the analyte-specific binding.

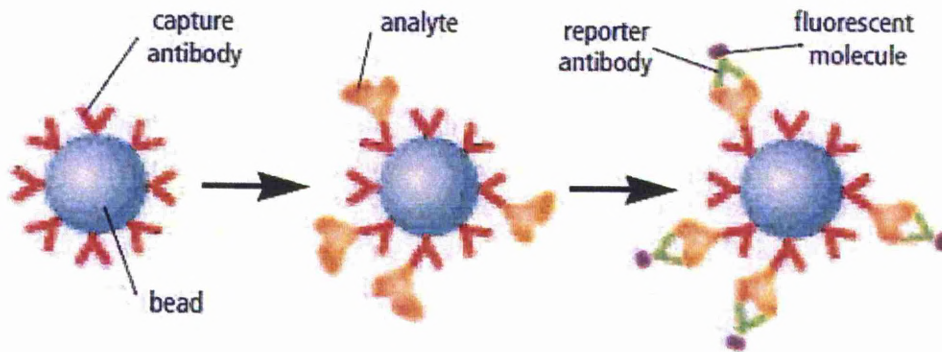


Figure 2. 1 FCAP Bead assays

2.7.3.1 Quantitative analysis

Quantitative analysis was determined by analyte concentrations based on the known concentration values of a set of standards with FCAP Array *soft*ware. The number of concentration levels was used to calibrate the standard curve and number of sample and standard replicates were adjusted. FCAP Array *soft*ware read the FCS 2.0 data files from the experiment, locates clusters (to which analytes have been assigned), and then determined the median fluorescence intensity (MFI) of the detector antibody for each analyte. The software fit a standard curve to the data from the concentration standards. The standard curve was used to calculate concentration values for each measured analytes in each sample bead capture analyte

2.7.3.2 Reagents provided

The BD CBA system uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. Eachbead in a BD CBA Kit provides a capture surface for a specific protein similar to a coated well in an ELISA plate. BD CBA capture bead mixture is in suspension to allow for multiple analytes detection in small volume sample. BD CBA Human Inflammatory Cytokines

Kit was used to quantitatively measure the different cytokines and chemokines in this study Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interleukin-7 (IL-7), Interleukin-8 (IL-8), Interleukin-9 (IL-9), Interleukin-10 (IL-10), Interleukin-12p70 (IL-12p70), Tumor Necrosis Factor (TNF-α), Interferon gamma (IFN-γ), chemokines Monocyte Chemotactic Protein-1(MCP-1), Macrophage Inflammatory Protein-1α (MIP-1α), Macrophage Inflammatory Protein-1β (MIP-1β), Human interferon inducable protein-10 (IP-10) and Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES) protein levels in a single sample. The kit performance has been optimized for analysis of specific proteins in serum samples using the protocol BD™ Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit.

2.7.3.3 Serum Assay Procedure

10 µl/test of each Human Inflammatory Cytokine Capture Bead suspension was mixed and the pellet mixed beads were centrifuged and the supernatant aspirated. It was resuspended in serum enhancement buffer and incubated for 30 min at RT. Then 50 µl mixed beads were transferred to each assay tube. Reconstitute Human Inflammatory Cytokine Standards were reconstituted in assay diluent for 15 min and dilute standards were formed by serial dilutions using assay diluents. The standard dilutions and test samples were added to the appropriate sample tubes (50 µl/tube) and incubated for 1.5 hour at RT. The samples were washed with 1 ml wash buffer and centrifuged with 100 µl residual volume left after aspiration. PE Detection Reagent (50 µl/test) was added to the samples and incubated for another 1.5 hours at RT. The samples were washed with 1 ml wash buffer and centrifuged. Then an additional 300 µl of wash buffer was added to each assay tube and the samples analyzed.

2.7.3.4 Cytometer Setup Bead Procedure

Three 50 µl/tube tubes A, B and C were setup and Cytometer Setup Beads were added to them. This was followed by adding 50 µl of FITC positive control to tube B and 50 µl of PE positive control to tube C and incubated for 1.5 hours at RT. Then 400 µl of wash buffer was added to tubes B and C while 450 µl of wash buffer was added to tube A. The tubes A, B and C were used for cytometer setup. The Human Inflammatory Cytokines Standards were lyophilized and reconstituted and serially diluted 1:1, 1:2 and 1:4 before mixing with the Capture Beads and the PE detection reagent.

2.7.3.5 Preparation of Human Inflammatory Cytokines capture beads for serum sample analysis

The number of assay tubes (including standards and controls) required for the experiment were 49 known and 9 standard dilutions and 1 negative control = 59 assay tubes. Each capture bead suspension was vigorously vortexd for a few seconds before mixing. Thena 10 μl aliquot of each Capture Bead was added for each assay tube to be analyzed, into a single tube labeled "mixed Capture Beads" (eg, 10 μl of IL-8 Capture Beads x59 assay tubes = 5900 μl of IL-8 Capture Beads required). The mixed Capture Beads were centrifuged at 200 × *g* for 5 minutes and carefully aspirated and supernatant was discarded. The mixed Capture Beads pellet was resuspended in Serum Enhancement Buffer equal to the same volume above and vortexed thoroughly. The assay tubes were incubated for 30 minutes at RT and protected from direct exposure to light. The mixed Capture Beads were then ready to be transferred to the assay tubes (50μl of mixed Capture Beads/tube) and samples analyzed by flow cytometer as described in BD CBA Human Inflammatory Cytokines Kit Assay Procedures using the BD FACS Calibur flow cytometers and the BD FACS Comp *soft*ware BD FACSArray™ bioanalyzer (FCAP Array software (Cat.No. 641488)).

2.7.4 Data analysis

The profiles of a selection of 14 cytokines and chemokines in serum of children with ARF and RRF are described. The cytokines and chemokines IL-1β, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p20, TNF-α, IFN-γ, MCP-1, MIP-1α, MIP-1β, IP-10 and RANTES were analyzed using FCAP Array Software with BD FACS Array Bioanalyzer. The analysis of cytokines and chemokines and their correlations included their comparisons in ARF and RRF patients. Data analysis was conducted using SPSS version 17 with descriptive statistics using median and inter-quartile ranges. The Mann-Whitney test was used to compare medians between ARF and RRF groups. Spearman correlation coefficient was conducted on the study of correlations between the different cytokines and chemokines and the level of significance was $p = \leq 0.05$.

Chapter 3

3 Prevalence of Rheumatic Fever and Rheumatic Heart Disease among Yemeni school children in Aden

3.1 Introduction

Rheumatic Heart Disease (RHD) is the leading form of acquired childhood cardiovascular disease and continues to be a common medical problem in industrializing countries. It is a major public health issue in areas where there is limited access to medical care of children living in poverty, overcrowding and poor housing conditions (Al-Eissa, *et al* 1993, Olgunturk, *et al* 2006). RHD is clearly an important and preventable cause of ill health and death (Nkomo 2007, Omurzakova, *et al* 2009, WHO 2004).

RHD is triggered by an auto-immune reaction to an untreated throat infection with GAS; initially giving rise to ARF within days to weeks and then disappearing, but leaving behind damage to the heart valves (Carapetis, *et al* 2005a, Kafetzis, *et al* 2005, Majeed, *et al* 1992a). The cumulative damage of recurrent and persistent episodes of ARF results in RHD (Omokhodion 2006, Oran, *et al* 2000). Primary school screening surveys appear to be the most informative source to assess the prevalence of RHD when echocardiogram is available for confirmation (Padmavati 2001, Robertson, *et al* 2006).

The prevalence of RHD has been estimated in surveys of school-age children, with wide geographic variations ranging from 0.2 per 1000 school children in Pinal Del Rio, Cuba (Nordet, *et al* 2008), 1.2 per 1000 in India (Bahadur, *et al* 2003), 2.3 per 1000 schoolchildren in Mozambique (Marijon, *et al* 2007), 3.4 per 1000 in Egypt (Refat 1994), 14 per 1000 schoolchildren in Kenya (Longo-Mbenza, *et al* 1998), 21.9 per 1000 schoolchildren in Pakistan (Sadiq, *et al* 2008). The prevalence of RHD was 26/1000 in children in New Zealand and 8.5/1000 in Australia (Jaine, *et al* 2008, Parnaby and Carapetis 2010, White, *et al* 2010, Wilson 2010) and 8.4/1000 in Fiji (Steer, *et al* 2009c).

In Yemen symptomatic patients with RHD are usually detected through paediatric clinics and hospitals and RHD accounts for a significant cause of acquired heart morbidity and mortality.

The school survey presented in this chapter is the first of its kind in Yemen and aimed to estimate the prevalence of RHD among schoolchildren aged 5-16 years in Aden city. The survey was based on cardiac auscultation with echocardiographic confirmation of the diagnosis.

3.2 Objective

To determine the prevalence of RHD among primary school children in Aden.

3.3 Literature review

ARF is a clinical syndrome without a specific diagnostic test or single pathognomonic feature. Dr. T. Duckett Jones proposed guidelines for ARF diagnosis (1944) that were revised by the American Heart Association (1992) and the World Health Organization (WHO 2004) (Table 3.1).

ARF is unique among infectious diseases in having a tendency to recur and the only cardiac disease that is clearly preventable (Taranta 1967, Taranta and Markowitz 1989). ARF still remains highly prevalent in developing nations with presence of unfavourable conditions such as overcrowding, poor hygiene and limited access to health care (Alkhalifa, *et al* 2008, Shulman 2007). RHD continues to be an important and significant cause of congestive heart failure (CHF) in Africa, Central and South America, and Asia (Mendez and Cowie 2001, Shaddy and Wernovsky 2005).

Disability-adjusted life years (DALYs) lost to RHD ranged from 27.4 to 173.4 per 100 000 population in WHO Regions of America and South -East Asia with an estimated 6.6 million DALYs lost per year worldwide (Carapetis, *et al* 2005a, Hart 1993).

An approach to the problem of ARF/RHD must be guided by a plan which coordinates medical and non-medical community leaders for mass primary prevention, providing care for high-risk group of susceptible children and for checking the accuracy of secondary prevention (Alkhalifa, *et al* 2008, Robertson, *et al* 2006). This important

public health problem requires improvement in the detection and treatment of sore throats and regular screening of school-children for ARF/RHD. There is an urgent need for early identification of children with ARF/RHD for penicillin prophylaxis, thus preventing recurrence of ARF and progression of RHD (Carapetis, McDonald et al. 2005).

It is of utmost importance to provide health education to parents, children and to teachers, as well as to medical and non-medical health providers in order to increase awareness and understanding of the disease (Steer, *et al* 2011). The Director of health care centres should facilitate the introduction of secondary prophylaxis strategies into existing health care systems. A simple registry program should be introduced at the polyclinics, hospitals and public and private hospitals in order to facilitate regular and continuous follow up and secondary prophylaxis(Robertson, *et al* 2006,Steer and Carapetis 2009).

RHD is the most common acquired heart disease worldwide leading to incapacitating haemodynamic changes in children, adolescents and young adults (McDonald, *et al* 2005). Its public health importance is not only a direct result of its high occurrence rates (mortality, prevalence and incidence), but also the population affected (children and young adults) and its economic consequences, both in health care related costs and its indirect cost to society, often resulting in premature death or disability (Carapetis 2008a, Demina, *et al* 2005, Kaplan 2005).

Table 3. 1Diagnosis of Rheumatic fever

Duckett Jones criteria (1992)

Two major or one major and two minor manifestations must be present, plus evidence of antecedent GAS infection.

Chorea and indolent carditis do not require evidence of antecedent GAS infection.

Recurrent episode requires only one major or several minor manifestations, plus evidence of antecedent GAS infection.

Major manifestations:

Carditis

Polyarthritis

Chorea

Erythema marginatum

Subcutaneous nodules

Minor manifestations:

Arthralgia

Fever

Raised erythrocyte sedimentation rate or C-reactive protein concentrations

Prolonged PR interval on electrocardiogram

Evidence of antecedent GAS infection

Positive throat culture or rapid antigen test for GAS

Raised or rising streptococcal antibody titer

WHO criteria (2002-03)

Chorea and indolent carditis do not require evidence of antecedent GAS infection.

First episode

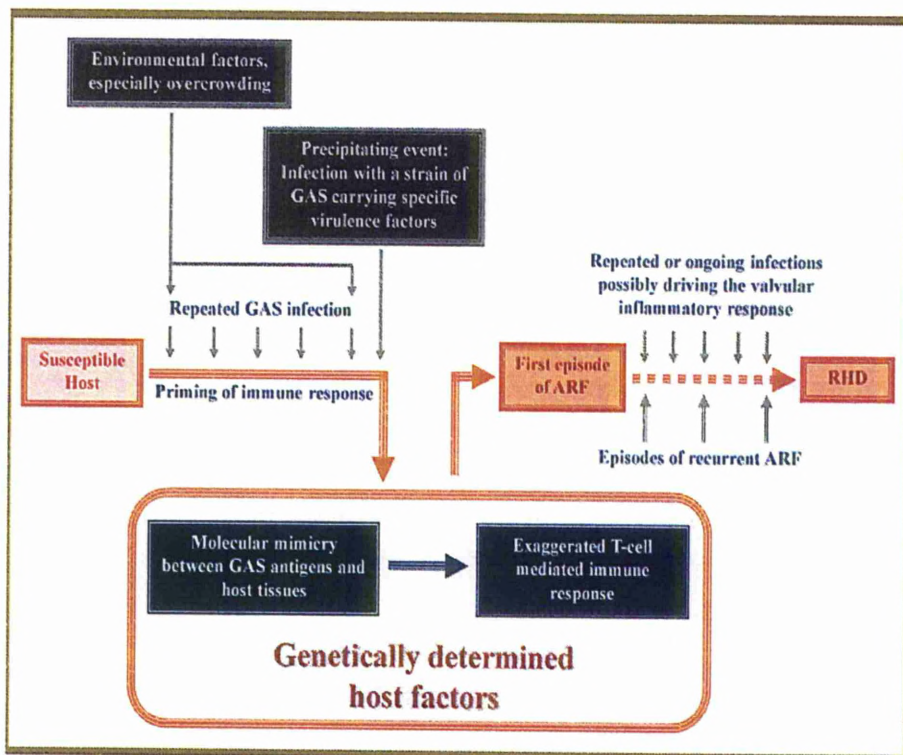
As per Jones criteria

Recurrent episode

In a patient without established RHD: as per first episode.

In a patient with established RHD: requires two minor manifestation, plus evidence of antecedent GAS infection. Evidence of antecedent GAS as per Jones criteria, but with addition of recent scarlet fever.

Modified from (Carapetis Jonathan et al 2005)



Ref. From Carapetis et al (2007)

Figure 3. 1 Figure Prerequisities for the development of ARF/RHD

ARF is an immunoinflammatory delayed non-suppurative sequelae of GAS upper respiratory tract infection (Kaplan 2005, Majeed 2002). RHD is the end result of a number of factors including neglect, late diagnosis, improper treatment, atypical presentations or repeated recurrences of ARF (Robertson and Mayosi 2008). In low income countries, it is a serious cause of chronic health problem during the school age period (Figure 4.1) (Guilherme, *et al* 2006).

Even though substantial efforts have been made to understand the pathogenesis of GAS infections in triggering RHD, it still remains an intricate network of events that have a complex immunologic basis. It is ironic that a rather innocuous "sore throat" should extract such a high price from the host (WHO 2004).

The clinical manifestations of RHD reflect the response to GAS and the severity of ARF in an individual(Commerford and Mayosi 2006). These are determined by host genetic susceptibility, virulence of the infecting organism and a conducive environment (Stollerman 2001a).A high proportion of people with RHD do not recall a history of ARF (Carapetis, *et al* 2005b), and they are not aware of their problem until an incidental detection occurs during a medical check up or when the disease progresses with the development of symptoms and signs of cardiac failure (Giannoulia-Karantana, *et al* 2001).

Genetic susceptibility and autoimmune features play an important role in RHD along with familial disease occurrence and the relationship with HLA antigens of the major histo-compatiblity complex related to racial and ethnic origin (Kudat, *et al* 2006, WHO 2004). Disease susceptibility factors including the major histocompatibility antigens and tissue-specific antigens are presently under investigation to assess their role as disease determinants (Kaplan 2005).

Toll-like receptors (TLRs) are key cell-associated pattern recognition receptors (PRRs) in the innate immune system able to recognize a wide range of pathogens (Liadaki, *et al* 2011). The classification depends on the cell surface/intracellular site and type of pathogen-associated molecular patterns (PAMPs). Bacterial cell surface components combine with PRRs such as the ficolin family of proteins, or Mannan binding lectins that form complexes leading to complement activation causing phagocytosis of the invading pathogen, apoptosis, or modulation of inflammation. *TLR2* and *TLR4* are located on the cell surface and recognize bacterial products unique to GAS. Mutations and polymorphisms in TLRs have shown their essential role in human defense against diseases.

TLR lead to production of proinflammatory cytokines which activate macrophages and dendritic cells (DC) to remove the pathogens. DCs couples TLR-mediated innate immune recognition leading to the initiation of T and B cell activation. These molecules act as signals to activate adaptive immune responses (Schumann and Keitzer 2005). DCs act as the antigen presenting cells that express CD80 and CD86 and MHC molecules on their cell surface allowing these cells to present processed antigens to T cells through the T cell receptor (TCR). Class I MHC molecules present peptides from

intracellular pathogens to CD8+ T cells, while class II MHC molecules present peptides derived from extracellular pathogens to CD4+ T cells. These in turn secrete cytokines, as TNF-α and IFN-γ that affect pathogen survival and control the immune response. CD4+ T cells cause expansion of CD4+ effector cells, CD8+ T cells and B cells (Liadaki, *et al* 2011).

These molecules involved in the innate and adaptive immune responses are encoded by genes that may be associated with ARF/RHD. TLR2 is encoded by a gene located on chromosome 4 in the 4q32 region with a single nucleotide polymorphism (SNP) in exon 3 (2258 G>A) that leads to the replacement of arginine with glutamine in codon 753. The polymorphism in TLR-2 genotype 753 Arg/Gln was reported to be more frequent in Turkish children with ARF. *TLR4* polymorphisms as *TLR4*-D299G SNP, alone or together with *TLR4*-T399I, were reported to be associated with an increased risk of recurrent GAS pharyngotonsillitis.

Ficolins are molecules that play a role in the innate immune response. They are able to bind to specific PAMPs and trigger the complement lectin pathway. Ficolins bind to collectin receptors and activate immune cells to produce cytokines like TNFα, IL-1 and IL-8. Three ficolin genes were found in humans that encode ficolin-1 (M-ficolin), ficolin-2 (L-ficolin) and ficolin-3 (H-ficolin or Hakata antigen). The role of ficolin-2 is involved in the clearance of the pathogen by binding to the specific GAS antigen, lipoteichoic acid; hence low levels predisposes the patients to recurrent GAS infections leading to ARF and RHD (Messias-Reason, *et al* 2009) . Polymorphisms in the ficolin genes may produce different serum ficolin protein levels which lead to prolonged or repeated GAS infections and are important in ARF pathogenesis. Polymorphisms in the FCN2 gene promoter for L ficolin protein that binds to GAS also may be important. *FCN2* gene polymorphism was shown to be associated with CRHD. CRHD patients express the haplotype -986/ 602/-4 *G/G/A*, with decreased serum ficolin-2 levels. The haplotype -986/-602/-4*A/G/G* was associated with protection against CRHD. MBL binds to N-acetyl-D-glucosamine present on the cardiac valves. *MBL2* genotypes were reported to be associated with risk of carditis in patients with ARF/RHD. Haplotype GGA was found more frequently in CRHD patients in Brazil (Hernández-Pacheco, Flores-Domínguez et al. 2003).

MBL is a lectin pathway member of the complement system involved in innate immune responses that is involved in the clearance of the bacteria. MBL2 gene was reported to be involved in the development of valvular lesions (Chou and Tsai 2006). SNPs were found in the promoter site of the MBL2 gene (-550H/L, -221X/Y and +4P/Q) and in exon1 (codons 52A/D,54A/B and 57A/D) of ARF/RHD patients (Bryant, *et al* 2009).

Different alleles were associated with valvular lesions in RHD. A allele (52A, 54A and 57A) codes for high production of MBL was reported to be linked to mitral stenosis while the O allele (52D, 54B and 57D) which encodes for low production of MBL was found in AR. Fc fragments receptor of immunoglobulin G (FcgRIIA) eliminates antigen-antibody complexes from the blood and protects the host against foreign antigens. The gene for this receptor is expressed on mononuclear phagocytes, neutrophils, and platelets and shows SNP in exon 4. The 131R/R genotype was reported to be associated with a high risk of ARF in Turkish children (Sallakci, *et al* 2005, Akcurin et al. 2005).

Several studies have reported the role of TNF-α gene polymorphisms in RHD(Berdeli, *et al* 2005)(Düzgün, *et al* 2007). TNF-α gene in the alleles TNF-α -308A and -238G and genotype was associated with ARF/RHD in Mexico, Brazil, Egypt but not in Turkey (Berdeli, *et al* 2006, Bryant, *et al* 2009, Hernández-Pacheco, *et al* 2003, Sallakci, *et al* 2005) There may be a risk of RHD associated with polymorphisms in the TNF-α promoter region and TNFAIP3 (Hua, *et al* 2009, Mohamed, *et al* 2010, Settin, *et al* 2007). Another study reported immunoglobulin FcRIIA gene polymorphisms with risk to ARF by a genetic failure of removal of immune complexes (Berdeli, *et al* 2004, Guilherme, *et al* 2011).The MEFV polymorphism was reported to cause impaired control of Th1 helper cell immune response in pathogenesis of ARF (Col-Araz, *et al* 2012). Polymorphisms of the ACE gene have been shown to be related with CRHD (Berdeli, *et al* 2005, Bryant, *et al* 2009). These polymorphisms were observed to be linked to valvular damage (Ramasawmy, *et al* 2007, Smeesters, *et al* 2009).

Extensive research is in progress to advance knowledge on the genetic and host susceptibility factors for ARF/RHD, disease pathogenesis and distribution and frequency of toxin-producing strains of GAS with a view to the development of

vaccines against GAS disease, and these efforts should improve prophylaxis and treatment strategies (Medina and Chhatwal 2002, Thomas 2001).

Prevalence rates of RHD vary between and within populations which may be attributed to genetic predisposition and/or socioeconomic standards (Jose and Gomathi 2003, Marijon, *et al* 2007, Zhimin, *et al* 2006). These differences also depend on the age of the studied population and the survey techniques (Table 3.2).

It is estimated that at least 15.6 million people have RHD and 300,000 of 500,000 new individuals with ARF develop RHD annually. There may be up to 233,000 deaths worldwide due to ARF or RHD per year and 95% of these occur in developing countries (Oli and Asmera 2004, WHO 2004). However, the burden of RHD is likely to be higher than these estimates given that epidemiological data from developing countries are poor (Isaacura and Granero 1998, Mincham, *et al* 2002, Ravisha, *et al* 2003).

Most surveys are conducted in schools that include clinical cardiac examination with the subsequent assessment of RHD through performance of echocardiography and Doppler ultrasonography (Carapetis 2008a, Marijon, *et al* 2007, Meira, *et al* 2005). The utilization of these techniques is acceptable to the population and is appropriate for screening and has a high sensitivity and specificity (Steer and Carapetis 2009b, Vijayalakshmi, *et al* 2005). In low income countries, RHD accounts for 30% to 40% of cardiac patients admitted to hospital and are an important reason for heart surgery (Alkhalifa, *et al* 2008, Essop and Nkomo 2005).

RHD follows the prevalence ARF which increases with age, starting in childhood and peaking in adulthood (Berry 1972, Chockalingam, *et al* 2004). This profile has been well documented in populations having both low and high rates of RHD (Ahmed, *et al* 2005, Kumar, *et al* 2002, Oli and Asmera 2004).

Several reports from South Asia, the Middle East and North Africa have highlighted unacceptably high sex predilection pattern of RHD with an association of severe manifestations in girls and women (al-Sekait, *et al* 1990, Diao, *et al* 2005, Rizvi, *et al* 2004). It has been postulated that this gender preference may be explained by innate

susceptibility, limited access to preventive health care or increased exposure to GAS infections (Padmavati 2001).

Table 3. 2 Prevalence of RHD per 1000 in school-aged children by ehocardiography

Reference	Year of study	Place	Age (yrs)	No. screened	RHD/ 1000	No of RHD children
Wilson *et al*	2010	New Zealand	5 – 15	1142	26	29
Jaine *et al*	2008	Australia	5 - 14	-	8.5	-
Periwal *et al*	2005	India	5 - 14	3002	0.67	2
Sadiq *et al*	2002	Pakistan	5 - 15	24,980	21.9	546
Bahadur *et al*	2002	Nepal	5 - 18	9420	1.2	11
Marijon *et al*	2001	Mozambique	6 - 17	2170	2.3	5
Marijon *et al*	2001	Cambodia	6 - 17	3677	2.2	8
Mebenza *et al*	1998	Kenya	5 - 16	4848	14	59
Hasab *et al*	1997	Oman	5 - 16	9904	0.8	8
Nordet *et al*	2000	Cuba	5 - 15	25,159	0.2	6
Refat *et al*	1994	Egypt	6 - 12	8000	3.4	27
Mahmodi *et al*	1992	Iran	7 - 18	167,786	0.14	23
Khalil *et al*	1992	Sudan	5 - 15	13,332	3	40
Al-Sekait *et al*	1990	Saudi Arabia	6 - 15	9418	2.4	22

References: (Alkhalifa, *et al* 2008, Carapetis, *et al* 2005a, Marijon, *et al* 2007, Nordet 2000, Sadiq, *et al* 2008, Steer and Carapetis 2009b)

The most common valvulopathies in RHD are mitral regurgitation, which is the predominant cardiac lesion, and mitral stenosis. Mitral stenosis becomes more common with advancing age (Choudhary, *et al* 2001, Mahmoud U Sani 2007).

It is well known that low income, poverty, overcrowding, poor housing conditions and inadequate sanitation increase the impact of the disease (Abella-Reloza 1998, Jose and Gomathi 2003, Paul 1930). In addition, inadequate expertise of primary healthcare providers, shortage of health care resources and low disease awareness in the community further affect both the occurrence and recurrence of the disease. Another contributing factor is the fear of allergic reactions to penicillin for primary and secondary prophylaxis. This is of great concern as prophylaxis is needed to minimize

the risk of exposure of children and adults to GAS (Cilliers 2006, Kumar, *et al* 2002, Lennon 2004, Robertson, *et al* 2005).

Although, in most developing countries, cost-effective strategies for ARF/RHD control programmes are available they remain under-utilised (Cilliers 2003, McDonald, *et al* 2005). RHD is now very rare in developed countries and remains mainly a disease of poverty. The diagnosis of RHD may subject the patient to high cost cardiac surgery, although this may not be available for populations of low socioeconomic settings (Demina, *et al* 2005, Hillman, *et al* 2004).

3.3.1 Components of a ARF/RHD Prevention Programme

Penicillin remains the drug of choice in primary and secondary prophylaxis of ARF/RHD since there are no reports of resistance of GAS isolates to penicillin (Carapetis, *et al* 2005a, Robertson, *et al* 2006, Steer and Carapetis 2009a). Primary prevention is the first line of treatment for acute GAS pharyngotonsillitis with appropriate antibiotics. Primary prophylaxis is to prevent subsequent attacks of GAS pharyngotonsillitis which could trigger ARF and recurrence with increasing severity for cardiac disease (Steer, *et al* 2011).

Antibiotic therapy started within 9 days of onset of acute pharyngotonsillitis is effective to prevent ARF. Oral penicillinV for 10 full days is still the first line treatment in patients with good compliance. In patients with poor compliance to an oral regimen, they can be given a single intramuscular dose of benzathine penicillin G (BPG) at time of GAS diagnosis. Patients who show hypersensitivity to penicillin may be treated with erythromycin (Madden and Kelly 2009). First generation cephalosporins maybe used but 5% of patients allergic to penicillin may also show allergy to cephalosporins. If erythromycin resistance is documented then alternatives include clarithromycin or azithromycin. Some measures likely to be effective but difficult to implement include decreasing overcrowding and improving access to medical care and antibiotics (Warren 2010).

Secondary prophylaxis is given to patients after the initial attack of ARF. They are predisposed to recurrent attacks of ARF with high risk to develop carditis, if not yet developed or worsen with an existing RHD (Kerdemelidis, *et al* 2010). The choice of

antibiotics is similar to the primary prophylaxis.Secondary antibiotic prophylaxis is only effective with good compliance and given as intramuscular BPG every 4 weeks to ensure adequate serum penicillin levels. In high risk areas of ARF the BPG dose should be given every 3 weeks for better prevention of recurrent ARF(Commerford and Mayosi 2006). Unless patients are compliant with oral regimens, these can also be effective.

The duration of secondary prophylaxis depends on clinical symptoms of the disease and presence or absence of heart valvular defects Patients with history of ARF without carditis should be treated until the age of 21 years or 5 years after their last attack. Patients with RHD should be treated until 40 years of age or 10 years after their last attack of ARF. Patients without CRHD are recommended to receive at least 10 years of therapy or until well into adulthood (Gerber, *et al* 2009). Patients who live in areas with continuous high exposure to GAS may require lifelong prophylaxis (Seckeler and Hoke 2011). It is also recommended to continue prophylaxis after heart valve replacement surgery, as any of the four cardiac valves can be affected by ARF. Patients with RHD require antibiotic prophylaxis to prevent endocarditis before minor surgical and dental procedures with short term antibiotics to be continued for one week after the procedures (Ananda and Purushottam 2008, Carapetis, *et al* 2007).

Screening surveys in combination with a national registry form the framework of a ARF/RHD prevention programme. Although primary prophylaxis preventing initial attacks of ARF is ideal, it is rather difficult to implement in large populations, hence secondary prophylaxis to prevent recurrent attacks of ARF remains the most practical solution in underprivileged countries (Tandon 2004).

A registry program has been shown to be inexpensive and to facilitate the delivery of secondary prophylaxis through continuous and regular follow up (Bassili, *et al* 2002, Kumar, *et al* 2002, Marijon, *et al* 2007). It can be conducted in school health centres, maternal and child health services and paediatric clinics. There is no registry program in operation in Yemen.

3.4 Patients and methods

This was a cross sectional survey of school children in Aden. Aden city has 59 primary public schools with 1796 classrooms segregated by gender, of which 47683 are male and 40952 female students. A cross-sectional survey of RHD was conducted on a representative sample of 6000 school children aged 5 – 16 years of both sexes from February 2004 through April 2005.

3.4.1 Sample size

The sample size for the survey was based on data obtained from previous studies that reported prevalence rates per 1000 school children of 3.6 in Sana'a, Yemen. If the RHD prevalence accepted was 3.6/1000 taking the CI of 95%, power 80%, and a precision of ±10%, then the number of students which would be a representative of one million was 6000. The target population was drawn from 12 governmental primary schools of eight districts at Aden selected by systematic sampling.

A written approval acceptance form of school visits was obtained from the Director of the Ministry of Education, the School Health Care Doctor and eight School Vice-Directors. Informed verbal or written consent was obtained from the parents. All students enrolled underwent a careful cardiacauscultation in a quiet room by the principal investigator and two paediatric specialists. They were examined in both the supine andleft lateral decubitus positions. A social worker recorded their identification data.

3.4.2 Clinical asssessment

Children diagnosed with organic/pathological murmurs were auscultated for a second time by the same investigator to confirm the clinical diagnosis (Chapter 2).During the clinical examination if a pathological murmur was diagnosed; the patient was offered echocardiography. In students with doubtful murmurs for a second examination were also given appointment for echocardiography. Children with pathological murmurs were then given appointments for echocardiography in a specialty hospital to which they were taken by bus after obtaining a second informed consent from their parents/guardians. These children also had a complete blood count, ASO and CRP investigations which were done in a specialized diagnostic centre.

Two-dimensional and colour flow Doppler echocardiography was used for detecting abnormal blood flow and valvular regurgitation. Independent echocardiography was performed by two paediatric cardiologists who were present for all the echo Doppler examinations. The echocardiography Doppler findings were then discussed by the two cardiologists and a consensus diagnosis of RHD was achieved prior to the enrolment into the further components of the study. The diagnosis of definite RHD was based on the modified Duckett Jones' criteria which included the presence of a significant organic mitral or aortic murmur.

Echocardiographic evidence of significant valvular incompetence was defined as the presence of evidenceof mitral or aortic valve regurgitation seen in two Doppler echocardiography planes, accompanied by at least two ofthe following three morphologic abnormalities of the regurgitantvalve: restricted leaflet mobility, focal or generalized valvularthickening, or abnormal subvalvular thickening (Ferrieri 2002, Folger, *et al* 1992). These features had tobe identified concordantly by each cardiologist.

A diagnosis of suspected RHD was made in the presence of significant mitral or aortic organic murmurs with trivial or simple mitral (MR) or aortic valvular regurgitation (AR) without morphologic abnormalities of the regurgitant valve in the presence of normal heart chambers. Mitral valve prolapse (MVP) was categorized as CHD, if it was a mitral valvulopathy with an underlying non-rheumatic structural abnormality; and as RHD when MVP was associated with MR and thickened valves.

3.4.3 Socio-economic assessment

Regarding the demographic characteristics, overcrowding was defined as > 4 members per bedroom(Sana'a, Yemen: Ministry of Planning 2006). The housing conditions were classified as "good" when the house was made of stone with $\geq$ four rooms and had a water supply; "satisfactory" if the house was made of stone and had water supply but was small; and "bad" if was made of other materials (e.g. wood) and had no water supply. Water supply included treated pipe water or untreated water from protected bore holes, springs and sanitary wells, either in the home or within 15 minutes walking distance.

The socio-economic status was assessed by obtaining the income of each household member for one month and calculating the total monthly money income of the household/total members in the household. Family income was graded as low (<5000 Yemeni Riyals (YR), intermediate (5000 – 10,000 YR) and high (>10,000 YR) and around 340 Riyals equal to a £1(MOPIC2006).

A questionnaire which included demographic details, past medical history; family socio-economic background and income per capita was completed for all students. Prophylactic benzathine penicillin G every three weeks was prescribed to suspected and confirmed patients with RHD. This prophylactic treatment was to be undertaken regularly in primary health centres in their neighbouring districts.

3.4.4 Statistical analysis

Data was analyzed using the Statistical Package for Social Sciences (SPSS-PC) version 17. Statistical analysis included quantitative descriptive analysis and summary statistics (mean, percentages, standard deviations, 95% CI and rates) stratified by gender, age and valvulopathies. Analysis was based on chi-square tests and Fisher's exact tests.

3.5 Results

3.5.1 General characteristics of children in the survey

A total of 6000 primary school children of both sexes in twelve schools representing 7.4% of the school population underwent clinical screening followed if necessary by Doppler echocardiography. The primary level starts from class 1 to class 6 which includes children from approximately 5 to 12 years of age while the preparatory level from class 7 to class 9, includes children from 13 to 16 years.

The male to female ratio was 1.1:1 with a mean (SD) age of 11.2 (3.1) years. Children 10 – 16 years old comprised 53% of the participants. About half, 3203 (53.4%) children attended primary school and 2797 (46.6%) preparatory school. Males comprised 1562 (49%) and 1506 (53.8%) participants who attended primary and preparatory level, respectively (Table 3.3).

The prevalence of RHD was probably underestimated since 514 had an organic murmur and out of them 168/514 (32.7%) parents of children (of whom 105 were females

comprising a female-to-male ratio of 1.7: 1) considered to have organic murmurs refused permission for further investigation. An important limitation of the study was that only 346 of the 516 children with a clinically detected cardiac abnormality consented to echocardiography; one-third refused. This systematic error could partly explain the lack of gender association in our study.

Table 3. 3 Characteristics of 6000 study participants

Variables		n = (%)
Age/years	Mean (SD)	11.16 (3.1)
Age groups (years)	5 – 10	2817 (47.0%)
	>10- 16	3183 (53.0%)
Gender	Male: female (male %)	3068 : 2932 (51.1%)
Education	Primary	3203 (53.4%)
	Preparatory	2797 (46.6%)
Class and sex	Primary (male %)	1562/3203 (48.8%)
	Preparatory (male %)	1506/2797 (53.8%)
School	Almasmoum	297 (5.0%)
	Alshukany	699 (11.6%)
	Basateen	432 (7.2%)
	Fatehh	322 (5.4%)
	Gala	513 (8.6%)
	IbnZaidoon	570 (9.5%)
	Kadicia	265 (4.4%)
	Raydan	452 (7.5%)
	Rowdah	522 (8.7%)
	SevenJuly	744 (12.4%)
	Shamsan	686 (11.4%)
	Wahdda	498 (8.3%)

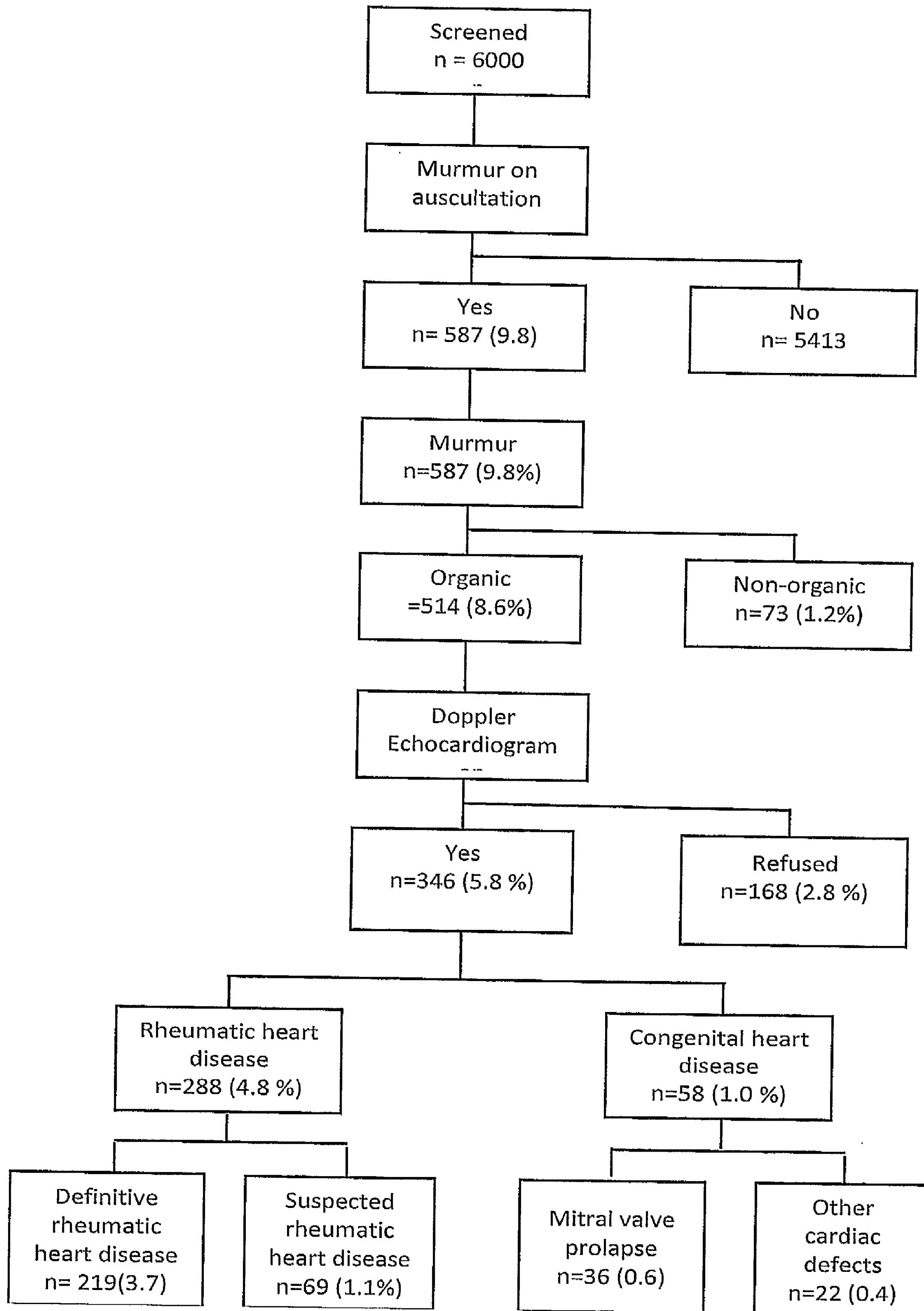


Figure 3. 2Flow diagram of total participants in the study

3.5.2 Cardiac findings of screened children

Heart murmurs were documented in 587 (9.8%) students. Of these 514 (8.6%) were deemed to be organic murmurs. One hundred and sixty eight (2.8%) parents refused further investigations or did not attend school during the day of the echocardiography examinations.

Cardiac defects were diagnosed by echocardiography in 346 (5.8%) children. RHD was diagnosed in 288 (4.8%), 219 (3.7%) with definite RHD and 69 (1.2%) with suspected RHD. The overall prevalence of definitive RHD comprised 36.5/1000 children (95% CI 32% - 41.6%) (Table 3.4).

Children with suspected RHD were discharged from the study. These children with suspected RHD were given prophylaxis with antibiotics as penicillins or if allergy give erythromycin and were instructed to attend health services and to be assessed every 6 months with a repeat echocardiography one year later. Fifteen students were diagnosed with ARF including three with recurrent rheumatic fever (RRF) and RHD. These were referred for hospital admission.

Table 3.4RHD among the school children screened

Variables		n (%)
Children screened		6000
Murmur		587 (9.8)
	Organic	514 (8.6)
	Non-organic	73 (1.2)
Echocardiography	Done	346 (5.8)
	Refused	168 (2.8)
Rheumatic heart disease		288 (4.8)
	Definitive	219 (3.7)
	Suspected	69 (1.1)
Congenital heart disease		58 (1.0)
	Mitral valve prolapse	36 (0.6)
	Others*	22 (0.4)

*Other CHD included 7 PDA, 4 ASD, 4 PS, 3 AS, 2 VSD, 1 TOF and 1 TA

Congential heart diseases (CHD) included seven patent ductus arteriosus (PDA), four atrial septal defects (ASD), four pulmonary stenosis (PS), three atrial stenosis (AS), two ventricular septal defects (VSD), one Tetrology of Fallot (TOF) and one tricuspid atresia (TA).

3.5.3 Prevalence of cardiovascular disease

The survey documented 277 students with echocardiographically confirmed cardiovascular disease (219 with definitive RHD, 36 with MVP and 22 with CHD) There was a higher prevalence of RHD in preparatory than in primary school children(59.3 vs 16.6/1000, $p = 0.001$)

The mean age (SD) of children with and without definite RHD was 14 (1.8) and 10.8 (3.2) respectively (p = 0.001). The prevalence of RHD among males was 40.4/1000 (95% CI 33.7–48.0) which was higher than females 32.4/1000 (95% CI 26.3 - 39.5), but this was not statistically significant (p=0.97) (Table 3.5).

Table 3.5Prevalence of Cardiovascular Diseases per 1000 school children by the education level and sex

Cardiovascular defects	N	CHD n (‰)	RHD n (‰)	All n (‰)	95% CI
Education					
-Primary (1 – 6 class)	3203	32 (10.0)	53 (16.6)	85 (26.5)	(21.3 – 32.8)
-Preparatory (7 – 9 class)**	2797	26 (9.3)	166 (59.3)	192 (68.6)	(59.6 – 78.8)
Sex					
-Male***	3068	40 (13)	124 (40.4)	164 (53.5)	(45.9 – 62.2)
- Female	2932	18 (6.1)	95 (32.4)	113 (38.5)	(32.0 – 46.3)
All cardiac defects	6000	58 (9.7)	219 (36.5)*	277 (46.2)	(41.1 – 51.9)

*Prevalence of RHD 36.5/1000 school children ** p = < 0.0001 comparing CHD and RHD between primary and preparatory levels ***p = <0.005 when comparing CHD and RHD between males and females

Echocardiographic evidence of CHD was obtained in 58 (1%) children resulting in a prevalence of 9.7/1000 (95% CI 7.4 – 12.6%). The male to female ratio of CHD was 2.2: 1 but this difference was not statistically significant (p = 0.08).

Thirty-six children with CHD had mitral valve prolapse, which was three times more common in males than females (27 versus 9) ($p < 0.02$). The main types of other CHD are listed in Table 3.4. The majority had MVP followed by PDA, then by ASD and PS.

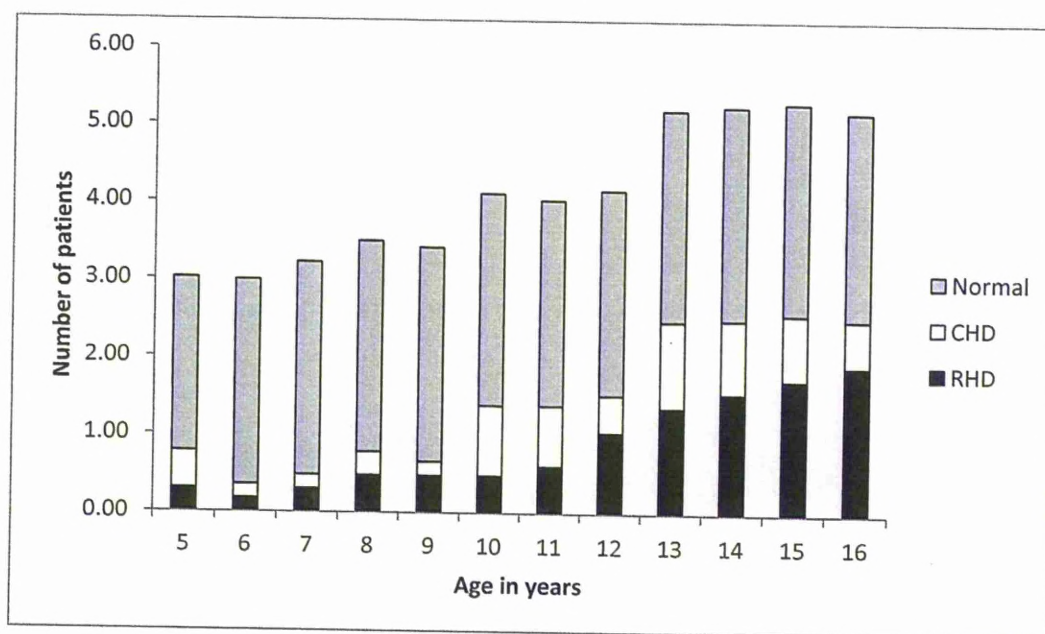


Figure 3. 3Distribution of RHD and CHD by age

3.5.4 Medical background

A history of RHD was obtained from more than one family member in 53 (24.2%) of all children with RHD. RHD was diagnosed in 20 siblings of the children with definite RHD. One family had four siblings affected, another three siblings in two families, two siblings in two families and one sibling in six families all attending the same schools. In addition there was a positive family history of one of the parents or a close relative in 33 children. Only 36 (16.4%) out of 219 children with RHD were aware of their cardiovascular disease before the study and 18 (8.2%) were on irregular secondary rheumatic prophylaxis.

3.5.5 Socio-economic status of children with and withoutcardiac defects

Low income was observed most frequently among children with RHD (49.3%), followed by children with CHD (44.8%), compared with children without these

conditions (21.6%) X^2 for trend (13.2). Similarly poor housing conditions was reported most frequently in children with RHD (39.3%) followed by CHD (32.8%), compare to (21.6%) in normal children X^2 for trend (12.9).

An overcrowding index was assessed by dividing the total number of family members by the number of bedrooms used and overcrowding considered when there were four or more persons per bedroom. A higher overcrowding index was reported in children with RHD (64.8%) while in CHD it was 48.3% and those without any cardiac defects 37.1% X^2 for trend (22.2). The difference between low income, bad housing conditions and overcrowding between the three groups were all statistically significant (p=0.001) (Table 3.6).

Table 3.6Socio-economic status among children with cardiovascular disease

Socio-economic status		Cardiovascular defects		No defects n =5723	p value
		CHD n = 58	RHD n = 219		
Income	Low	26 (44.8%)	108 (49.3%)	1236 (21.6%)	0.001
	Middle	22 (37.9%)	62 (28.3%)	2175 (38%)	
	High	10 (17.2%)	49 (22.4%)	2312 (40.4%)	
Housing conditions	Bad	19 (32.8%)	86 (39.3%)	1234 (21.6%)	0.001
	Satisfactory	21 (36.2%)	82 (37.4%)	2225 (38.9%)	
	Good	18 (31%)	51(23.3%)	2264 (39.6%)	
Overcrowding	Yes	28 (48.3%)	142 (64.8%)	2123 (37.1%)	0.001
	No	30 (51.7%)	77 (35.2%)	3600 (62.9%)	

3.5.6 Cardiovascular disease by age and sex

There was a higher frequency of heart disease among males than females > 10 years old (145 (52.3%) and 104 (37.5%)), respectively compared with that among children < 10 years old, (19 (6.9%) and 10 (3.6%)) for males and females, respectively (p = 0.04). Older children were more likely to have confirmed RHD ($p < 0.05$) (Table 3.7).

Table 3.7Children with cardiovascular disease by sex and age

Sex	Age	CHD n (%)	RHD n (%)	P value
Male n=164		40 (24.4)	124 (75.6)	
	5 – 10 year (19)	9 (47.4)	10 (52.6)	
	*11 – 16 year (145)	31 (21.4)	114 (78.6)	0.02
***Female n=113		18 (15.9)	95 (84.1)	0.06
	5 – 10 year (10)	7 (70%)	3 (30%)	
	11 – 16 year (104)	11 (11.5)	92 (88.5)	0.001
**All	(277)	58 (20.9)	219 (79.1)	0.001

*p = 0.02 between CHD and RHD and sex in the 11 – 16 year age
** p = 0.001 between CHD and RHD in both age groups
*** p = 0.06 between male and female in CHD and RHD

3.5.7 Blood investigations in children with RHD

Ninety-three school children (42.5%) with RHD had a positive CRP and 139 (63.5%) had raised ASO titers > 200 Todd units, of whom 27 (12.3%) had ASO titers over 600 Todd units. (Table 3.8).

Table 3. 8Blood investigations in 219 children with RHD

Blood Test		RHD n (%)
ASO Todd units		
(normal=<200)	< 200	80 (36.5)
	> 200 - 400	62 (28.3)
	>400 - 600	50 (22.8)
	> 600	27 (12.3)
CRP		
(normal=<1mg/dL)	Normal	126 (57.5)
	Raised	93 (42.5)

The median CRP was 3 mg/dL (IQR 2-5 mg/dL) among patients with a single valve involvement and 6 mg/dL (IQR 4-8mg/dL) with multiple valve involvement. A higher

proportion of students with multiple valve involvement 21 (65.6%) had a raised CRP level than students with single valve involvement 11(35.4%) and the difference was statistically significant (p=0.001).

Table 3. 9C-reactive protein levels among patients with valve involvements

CRP mg/dL	Single valve n=54	Multiple valves n=39	p
≤5	43 (70.5)	18 (29.5)	0.001
>5	11 (34.4)	21 (65.6)	

3.5.8 Cardiac valve lesions in school children with RHD

The commonest valves affected among children with RHD were the mitral valve in 209 (95.4%) and the aortic valve in 44 (20.1%). The mitral valve was predominantly involved in more than half of the children, with isolated MR in 109 (50.9%), combined MVP with MR in 57 (26.6%) and combined MR and AR in 39 (17.8%). Isolated aortic valve regurgitation was diagnosed in nine patients (2.3%) and pure mitral stenosis was detected in five (1.9%) 11 – 16 year old students, four boys and one girl (Table 3.9).

Although the proportion of males with RHD was higher than in females, the difference was not statistically significant. Valvulopathies were more frequent in 11 -16 years old children (P< 0.001). In the 13 children with RHD < 10 years of age isolated MR with no evidence of other valvulopathies was the only finding.

Table 3. 10Type of cardiac valve lesions in children with RHD by sex and age

Sex	Age	
	5 – 10 year n(%)	11 – 16 year*** n(%)
Male n= 124 (%) *		
Mitral regurgitation	10 (8.1)	48 (38.7)
Aortic regurgitation		7 (5.7)
Mitral stenosis		4 (3.2)
Mitral regurgitation+ aortic regurgitation		21 (17.0)
Mitral regurgitation + mitral valve prolapse		34 (27.4)
Sub total	10 (8.1)	114 (91.9)
Female n=95 (%) **		
Mitral regurgitation	3 (3.2)	48 (50.5)
Aortic regurgitation		2 (2.1)
Mitral stenosis		1 (1.1)
Mitral regurgitation+ aortic regurgitation		18 (19.0)
Mitral regurgitation + mitral valve prolapse		23 (24.2)
Sub total	3 (3.2)	92 (96.8)

*p = 0.002 between valvulopathies and both age groups in males

**p = 0.4 between valvulopathies and both age groups in females

***p = 0.001 between valvulopathies and both age groups in both sexes

3.6 Discussion

RHD remains a major contributor to cardiovascular disease in children and adults in low and middle income countries accounting for recurrent hospital admissions, premature disability and early death (Canter, *et al* 2004, Golbasi, *et al* 2002, Olgunturk, *et al* 2006, Sadiq, *et al* 2008).

This school survey is the first to assess the prevalence of RHD among school children in Aden. The lack of similar surveys was largely due to limited funding for public health issues. The prevalence of RHD reported in this study in Aden is 36.5/1000 school children and is one of the highest reported in the world among the school surveys that have used echocardiography (Al-Munibari, *et al* 2001) . Despite the high prevalence, it is likely that this is an underestimate of the true prevalence in this population, as students with poor health are more likely to be kept at home than healthy children.

A large proportion of the participants were diagnosed for the first time. This is attributed to some families being totally unaware of their children's illness or its severity and as they lack access to proper diagnostic tests. This may be explained by limited financial resources for families to access health services and thus they seek medical advice mostly for emergency purposes only.

This high prevalence confirms that RHD is an alarming public health program in Yemen with long-term health and economic consequences. General health screening programs and a preventive RHD prophylactic program are an urgent and essential necessity which should be undertaken as a regular basis.

Routine screening programs should lead to earlier detection and monitoring of asymptomatic and symptomatic cardiac lesions in patients through primary school surveys which are the mainstay of childhood screening programs around the world (Carapetis 2008a, Chen, *et al* 2003, Finau and Taylor 1988, Jose and Gomathi 2003).

In this study there was an inconsistent sex pattern among patients with RHD. Previous studies have reported that females are more likely to experience RHD than males. This might reflect behavioural patterns such as being more often housebound and to have closer involvement with mother and child care, thus increasing their exposure to GAS

infections and overcrowding. Females also face constraints due to socio-cultural norms in health care seeking thus limiting their access to preventive medical care. However, this is not a universal pattern, as other studies have not confirmed these sex differences as was the case in this study (Thakur, *et al* 1996, Vashistha, *et al* 1993). It is difficult to determine whether the lack of gender differences was due to similar backgrounds among males and females, or a preferential pattern of healthy school children being overrepresented in schools.

The prevalence of RHD was probably underestimated since 514 had an organic murmur and out of them 168/514 (32.7%) parents of children (of whom 105 were females comprising a female-to-male ratio of 1.7: 1) considered to have organic murmurs refused permission for further investigation. An important limitation of the study was that only 346 of the 516 children with a clinically detected cardiac abnormality consented to echocardiography; one-third refused. Refusal means that the parents did not agree for the child to have an echocardiogram performed or the child did not attend school on the day the echo was performed. The availability of a portable echocardiographic machine in the field might have minimised some of this loss. The majority of those for whom echocardiography was refused were female (62.5%) and the explanation given by their parents was reluctance to accept that the child might have a heart disease. If it had been proved by echocardiography, it would affect the family's reputation and damage the child's prospects of marriage. Some families were suspicious of the fact that the echocardiogram, an expensive investigation, was free of charge. They might have thought that it was not for the purposes of research but for some other unspecified reason.

The more frequent refusal of females to accept the echo could have led to a systematic error. This systematic error could partly explain the lack of gender association in our study. In other settings females are reported to have a higher prevalence of RHD.Their higher refusal rate could have preferentially lead to the exclusion of selected participants aware of their illness and thus an under representation of the true prevalence of RHD in females.

As accepted, the long term characteristic of RHD results in an increasing prevalence with age. In various school surveys the highest prevalence is reported between 11 and 16 years with mean ages of 10 and 11 years, which was confirmed in this study. Similarly, rates were not different among boys and girls as reported from other developing countries (Bahadur, *et al* 2003, Refat 1994, Rizvi, *et al* 2004, Sadiq, *et al* 2008). The lower prevalence rate of RHD in primary schools indicates that there is an opportunity for the early identification of patients and commencement of secondary prophylaxis at this age (Grover, *et al* 2002, Veasy, *et al* 2004).

RHD is a disease of poverty and has been nearly eradicated in industrialized countries due to improved housing and economic conditions and health care facilities (Kutumbiah 1958, Steer, *et al* 2002, Taranta and Markowitz 1989, Vendsborg, *et al* 1968). Socioeconomic and environmental factors play an indirect but important role in the magnitude and severity of ARF and RHD (Kurahara, *et al* 2006, Leirisalo, *et al* 1980, Meira, *et al* 2005). Overcrowding, poverty and poor housing conditions were documented in 56.1%, 71.4% and 3.3% of school children, respectively, which is similar to that reported in the literature (Alkhalifa, *et al* 2008, Longo-Mbenza, *et al* 1998, Steer, *et al* 2002). These factors facilitate the high prevalence and spread of streptococcal infections, and may also enhance their virulence and rheumatogenicity (Groves 1999, Lennon 2004, Schaffer, *et al* 2003).

Previous reports indicate that the mitral and aortic valves are the usual valves affected in RHD, with isolated involvement of mitral valve regurgitation in more than half of the patients (Bitar, *et al* 2000, Carapetis, *et al* 2005a, Hillman, *et al* 2004, Khriesat, *et al* 2003). This is consistent with this study with 49.8% of the children presenting with a mitral valve lesion (Table 4.9).

Rapid progression of the disease in children and adults leads to severe haemodynamic compromise with combined mitral and aortic valve involvement (Schaffer, *et al* 2003, Tani, *et al* 2004, Venugopalan, *et al* 2002).This was also documented in 17.8% of the study participants with valve disorders with no sex predilection.

In developing countries including South Africa, Egypt and India, isolated mitral stenosis is commonly reported among female adolescents and young women (Andy and Soomro 2001) ,but was not detected among females in this study. However, there were only five cases of mitral stenosis in this study altogether (4 males and 1 female).

It is important to differentiate rheumatic mitral valve prolapse from degenerative or myxomatous mitral valve disease. Rheumatic prolapse always involves the anterior leafletwhereas myxomatous prolapse involves the posterior leaflet (Pomerantzeff, *et al* 2000). Myxomatous leaflets are thick, huge with significant systolic bulge toward the left atrium. In contrast, the leaflets in rheumatic carditis with MR have minimal thickening or bulge (Essop and Nkomo 2005). MVP may have a variable course; the pathologic changes in the valves and chorda tendineae cordis may occur at different stages after the inflammation or may disappear with the inflammation (Talwar, *et al* 2005, Zhou and Lu 1997).

The mechanisms involved in combined mitral valve regurgitation and mitral valve prolapse appear to differ when associated with rheumatic as opposed to a congenital floppy mitral valve origin (Kaymaz, *et al* 2005, Perier 2004). Mitral valve abnormalities with resultant anterior leaflet thickening of mitral prolapse are reported to occur in severe mitral regurgitation due to the result of annular dilatation and chordal elongation in patients with RHD, and are not caused by congenital abnormalities of the mitral leaflets per se (Essop and Nkomo 2005, Kaymaz, *et al* 2005).

The posterior leaflet of the mitral valve is protected from involvement and rupture in patients with rheumatic mitral valve disease due to its leaflet thickening and retraction and chordal elongation (Kalangos, *et al* 2000, Jonkaitienė, *et al* 2005, Turri, *et al* 1989). In this study, a combination of mitral regurgitation with mitral valve prolapse involving the anterior leaflet with minimal thickening was diagnosed in 26.6% of the children in total, which is comparable to Wou et el (30%), but significantly lower than Lembo et el (80%) (Avierinos, *et al* 2002, Cheng 1997, Gersh, *et al* 1985).

These complex lesions need to be further investigated, for example with follow-up studies to predict the effect of mitral valve prolapse on the outcome of children with RHD. Further consideration should be given to include MVP as one of the common

cardiac valve lesions in ARF/RHD and specifically correlate its origin of occurrence whether it still stands as a primary or a secondary abnormality and determine its course of development with the progress of the disease.

CRP is not specific enough to diagnose a specific disease but it serves as general marker for infection and inflammation (Elliott, *et al* 1997). The CRP test does not indicate where the inflammation is located or what causes it. Higher-than-normal levels of CRP may indicate ARF. CRP is useful mainly in monitoring inflammatory conditions since CRP drops as inflammation subsides (Yeh 2004). CRP increases above 10 mg/L in an inflammatory or infectious disease (Cengić, *et al* 2002). When CRP drops below 10mg/L it indicates the patient does not have clinical active inflammation (Reitzenstein, *et al* 2010). CRP appears and disappears more rapidly than ESR changes (Sarzi-Puttini and Atzeni 2004). Normal values may vary from laboratory to laboratory from CRP 0–1.0 milligrams per deciliter (mg/dL), or less than 10 mg/L (SI units) (Cengić, *et al* 2002, Rerkasem, *et al* 2002, Ridker, *et al* 2003). The highest values for CRP were up to 10mg/dL equivalent to 100mg/L in this study.

High CRP concentrations indicate an acute phase response to a previous inflammatory process and also a reaction to existing damaged tissue. High concentrations of CRP were observed in 39.3% of the students with RHD. A higher proportion of students with multiple valve involvement 21 (65.6%) had a raised CRP level than students with single valve involvement 11(35.4%) ($p=0.001$). The severity of valvular involvement may result in increased production of CRP (Golbasi, *et al* 2002). Thus CRP monitoring should be included in the follow up of individuals affected with not only ARF but also in chronic RHD.

The occurrence of RHD in more than one family member has long been reported but specific patterns of inheritance have not yet been identified. HLA Class II associations with RHD are more evident and consistent among clinically homogenous patients (Guedez, *et al* 1999). Interesting trends and associations between DR6 haplotypes and RHD have been reported (Guedez, *et al* 1999). A significant increase in frequency of DRB1*0701 allele in RHD patients has been detected (Kudat, *et al* 2006). Carreno et al (2000) documented increased frequency of HLA-DR4 and HLA-DR2 with RHD in both white and black patients respectively (Carreno-Manjarrez, *et al* 2000). Sudies in

Brazilian patients have implicated DR1 and DRw6. DR7 and Dw53 . These conflicting results have raised speculation that the observed associations might be of Class II genes close or in linkage disequilibrium with a putative RF-susceptibility gene (Kaplan 2005, Quinn, *et al* 1967). RHD was diagnosed in more than one family member in 53 (24.2%) of the children with RHD, all of whom were also attending the school which included 20 siblings. One family had four siblings affected; three were in two families, two siblings in two families and one sibling in six families. In addition in 33 children, there was a positive family history of RHD in one of the parents or a close relative. There are no recent studies documenting the familial occurrence of ARF with or without RHD. Reports of familial RHD exist from the 19th century and early 1940s (Davis, *et al* 1954, Saksena, *et al* 1969, Wilson and Morton 1937, Winter 1972)

Prior to the survey only 36 (16.4%) of the parents were aware of their children having RHD and they only volunteered this information after the echocardiography. Parents of students with known RHD wanted to have a follow up echocardiography to know if the damaged valve had resolved and thus were subjected to echocardiography examination for reassurance.

Eighteen of these children were taking secondary prophylaxis on an irregular basis while the other eighteen had refused prophylactic treatment due to their parents' fear of penicillin hypersensitivity reactions and long term compliance. Health professionals should stress the consequences of RHD with the families and create a good parent-doctor relationship to gain their confidence and provide continuous reassurance (Davis 1970, Kurahara, *et al* 2006).

There is an urgent need for the early identification of children with ARF/RHD so that penicillin prophylaxis is commenced thus preventing both recurrence of ARF and progression of cardiac valve lesions (Kimbally-Kaky, *et al* 2002, Lue, *et al* 1988, Ralph, *et al* 2006, Stollerman 2001a). Shortage of resources for providing quality health care, inadequate expertise of primary health care providers and a low awareness of ARF/RHD all impact on the expression of the disease and put a large burden of a severely disabling condition on a young productive population (Kumar, *et al* 2002, Kung, *et al* 2005).

3.7 Conclusions

There is a high prevalence of RHD among Yemeni school children (36.5/1000) which is higher than in neighbouring countries. RHD is a major and serious health problem in Yemen. The prevalence of RHD increased with age, having a high preponderance in 10-16 years old students. There was no sex specific difference. MR either isolated or combined with other RHD valve lesion was the most common lesion followed by MR combined with MVP. The disease was reported in one or more than one family member in 24.2% of siblings. This survey identified new cases of RHD who were likely to go undetected until severe progressive damage had occurred.

3.8 Recommendations

Schools can be used to educate and motivate teachers, parents and children about the importance and treatment of sore throat. It is time for the public health authorities to understand the burden of this hazardous preventable heart disease and realise the cost incurred by the curative health care of patients with RHD. Sufficient human and financial resources need to be provided to promote a successful program for control of ARF and RHD.

A major goal of the Ministry of Public Health in Sana'a should be the development of national ARF and RHD prevention strategies tailored towards the local characteristics of the population. The success of the programme will depend on the cooperation, effectiveness and dedication of health personnel, policy health makers, educational administrators, community leaders and as an increased awareness of the risk by parents.

It is of utmost importance to introduce primary and secondary prophylaxis at a public health level. The strategy of primary prophylaxis programs needs to be effective and successful. It should take into consideration certain requirements that might pose some difficulties in successful achievement. The prerequisites are that patients with sore throat will attend a health center for care; primary health care providers should have the ability to diagnose GAS pharyngotonsillitis and when diagnosis is made the respective patients must be treated adequately and should adhere to the treatment regimen. It should be emphasized that such prophylactic programs should be started and continued

to further promotion of investigation and treatment of GAS pharyngotonsillitis in settings with feasible facilities.

Secondary prophylaxis programs should be continued and strengthened due to their feasibility and cost-effectiveness. Benzathine penicillin G injections given every 2 to 4 weeks are superior to daily oral penicillin. The duration of prophylaxis depends on the severity of RHD and the patient's age. Secondary prophylaxis is recommended for at least 5 years for patients with a history of ARF but without carditis and a minimum of 10 years or until age 21 years whichever is longer, with even longer duration for CRHD patients. Secondary prophylaxis requires a successful delivery through effective coordination of a central registry program with the public health authorities. It should include strategies for increasing and continuous adherence to secondary prophylaxis, screening programs through clinical and echocardiography examination to enhance early detection of ARF and RHD. Health education and health promotion programs should be conducted regularly to increase awareness of GAS sore throat and its complications of ARF and RHD for primary health care providers, teachers, patients and their families.

3.9 Limitations of the study

The availability of a portable echocardiography in the field work could have minimized the loss of participants after the clinical examination. The prevalence of RHD among the school children was probably underestimated since of the 514 children with an organic murmur, 168 (32.7%) refused to come for the echocardiography appoinment. The majority of refusals were females 150 (29.2%) and the explanation for refusal given by their parents was reluctance to accept the fact that their child might turn out to have a heart disease. If it was proved by echocardiography, it would affect their familial reputation and hinder the marital status of their children. Some families were suspicious as to why echocardiography, an expensive investigation, was free of charge. They may have thought that it was not done for the intention of research but for other unspecified reasons.

Chapter 4

4 Prevalence of Group A beta hemolyitc streptococcal infections among Yemeni school children with acute pharyngotonsillitis

4.1 Introduction

Group A beta hemolyitc *Streptococcus (*GAS) is the most important human pathogenic bacterium of the genus *Streptococcus,* encountered in paediatric clinical practice (Dale 2008, Kaplan, *et al* 2001, Martin and Green 2006).

GAS is the most common cause of bacterial pharyngotonsillitis accounting for 10 to 30% of episodes in children and 5 to 10% of episodes in adults (Barzilai, *et al* 2001, Bisno 2001, Rajkumar and Krishnamurthy 2001). A clinician has to decide if a child with acute sore throat has a GAS infection or a viral infection and whether to prescribe antimicrobial therapy (Loganathan, *et al* 2006, Menon, *et al* 2008).

Due to lack of epidemiological data from low income countries, the global burden of GAS disease is not yet well established. GAS disease may be influenced by multiple factors and the strains responsible for ARF/RHD exhibit geographical variations (Pichichero 1998, Tanz, *et al* 2006).

This study estimated the prevalence of GAS infections among Yemeni children with pharyngotonsillitis.

4.2 Objective

To determine the prevalence of GAS infection among children with pharyngotonsillitis in Aden, Yemen.

4.3 Literature review

GAS colonizes superficial tissues leading to pharyngotonsillitis or impetigo and is associated with the development of the non-suppurative sequelae ARF, RHD, acute glomerulonephritis and reactive arthritis (Bassili, *et al* 2002, Rajkumar and Krishnamurthy 2001). GAS occupy a narrow ecological niche with humans as the primary biological host and the sole environmental reservoir. The bacteria are able to replicate and withstand adverse conditions facilitated by the presence of virulence factors that promote survival (Martin, *et al* 2004).

Changes in the disease epidemiology of GAS might be associated with the introduction and reappearance of serotypes against which a population lacks immunity or to specific serotypes becoming more prevalent within populations at risk (Martin and Green 2006). Despite the advent of antimicrobials, GAS infections and their sequelae still pose a significant public health problem in many countries (Leung and Kellner 2004, McDonald, *et al* 2006, Smeesters, *et al* 2006).

4.3.1 Biology of GAS

Louis Pasteur first described *Streptococcus pyogenes*, as 'chains of beads' in 1879 and Rossenbach (1884) proposed the name Group A *Streptococcus*. Schotmuller (1903) reported distinct changes in blood media with clear zones of lysis called Group A Streptococcus β-haemolyticus(Carapetis, *et al* 2005b, Cunningham 2000). Rebecca Lancefield in 1920 discovered the GAS group polysaccharide and the cell wall M protein which are used to separate serotypes (Facklam 2002). In 1930, Fred Griffith, worked on the T protein and in 1990, together with Lancefield, developed the classic scheme for the pathogenic and phenotypic GAS characterization used globally (Efstratiou, *et al* 2003, Tagg, *et al* 1990). There was a resurgence of severe clinical GAS infections and RHD during 1980 and 1990 in many countries highlighting the importance of strain characterization (Dale 2008, Kaplan, *et al* 2001, Krause 2002).

4.3.2 Serological classification of GAS

GAS is serologically classified into specific types based on the detection of T and M cell wall protein antigens. T protein forms the basis of the agglutination typing system but its function is unknown. M protein is the basis of the precipitation typing system

and has major significance in the pathogenicity of the organism (Banks, *et al* 2002). It is quite difficult for the organism to survive in normal human blood without the M protein against which a type specific antibody can be produced (Cunningham 2000). One hundred and three different M types have been validated and internationally recognized with only one M-type antigen usually expressed by each strain. Currently a molecular approach for the identification of *emm* (M protein) genes is available (Beall, *et al* 2000).

Approximately 50% of GAS strains produce an enzyme that causes mammalian serum to increase in opacity, called serum opacity factor (*SOF*) (Oehmcke, *et al* 2004) . Antibody to OF is specific in its inhibition of the opacity reaction of the M type producing it, a characteristic that is useful as a supplementary aid to the typing scheme (Almengor, *et al* 2006, Gillen, *et al* 2002, Kanellopoulou, *et al* 2000). The typing of strains is usually based on the M and T proteins and production of *SOF*(Prakash and Dutta 1991, Shah, *et al* 1995). Genotypic methods such as *emm* and *sof* gene sequencing and *vir* typing, are newer highly discriminatory methods (Lechot, *et al* 2001, Oehmcke, *et al* 2004).

Modern techniques have advanced understanding of the virulence factors for GAS and the genomes of several GAS types are now determined (Tart, *et al* 2007). The prevalence of highly virulent clones vary across populations and further epidemiologic, laboratory and molecular studies are necessary for its full characterization (Johnson, *et al* 2006).

4.3.3 Prevalence

The prevalence of severe GAS-associated disease worldwide is estimated to be 18·1 million cases, with 1·78 million new cases annually (WHO 2004). It is estimated that 517, 000 deaths occur each year due to GAS-associated diseases. The prevalence of GAS pharyngotonsillitis varies across countries, season, age and quality of health care (Katz, *et al* 2002, Nanda Kumar, *et al* 1992).

GAS carriage in the throats of asymptomatic primary school children ranges from 10% to 50%. Recovery rates from children with GAS pharyngotonsillitis vary from 0.3 to 37% in locations such as Kuwait, Egypt, Ethiopia, Tunisia and India, with peaks in late winter and early spring (Durmaz, *et al* 2003, McIsaac, *et al* 2000, Nanda Kumar, *et*

*al*1992, Nandi, *et al* 2002b, Steinhoff, *et al* 1997). However, this is likely to be an underestimate as some patients may not seek medical advice for a sore throat due to economic or cultural reasons.

GAS pharyngotonsillitis is common in children between 3 – 15 years of age with a peak age of 11 years (Ebell, *et al* 2000). It is likely that there are no differences by sex (Barzilai, *et al* 2001). Most children have at least one episode of pharyngotonsillitis per year with 15% – 25% of these being caused by GAS (Bisno 1996, Kaplan, *et al* 2001, Martin and Green 2006).

GAS is highly transmissible and spreads rapidly in overcrowded settings through respiratory droplets or close contact within pupils, families and guests (Barzilai, *et al* 2001). The predominant serotypes constantly change in terms of their severity, virulence and sequelae.. ARF attacks vary with the severity of the host's immune response and the virulence of the GAS strain (Martin and Barbadora 2006, Veasy, *et al* 2004). Different *emm* types in GAS isolates have varied genotypes and variable virulent antigens (Bisno, *et al* 2003, Chaussee, *et al* 2008, Martin, *et al* 1994). GAS produce several surface-exposed and secreted proteins that are important for virulence at different stages during a GAS infection (Beyer-Sehlmeyer, *et al* 2005, Johnson, *et al* 2006). Variation in GAS virulence is associated with their M protein content and their degree of hyaluronate encapsulation (Bisno, *et al* 2003, Martin and Green 2006, Metzgar and Zampolli 2011). Certain environmental conditions may affect the transcription of emm in GAS (Beyer-Sehlmeyer, *et al* 2005). Bacterial growth in high salt, ambient atmosphere, or restricted iron cause a decreased activity of the virulence factors compared with growth in active carbon dioxide atmosphere (Rothbard and Watson 1948, Stollerman 1975)

GAS strains causing throat infections do not all lead to ARF but some strains that do so are unusually virulent (Bisno, *et al* 1977, Erdem, *et al* 2007, Robinson and Kehoe 1992, Stollerman 1993). Virulent fresh GAS isolates attenuate quickly during convalescence from pharyngotonsillitis, and also when grown on blood agar plates, losing their M protein content and hyaluronate capsules and resistance to phagocytosis (Martin, *et al* 1983, Stollerman 2001b). These attenuated strains of GAS survive commonly as carriers in the throats of children and remain transmissible (Kaplan 1980, Stollerman

2001a). Thus throat cultures from sporadic pharyngitis or asymptomatic carriers show varied pathogenicity of GAS strains (Johnson 1992, Stollerman 2001b). During severe epidemics of pharyngotonsillitis some GAS strains with virulent serotypes became prevalent leading to ARF and their virulence can be identified (Majeed, *et al* 1992b, Steer, *et al* 2009d). Some affected patients may not remember having an acute episode of GAS pharyngotonsillitis (Kaplan 1980). So by the time the diagnosis of ARF is confirmed, the infecting GAS clones may have gone or the persisting strain may have become attenuated and the M type may not be recognized, or new GAS strains may be acquired during the latent period (Adanja, *et al* 1991, Martin, *et al* 1994).

The resurgence of ARF in the developed world was attributed to the epidemiologic changes in the virulence of circulating GAS strains, possibly host susceptibility and the changing patterns of antibiotic use (Bisno 1990, Chang 2011, Cunningham 2000, Kaplan 1991) .

4.3.4 Clinical Diagnosis

It is difficult to differentiate the clinical features of GAS from viral upper respiratory infections or other causes of pharyngotonsillitis (Magdy Attia 1999, Santos and Berezin 2005). Headache, nausea, vomiting and abdominal pain may be seen in children with GAS (Meland, *et al* 1993, Shet and Kaplan 2004).The presence of cough, running nose, hoarseness, diarrhoea and conjunctivitis usually favour viral infections but in patients with GAS pharyngotonsillitis, respiratory symptoms may be more common than previously thought (Leung and Kellner 2004).

Tonsils are reddened and swollen and exudates appear on the posterior pharyngeal and tonsillar pillars with fine petechial lesions on the roof of the mouth. Around 50% of GAS pharyngotonsillitis in children present with tender anterior cervical lymph nodes.

Several clinical prediction rules with scoring points have been proposed to aid primary health workers in the diagnosis of GAS pharyngotonsillitis (Edmonson and Farwell 2005). One of these scoring systems is the McIsaac modification of the Centor criteria (McIsaac, *et al* 2000). This scheme is based on the following criteria and points:

Table 4. 1McIsaac Scoring System

McIsaac Score	Score
history of fever or temperature >38°C	+1
absence of cough	+1
tender anterior cervical adenopathy	+1
tonsillar swelling and exudates	+1
age ≤15 years	+1
age ≥ 45 years	-1
Maximum	5 points

Children with no points are unlikely to have GAS infection and do not need to be tested. Those with 1-3 points should be tested and treated based on the test result. Those with 4-5 points have a high likelihood of having GAS and may be treated empirically or treated if the test is positive (Edmonson and Farwell 2005, McIsaac, *et al* 2000). Clinical scoring systems can help to determine which patients should undergo diagnostic testing and potentially reduce unnecessary use of antibiotics (Warren, *et al* 1998).

Leung and Kellner reported that the sensitivity and specificity of the McIssac scoring system was 80% (Leung and Kellner 2004). In a community based study McIssac et al (2000) found that the sensitivity was 92.6% and the specificity 72.3%, while in a family medicine centre it was 96.9% and 67.2% respectively (McIsaac, *et al* 2000). Another study by Hall et al reported that the sensitivity of rapid antigen detection tests increased with the number of the scores, 47% with score of 1, 65% with a score of 2, 82% with a score of 3 and 90% with 4 and 5. Diametto et al stated that the sensitivity increased from 0 to 97% for those with 1 to 5 criteria (Hall, *et al* 2004, Leung, *et al* 2006).

4.3.5 **Diagnostic**strategies

Data from low income countries suggest that the frequency of the classical clinical presentation of GAS pharyngotonsillitis in children is estimated to be low (Kaplan, *et al* 2001). This may be attributed to regional differences in the organism and host response or limited access to health care. Re-evaluation of the clinical criteria is required taking into consideration the continuous changes that occur with the epidemiology and

demographic characteristics of GAS infection in a specific community. Hence, GAS pharyngotonsillitis in these settings may not be diagnosed in patients presenting with atypical clinical features and this reduces the clinician's confidence to diagnose GAS on clinical grounds. Under such circumstances microbiological studies are required to reach a correct diagnosis.

4.3.6 Rapid antigen detection test

In patients with acute pharyngotonsillitis, a rapid antigen detection test (RADT) can provide results within 10 to 15 minutes (Cohen 2004, Tanz, *et al* 2009). Most of the RADT have a specificity of 90% and a sensitivity > 90% (Araujo Filho, *et al* 2005, Camurdan, *et al* 2008, Mayes and Pichichero 2001, Santos and Berezin 2005). Due to the high specificity of the RADT, a positive test is accepted to be diagnostic for GAS (Cohen, *et al* 2004, Tanz, *et al* 2009). Conversely, a negative RADT should be confirmed by a throat culture in the presence of a suggestive clinical diagnosis (Johansson 2003, Leung, *et al* 2006). The rapid identification and treatment of patients with pharyngotonsillitis can reduce the risk of spread of the infection, shorten the symptom duration, decrease the occurrence of complications, reduce school absenteeism and misuse of antibiotics (Camurdan, *et al* 2008, Kaplan 2004, Kim 2009).

4.3.7 Throat culture

GAS pharyngotonsillitis is usually diagnosed in symptomatic patients by swab culture, which remains the reference standard. Culture has 90% – 97% test accuracy (Johansson 2003, Shet and Kaplan 2004, Tanz, *et al* 2009). A disadvantage of culture is the delay in confirming a diagnosis as it usually takes 24 – 48 hours to obtain a definitive result and further typing with latex agglutination kits (Martin and Green 2006). In a child with compatible history and clinical evidence of pharyngotonsillitis, a positive culture confirms a GAS diagnosis (Tanz, *et al* 2009). However in low income countries bacteriological culture is not readily available and primary health care providers depend on a clinical diagnosis to prescribe treatment (Lindbaek, *et al* 2005).

4.3.8 Treatment

It is essential to treat GAS pharyngotonsillitis with appropriate antibiotics. Appropriate treatment results in the resolution of clinical manifestations within 24 to 48 hours

(McIssac et al 2004). Penicillin remains the drug of choice and its efficacy for preventing ARF is well established (Dale 2008, Linder, *et al* 2005). This antibiotic has low cost, few side effects, narrow antimicrobial spectrum, and there is so far no documentation of penicillin resistance in GAS (Martin and Green 2006). Intramuscular benzathine penicillin G is preferred for patients who are unlikely to complete a 10-day oral course of pencillinV (Brahmadathan and Gladstone 2006).

Amoxicillin has been reported to be effective in treatment of GAS and a better solution for children, with easier dosing and better taste and less side effects (Brook 1989, Leelarasamee, *et al* 2000). In patients allergic to penicillin or amoxicillin, erythromycin remains the first choice with high efficacy (Bingen, *et al* 2000). In communities with erythromycin resistance to GAS, azithromycin or clarithromycin may be used (Cohen 2004, Leung and Kellner 2004). Oral cephalosporins are more effective in the eradication of the GAS carriage than penicillin but have more side effects, are more expensive and thus their routine use cannot be endorsed (Camurdan, *et al* 2008, Kim 2009, Wong and Chung 2002).

4.4 Patients and methods

4.4.1 Sample size and strategy

The principal investigator (PI) was informed about all Yemeni children with acute pharyngotonsillitis attending public, private and school polyclinics by the attending physicians. The PI then went to the reported site and recruited the required patients. The PI generally visited five to six clinics per day. The patients who fulfilled the inclusion criteria of age 1– 16 years, both sexes, symptoms of sore throat with evidence of fever, anterior tonsillar exudates and anterior cervical adenitis were selected, after obtaining verbal consent from their parents or through the health social worker at the school. Patients with coryza, cough, conjunctivitis and those who had used antibiotics in the two-week period prior to onset of current illness were excluded.

A sample size of 730 children was estimated. This was based on published prevalence figures for GAS 45% from neighbouring countries with similar climatic conditions at 95% CI and accepting a maximal error of $\pm$ 2 in the estimate with a power of 80% to determine the prevalence of GAS sore throat infections.

4.4.2 Collection of throat swab

Two throat swabs were collected by rubbing sterile cotton tipped swabs over the tonsillar area without touching the tongue or lips. The first swab was tested by a RADT to detect GAS antigens in pharyngeal swabs. Results were obtained in 10 – 15 minutes and graded as positive, negative or non-specific.

The second swab was rolled on a Strept Selective Agar plate containing 5% sheep blood. There was a laboratory technician who helped with these cultures. The plates were incubated at 35°C under anaerobic conditions for 24 to 48 hours and checked for β haemolytic streptococi. Bacitracin was placed culture plates with a sub-culture of the initial isolateand those that gave a zone of inhibited growth around the disc of > 15 mm diameter were considered potential GAS. Bacitracin sensitive GAS that were isolated from patients with a negative RADT were subsequently tested for the Lancefield group by the latex agglutination test, the Oxoid streptococcal grouping kit, used for the identification of the streptococcal group and reagents provided for groups A, B, C, D, F and G (Diagnostic Reagents Streptococcal Grouping Kit Code: DR0585).

4.4.3 Treatment of patients

The patients were treated with penicillin if positive by either RADT or culture. If patients had a positive clinical McIssac score but RADT was negative, they were given an appointment to return after 48 hours for their culture results and then received their treatment. School children were revisited by the PI within the next 48 hours with the culture results. Patients were given penicillin for 7 – 10 days either orally or parenterally based on their clinical condition. Those having history of pencillin allergy were treated with erythromycin for 7 days.

4.5 Results

4.5.1 General characteristics of the participants

A total number of 730 children were enrolled, with a mean (SD) age of 11.8 (3.4) years between August 2006 and July 2007. The predominant age was 11 -15 years which included more than half of the patients (406, 55.6%), followed by 6-10 years' old

children (163, 22.3%). Male comprised 319 (43.7%) and female 411 (56.3%) of the children with pharyngotonsillitis, with a ratio of 1:1.3. The participants recruited from schools comprised 553 (75.8%) of the patients and those recruited from a public clinic were 96 (13.1%) and those from a private clinic comprised 81 (11.1%).

A history of prophylactic penicillin was recorded in 83 (11.4%) of the participants who were either previously diagnosed as ARF or RHD. A history of tonsillectomy in another family member was obtained in 95 (13.1%). A history of ARF and RHD was elicited in 62 (8.5%) and 49 (6.7%), respectively and a family history of ARF and RHD was reported in 44 (6%) and 45 (6.2%), respectively.

A greater proportion of the patients (536, 73.3%) were enrolled during the winter months (October to February) while the lowest frequency (194 (26.7%)) occurred in the summer (March to September) (Table 4.1). 297 (40.7%) children reported having 5 – 8 acute pharyngotonsillitis attacks per year followed by 224 (30.7%) with 9 to 12 attacks per year.

Table 4. 2Demographic characteristics of participants with acute pharyngotonsillitis

Demographic factors		n=730 (%)
Sex	Male	319 (43.7%)
	Female	411 (56.3%)
Age (years)	Mean (S.D.)	11.8 (3.4)
Age group (years)	0 – 5	69 (9.5%)
	6 – 10	163 (22.3%)
	11- 15	406 (55.6%)
	> 15	92 (12.6%)
Season	Winter (October –February)	536 (73.3%)
	Summer (March- September)	194 (26.7%)
Medical history	History of ARF	62 (8.5%)
	History of RHD	49 (6.7%)
Family history	ARF	44 (6%)
	RHD	45 (6.2%)
	Tonsillectomy	95 (13.7%)
Sore throat episodes reported/year	< 4	205(28.1%)
	5 – 8	297 (40.7%)
	9 – 12	224 (30.7%)
	>12	4 (0.5%)
Prophylactic penicillin		83 (11.4%)
Clinic location	School	553 (75.8%)
	Private	81 (11.1%)
	Public	96 (13.1%

4.5.2 Acute pharyngotonsillitis attacks among participants by age and sex

The frequency of pharyngotonsillitis episodes per year varied by age and sex. Females had a mean (SD) 7.3 (3.5) episodes which was higher than that for males who had a mean (SD) 6.7 (3.5) episodes (p=0.02). There was no statistically significant difference in the number of episodes of pharyngotonsillitis in the different age groups.

Table 4. 3Pharyngotonsillitis episodes reported by sex and age group

Variable		Pharyngotonsillitis episodes per year	
		Mean (SD)	p
Gender	Female	7.3 (3.5)	0.02
	Male	6.7 (3.5)	
Age groups (years)	< 5	7.1 (3.1)	0.06
	6 – 10	6.6 (3.3)	
	11 – 15	7.4 (3.7)	
	>15	6.5 (3.3)	

4.5.3 Culture and RADT results

RADTs followed by culture on sheep blood agar media were performed on all patients with clinical manifestations of acute pharyngotonsillitis (figure 4.1). A total of 267 (36.6%) patients had positive RADT.

GAS cultures were positive in 238 (89.1%) patients with positive RADT compared to 33 (7.1%) patients with negative RADT. Among the patients with negative RADT but positive cultures, 20 (60.6%) were GAS positive by culture. The cultures were positive for streptococci other than GAS in 13 (39.4%) patients with acute pharyngotonsillitis. Cultures were contaminated in 10 patients with positive RADT but were included in the analysis as having GAS since the RADT is only capable to detect the carbohydrate antigen only for GAS. There were 39 contaminated cultures of patients with negative RADT and these were excluded from the study.

There were a total of 49 cultures that were contaminated because of overgrowth with other bacteria. The likely reason is laboratory plate contamination. We attempted unsuccessfully to recover GAS from these cultures.

The proportion of contaminated cultures in the RADT positive cases was 10/267 (3.7%), significantly less than the 39/463 (8.4%) contaminated cultures in the RADT negative group (p=0.02). If the all contaminated cultures are excluded from the analysis the sensitivity of the RADT is 92.6% and specificity 95.3%. If the contaminated cultures are all included and assumed to be GAS positive (for the RADT positive samples) and GAS negative (for the RADT negative samples) the sensitivity and specificity would be little changed at 92.9% and 95.6%. In contrast, if the worse case scenario is assumed, contaminated culture were included and assumed to be GAS culture negative (for the RADT positive samples) and GAS culture positive (for the RADT negative samples) the sensitivity and specificity would be 89.1% and 86.5%. The true sensitivity and specificity is likely to rest between these values with a bigger variation in the possible specificity.

Overall a total of 691 patients with acute pharyngotonsillitis of both sexes had valid GAS information and were included in subsequent analysis.

4.5.4 Composite diagnosis

A composite diagnosis was formed with the terms "with" GAS and "without" GAS pharyngotonsillitis. Patients with GAS pharyngotonsillitis included all patients with positive RADT and patients with negative RADT but positive culture for GAS comprising a total of 287 (41.5%) patients. Patients without GAS pharyngotonsillitis were defined as patients who had both negative RADT and negative GAS culture, or a negative RADT with a positive culture for other non GAS bacteria. A total of 404 patients were classified as not having GAS pharyngotonsillitis (Figure 4.1).

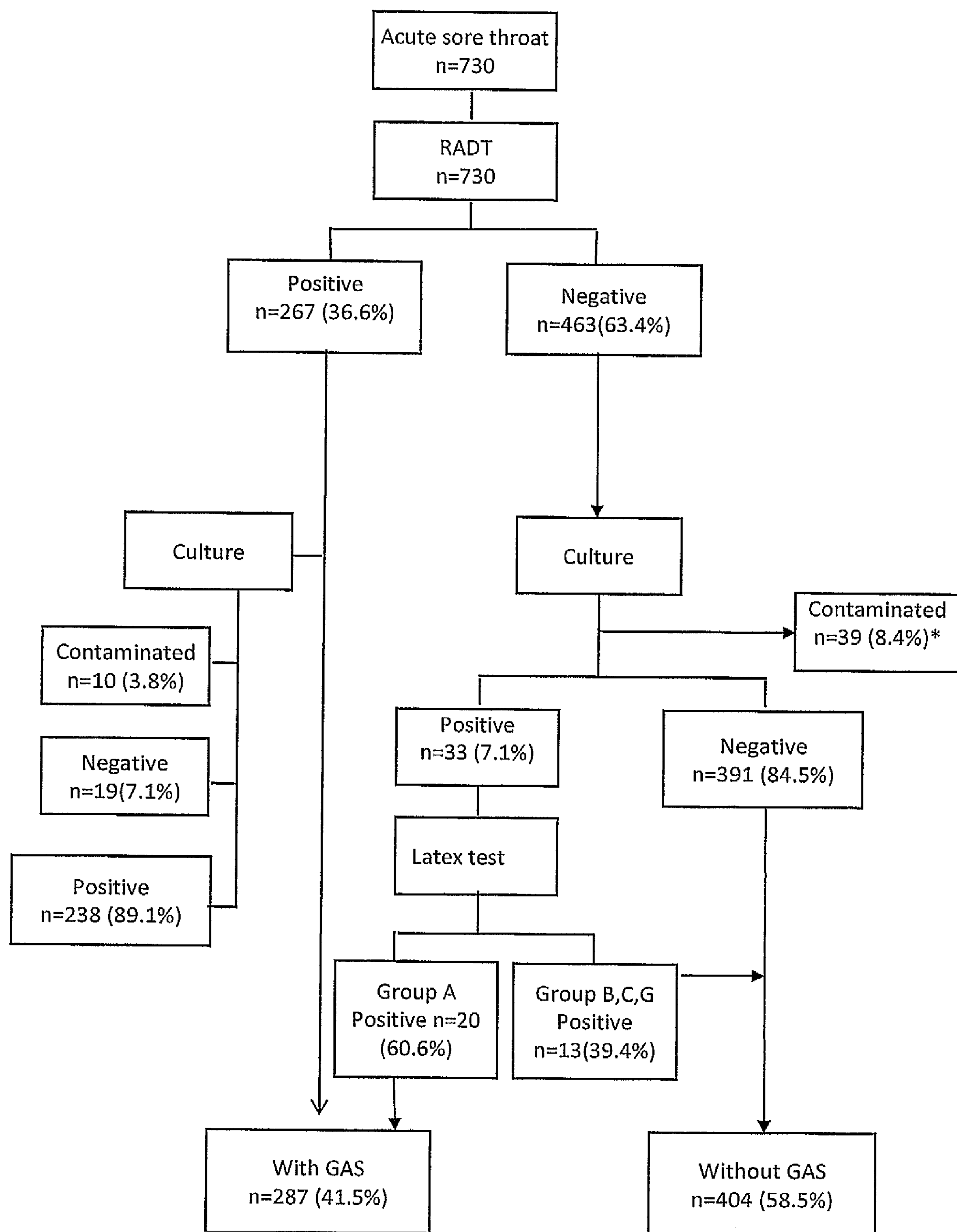


Figure 4. 1Flow chart of participants with acute sore throat. *Contaminated cultures 39 (8.4%) were excluded from the detailed analysis

4.5.5 Demographic characteristics of patients with and without GAS pharyngotonsillitis

Patients with GAS pharyngotonsillitis were more frequently observed among 11 -15 years old children (p=0.001) (Table 4.3). Acute pharyngotonsillitis with GAS was more frequent among females than males (219 (54.2) and 185 (45.8%), respectively), but this was not statistically significant.

Children with GAS reported a higher frequency of pharyngotonsilltiis episodes per year with a mean (SD) 8.2 (3.6) compared to children without GAS pharyngotonsillitis who had a mean (SD) 6.2 (3.2) episodes per year. The difference between the children with and without GAS pharyngotonsillits was statistically significant (p=0.001) (Table 4.3).

Table 4. 4Demographic characteristics in 691 children with and without GAS

Variable		With GAS n=287 (%)	Without GAS n=404 (%)	p
Age (years)	< 5	17 (5.9%)	51 (12.6%)	
	6 -1 0	51 (17.8%)	105 (26%)	
	11 – 15*	196 (68.3%)	182 (45%)	0.001
	>15	23 (8%)	66 (16.3%)	
Sex	Male**	113(39.4%)	185 (45.8%)	0.1
	Female	174 (60.6%)	219 (54.2%)	
Pharyngotonsillitis episodes***	Mean (SD)	8.2 (3.6)	6.2 (3.2)	0.001

*p=0.001 The age difference in children with and without GAS was statistically significant in the 11 – 15 years group

**p= 0.1 The differences between sexes in patients with GAS and without GAS was not significant statistically

***p=0.001 Pharyngotonsillitis episodes was statistically significant in children with and without GAS

4.5.6 Seasonal variation of GAS pharyngotonsillitis

Cases of GAS pharyngotonsillits were observed all year round. A total of 209 (72.8%) patients with GAS had throat infections during the winter months which were higher than in the summer months. However, a higher proportion of patients had GAS pharyngotonsillitis during the months of November to February with a peak in January 71 (25%), followed by December 53 (19%), February 44 (15%) and November 41 (14%).

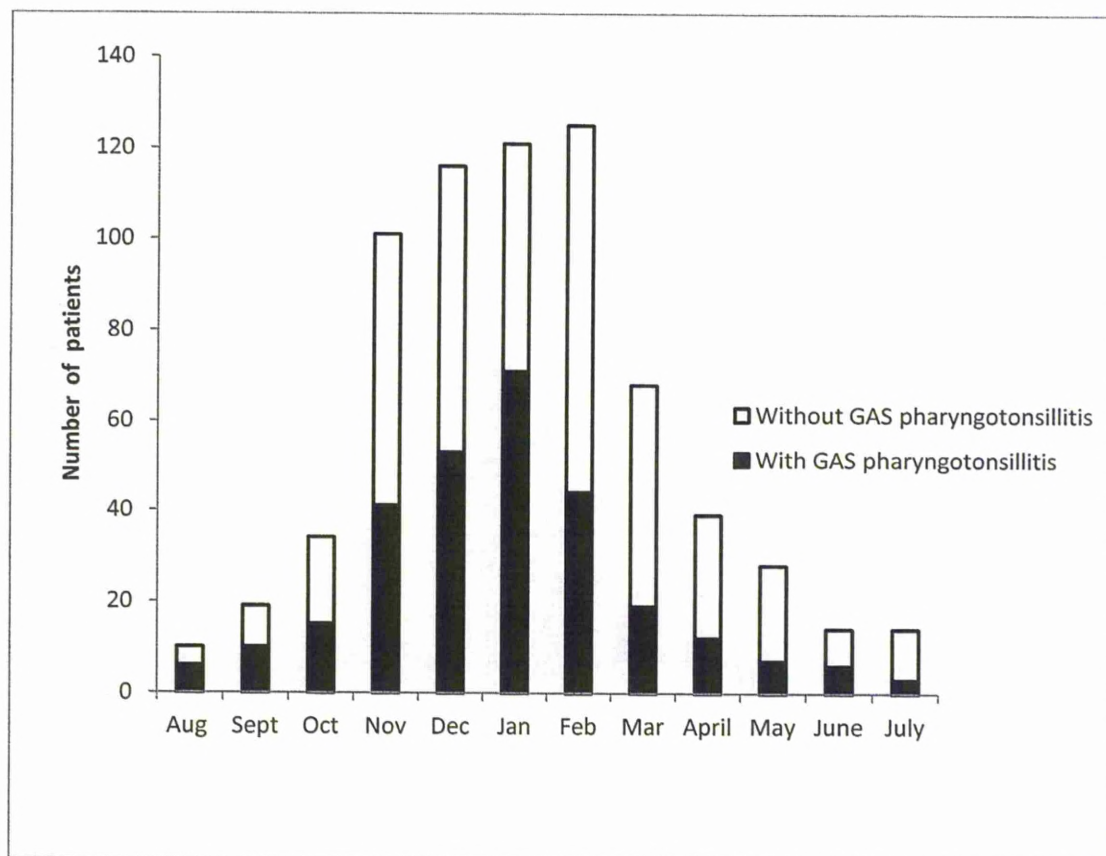


Figure 4. 2Distribution of patients with and without GAS pharyngotonsillitis by months (2006-2007)

4.5.7 Clinical symptoms of patients with and without GAS pharyngotonsilltis

A history of sudden onset with fever, pain on swallowing, headache and nausea were obtained with a higher frequency in the patients with GAS pharyngotonsillitis than in children without GAS (p=0.001). In contrast the presence of cough, rhinorrhoea and hoarseness were more frequent in patients without GAS pharyngotonsillitis (p=0.001) (Table 4.4).

Table 4. 5Clinical symptoms of patients with and without GAS Pharyngotonsillitis

History of	With GAS n=287	Without GAS n=404	OR (95% CI)	P
Sudden onset	267 (93)	307 (76)	4.2 (2.5 – 7.0)	0.001
Sore throat	285 (99.3)	400 (99)	1.4 (0.3 – 7.8)	0.7
Fever	280 (97.6)	293 (72.5)	15.2 (6.9 – 33.1)	0.001
Headache	149 (51.9)	150 (37.1)	1.8 (1.4 – 2.5)	0.001
Pain on swallowing	279 (97.2)	349 (86.4)	5.5 (2.6 – 11.7)	0.001
Vomiting	34 (11.8)	42 (10.4)	1.2 (0.7 – 1.9)	0.6
Nausea	123 (42.9)	114 (28.2)	1.9 (1.4 – 2.6)	0.001
Abdominal pain	20 (7)	13 (3.2)	2.3 (1.1 – 4.6)	0.03
Cough	27 (9.4)	144 (35.6)	0.2 (0.1 – 0.3)	0.001
Rhinorrhoea	10 (3.5)	88 (21.8)	0.1 (0.1 – 0.3)	0.001
Hoarseness	10 (3.5)	81 (20.0)	0.2 (0.1 – 0.3)	0.001

4.5.8 Clinical signs of patients with and without GAS pharyngotonsillitis

Erythema with exudate in the anterior tonsillar pillar was observed in 251 (87.5%) children, red uvula in 113 (39.4%), petechiae on the soft palate in 90 (31.4%) and tender anterior lymph nodes in 196 (68.3%) patients with GAS pharyngotonsillitis. In contrast erythema with exudate in the anterior tonsillar pillar in 235 (58.2%), red uvula in 38 (8.9%), petechiae on the soft palate in 18 (4.5%), tender anterior lymph nodes in 57 (14.1%) were less frequent in patients without GAS pharyngotonsillitis (p=0.001). Skin rash was observed in 2 patients with GAS and in 4 patients without GAS (Table 4.5).

Table 4. 6Clinical signs of patients with and without GAS pharyngotonsillitis

Clinical signs	With GAS n=287	Without GAS n=404	OR (95% CI)	p
Erythema	284 (99)	393 (97.3)	2.7 (0.7 – 9.6)	0.14
Oral ulcers	1(0.3)	10 (2.5)	7.3 (0.9 – 57)	0.06
Anterior tonsillar erythema	251 (87.5)	235 (58.2)	5.0 (3.4 – 7.5)	0.001
Exudates	277 (96.5)	363 (89.9)	3.1 (1.5 – 6.4)	0.002
Anterior tonsillar exudate	209 (72.8)	187 (46.3)	3.1 (2.3 – 4.3)	0.001
Red uvula	113 (39.4)	38 (9.4)	6.6 (4.4 – 10.1)	0.001
Soft palate petechiae	90 (31.4)	18 (4.5)	9.8 (5.7 – 16.7)	0.001
Anterior cervical lymph node	196(68.3)	57 (14.1)	13 (9.0 – 19)	0.001
Skin rash	2 (0.7)	4 (1.0)	1.9 (0.8 – 4.5)	0.15

A logistic regression model with the best fit based on the clinical criteria to identify children with GAS infections is shown in Table 4.6. The clinical characteristics independently associated with GAS pharyngotonsillitis included fever (AOR=6.1), anterior tender cervical lymphadenopathy (AOR=13.1), petechiae on soft palate (AOR=4.2) and the presence of a red uvula (AOR=4.0). Conversely, clinical symptoms

less likely to be associated with GAS pharyngotonsillitis were rhinorrhoea (AOR= 0.4), hoarseness (AOR=0.6) and cough (AOR= 0.4) (Table 4.6).

Table 4. 7Logistic regression analysis of clinical criteria of acute pharyngotonsillitis to identify children with GAS

Clinical criteria	OR	AOR	95% CI	P value
Fever	15.2	6.1	2.7 – 14	0.001
Anterior cervical lymph node	13.1	9.1	6 – 13.7	0.001
Soft palate petechiae	9.8	4.2	2.3 – 7.9	0.001
Red uvula	6.6	4.0	2.6 – 6.2	0.001
Cough	0.2	0.4	0.2 – 0.6	0.001
Anterior tonsillar erythema	5.0	3.4	1.9 – 6.0	0.001
Nausea	1.9	1.9	1.3 – 2.7	0.001
Headache	1.8	1.7	1.2 – 2.4	0.003
Rhinorrhoea	0.1	0.4	0.2 – 0.7	0.002
Hoarseness	0.2	0.6	0.3 – 0.9	0.002
Anterior tonsillar exudate	3.1	1.8	1.0 – 3.0	0.034
Sudden onset	4.2	1.2	–	–
Pain on swallowing	5.5	2.1	–	–
Sore throat	1.4	0.3	–	–
Vomiting	1.2	0.9	–	–
Abdominal pain	2.3	1.9	–	–

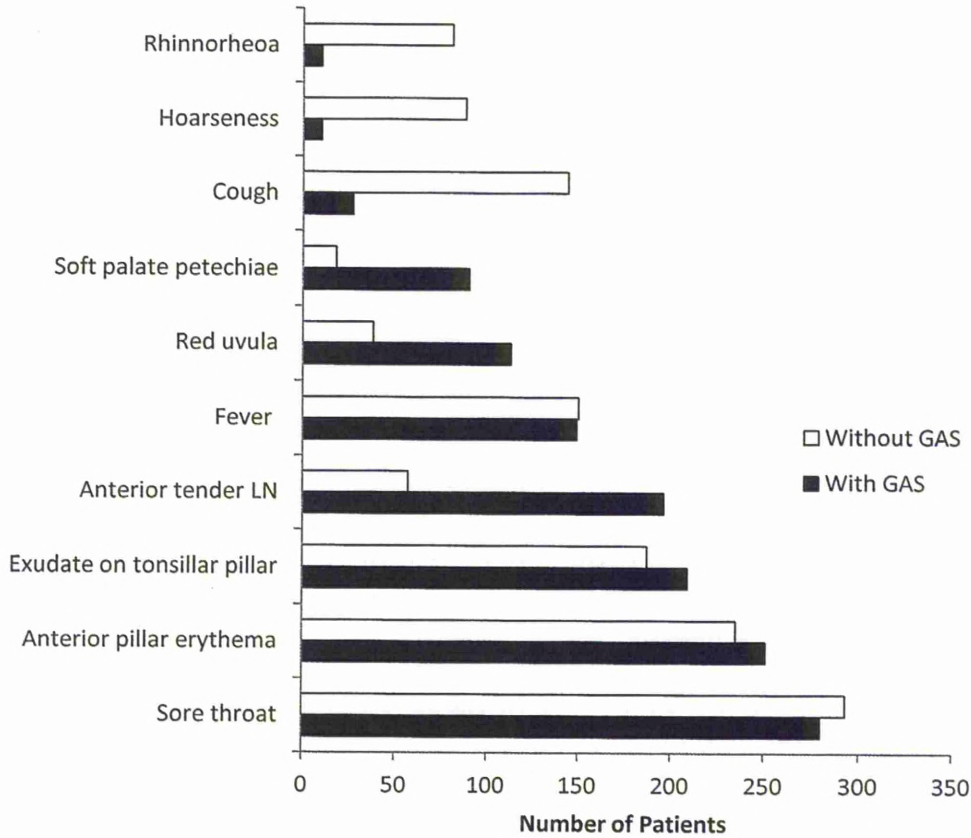


Figure 4. 3Clinical characteristics of patients with and without GAS

4.5.9 McIssac clinical score (McIsaac et al. 2000)

The McIssac score was assessed among patients with positive RADT having negative throat cultures in comparison with patients with positive RADT and positive throat cultures. The results are shown in Table 4.7. The difference was statistically significant between the two groups (p=<0.02).

Table 4. 8McIssac score in patients with positive RADT and negative/positive cultures

McIssac score	Positive RADT Negative culture n=29	Positive RADT Positive culture n=238	Total n=267
1	0	2 (0.8%)	2 (0.75%)
2	0	2 (0.8%)	2 (0.75%)
3	11(38%)	17 (7.2%)	28 (10.5%)
4	13 (44.8%)	76 (32%)	89 (33.3)
5	5 (17.2%)	141 (59.2%)	146 (54.7%)

$p < 0.02$ McIssac score was significantly different between patients with positive RADT and negative culture compared to those with both positive RADT and culture

The McIssac score was also assessed among patients with positive RADT having negative throat cultures compared to patients with negative RADT and positive throat cultures. The results are in Table 4.8. The difference was not statistically significant between both groups.

Table 4. 9McIssac score in patients with positive RADT and negative cultures and negative RADT with postive cultures

McIssac score	Positive RADT Negative culture n=29	Negative RADT Positive Culture n=20	Total n=49
1	0	1 (5%)	1 (2%)
2	0	1 (5%)	1 (2%)
3	11(38%)	5 (25%)	16 (32.7%)
4	13 (44.8%)	7 (35%)	20 (40.8%)
5	5 (17.2%)	6 (30%)	11 (22.5%)

$p<0.5$ McIssac score was not significant between the two groups

Among patients with a positive RADT having negative cultures 10 (12.5%) were receiving prophylactic benzathine penicillin compared with 42 (17.6%) patients with positive RADT and positive throat cultures receiving prophylactic benzathine penicillin. There was no significant difference in the proportion of RADT positive patients receiving penicillin prophlaxis with either positive or negative cultures.

Table 4. 10Positive RADT in patients with negative or positive cultures in 52 patients on antimicrobial prophylaxis

Antimicrobial prophylaxis	Positive RADT Negative culture n=29	Positive RADT Positive culture n=238	Total n=267
No	19 (65.5%)	196 (82.4%)	215 (80.5%)
Yes	10 (34.5%)	42 (17.6%)	52 (19.5%)

$p < 0.4$ = the difference in RADT between positive and negative cultures in patients on penicillin prophylaxis was not significant

The McIssac clinical score was assessed according to the presence or absence of GAS pharyngotonsillitis and the sensitivity and specificity were calculated (Table 4.10). A ROC curve was drawn to identify the optimal cut off that resulted in the highest combination of sensitivity and specificity (Figure 4.4). The sensitivity and specificity at the cut off point of 3 which is currently recommended showed a sensitivity of 98.6% but a very low specificity of 17% in this analysis. A sum score of 4 had a sensitivity of 93% and a specificity of 82% while a sum score of 5 was 66% sensitive and 89% specific.

The estimate of the area under the smooth ROC curve (AUC) is 0.839 and its 95% confidence interval is 0.808 and 0.869

Table 4. 11Sensitivity and specificity of McIssac score in children with /without GAS

McIssac score Cut off points	Total	With GAS N=287	Without GAS N=404	Sensitivity	pecificity
≥1	13	287	404	100	1
≥2	61	285	393	99	3
≥3	279	283	334	99	17
≥4	102	266	72	93	82
5	236	190	46	66	89

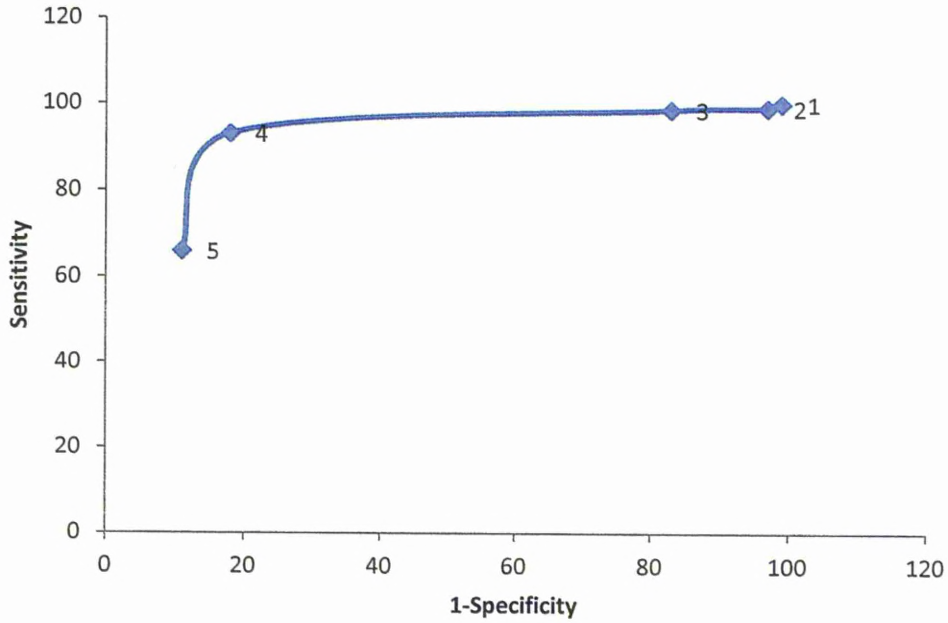


Figure 4. 4ROC curve of McIssac score to identify Definitive GAS pharyngotonsillits

4.5.10 Characteristics of patients who had GAS with positive and negative RADT

The RADT was interpreted as positive in 267 (93%) patients of whom 238 had positive GAS cultures and in 19 who had negative GAS cultures. The RADT was false negative in 20 (7%) patients with culture confirmed GAS pharyngotonsillitis. The mean age (SD) of patients with RADT true positives was 12 (3.2) and the mean age (SD) for RADT false negative was 13 (1.3) (p=0.005).

Males had 111 (98.2%) RADT true positives and 2 (1.8%) false negatives compared to 156 (89.7%) females having RADT true positives and 18 (10.3%) false negatives.The difference between males and females was statistically significant (p=0.004).

McIssac scoring with a 5 cut off point was observed in 153 (57.4%) patients with GAS pharyngotonsillitis who had true positive RADT and in 20 (100%) patients with GAS false negative RADT (P=0.01) as shown in Table 4.10.

Table 4. 12Clinical characteristics of patients with GAS and positive or negative RADT

Variable		RADT		p
		True positive n=267	False negative n=20	
Age (years)	Mean (SD)	12 (3.2)	13 (1.3)	0.005
Sex	Female	156 (89.7%)	18 (10.3%)	0.004
	Male	111 (98.2%)	2 (1.8%)	
McIssac score	1	3 (1.1%)	0	
	2	3 (1.1%)	0	
	3	22 (8.2%)	0	
	4	86 (32.2%)	0	
	5	153 (57.4%)	20 (100%)	0.01

4.6 Discussion

Globally GAS accounts for 10% to 30% of acute pharyngotonsilltis and is the only type of sore throat that requires antibiotic treatment to prevent ARF and reduce the occurrence of local non suppurative complications (Johansson 2003, Park, *et al* 2006, Tanz, *et al* 2009). Despite its clinical significance and the high prevalence of 36.5 cases of RHD per 1000 population among Yemeni school children (see Chapter 3), there is no information on the prevalence of GAS pharyngotonsillitis in Yemen.

This is the first major study of the prevalence of GAS isolated from Yemeni children with acute pharyngotonsillitis. The prevalence of GAS pharyngotonsillitis in children was 41.5%, which is higher than the rates reported from both neighbouring countries and other parts of the world. Studies include India (4.2% -13.7%; (Menon, *et al* 2004), Tunisia (17.7%; (Mzoughi R2004), Egypt (17% -24%), China (21.4%; (Meng-Hsun Lin2003), Brazil (24%; (Santos and Berezin 2005), Canada (34.8%; (McIsaac, *et al* 2000) and Turkey (35.9%) (Rimoin, *et al* 2008). These variations in the prevalence of GAS pharyngotonsillitis might be attributed to the differences in geographical sites and the methods of diagnosis.This finding of high prevalence confirms that GAS is an important cause of pharyngotonsillitis among Yemeni children and requires prompt diagnosis and appropriate antibiotic therapy to shorten the symptom duration and reduce the risk of ARF and RHD.

In general the literature does not demonstrate a gender association with the risk of GAS pharyngotonsillitis (Danchin, *et al* 2007, Rimoin, *et al* 2005). In this study even though pharyngotonsillitis was more frequent among females, this was not a statistically significant association. It is a comparable observation to two similar studies in India (Nandi, *et al* 2002b) and Brazil (Santos and Berezin 2005). In contrast to China there was a higher prevalence in males than females (Meng-Hsun Lin2003). The differences in these results may be attributed to the peculiarities in the study location.

Age is reported to be an important factor in the aetiology of GAS pharyngotonsillitis. This study found the highest prevalence of GAS pharyngotonsillits was in the 11 – 15 years group (68.3%) versus an overall prevalence of (41.5%) in all age groups. The prevalence varies in different age groups from one study to another as reported in Rimion's study, although this is partly due to use of different age groups. A peak age of 5 – 9 years was reported in Egypt, Brazil and Croatia, and in China it was 6 -11 years (Meng-Hsun Lin2003), while in Latvia it was 10 – 12 years of age (Bassili, *et al* 2002, Rimoin, *et al* 2008).

Comparison with studies in other low income countries is currently difficult due to the limited data on the age distribution of GAS pharyngotonsillitis in children. But the occurrence of GAS pharyngotonsillitis in an older age group among Yemeni patients could be explained by the probability that it coincides with the age of transfer from the primary to preparatory classes with more overcrowding and increased rate of sore throat infections among older students.

The lowest frequency of GAS pharyngotonsillitis was detected among 68 (9.8%) children less than 5 years of age in this study. This result was still higher than the WHO report on the clinical evaluation of GAS pharyngotonsillitis among children under 5 years that found low rates of 4.6%, in Egypt, 3.6% in Croatia and no case below 5 years in Brazil (Rimoin, *et al* 2005). The low frequency in this age group in this study might be attributed to the fact that children may not present with the classical symptoms of GAS infections making the clinical diagnosis difficult. Hence in this group the diagnosis would be better based on the diagnostic laboratory tests in conjunction with clinical findings.

The frequency of acute sore throat attacks are reported to be higher in low income countries than high income countries. Danchin *et al*(2007) in Australia reported one-third of children experiencing a confirmed GAS positive culture sore throat every year . They estimated that approximately 7 sore throat episodes occur per child per year with 13.5 % of these being caused by GAS (Pichichero 1998, Sauver, *et al* 2006). These results are similar to this study with the majority having 6 episodes although we obtained a higher rate of 19.9% GAS pharyngotonsillitis.

There are inconsistent reports of seasonal variation in GAS pharyngotonsilltis. In temperate regions the general observation is that the peak is during winter and early spring while in the tropics it is in late autumn and early winter and and declines in summer (Brahmadathan and Gladstone 2006, Kaplan 2004, Sauver, *et al* 2006). The results of this study revealed a high occurrence of GAS pharyngotonsillitis of 84.7% during the cool months of November, December, January and February with the peak in January. These results are similar to a study in Latvia which showed a similar high frequency between December to February (Rimoin, *et al* 2008). In Brazil, the peak was during the cool months of March to August (Santos and Berezin 2005). This study was quite different, however, from a study in Tunisia where the isolation rate of GAS peaked twice during the year from October to December and in June (Mzoughi R2004). In Egypt and Croatia the peak was from September to November (Gove, *et al* 1998, Rimoin, *et al* 2008). These differences might be explained by the variations in climatic conditions of each country.

The accurate diagnosis of GAS pharyngotonsillitis is repeatedly reported to be quite difficult since the symptoms and signs of sore throat due to GAS or non GAS infections overlap widely and make it unreliable to base a diagnosis only on clinical assessment (Bisno, *et al* 2003, Gieseker, *et al* 2003). In this study, the clinical characteristics of fever, sore throat, anterior tonsillar erythema and exudates with enlarged anterior tender lymph nodes were detected with a higher frequency in children with GAS pharyngotonsillitis compared with those without GAS pharyngotonsillitis.

In contrast, the symptoms of cough, hoarseness and rhinorrhea favoured the diagnosis of those without GAS pharyngotonsillitis that are more likely to be due to a viral

aetiology (Bisno, *et al* 2002, Johansson 2003). These clinical characteristics were not significantly different from the reports from various countries history of fever, absence of cough, tender cervical lymph adenopathy, tonsillar exudates and age $\leq$15 years associated with GAS infection (Lazar R. 2004, Lindbaek, *et al* 2005, Magdy Attia 1999).

Some symptoms and signs of GAS pharyngotonsillitis may vary by site and clinical status. Even though the clinical symptom of difficulty in swallowing was statistically associated with GAS pharyngotonsillitis in Brazil, Egypt and Latvia (Rimoin, *et al* 2008) but there was no clinical association among Yemeni patients with GAS pharyngotonsillitis. A significant association of skin rash with GAS pharyngotonsillitis had been reported which was not evident in this study (Gieseker, *et al* 2003, Santos and Berezin 2005). The patients with GAS pharyngotonsillitis in this study who presented with petechial lesions on the soft palate comprised 31.4% which was three times higher than the literature reports of 10% (Bisno, *et al* 2002). The presence of red erythematous uvula was also observed with higher frequency in this study when compared to other reports (Bisno, *et al* 2003, Kaplan 2005).

The presence of red erythematous uvula and petechiae on the soft palate were significantly more common in patients with GAS pharyngotonsillitis compared with those without GAS (P=0.001). In various other literature reports, these two specific significant signs did not help to differentiate between the clinical predictor variables of acute pharyngototnsillitis due to GAS and those without GAS (Martin, *et al* 2004, Steinhoff, *et al* 1997).

It is recommended that further studies should be conducted with the possibility of introducing these two particular signs into a scoring system to improve identification of GAS pharyngotonsillits. Moreover the re-evaluation and improvement in this scoring scale based on clinical criteria alone would of great help in underdeveloped countries with poor financial resources that limits the widespread use of laboratory test to confirm GAS diagnosis in patients with acute pharyngotonsillitis.

In patients with acute GAS pharyngotonsillitis, the recommended strategy is that if the RADT is positive it is considered reliable and appropriate treatment is given (Armengol,

et al 2004, Gieseker, *et al* 2003, McIsaac, *et al* 2000). This is because the occurrence of false-positive results is unusual and so therapeutic decisions can be taken with confidence without requiring a throat culture confirmation (Chapin, *et al* 2002, Fox, *et al* 2006). There were 33 patients with negative RADT on whom backup throat cultures were performed of whom 20 (60.6%) patients showed positive GAS cultures while 13 (39.4%) patients grew cultures positive for Non-GAS isolates.This is comparable to the study of McIssac et al in Canada and Edmonson in USAwho confirmed that 70% with negative RADT required backup cultures (Edmonson and Farwell 2005, McIsaac, *et al* 2004). If the RADT is negative a confirmatory culture is required to avoid the risk of missing GAS pharyngotonsillitis that may necessitate treatment especially in regions with high prevalence of ARF and RHD.

RADT is unlikely to yield a positive result in children with low McIssac scores ≤ 2 where if a backup culture is done it will have a great likelihood of giving a negative result. We found that a false negative RADT was significantly more common in older children, females and in those children with a McIssac score of 5. A similar study by Edmonson and Farwell (2005) in Wisconsin USA detected difference in age but there was no sex difference.

The McIssac score of clinical criteria for GAS diagnosis of acute pharyngotonsillitis used in this study showed that the usual cut off point of 3 was unreliable. The application of the cut off point of 4 or more had a higher sensitivity (93%) and specificity (82%) [Figure 4.4]. These results suggest that RADT could be selectively used in children with McIssac Score of 4 and above with a positive result removing the need of a backup culture for confirmation of the diagnosis.

Throat culture has long been accepted as the gold standard for GAS diagnosis of acute pharyngotonsillitis. RADT has been recently introduced to overcome the time delay in throat cultures (Bassili, *et al* 2002, Van Howe and Kusnier 2006). RADT has an advantage in that by confirming the presence of GAS carbohydrate antigen on a throat swab with results available within 10 – 15 minutes in the clinic, they provide an effective way to differentiate between patients having acute pharyngotonsillitis that need antibiotics and those who do not (Leung, *et al* 2006, Park, *et al* 2006).

RADT has been utilized in different populations and it has been shown that a significantly increased number of patients were treated for GAS pharyngotonsillitis on time when compared to the use of traditional cultures (Gerber and Shulman 2004, Gonzalez-Rey, *et al* 2003, Van Howe and Kusnier 2006). The use of RADT can reduce the home stay of the patient and allow their early return to school and further reduce the acute disease state (McIsaac, *et al* 2004). It will further decrease the number of consultations with general practitioners and paediatricians (Araujo Filho, *et al* 2005).

Furthermore the prompt management of GAS pharyngotonsillitis with antimicrobial therapy will help to reduce the risk of spread to close contacts and may also hasten clinical improvement and prevent the suppurative and nonsuppurative sequelae which are key therapeutic goals (Cooper, *et al* 2001, Darrow and Buescher 2002, Shet and Kaplan 2004). RADTs, however, are quite expensive (De Socio, *et al* 2004). This limitation needs to be taken into consideration before they are widely distributed and applied as a routine practice in poor developing countries for the diagnosis of patients with acute pharyngotonsillitis due to GAS.

There were differences in the symptoms and signs of GAS pharyngotonsillitis in this study when compared to those in other countries. However, it is rather difficult to create and follow a strict single prediction rule for all countries with the changing epidemiology of GAS infections.

It is of great concern to correctly diagnose GAS pharyngotonsillitis so as to minimize the inappropriate use of antibiotics and occurrence of antimicrobial resistance (Dey, *et al* 2005, Ebell, *et al* 2000, Hsin Chi 2003). However, it is essential to conduct further studies in neighbouring regions to assess the valid clinical indicators for GAS pharyngotonsillitis especially in countries with high prevalence of ARF and RHD.

It is of paramount importance to instruct parents with children suffering acute sore throat to seek medical advice through visits to health care facilities. It is also essential to increase awareness regarding the importance of GAS pharyngotonsillitis and its implications through a school health programme.

4.7 Conclusions

A high prevalence of GAS pharyngotonsillitis was detected particularly in the 11 – 15 year age group but there was no significant difference between both sexes. A high proportion of GAS pharyngotonsillitis was observed during the winter with the highest peak in January. The cut off point for McIssac score of a value of 4 and above demonstrated better diagnostic accuracy for GAS pharyngotonsillitis.

The clinical characteristics associated with GAS pharyngotonsillitis included fever, anterior tender cervical lymphadenopathy, petechiae on the soft palate and the presence of a red uvula. The clinical symptoms less likely to be associated with GAS pharyngotonsillitis were rhinorrhea, hoarseness and cough.The RADT was positive in 93% of the patients of whom 89% had positive GAS. The RADT was false negative in 7% of patients with GAS pharyngotonsillitis. The McIssac score was significantly different between patients with positive RADT having negative throat cultures compared to positive RADT and positive culture.

Two additional signs, petechiae on the soft palate and a red erythemaous uvula were significantly associated with GAS pharyngotonsillitis. In future studies it is important to include and further assess these two clinical findings in the predictive clinical criteria for GAS pharyngotonsillitis in children. This tool will enhance a simple and practical approach for the primary care physician and paediatricians in the identification and management of GAS pharyngotonsillitis.

4.8 Recommendations

RADT has an advantage over throat cultures with immediate positive results for GAS pharyngotonsillitis. This allows decisions to be taken on the spot and antibiotics prescribed but its high cost may limit its practical use in poor underdeveloped countries.In Yemen it is essential to implement a public health program for the appropriate diagnosis and management of GAS pharyngotonsillitis together with control and prevention of ARF and RHD.

GAS pharyngotonsillitis is a major problem in Yemen and the main indication for antibiotic treatment in sore throats among children and families. It would be beneficial

to introduce a clinical scoring system based on epidemiological criteria without corroborative laboratory investigations. If this was linked to specific management recommendations for patients with sore throat and could reliably identify GAS infection this would be a practically important development. In health centers where laboratory facilities exist, clinical scoring systems can be used to select cases for confirmation of diagnosis either using culture or rapid antigen tests.

When antimicrobial therapy is required, the safest, narrowest-spectrum, and most cost-effective drugs should be used, e.g.penicillin. Efforts are required to be directed at health policy and educational approaches for the continuous training of health care providers on adherence to clinical guidelines and to improve practice on antibiotic prescribing for acute pharyngotonsillitis in children. Public health authorities must be involved in providing medical and financial resources and necessary laboratory facilities for the proper diagnosis of GAS. It is essential to establish a public health program that will focus on the establishment of registers in primary prophylaxis to prevention of ARF/RHD and compliance associated with improvement in clinical follow-up of patients receiving secondary prophylaxis regimens. These are the major priorities for control of GAS diseases. Future strategies need substantial commitment from the policy health makers and the Ministry of Public Health to concentrate on ensuring that programs are properly resourced, but not overly ambitious in their early stages, and are systematically evaluated.

4.9 Limitations of this study

During the collection of the throat samples, they were ideally taken straight away to the central diagnostic laboratory for culturing. Sometimes due to transport problems there was a delay in culturing the isolates that might have lead to some negative cultures in positive RADT isolates.

Chapter 5

5 Non-group A beta-haemolytic streptococci, Lancefield groups C, G and B

5.1 Introduction

Non-Group A β-haemolytic streptococci (SNA), particularly Lancefield groups C (GCS), G (GGS) and B (GBS), although initially recognized as animal pathogens, are part of the normal human flora (Facklam 2002, Kalia, *et al* 2001, Vieira, *et al* 1998). GAS is a completely human adapted organism but the human strains of GGS and GCS are more likely to be related to their animal origins (Pinho, *et al* 2006, Vandamme, *et al* 1996, WHO 2005). Human seemed to have acquired some strains of GGS and GCS during the process of animal domestication (Lindbaek, *et al* 2005, Meland, *et al* 1993, Turner, *et al* 1993).

Recently the pathogenic potential for humans of SNA have been clarified, as they have been implicated in acute pharyngotonsillitis in children and adults (Darrow and Buescher 2002, Johansson 2003).

A higher rate of isolation of these organisms has been described in recent years. This may be related to the improved diagnostic methods, an increase in virulence or an expanding population of compromised hosts (Pinho, *et al* 2006). It is not possible to differentiate between infections caused by GAS, GBS, GCS or GGS solely on the clinical presentation (Davies, *et al* 2006, Kalia, *et al* 2001).

This aim of this study was to determine the prevalence of (SNA) pharyngotonsillitis among Yemeni children attending schools and clinics in Aden city.

5.2 Objective

To determine the prevalence of (SNA)among children with acute pharyngotonsillitis

5.3 Literature review

Bacterial genome typing has demonstrated that human phenotype GCS and GGS are closely related and members of one species, *Streptococcus. dysgalactiae subsp. equisimilis* GCS/GGS (Davies, *et al* 2006, Faden 2005, Pinho, *et al* 2006). Human strains of GCS and GGS tend to have large colonies (Lopardo, *et al* 2005). They are often termed 'pyogenes-like', because they share virulence factors, including haemolysins, extracellular enzymes and M-proteins that have structural, biologic and immunochemical features similar to GAS (Liu, *et al* 2006, Zaoutis 2004).

The reported prevalence of GCS/GGS carriage and related disease varies worldwide. Most studies originate from temperate climate regions with limited data available from low resources and tropical regions (Steer, *et al* 2009b). Reported rates for pharyngeal carriage of GCS ranged from 0% to 12% in a Finnish community, 0% to 9.3% in Indian school children and 20% in an Aboriginal population (Haidan, *et al* 2000, Kalia, *et al* 2001, Menon, *et al* 2008). GGS throat carriage was reported to be more common than GAS in Tunisian, Bangladeshi and Nigerian schoolchildren (Ahmed, *et al* 2003, Hashikawa, *et al* 2004, McDonald, *et al* 2006, Zaoutis 2004).

The age distribution of GCS/GGS throat carriage is similar to that of GAS, with the highest recovery rates in 5 to 9 year and 10 to 14 year old children (Portillo, *et al* 2003, Steer, *et al* 2009b). Among symptomatic patients with sore throat, those with positive cultures for GCS and GGS are significantly more likely to have fever, exudates, anterior cervical adenopathy and higher mean serum ASO titres than those with negative culture (Benjamin and Perriello 1976, Bisno 2001, Gerber 1996, Gerber and Shulman 2004). SNA cannot be detected using the current RADTs for GAS (Facklam 2002, Leung and Kellner 2004).

SNA are Gram-positive bacteria that grow as pairs or chains of variable length in culture. On sheep blood agar they appear as transparent to opaque, round, small colonies surrounded by a zone of complete haemolysis (β of red cells (Vieira, *et al*1998,

Zaoutis 2004). β- haemolysis is characteristic of GAS but strains of GBS, GCS and GGS are most often also β –haemolytic (Liu, *et al* 2006, Vandamme, *et al* 1996). Bacitracin susceptibility is a widely used screening method for GAS identification. About 3% -5% of GBS, GCS and GGS are also susceptible to bacitracin (Brahmadathan and Gladstone 2006, Lopardo, *et al* 2005).

Although there are no published cases of ARF proven to have been caused by GCS/GGS, the M protein of GCS/GGS share the surface-exposed, conserved domain region common to the M proteins of GAS types epidemiologically associated with ARF. This may play a role in clinical disease, tissue tropism and suggests the potential for rheumatogenicity (Davies, *et al* 2006, Haidan, *et al* 2000, Liu, *et al* 2006).

Interestingly, however, investigators in Australia recently reported a high carriage rate of GCS and GGS in an Aboriginal population that had a high rate of ARF but a low incidence of GAS disease. It has been demonstrated *in vitro* that GCS and GGS have the potential to elicit an autoimmune response that may trigger ARF (Haidan, *et al* 2000).

5.4 Patients and methods

All Yemeni children with acute tonsillitis attending public polyclinics and primary elementary schools who fulfilled the inclusion criteria of age 1– 16 years of age, both sexes, symptoms of sore throat with evidence of fever, anterior tonsillar exudates and anterior tender cervical adenitis were recruited after obtaining a verbal consent from their guardians. Patients with coryza, cough, conjunctivitis and use of antibiotics in the last two-weeks were excluded from the study. The patients included in this objective were recruited from the participants of chapter 4

5.4.1 Diagnostic methods

The process followed the same procedures as described in objective 2. Bacitracin sensitive β haemolytic streptococci that were isolated from patients with a negative RADT were subsequently tested with the Oxoid streptococcal grouping kit, used for the identification of the streptococcal group according to the manufacturer's instructions

5.4.2 Statistical analysis

Statistical analysis included quantitative descriptive analysis and summary statistics for describing the prevalence of SNA. Univariate analysis were based on "odds ratio" and p values <0.05. Analyses were further based on chi-square tests, exact Fisher tests followed by logistic regression analysis for categorical variables.

5.5 Result

5.5.1 Baseline characteristics of children with SNA- positive cultures, GAS-positive cultures and negative cultures

A total of 13 patients had positive cultures for SNA comprising 1.9% of 691 children with acute pharyngotonsillitis and 4.3% of the 300 children with positive cultures.

Six of the 13 SNA pharyngotonsillitis cases were male comprising 5% of the total 119 male patients with positive cultures. There were 7 females with SNA pharyngotonsillitis representing 3.9% of 181 females with positive cultures (Table 5.1).

The mean (SD) age of the patients with SNA pharyngotonsillits was 13.1 (1.5) years compared to a mean (SD) of 11.4 (3.8) years for all children and 12 (3.1) years for children with GAS pharyngotonsillitis. The difference in means between patients with SNA pharyngotonsillitis and those with positive GAS and negative GAS cultures was statistically significant (p=0.001). The predominant age group affected in children with SNA positive cultures was between 11 – 15 years in 92.3% of the children compared to 68.3% in children with positive GAS and 45% with negative cultures (Table 5.1).

All the 13 SNA patients were diagnosed during the winter months of December and February compared to 84.7% of children with GAS pharyngotonsillitis (Figure 5.1).

A history of ARF and RHD were elicited in 2 (15.4%) and 11 (84.6%) children with SNA pharyngotonsillitis compared to 41 (5.9%) and 46 (6.5%) patients with GAS pharyngotonsillitis (p=0.001).

A positive family history of ARF and RHD was obtained in 2 (15.4%) and 11 (84.6%) patients with SNA pharyngotonsillitis compared to 32 (4.6%) and 43 (6.2%) of patients with GAS pharyngotonsilitis (p=0.001). The mean (SD) frequency of

pharyngotonsillitis episodes experienced by the patients with SNA pharyngotonsillitis was 10.2 (1.9) episodes compared to 8.2 (3.6) episodes for children with GAS pharyngotonsillitis (p=0.001).

All the 13 patients with SNA pharyngotonsillitis with recurrent episodes of sore throat were on parenteral benzathine penicillin injections on an irregular basis with the exception of one who received injections every 21 days. All patients with positive SNA pharyngotonsillitis gave a history of receiving prophylactic benzathine penicillin.

A higher proportion of children (10, 76.9%) with SNA pharyngotonsillitis than children with GAS pharyngotonsillitis (188, 65.5%) were diagnosed from the schools (Table5.1).

Table 5. 1Baseline characteristics of children with non-GAS compared to those with positive GAS and negative cultures

Demographic factors	Total n=691patients	Non-GAS n=13 (%)	GAS n= 287 (%)	Negative cultures n=391(%)	P
Sex	Male	6 (46.2%)	113 (39.4%)	179 45.8%)	0.2
	Female	7 (53.8%)	174 (60.6%)	212 54.2%)	
Age (years)	Mean (S.D.)	13.1 (1.5)	11.4 (3.8)	12 (3.1)	0.001
Season	Winter (Oct –Feb)	13 (100%)	241 (84.7%)	323 80.2%)	0.001
Medical history	History of RF	2 (15.4%)	41 (5.9%)	20 (4.9%)	0.001
	History of RHD	11 (84.6%)	46 (6.5%)	26 (6.6%)	0.001
Family history	RF	2(15.4%)	32 (4.6%)	12 (3.1)	0.001
	RHD	11 (84.6%)	43 (6.2%)	3 (0.8)	
Pharyngotonsillitis episodes/year	Mean (SD)	10.2 (1.9)	8.2 (3.6)	6.2 (3.2)	0.001
Drug history	Prophylactic penicillin	13 (100%)	80 (27.9%)	29 (7.4%)	0.001
Clinical location	School	188 65.5%)	10 (76.9%)	322 82.4%)	0.001
	Public	31 (10.8)	2 (15.4%)	45 (11.5%)	
	Private	68 23.7%)	1 (7.7%)	24 (6.1%)	

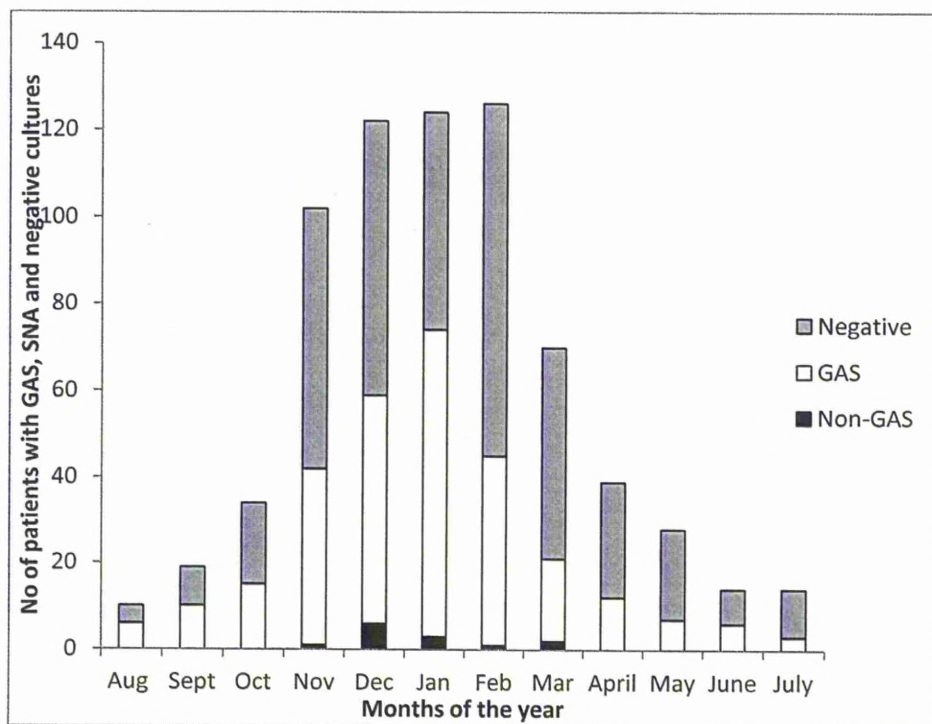


Figure 5. 1Distribution of patient with GAS and SNA pharyngotonsillitis and negative cultures by months

5.5.2 Clinical symptoms of patients with GAS and non-GAS pharyngotonsillitis

A history of sudden onset of sore throat, fever and pain on swallowing was documented in all the thirteen (100%) patients with positive throat cultures for SNA pharyngotonsillitis compared to those with GAS PT in 93%, 99% and 97.6%, respectively

Headache was reported in 10 (76.9%) versus 149 (51.9%), nausea in 7 (53.8%) versus 123 (42.9%) and vomiting in 2 (15.4%) versus 34 (11.8%) patients with SNA and GAS pharyngotonsillitis, respectively (Table5.2)

Table 5. 2Clinical symptoms of patients with GAS and SNA Pharyngotonsillitis

History of	SNA n=13	GAS n=287	OR (95% CI)	P
Sudden onset	13 (100)	267 (93)	-	1.0
Sore throat	13 (100)	285 (99.3)	-	1.0
Fever	13 (100)	280 (97.6)	-	1.0
Headache	10 (77)	149 (51.9)	0.3 (0.08 – 1.1)	0.07
Pain on swallowing	13 (100)	279 (97.2)	-	1.0
Vomiting	2 (15.4)	34 (11.8)	0.6(0.1 – 3.1)	0.6
Nausea	7 (53.8)	123 (42.9)	0.5 (0.2 – 1.6)	0.3

5.5.3 Clinical signs of patients with GAS and SNA pharyngotonsillitis

The clinical signs of red uvula (OR= 0.3) and petechiae on the soft palate (OR=0.2) in patients with positive SNA and GAS pharyngotonsillitis revealed statistical significance of (p=0.05 and p=0.008) between the two groups as shown in Table 5.3

Table 5. 3Clinical signs of patients with GAS and SNA pharyngotonsillitis

Clinical signs	GAS n=287	SNA n=13	OR (95% CI)	P
Erythema	284 (99)	12 (92.3)	12 (1.1 – 140)	0.05
Anterior tonsillar erythema	251 (87.5)	13 (100)	-	1
Exudates	277 (96.5)	13 (100)	-	1
Anterior tonsillar exudate	209 (72.8)	13 (100)	-	1
Red uvula	113 (39.4)	9 (69.2)	0.3 (0.1 – 1)	0.05
Soft palate petechiae	90 (31.4)	9 (69.2)	0.2 (0.06 – 0.7)	0.008
Anterior cervical lymph node	196(68.3)	13 (100)	-	1

The best fitting logistic regression model based on the clinical criteria to distinguish children with SNA and GAS is depicted in Table 5.4. The clinical characteristics independently associated with SNA were erythema (AOR=14.5, p=0.05) and petechie on the soft palate (AOR = 3.4, p=0.008).

Table 5. 4 Logistic regression analysis of clinical criteria of acute pharyngotonsillitis to identify children with GAS and non-GAS

Clinical criteria	OR	AOR	95% CI	P value
Headache	0.4	2.9	0.8 – 11	0.1
Erythema	0	14.5	1 - 210	0.05
Soft palate petechie	0.3	0.2	0.06 – 0.6	0.008

5.5.4 McIssac score in patients with SNA and GAS pharyngotonsillitis

All the thirteen patients with non-GAS pharyngotonsillitis had a McIssac score of 5 presenting with all the definitive clinical criteria suggestive for GAS acute pharyngotonsillits. The RADT was negative in all these patients whereas the cultures grown both on sheep and on horse blood agar culture media were all positive for non-GAS. Bacitracin disc were added to these culture isolates that were all observed with an inhibition zone of >15mm.

Table 5. 5 Diagnostic tests of isolates with non-Gas and GAS pharyngotonsillitis

McIssac score Cut off points	GAS N=287	Non-GAS N=13	p
1	2	0	
2	2	0	
3	17	0	
4	76	0	
5	190	13	0.1

5.5.5 Culture isolates of GBS, GCS and GGS by age and sex

The latex agglutination test for Lancefield group was performed on the 13 non-GAS isolates of which 1 (7.7%) isolate was GBS, 6 (46.2%) isolates were GCS and the other six (46.2%) were GGS.

The predominant age of occurrence for SNA was 13 years in almost half of the patients. Six patients with GCS, five patients with GGS and one patient with GBS were within the age group of 11 – 15 years.

The patient who had GBS infection was a female while the isolates of GCS and GGS were equally distributed between both genders.

Table 5. 6 Culture isolates of GBS, GCS and GGS between different ages and sex

Variables		GBS N=1(%)	GCS N=6(%)	GGS N=6 (%)
Age (years)	Mean (SD)	11(0)	13.7 (1.2)	12.8 (1.6)
Sex	Female	1 (100)	3 (50)	3 (50)
	Male	0	3 (50)	3 (50)

5.5.6 Clinical symptoms and signs of patients with the different strains of SNA

The clinical manifestations and signs of patients with SNA within the three different SNA strains of GBS, GCS and GGS are shown below for completness. Frequencies are too small for further statistical analysis.

Table 5. 7Clinical symptom/sign of patients with GBS, GCS ,GGS pharyngotonsilltiis

Clinical findings	GBS N=1(%)	GCS N=6(%)	GGS N=6 (%)
Symptoms			
Sudden onset	1(100%)	6 (100%)	6 (100%)
Sore throat	1(100%)	6 (100%)	6 (100%)
Fever	1(100%)		
Headache	1(100%)	4 (66.7%)	5 (83.3%)
Pain on swallowing	1(100%)	6 (100%)	6 (100%)
Nausea	-	4 (66.7%)	3 (50%)
Vomiting	-	-	2 (33.3%)
Signs			
Erythema	1(100%)	6 (100%)	6 (100%)
Anterior tonsillar exudates	1(100%)	6 (100%)	6 (100%)
Ant cervical lymph node	1(100%)	6 (100%)	6 (100%)
Red uvula	-	5 (83.3%)	4 (66.7%)
Petechiea of soft palate	-	5 (83.3%)	4 (66.7%)
RADT	Negative	Negative	Negative

5.6 Discussion

Thirteen patients were diagnosed with SNA based on clinical throat isolates comprising a prevalence of 1.9% of the total 691 patients with acute pharyngotonsillitis, but from the total 300 positive cultures the prevalence was 4.3%. The result of this study were not different from the 3% rate of isolation of SNA pharyngotonsillitis in children having acute sore throat reported by Zaoutis in USA 3.1% GCS (65/2085) and 2.6% GGS (38/1449) and Mzoughi in Tunisia 3% (14 GCS and GGS / 474 patients) but it was higher than what was reported by Wong et al in Hong Kong 0.3% GCS (5/1449)

(Mzoughi R2004, Wong and Chung 2002, Zaoutis 2004). In addition Thangam Meneon et al in India reported 5 GCS and 3 GGS (8/310 patients) and Tewodros in Ethiopia 6 GCS and 2 GGS (8/143 patients) comparable with six patients each of GCS and GGS in this study (12/300 patients) (Menon, *et al* 2004, Tewodros, *et al* 1992).

Patients with SNA pharyngotonsillitis had a male to female ratio of 1.2:1 and 12 (92.3%) fell in the age range of 11 – 15 years. This age range is higher than the age range of 0 – 5 years and 5 – 9 years reported in two Egyptian studies in children (Bassili, *et al* 2002, Tamburlini and Ronfani 1998). The mean age for SNA in this study was 13 whereas Faden et al reported a median of 17 years (Faden 2005). Various reports showed an increasing prevalence of SNApharyngotonsillitiswith increasing age in paediatric age groups (Hofkosh, *et al* 1988). A trend of increasing prevalence with age was reported as follows: 12% in 3-6 years age group , 20% in 6-12 years, 40% in 12-18 years and 61% in those more than 18 years of age(Cimolai, *et al* 1990, Gove, *et al* 1998, Mzoughi R2004, Wong and Chung 2002).

The number of sore throat episodes reported in SNA pharngotonsillitis per year in males was higher than in females. It is difficult to comment on gender differences due to the small sample size of the study.Bassili et al in Egypt reported that the peak of non-GAS pharyngotonsillitis occurred in March and April which is in disagreement with this study where the peak was in December and January. These differences may be explained by the difference in the site of study and geographical climate (Bassili, *et al* 2002, Davies, *et al* 2006).

All 13 patients with SNA pharyngotonsillitis had a score of five on the McIssac scoring system criteria with a clinical score of 100%. This is in contrast to Lindbaek in Norway, who found that only 45% of the patients with SNA met three or four of the criteria (Lindbaek, *et al* 2005).The RADT were negative in patients with SNA which was in line with the reports of many authors (Gerber and Shulman 2004, Lopardo, *et al* 2005). Latex agglutination test was further conducted on clinical isolates of patients with McIssac score of five and negative RADT. Six patients had GCS and six had GGS. Only one patient was indentified with SNA GBS. Mzoughi in Tunisia and Meland in

Norway did not reveal GBS strain among their patients (Meland, *et al* 1993, Mzoughi R2004).

The clinical manifestations of patients with non-GAS pharyngotonsilliits in this study were similar to those with GAS pharyngotonsillitis and did not reveal any difference. This similarity was reported by Bassili et al in Egyptian children (Bassili, *et al* 2002) and in various other studies (Faden 2005, Gerber and Shulman 2004, Liu, *et al* 2006, Portillo, *et al* 2003).Symptomatic patients with sore throats and positive cultures for GCS and GGS were more likely to present with fever, pain on swallowing, erythematic and exudates on anterior pillar regions. Ths is in line with the results of Benjamin et al in Virginia, Tunner et al in Columbia and Meland et al in Norway (Benjamin and Perriello 1976, Meland, *et al* 1993, Turner, *et al* 1997). Lindbaek et al found that pain on swallowing was not significantly associated with GCS and GGS pharngotonsillitis which was quite different from the findings in this study, where 100% of the patients presented with pain on swallowing (Lindbaek, *et al* 2005)

The finding of GCS and GGS in patients with acute pharyngotonsillitis with underlying history of ARF and RHD may be proposed to be a prerequisite to further prove its autoimmune pathogenicity. Hiadan and colleagues in the Aboriginal population of Australia suggested that GCS and GGS pharyngeal carriage may be capable of causing ARF by conducting a *in vitro* research on mice. They demonstrated entry into the pharyngotonsillar cells with an increase in the ASO titre and a high invasive reactivity eliciting an autoimmune response with human heart myosin, a major cardiac antigen in ARF and RHD (Haidan, *et al* 2000). They proposed that these SNA C and G may be capable of causing ARF by acquiring rheumatogenic factors from GAS by horizontal transfer of genetic material (Johnson, *et al* 2006).

A few other studies have suggested that there could be a link between SNA and ARF and RHD, but there are no reports to this date confirming that SNA are implicated in the pathogenesis of ARF and RHD in humans (Saslaw and Jablon 1960, WHO 2004, Zaoutis 2004).

It is important to document that in these thirteen patients with SNA pharyngotonsillitis, 2 patients gave a history for ARF and 11 patients were echo proven cases of RHD. A

positive family history of ARF/RHD was also obtained in one, or more than one, family member in all these patients with SNA pharyngotonsillitis. It is possible that these positive throat cultures for SNA, particularly GCS and GGS, in these patients who also gave a positive history of ARF and were echocardiographically proven cases of RHD may suggest the possibility of their clinical association with ARF and RHD. Stronger evidence for this rheumatogenic potential could have been obtained if these patients were followed up during the course of their acute pahryngotonsillitis and observed to see if during this sore throat attack they developed any recurrence of ARF. All these patients were on secondary prophylaxis with benzathine pencillin.

This is the first study conducted in Yemen to document the SNA pathogens in children with acute pharyngotonsillitis mainly GCS and GGS. Even though SNA GCS and GGS may share many of the cell surface and extracellular pathogenic factors associated with GAS, they need further investigations (Kalia, *et al* 2001, Portillo, *et al* 2003, Zaoutis 2004).Global differences are emerging in the epidemiology of GAS and SNA infections and their sequelae and this requires comparisons to be made in different countries.

The most productive way is likely to be gene expression profiling of both bacteria and patients and their immune responses in areas where ARF/RHD still remain major health issues.The question needs to be resolved because current approaches to controlling ARFand RHD in some communities have clearly been ineffective. Resolving these uncertainties is likely to require collaborative studies. This would help to broaden the spectrum of causative bacteria and have an immediate effect on primary prevention strategies and possible vaccine development.

5.7 Conclusion

GCS, GGS and GBS are an infrequent cause of pharyngotonsillitis compared with GAS. The clinical criteria for diagnosing SNA did not differ from that characteristic for GAS pharyngotonsillitis and the reference standard for provisional diagnosis is the throat culture. In the presence of McIssac clinical score of five points and negative RADT, it is recommended to conduct a throat culture that should be followed by Lancefield Grouping latex agglutination test for identifying SNA types.

5.8 Recommendations

Further epidemiological studies on exudative pharyngotonsillitis in countries such as the Yemen should include grouping of GAS isolates to differentiate them from SNA isolates. Such studies will help to broaden knowledge on the spectrum of causative bacteria and improve understanding of possible characteristics and virulence factors potentially leading to ARF pathogenesis.

The question of whether SNA could be involved in eliciting an autoimmune response leading to ARF must be taken into consideration. Studies should be designed in which patients with SNA are monitored during the course of their illness in order to diagnose the development of ARF and RHD. The successful prevention of ARF/RHD may require thorough treatment not only of GAS but also of SNA pharyngotonsillitis which would have an immediate effect on primary prevention strategies and vaccine development.

5.9 Limitations of this study

This prevalence of SNA pharyngotonsillitis among children with acute sore throat could be an underestimate of the SNA contribution since the latex agglutination test for LancefieldStreptococci Grouping kits was not available during the time of the field work and there is high probability that cases may have missed. In particular β-haemolytic Streptococci isolated from throat swabs that were bacitracin resistant were not pursued in this study.

Chapter 6

6 Emm serotypes, exotoxin genes and serum opacity factor gene of GAS and SNA pharyngotonsillitis

6.1 Introduction

GAS isolates are characterized by numerous typing schemes that measure their genetic diversity (Sobhan, *et al* 2008). The M protein, a primary virulence factor, is a long coiled protein projecting from the GAS cell wall surface (Darmstadt, *et al* 2000, Fischetti 1989, Manjula, *et al* 1984, Pancholi and Fischetti 1992). The basis for determination of the M type depends on the type of amino acid at the amino-terminal portion of the protein and this has a high degree of variability (Lancefield 1962, Relf, *et al* 1996, Yoonim, *et al* 2005, Zimmerlein, *et al* 2005).

The *emm* gene code for the M protein is referred to as the *emm* gene sequence and this is another method for typing GAS (Martin and Green 2006, Sunaoshi, *et al* 2009). This can be performed using the automated DNA sequencing with an expanding reference database (Beall, *et al* 2000, Espinosa, *et al* 2003).

Molecular epidemiology is a sensitive method that is essential to detect the genetic relatedness of different GAS pathogens or other disease agents (Massey, *et al* 2001, McDonald, *et al* 2007b, Olive 2007, Sobhan, *et al* 2008). The distribution and genetic variations of these important pathogens should be regularly monitored to better understand the epidemiology of specific GAS and non-GAS strains (Alberti, *et al* 2003, Sakota, *et al* 2006, Seppala, *et al*1994b, Shulman, *et al* 2009).

The most important new insight has probably been the recognition that numerous diseases are caused by a limited number of clonal groupings and molecular types (Carapetis, *et al* 2005b, Seppala, *et al* 1994a). Molecular epidemiology also gives a global overview of changes in disease patterns and would help to distinguish the details of person-person transmission (Brandt, *et al* 2000, Single and Martin 1992).

This study was undertaken to determine the genotypes and exotoxin and *sof* genes of GAS and SNA among a group of patients with ARF and RHD in Aden.

6.2 Objective

To determine the prevalence of *emm* serotypes, exotoxin gene and serum opacity factor gene among GAS and SNA isolated from children with pharyngotonsillitis in Aden, Yemen

6.3 Literature review

6.3.1 GAS classification and *emm* typing

GAS strains are now commonly classified on the basis of sequence variation in the *emm* gene, which encodes the M protein (Bisno, *et al* 2003, Steer, *et al* 2009b). This *emm* region shows the highest level of polymorphism known for GAS genes (Koh, *et al* 2008, Rogers, *et al* 2007). Currently GAS are referred to as *emm* types rather than M types due to the limited availability of M antisera required to characterise the new *emm* types (Beall, *et al* 2000, Facklam, *et al* 1999). This methodology has lead to the description of almost 200 different GAS sequence types and clarified the understanding of the phylogenetic relationships between different GAS serotypes (Bernard, *et al* 2002, Kim and Lee 2004a, Metzgar, *et al* 2009, Nandi, *et al* 2008). This type-specific epitope shows strong host protective immunity for many M proteins (Kaplan, *et al* 2001, Rogers, *et al* 2007).

The *emm* sequence typing is based on the 5' end of the central *emm* gene within the *emm* chromosomal region present in all isolates. A unique *emm* type is defined as having 95% sequence identity to any other known *emm* type over 160 bp near the 5'end. In other words two isolates are considered to share the same type of sequence if they are 95% identical between their 5'end 160 nucleotide as specified (Beall, *et al* 2000). There is a very strong correspondence between M type, as determined by serology, and the *emm* type that meets the stated definition (Jing, *et al* 2006, Tyrrell, *et al* 2002).

There has been a need to reassess the typing strategies for GAS during the resurgence of ARF and emergence of severe GAS infections (Doktor, *et al* 2005, Efstratiou 2000). The nucleotide sequence at the 5' ends of *emm* genes has now been reported for many

strains (Beres, *et al* 2004, Durmaz, *et al* 2003). GAS infections are said to be in a continuous epidemiological change leading to the appearance and reappearance of serotypes to which the population lacks immunity (Enright, *et al* 2001). The differences in the strains may be explained by the geographical separation, the anatomical site of isolation and the time examined (Colman, *et al* 1993, Dey, *et al* 2005).

GAS strains responsible for ARF have distinct biological characteristics (McDonald, *et al* 2008, Stollerman 2001b). These strains are not all pathogenic and their ability to cause ARF depends on different biological properties such as the type and amount of M protein production and the epidemiological background that enhance person to person contact (Abdissa, *et al* 2006, Beres, *et al* 2002, Lamagni, *et al* 2005). There is a surface exposed antigenic site present on the M protein to which the IgG immunoglobulin released by ARF patients binds (Norrby-Teglund, *et al* 1996, Timmer, *et al* 2006). GAS strains responsible for ARF do not produce alphalipoproteinase serum opacity factor (*SOF*) and are heavily encapsulated with formation of mucoid colonies on blood agar plates (Gillen, *et al* 2008). It is still not clear whether each of these strains exhibit a unique rheumatic antigen (Erdem, *et al* 2009, McDonald, *et al* 2007b, Shulman, *et al* 2004).

There are various other genotypic methods used for the GAS typing. Vir-typing measures restriction fragment length polymorphisms within the *emm* chromosomal region (Ramachandran, *et al* 2004). Pulsed-field gel electrophoresis (PFGE) and arbitrary-primed PCR can provide high resolution levels between strains by measuring multiple loci for differences that are not necessarily under selection (Gonzalez-Rey, *et al* 2003, Martin and Green 2006). Even though various genotypic methods have been developed to determine the level of clonal relationship in GAS, the *emm* type was found to correlate with clone complex. Surveillance of M types causing ARF will be important during the development of future M type specific GAS vaccines (Espinosa, *et al* 2003).

6.3.2 Streptococcal M-protein

The GAS M-protein is one of the best characterized determinants of bacterial virulence. The M protein is a filamentous molecule with two protein chains in a coiled-coil configuration that extends to about 60 nm above the GAS surface (Bisno, *et al* 2003, Lancefield 1928). GAS may be divided into serotypes on the basis of antigenic differences in the M protein molecule with more than 100 known serotypes (Cunningham 2000, Lancefield 1962). The M protein coats GAS and acts as the primary antigen and determinant of type-specific immunity in man (Lancefield 1957).

M protein functions by preventing phagocytosis of the bacteria by PMNs allowing the bacterium to avoid the host immune response (Cunningham 2000). The protein is essential for the persistence of GAS in infected tissues and its survival in blood due to its anti-phagocytic function. Resistance to a GAS infection appears to be mediated, in part, to type-specific antibodies to M molecule (Guilherme, *et al* 2006).

The M protein is thought to play a major role in the development and progression of GAS disease (Cunningham 2000, Johnson, *et al* 2006). The surface-exposed, conserved C repeat domain of M protein in some GAS serotypes may be a virulence determinant for ARF (Guilherme and Kalil 2010, Robinson and Kehoe 1992). Specific regions of M protein serve as shared antigens, and cross-reactivity between these epitopes and human proteins lead to autoimmune RHD (Guilherme, *et al* 2006, Lancefield 1962). Thus, M protein is a major GAS virulence and immunological factor and antibody to this protein gives protection to the human host. This has made M phenotyping and genotyping the best epidemiological tool to detect outbreaks of GAS, and for measuring the general threat presented by GAS at any given time and place (Guilherme and Kalil 2010, Metzgar and Zampolli 2011, Smeesters, *et al* 2009). The M protein is the subject of intensive investigation and its potential as a vaccine against GAS infections (Steer, *et al* 2009d)

GAS are classified into Class I and II based on their M-protein reaction with a monoclonal antibody. The majority of Class I reactive M-proteins are associated with ARF including M types such as 1, 3, 5, 6, 14, 18, 19 and 24. Class II strains have non-reactive M proteins that produce an apolipoproteinase called opacity factor (Nwankwu and Njoku-Obi 1990). Children may be exposed to several GAS throat infections but

reinfections with the same serotype are less common due to the presence of homologous anti-M antibodies after an infection (Courtney, *et al* 2009). Some reports have identified certain M strains to be repeatedly associated with ARF while other prevalent strains do not cause ARF even in susceptible hosts. Not all strains of rheumatogenic types are equally pathogenic(Johnson, *et al* 2006, Menon, *et al* 2004)

6.4 Multilocus sequence typing

Multilocus sequence typing (MLST) is a new molecular epidemiological technique that helps to identify the global spread of virulent or antibiotic resistant isolates of bacterial pathogens and with the capacity for the typing information to be shared through the internet (Brian G 1999, Enright and Spratt 1999). MLST is a nucleotide sequence-based method that characterizes the genetic relationships between the organisms of a bacterial species (Enright, *et al* 2001). The nucleotide sequence of 450–500-bp internal fragments of seven housekeeping genes are determined for each isolate (Maiden, *et al* 1998). An allele is assigned for each locus according to the sequence. This length of DNA fragment can be sequenced on both strands using a single pair of primers and provides enough variation to identify many different alleles in most bacterial pathogens within the population (Urwin and Maiden 2003). Housekeeping loci are used for analysis because they are present in every organism, their products serve a vital function and the mutations within them are assumed to be selectively neutral (Meisal, *et al* 2010).

MLST is highly discriminatory as the accumulation of nucleotide changes in housekeeping genes is a relatively slow process (Richardson, *et al* 2011). The allelic profile of a bacterial isolate is sufficiently stable over time for the method to be ideal for global epidemiology (Urwin and Maiden 2003). The technique can identify clones, groups of isolates that are descendants of a recent common ancestor and have shared alleles at each of the housekeeping loci (Bessen, *et al* 2008). Currently the database contains the allelic profiles of isolates that represent the global diversity of GAS and other bacterial isolates (McGregor, *et al* 2004b). It has the advantage of being applied directly to clinical material and does not require laboratories to obtain the reference isolates of each of the important clones of a bacterial pathogen.

The major advantage of this approach is that sequence data are unambiguous and electronically portable, allowing electronic databases and molecular typing band global

surveillance studies of GAS and other bacterial pathogens (Cooper and Feil 2004, Maiden, *et al* 1998).

6.4.1 Emm genotyping system for GAS

A genetic marker for GAS is the *emm* pattern based on the chromosomal arrangement of *emm* genes that encodes surface protein fibrils. M protein is composed of two polypeptide chains. The chain comprises four repeat blocks A-D. The C (carboxy) terminal is highly conserved among GAS strains (Jenkinson and Lamont 1997). The N (amino) terminal portion extends into the environment with a series of 11 amino-acids that vary among different clinical isolates. This hypervariable region of the protein has antigenic differences that form the basis for the Lancefield classification of GAS (Beall, *et al* 1998, Facklam, *et al* 1999, Lancefield 1962). The classification has been expanded after cloning of the M-protein (*emm*) gene and the standardisation of the *emm* typing system (Beall, *et al* 2000, Facklam, *et al* 2002, Johnson, *et al* 2006).

PCR was introduced by Podbielski et al in 1991 to study the M protein gene family *emm* in GAS (Podbielski, *et al* 1991). This DNA sequencing has been used to describe more than 200 GAS sequence types. It also gave information on the phylogenetic associations between different serotypes of GAS (Brahmadathan and Gladstone 2006). *Emm* usually refers to the M protein gene although it may refer to any of three *emm*-like genes that are present at the *vir* locus (Shet and Kaplan 2004).

Only a few reference laboratories are able to prepare the type-specific M-typing antisera. It is frequently difficult to detect M proteins in this way due to the unavailability of typing reagents (Reed, *et al* 1980). No attempt has been made to produce them commercially due to their great expense. It is also believed that many GAS isolates are nontypeable because of the lack of M expression or lack of reactivity of expressed M protein with available antisera (Moses, *et al* 2003, Shet and Kaplan 2004).

6.4.2 emm GAS strains with "Rheumatogenic" characteristics

GAS strains have been evaluated by sequence analysis of *emm* gene-specific PCR products (*emm* typing). This methodology has allowed the recognition of several previously unknown GAS types in different geographic areas, demonstrating the usefulness of *emm* typing for detecting genetic diversity among GAS isolates and for tracing GAS infections (Doktor, *et al* 2005, Espinosa, *et al* 2003). GAS strains of certain *emm*/ M protein serotypes vary in their rheumatogenic potential (Dey, *et al* 2005, Erdem, *et al* 2009).

It is probable that not all GAS strains of rheumatogenic serotypes are equally pathogenic (Yoonim, *et al* 2005). The tendency of a given strain to provoke ARF depends on the type and production of M protein and conditions that favour person to person transmission (Kurahara, *et al* 2006, Shulman, *et al* 2006). Some strains are strongly and repetitively associated with outbreaks of ARF whereas other prevalent GAS strains do not provoke the disease or reactivate it in susceptible hosts (Ekelund, *et al* 2005, Lazar R. 2004, McDonald, *et al* 2007b). These rheumatogenic variations are responsible for the temporal and geographical variations of ARF/RHD (Bisno, *et al* 2003, Steer, *et al* 2009b).

The predominant serotypes associated with outbreaks of ARF in the United States and United Kingdom between1939 and 1971 were rheumatogenic strains of M1, M3, M5, M6, M14, M18, M19, M24 and M29. During the period 1970 to 1989, M1, M3, M5, M6, and M18 were the reported predominant rheumatogenic strains. The rheumatogenic strains M9, M22 and M33 were reported in the years 1970 to 1980 (Kaplan 2005).

Substantial heterogeneity in the M types was reported in India but the five most prevalent types were *emm* 74, *emm* 11 and StI129 with some areas having *emm* 5, *emm* 6, *emm* 18, *emm* 19,and *emm* 24 (Sagar, *et al* 2008, Sakota, *et al* 2006). In Australia *emm*14 *and st*3765 were reported as rheumatogenic but with lower prevalence of *emm* 1, 3, 12, 14, 18, 24, 75 and 89 (McDonald, *et al* 2007b, Richardson, *et al* 2010). In New Zealand rheumatogenic strains were diverse and *emm* 6,53,55, 58, 66, 74,75,76,92 and99 were associated with ARF (Dierksen, *et al* 2000, Safar, *et al* 2011).

There is supporting evidence of the concept of rheumatogenicty based on global epidemiologic studies but the nature of these strains needs to be further studied in countries with high prevalence of ARF and RHD to elucidate new rheumatogenic strains. It has been emphasized that changes in genotypic prevalence of GAS occurs which may account for the predominantly type-specific nature of immunity exhibited by individuals to GAS (McGregor, *et al* 2004a, Surdeanu, *et al* 2000) .

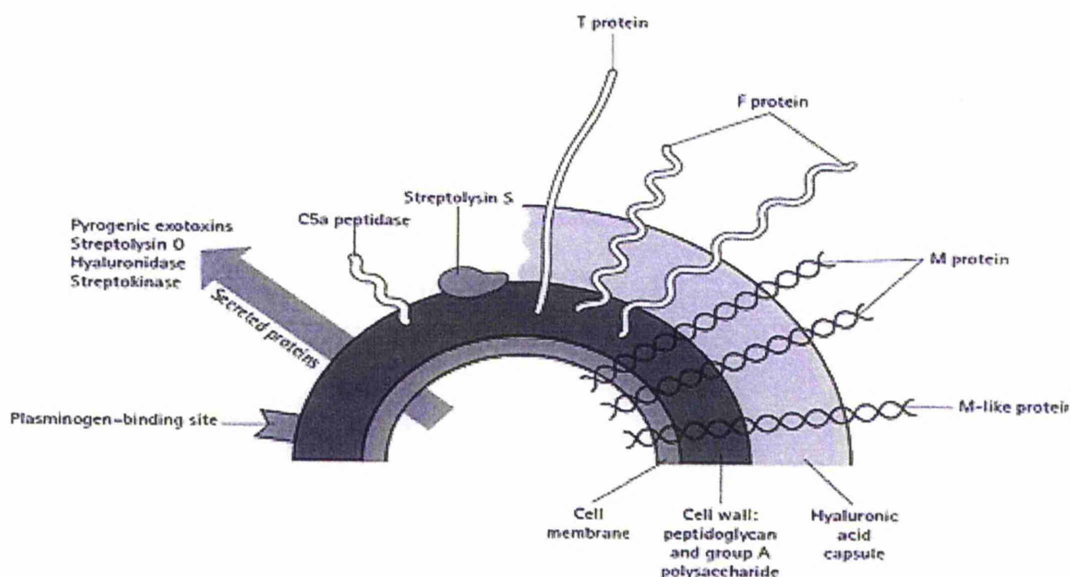


Figure 6. 1Virulence factors of GAS From Cunnigham et al (2002)

6.4.3 Serum opacity factor (SOF) and serum opacity factor (sof) gene

GAS has some surface adhesion molecules that can interact with extracellular host proteins such as fibronectin (Maxted, *et al* 1973). This interaction is important for the adherence of GAS to the epithelial host cells that makes it a prerequisite for colonization and subsequent disease(Courtney, *et al* 2006, Gillen, *et al* 2008). Serum opacity factor (SOF) is a large extracellular and surface-bound protein capable of binding bronectin which is encoded by the *sof* gene(Johnson 1993) . SOF is unique among GAS (Rosales, *et al* 2009). Almost half of the GAS strains produce an

apoproteinase, an enzyme that causes mammalian serum to increase in opacity (Bianco, *et al* 2006). The opacity factor enzymes are type specific for M types (Han, *et al* 2009).Opacity factor antibodies can be used in the inhibition tests (Timmer, *et al* 2006).

The two functional domains of SOF are located on separate regions of the protein(Johnson 1993) . The variable N-terminus of the enzyme domain of SOF is responsible for cleaving the ApoA1 portion of high-density lipoprotein (Rosales, *et al* 2009). The conserved C-terminus involves the fibronectin-binding domain of the SOF protein via the repeated homologous chains. SOF is a virulence determinant in GAS that expresses a class II M protein (Gillen, *et al* 2008). The GAS isolates that possess the class II M protein are the only ones able to express SOF (Brandt, *et al* 2001, Courtney, *et al* 1999). Some GAS strains are more likely to cause ARF and certain characteristics of individual GAS genotypes are associated with rheumatogenicity based on epidemiologic data (Carapetis 2008b). These include a high M protein content and mucoid colonial morphology indicative of a large capsule and lack of *in vitro* production of SOF (Courtney, *et al* 2009, Johnson, *et al* 2006).

Virtually all rheumatogenic *emm* types (1, 3, 5, 6, 14, 17, 18, 19, 24, 27 and 29) express a class I M protein and are SOF negative except *emm*11. Whatmore in 1995 demonstrated extensive genetic variation within certain individual M types and distinct *emm* sequence clusters associated with SOF status and previous rheumatogenecity, concluding that SOF-positive and SOF- negative M types represent distinct evolutionary lineages (Whatmore, *et al* 1995). These findings support the concept that there are specific rheumatogenic GAS strains that are encapsulated and associated with increased virulence and rheumatogenicity (Gillen, *et al* 2008, Shulman, *et al* 2006).

This complementary molecular methodology (*sof* typing) is also based upon sequence analysis of a hypervariable virulence gene (Perea-Mejia, *et al* 2006). However, recent studies have demonstrated that multiple *sof*-gene types do occur within a single M/*emm* type, and that a single *sof* sequence can be associated with more than one *emm* type (Beall, *et al* 2000, Jeng, *et al* 2003). Therefore, although SOF-inhibition serotyping remains a useful GAS classification tool, it must be interpreted carefully, with the

understanding that in some instances the SOF-inhibition type will not accurately predict M/*emm* type, especially when strains from diverse sources are analysed (Wolfe 2000).

Fibronectin-binding protein (FnBP) has been reported to be vital for endothelial invasion (Massey, *et al* 2001, Peacock, *et al* 1999). FnBP forms a link between bacterial pathogens and the cell of the host (Peacock, *et al* 2000). Sof is a unique bifunctional protein anchored to the cell surface of many GAS strains. *sof* is linked to a second gene encoding a new streptococcal fibronectin-binding protein(*sfbX*). Serum opacification is encoded by *sof gene* alone and *sfbX* encodes a fibronectin-binding function. *SfbX* protein was found to bind immobilized fibronectin and to partially inhibit GAS adherence to fibronectin. The *sof* and *sfbX* genes were found to be expressed on the same transcription unit. The *sfbX* gene was found to be present only in *sof*-positive strains where both genes influence the type of tissues and disease manifestations associated with *sof*-positive GAS(Jeng, *et al* 2003) . The presence of *prtF*, *sof*, and other genes encoding extracellular matrix binding proteins with GAS may be associated with antibiotic sensitivity and resistance patterns (Dicuonzo, *et al* 2002, Oehmcke, *et al* 2004)

Thus SOF is required for the virulence of GAS(Courtney, *et al* 2006, Timmer, *et al* 2006). The fibronectin-binding repeats of this streptococcal protein are highly conserved, required for fibronectin-binding by SOF and are capable of provoking an immune response not observed as a result of natural GAS infection (Courtney, *et al* 2009, Kreikemeyer, *et al* 1999). Some studies have suggested that the fibronectin-binding repeats of GAS SfbI (protein F) are protective (Oehmcke, *et al* 2004, Towers, *et al* 2003). Thus fibronectin-binding repeats of *sof* may prove to be a useful target for a vaccine against GAS (Goodfellow, *et al* 2000, Schulze, *et al* 2003).

6.4.4 Pyrogenic exotoxin superantigens

GAS produces a variety of exotoxins including streptococcal pyrogenic exotoxins (SPE). These belong to a larger group of pyrogenic toxin superantigens (PTSAgs) that stimulate T cells by binding to both regions on major histocompatibility complex (MHC) class II molecules and specific Vρ chains of the T-cell receptor (Ferretti, *et al* 2001). This reaction is called superantigenicity (Fraser and Proft 2008). Extensive T-

cell proliferation results in massive cytokine release. Structural and immunological characterization of the SPEs has revealed that they are low-molecular-weight proteins (24,000 to 28,000) that are relatively heat and protease resistant (Arcus, *et al* 2000, Proft, *et al* 2003). Other novel GAS superantigens made by virulent streptococci have been described (Green, *et al* 2005, Nakagawa, *et al* 2003, Norrby-Teglund 2001).

GAS strains possess mobile genetic elements like plasmids, bacteriophages, transposons and insertion sequences (Ferretti, *et al* 2001). The prophages or prophage-like elements comprise a small fraction of the GAS genome but may be responsible for 70% of the variation in the formation of GAS strains (Nagiec, *et al* 2004) .

GAS phages are considered to contribute to the characteristics of virulence and resistance (Smoot, *et al* 2002b). Many prophages encode one or two proven extracellular virulence factors and antibiotic resistance determinants (Kotb, *et al* 1990, Nandi, *et al* 2002a). These may play a role in the expression and horizontal transfer of genes (Beres and Musser 2007, Yang, *et al* 2006).

Prophage-encoded virulence factors are divided into two groups (McCormick, *et al* 2001, Schafer and Sheil 1995). The first group belongs to the pyrogenic toxin superantigens that consist of the streptococcal superantigen and the streptococcal pyrogenic exotoxins (SpeA, SpeC, SpeK, SpeH, SpeI, SpeM, and SpeL) (Chatellier, *et al* 2000, Schlievert 1993). The other group is composed of DNases (Spd1, Spd3, Spd4, Sda, and Sdn) and phospholipase A2 (Proft, *et al* 2001, Vitali, *et al* 2009).

6.4.5 Streptococccus invasive locus (*sil*)

Hidalgo-Grass et al., (2002) identified a DNA locus named *sil* in the invasive serotype M14 clone causing necrotizing fasciitis in Israel. Thus, *sil* may regulate virulence genes in GAS strains of different genetic backgrounds causing diverse types of human disease (Bidet, *et al* 2007, Billal, *et al* 2008, Hidalgo-Grass, *et al* 2004). The role of the superantigen-like activity of M-protein fragments, as well as the streptococcal pyrogenic exotoxin, in the pathogenesis of ARF is still the focus of study.

6.4.6 Pulsed field gel electrophoresis (PFGE)

PFGE is another powerful subtyping molecular method for epidemiologic investigations of GAS. PFGE is based on restriction length fragment polymorphisms of the whole microbial genome and allows the detection of variations among strains (Ghourchian and Elyasvandi 2005). Despite its usefulness, the results from PFGE generated between laboratories are not comparable unless a standardized protocol is followed(Bianco, *et al* 2006) . The typing patterns are then analyzed with computer software that permits their comparison online (Rydberg, *et al* 1994, Tenover, *et al* 1995).

PFGE of bacterial DNA provides genotyping information with a pattern of DNA fragments (Riapis, *et al* 2006). It is used to assess similarity among GAS. PFGE shows that within an outbreak of infections, single genetic events may occur randomly for months. The difference in PFGE patterns of 1 – 3 bands can result from a single genetic event. If the GAS isolates have bands that differ by 1–3 bands, they should be considered "closely related," those that differ by 4–6 bands "possibly related," and those that have six or more differing bands are distinct strains as outlined in Table 6.1 (Tenover, *et al* 1995). PFGE banding patterns (pulsotypes) are analyzed by visual inspection (Haukness, *et al* 2002, Riapis, *et al* 2007).

Table 6. 1Criteria for interpreting PFGE patterns (Tenover *et al.,* 1995)

Category	No. of genetic differences compared with outbreak strain	Typical no. of fragment differences compared with outbreak pattern	Epidemiologic interpretation
Indistinguishable	0	0	Isolate is part of the outbreak
Closely related	1	2 – 3	Isolate is probably part of the outbreak
Possibly related	2	4 – 6	Isolate is possibly part of the outbreak
Different	> 3	> 7	Isolate is not part of the outbreak

6.4.7 Future GAS Vaccine

Most of the recent isolates within *emm* types 1–89 share identical M type–specific sequences with corresponding reference strains that were isolated 20–70 years ago (Georgousakis, *et al* 2009, Nir-Paz, *et al* 2009). Despite considerable *emm* type heterogeneity, the majority of pharyngitis isolates are concentrated within relatively few M serotypes (Pichichero 2004, Steer, *et al* 2009d). An effective M protein–based vaccine encompassing the most prevalent serotypes may impact very significantly on GAS pharyngotonsillitis and its complications (Shulman, *et al* 2004, Steer, *et al* 2009b)

The 26-valent M-protein vaccine that is currently undergoing clinical trials includes immunogens for 86% of pharyngeal isolates and a highly conserved epitope (streptococcal protective antigen) that may protect against additional types. This vaccine includes 35–50 amino acid N-terminal segments of *emm* types 1–3, 5–6, 11–14, 18, 19, 22, 24, 28, 29, 33, 43, 59, 75–77, 89, 92, 101 and 114 (Dale 2008, Kotloff 2008, Loughlin 2007, Nir-Paz, *et al* 2009).

Continuous research is undertaken in the development of GAS vaccine for ARF prevention during over the past 40 years (Bryant, *et al* 2009) . A 26 valent –vaccine based on *emm* types and M subtypes collected across GAS diseases from the US was evaluated in a clinical trial(Dale, *et al* 2005) . Reports showed this 26-valent vaccine was associated with 79% *emm* coverage in the US, 69% in Europe, 40% in Fiji, 33% in New Zealand, 76% in Brussels and 32% in Brasilia (Hu, *et al* 2002, Lee, *et al* 1995, McNeil, *et al* 2006, Metzgar and Zampolli 2011, Nir-Paz, *et al* 2010, Shulman, *et al* 2009). Reports on this vaccine revealed seroresponse to 84.5% of GAS pharyngeal isolates, 92.5% isolates linked with ARF and 87.6% of invasive disease isolates and 100% of strains causing necrotizing fasciitis in US (Steer, *et al* 2009a).

Overall, the 26 *emm* types in the experimental multivalent vaccine accounted for less than 65% of all isolates in Africa, Asia, Middle East and Pacific region (Abdel-Aal, *et al* 2010, McNeil, *et al* 2005).This vaccine will not be applicable for countries with high risk of ARF due to the lack of data on rheumatogenic GAS strains(Seckeler and Hoke 2011). GAS vaccines, should be evaluated for their efficacy against GAS isolates of greatest importance to less developed countries (Dale, *et al* 2005). Moreover GAS vaccines face a number of challenges among which is the avoidance of cross-reactivity

with human tissue. It will take several more years for a possible GAS vaccine to be clinically effective and affordable in underdeveloped countries but it still remains a priority (Seckeler and Hoke 2011). The more urgent requirement is to implement available public health control measures against GAS diseases worldwide with special emphasis in low income countries.

6.5 Patients and methods

Thirty four throat culture isolates from patients with GAS and SNA pharyngotonsillitis required for the phenotype and genotype study were selected from a pool of samples described in chapters four and five who gave a history of ARF and were echo-cardiography-proven cases of RHD.

There was limited funding available for isolate typing and so it was only possible to type a small number of isolates. The strains that were chosen for typing with positive RADT and positive cultures were among the patients who gave a positive history of ARF and were echocardiographic proven cases of RHD. They showed raised anti-streptolysin body titers (>400 Todd units) with positive C-reactive protein (>50mg/L).

In this part of the study there were no controls that makes it somewhat difficult to determine if the genotype and phenotype of strains associated with GAS and SNA pharyngotonsillitis and a history of RHD reflected the general bacterial population circulating in the Yemen or a specific subset of this. Future studies should include a control group.

These included 21 patients with GAS and 13 patients with SNA pharyngotonsillitis. The 34 isolates were stored at -70°C until characterization. The GAS DNA extraction was performed according to the protocols described in Chapter 2. PCR amplification was carried out using specific "M" primers. The resulting PCR products were purified and sequenced on an automated sequencer. The nucleotide sequence with the N-terminal hypervariable portion of the M protein was subjected to homology searches against sequences in the Gen Bank molecular data bank.The *emm* sequence typing was performed as described by Beall *et al* on the CDC website http://www.cdc.gov/ncidod/biotech/strep/protocol_*emm* type.htm).The programs check the degree of similarity and attempt to obtain ≥ 95% identity with the 5' terminal portion

of a known *emm* genotype. This was followed by giving the nucleotide sequence the respective *emm* designation.

A PCR-based method was developed to determine the presence of phages and phage-associated virulence genes of *emm* genotypes,*speC, spd1, sdn, silC, silD,sof and sfbX*genes. The oligonucleotide primers and PCR conditions are listed in Table 6.2. The PCR-based method for the presence of the prophage-associated-*vir* genes: *speC, spd1, sdn* and the primer pairs for virulence genes were replicated from Matsumoto (Matsumoto, *et al* 2003). Multiplex PCR was used for toxin-gene profiling as described by Schmitz (Schmitz, *et al* 2003). PCR detection of the streptococcus invasive locus *(sil)* was performed with multiple sets of primers (Zimmerlein, *et al* 2005) .

6.5.1 PFGE interpretation

Interpretation of PFGE results was based on published guidelines (Tenover, *et al* 1995) as described in Table 6.1. The PFGE patterns were designated by their serial number identification in the study (Haukness, *et al* 2002).

6.6 Results

6.6.1 GAS and SNA pharyngotonsillitis

34 samples were subjected to PFGE, *emm* typing, and nucleic acid amplification for the pyrogenic exotoxin superantigen gene and serum opacity factor gene. These included 21 (61.8%) samples with GAS positive cultures and 13 (38.2%) with SNA positive cultures of GBS, GCS and GGS pharyngotonsillitis.

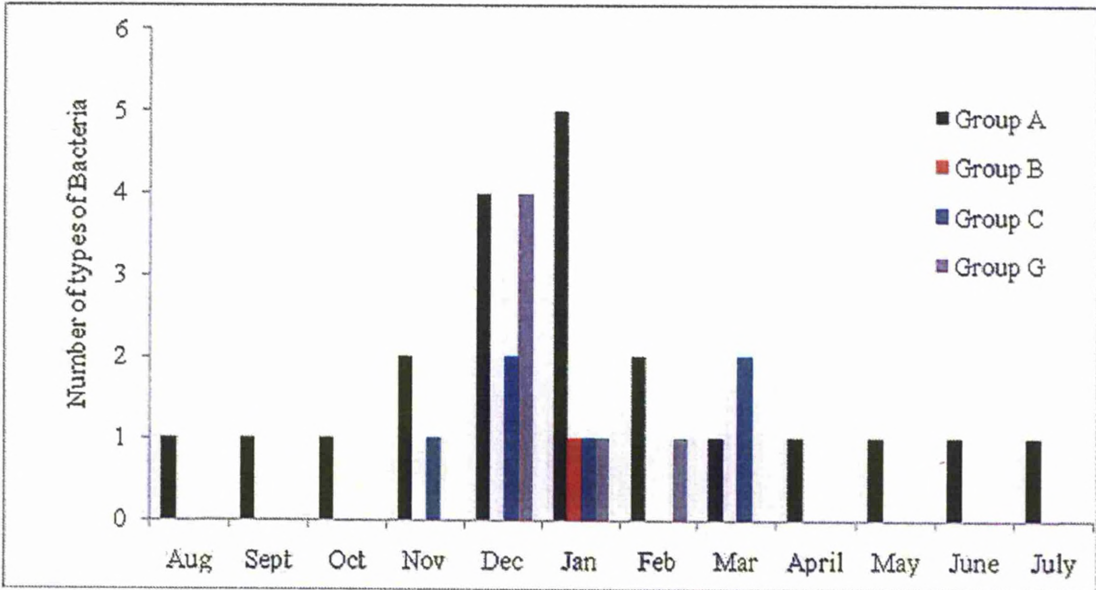


Figure 6. 2Distribution of 34 isolates of GAS and SNA by month (2006-2007)

6.6.2 GAS genotypes

Eleven (52.4%) GAS pharyngotonsillitis isolates encoded as *emm* 87, 6 isolates (28.8%) were *emm*12 genotype, 3 (14.3%) were *emm*28 and one (4.8%) *emm*5 genotype (Table 6.2).

Table 6. 2Distribution of GAS genotypes and *emm* sequence type

Strain	*Emm* type	No of *emm* isolates n=21 (%)
GAS	87	11 (52.4)
	12	6 (28.8)
	28	3 (14.3)
	5	1 (4.8)

6.6.3 SNA GBS, GCS and GGS genotypes

Four isolates (30.8%) of GCS pharyngotonsillitis were st2917 and two (15.4%) of the GCS isolates were *emm* stCK249 genotypes. Two (15.4%) GGS isolates were *emm* stG7882.2 genotypes, and the other four GGS were st2917, st7406, stG652.5 and stKNB7. One GBS isolate was stG4974. (Table 6.4)

Table 6. 3Distribution of SNA serotypes and *emm* typing

Strain	*Emm* type	No of *emm* isolates n=13 (%)
GBS	st4974	1 (7.7)
GCS	st2917	4 (30.8)
	stCK249	2 (15.4)
GGS	stG7882.2	2 (15.4)
	st2917	1 (7.7)
	st7406	1 (7.7)
	stG652.5	1 (7.7)
	stKNB7	1 (7.7)

6.6.4 Pyrogenic exotoxin superantigen genes in patients with GAS and SNA pharyngotonsillitis

The presence of pyrogenic exotoxin superantigenes among patients with GAS and SNA pharyngotonsillits is shown in Table 6.5. The *sof and sfbX* exotoxin gene amplification was positive in all the 21 (100%) patients with GAS but negative in all patients with SNA pharyngotonsillitis (p=0.001).

The superantigen gene *specC* was positive in 20 (95.2%) patients with GAS pharyngotonsillitis and negative in 13 (100%) patients with SNA pharyngotonsillitis (p=0.001). The *silC* virulence gene was positive in 21 (57.1%) GAS patients and 11 (77%) SNA patients with pharyngotonsillitis (p=0.048). The presence of the pyrogenic exotoxin superantigen gene *spd1*, *silD*, and *sdn* were positive in a higher proportion of

patients with GAS than SNA but the difference was not statistically significant between the two groups (p=<0.5) (Table 6.5).

Table 6. 4Distribution of pyrogenic exotoxin superantigen genes among patients with GAS and SNA Pharyngotonsillitis

Exotoxin superantigen gene	GAS n=21(%)	SNA n=13 (%)	p
sof	21 (100)	–	0.001
sfbX	21 (100)	–	0.001
speC	20 (95.2)	–	0.001
spd1	21 (100)	13 (100)	1.0
sdn	12 (57.1)	6 (46.2)	0.7
silC	21 (100)	11 (77)	0.048
silD	17 (81)	11 (77)	1.0

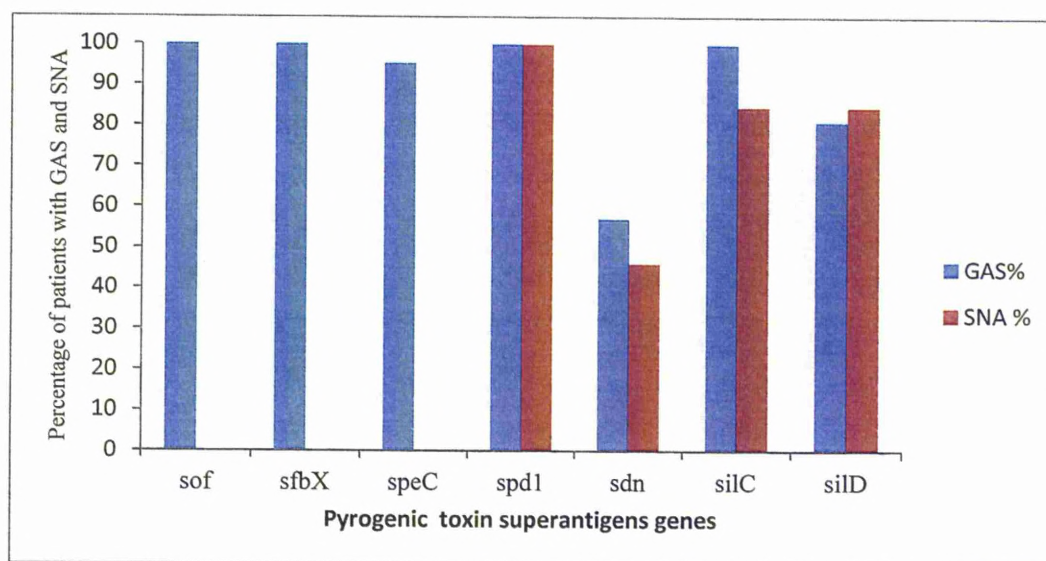


Figure 6. 3Distribution of pyrogenic toxin superantigen genes among isolates of GAS and SNA

6.6.5 Pyrogenic exotoxin superantigen genes in patients with GAS, GBS, GCS and GGS pharyngotonsillitis

The *sof* and*sfbX*gene was detectable in all patients with GAS and negative in all patients with GBS, GCS and GGS pharyngotonsillitis (p=0.001).

The pyrogenic exotoxin superantigen gene *specC* was positive in 20 (95.2%) patients with GAS pharyngotonsillitis but negative in all patients with GBS, GCS and GGS pharyngotonsillitis This difference of presence of *specC* among the four groups was statistically significant (p=0.001).

Table 6. 5Distribution of pyrogenic exotoxin superantigen genes among patients with GAS, GBS, GCS and GGS pharyngotonsillitis

Exotoxin Superantigen	GAS =21(%)	GBS n=1 (%)	GCS n=6 (%)	GGS n=6 (%)	p
sof	21 (100)	-	-	-	0.001
sfbX	21 (100)	-	-	-	0.001
speC	20 (95.2)	-	-	-	0.001
spd1	21 (61.8)	1(100)	6 (100)	6 (100)	1.0
sdn	12 (57.1)	-	6 (100)	-	0.001
silC	21 (100)	-	6 (100)	4 (67)	0.005
silD	17 (81)	-	6(100)	4 (67)	0.1

The exotoxin superantigen gene *sdnN* was positive in 12 (57.1%) patients with GAS and 6 (100%) patients with GCS pharyngotonsillitis. Patients with GBS and GGS pharyngotonsilltis were all negative for *sdnN* superantigen gene. This difference of the presence of *sdnN* was statistically significant (p=0.001).

The pyrogenic exotoxin superantigen gene *silC* was positive in all 21 (100%) patients with GAS and all 6 (100%) patients with GCS pharyngotonsillitis. It was positive in 4 (67%) of patients with GGS pharyngotonsillitis. The presence of *silC* exotoxin super-antigen gene showed a statistical significant difference within the groups (p=0.005).

6.6.6 Virulence prophage exotoxin superantigen gene among emm genotypes of GAS, GBS, GCS and GGS patients with pharyngotonsillitis

The *emm* genotypes observed among GAS isolates was *emm*87 in 11 patients, *emm*12 in six patients, *emm*28 in three patients and *emm*5 in one patient. All the eleven patients with *emm*87 GAS pharyngotonsilitis were positive for the pyrogenic exotoxin superantigens *sof*, *sfbX*,*speC*, *sdn*, *spd1*, *silC* and *silD*.

*emm*12 genotypes of GAS were present in six strains positive for the pyrogenic exotoxin superantigens genes of *sof*, *sfbX*, *speC*, *spd1*, *silC* and *silD*. None of the *emm* 12 genotype was positive for *sdn* pyrogenic exotoxin superantigen.

The *emm*28 genotype was identified in three isolates positive for the pyrogenic exotoxin superantigens gene *sof*, *sfbX*, *spec*, *spd1* and *silC*. One GAS isolate with *emm*5 genotype had positive pyrogenic exotoxin superantigen genes of *sof*, *spd1*, *sdn* and *silC*.

One GBS isolate *emm* st4974 had pyrogenic exotoxin superantigen gene positive for *spd1* and *silD*. GCS was identified with four isolates of st2917 and two stCK249 that were all positive for pyrogenic exotoxin superantigen gene of *spd1*, *sdn*, *silC* and *silD*.

There were six isolates with GGS serotypes. Four GGS identified as *emm* st652.5, st2917, st7406 and stKNB7 were positive for *spd1*, *silC* and *silD* exotoxin superantigen genes. GGS was also identified as *emm* stG7882.2 in two isolates that were positive for pyrogenic exotoxin superantigen gene *spd1* only.

Table 6. 6Distribution of the virulence genes among GAS and SNA pharyngotonsillitis isolates divided by *emm* genotype

Serotype	*emm* type	N	Sof	sfbX,	speC	Spd1	sdn	silC	silD
GAS	*emm*5	1	+	+		+	+	+	
	*emm*12	6	+	+	+	+		+	+
	*emm*28	3	+	+	+	+		+	
	*emm*87	11	+	+	+	+	+	+	+
GBS	st4974	1				+			+
GCS	st2917	4				+	+	+	+
	stCK249	2				+	+	+	+
GGS	st2917	1				+		+	+
	st7406	1				+		+	+
	st652.5	1				+		+	+
	stG7882.2	2				+			
	stKNB7	1				+		+	+

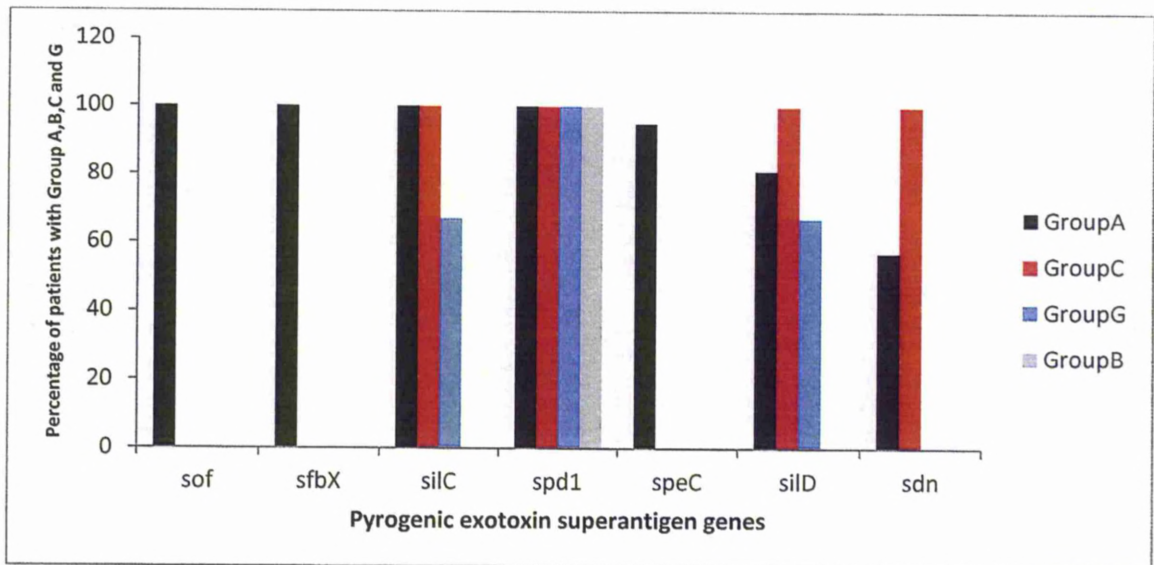


Figure 6. 4 Distribution of pyrogenic exotoxin superantigen genes among isolates of GAS, GBS, GCS and GGS

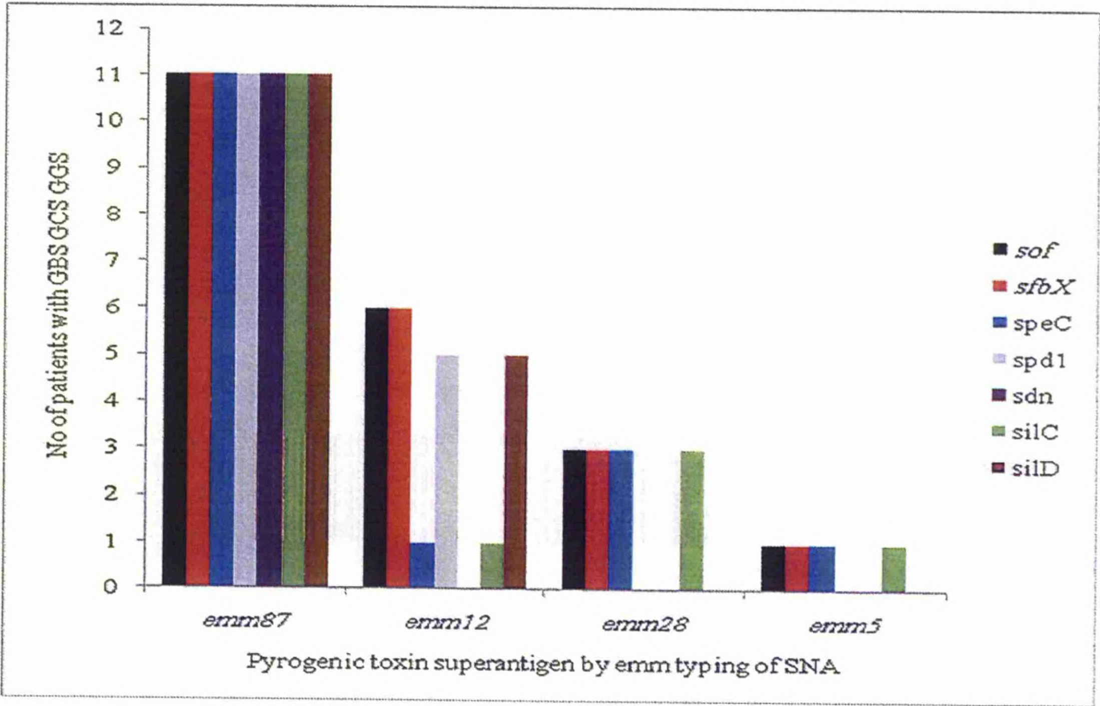


Figure 6. 5Pyrogenic exotoxin superantigen genes among GAS *emm* genotypes

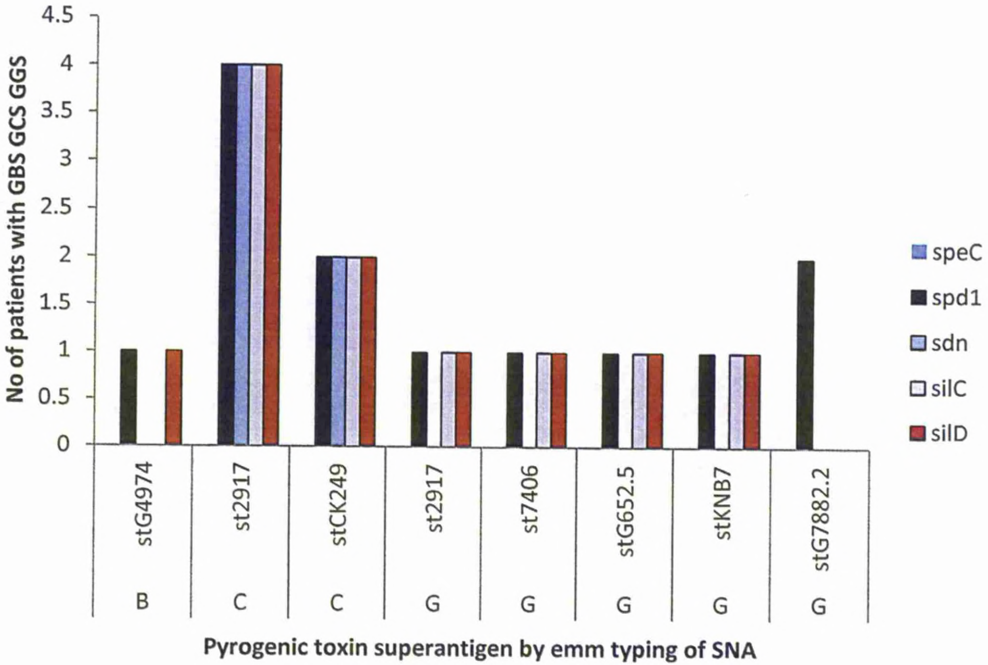


Figure 6. 6Pyrogenic exotoxin superantigen genes among GBS, GCS and GSS *emm* genotypes

6.6.7 Pulse field gel electrophoresis (PFGE) interpretation

The PFGE of a selection of GAS isolates is in Figure 6.7. PFGE representative band patterns analysis of genomic DNA restricted with *SmaI* enzyme. The nine Yemeni GAS isolates with the *emm*87 genotype in lane 5 to lane 13 share an identical pattern of chromosomal DNA bands and same size.

The control strain serves as a mean of comparison only. We were looking for the relatedness of the isolates (for example if one wanted to establish whether a particular set of organisms all belonged to an outbreak). We could establish whether strains examined were invasive or not if there was a control strain for either the invasive or the non-invasive (or both) strains. In Figure 6.7 the control strains in the PFGE were four lanes belonging to GAS isolates identified as *emm*12 genotypes from patients with acute pharyngotonsillitis at Alder Hey Children's Hospital, Liverpool.They merely served as a comparison with isolates from another site. The main purpose of the PFGE was to look for the relatedness of the isolates from the Yemen.

Table 6. 7 Comparison of PFGE genotypes and emm genotypes

Country	Serotype	*emm* genotype	No of isolates	PFGE profile	Lanes
Yemen	GAS	*emm* 87	9	A	1-9 *
		emm 87	1	A1	10**
Yemen	GAS	*emm* 12	5	B	7- 12**
	GAS	*emm* 12	1	B1	6**
Liverpool/UK	GAS	*emm* 12	4	B2	A-D*
Yemen	GAS	*emm* 28	2	C	3-4
Yemen			1	C1	2
Yemen	GAS	*emm* 5	1	D	5

*Figure 6.7, **Figure 6.8

A= *emm* 87 indistinguishable strains
B = *emm12* indistiguishable strains
B2= indistinguishable UK strains
C1 = closely related
A1= *emm* 87 closely related
B1 = closely related strains
C = indinsguishable strains
D = isolated strain

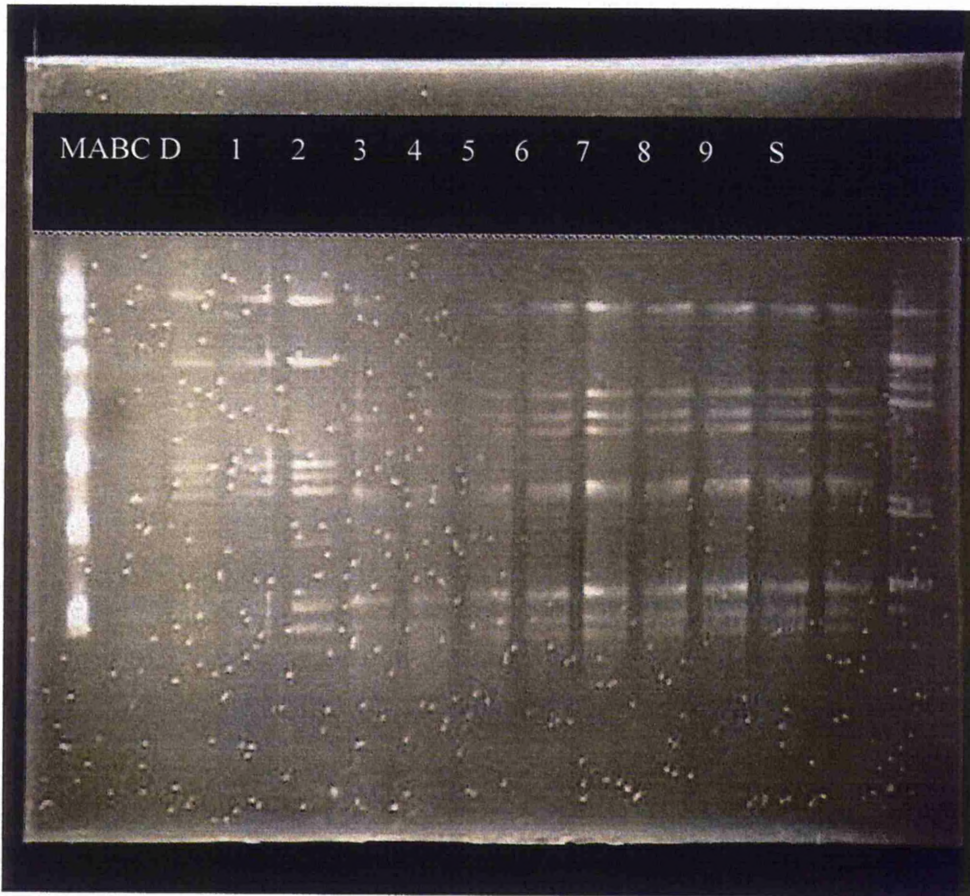


Figure 6. 7 PFGE interpretation of GAS isolates with acute pharyngotonsillitis digested with restriction enzyme *SmaI*

M lane is a reference molecular size marker.

A, B C and D lanes belong to GAS isolates from patients with acute pharyngotonsillitis at Alder Hey Childrens Hospital, Liverpool identified as *emm*12 genotypes.

Lanes 1 to 9 belong to GAS isolates from Yemeni patients with pharyngotonsillits identified as *emm*87 genotypes.

Lane S was the standard control for GAS isolate

Figure 6.8 shows the PFGE profile for the GAS isolates *emm*12 in lanes 6, 7, 8, 9, 11 and 12. They showed identical chromosomal patterns with no differences observed in the DNA strands and were closely related.

GAS isolates with *emm*28 showed identical patterns in lanes 2,3 and 4 of the PFGE profile in Figure (6.8). The chromosomal pattern of lane 5 belonged to GAS *emm*5. Lane 10 described a GAS *emm*87 that did not differ even in any DNA bands with the PFGE shown in figure 6.8

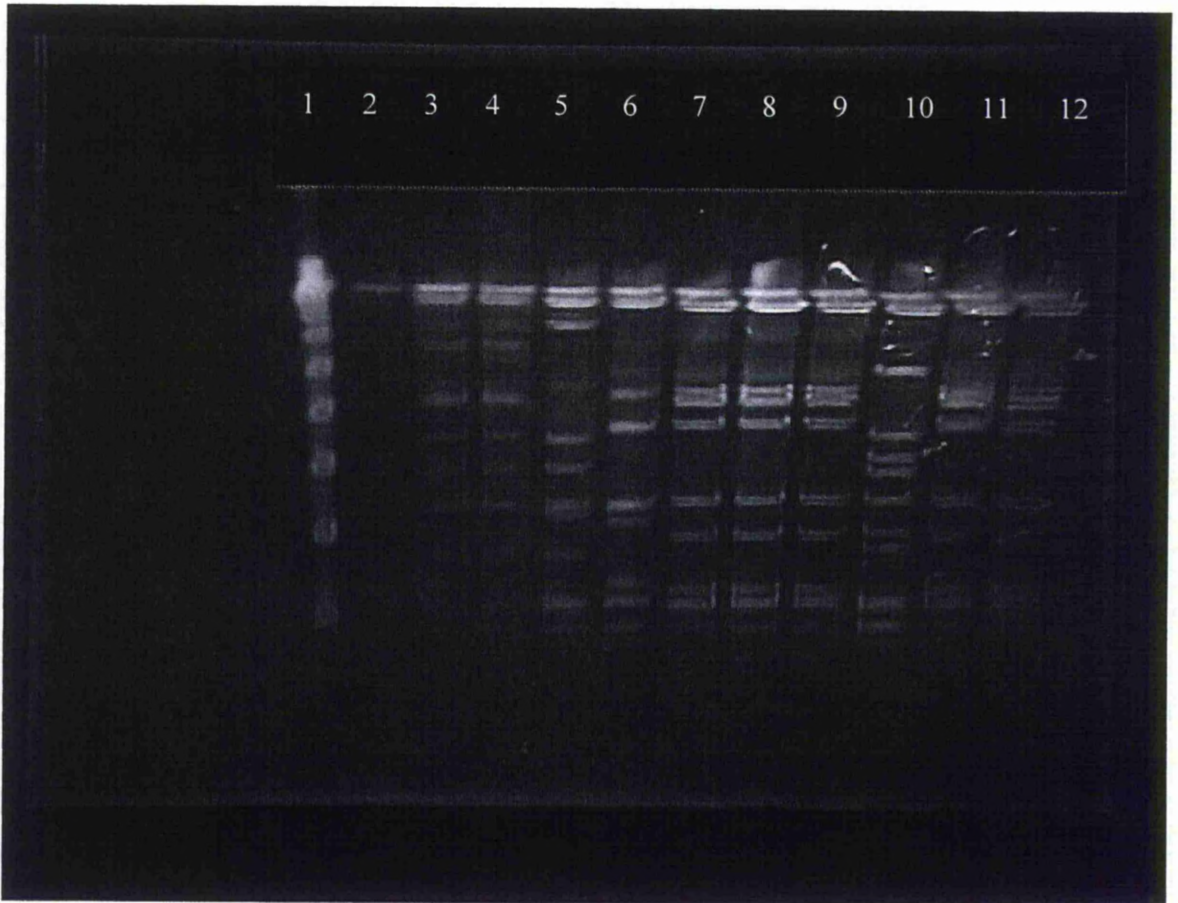


Figure 6. 8 PFGE of GAS isolates with *emm*5, 12, 28 and 87 genotypes using restriction enzyme S*maI*

Lane 1 molecular weight marker

Lane 3 and 4 belong to *emm*28 GAS isolates from Yemeni patients with acute pharyngotonsillitis. Lane 5 belongs to *emm*5 GAS isolate with acute pharyngotonsillitis

Lanes 6, 7, 8, 9, 11 and 12 belong to *emm*12 GAS pharyngotonsillitis

Lane 10 belongs to *emm*87 GAS isolate with acute pharyngotonsillitis

6.6.8 PCR interpretation of the prophage exotoxin superatigen genes

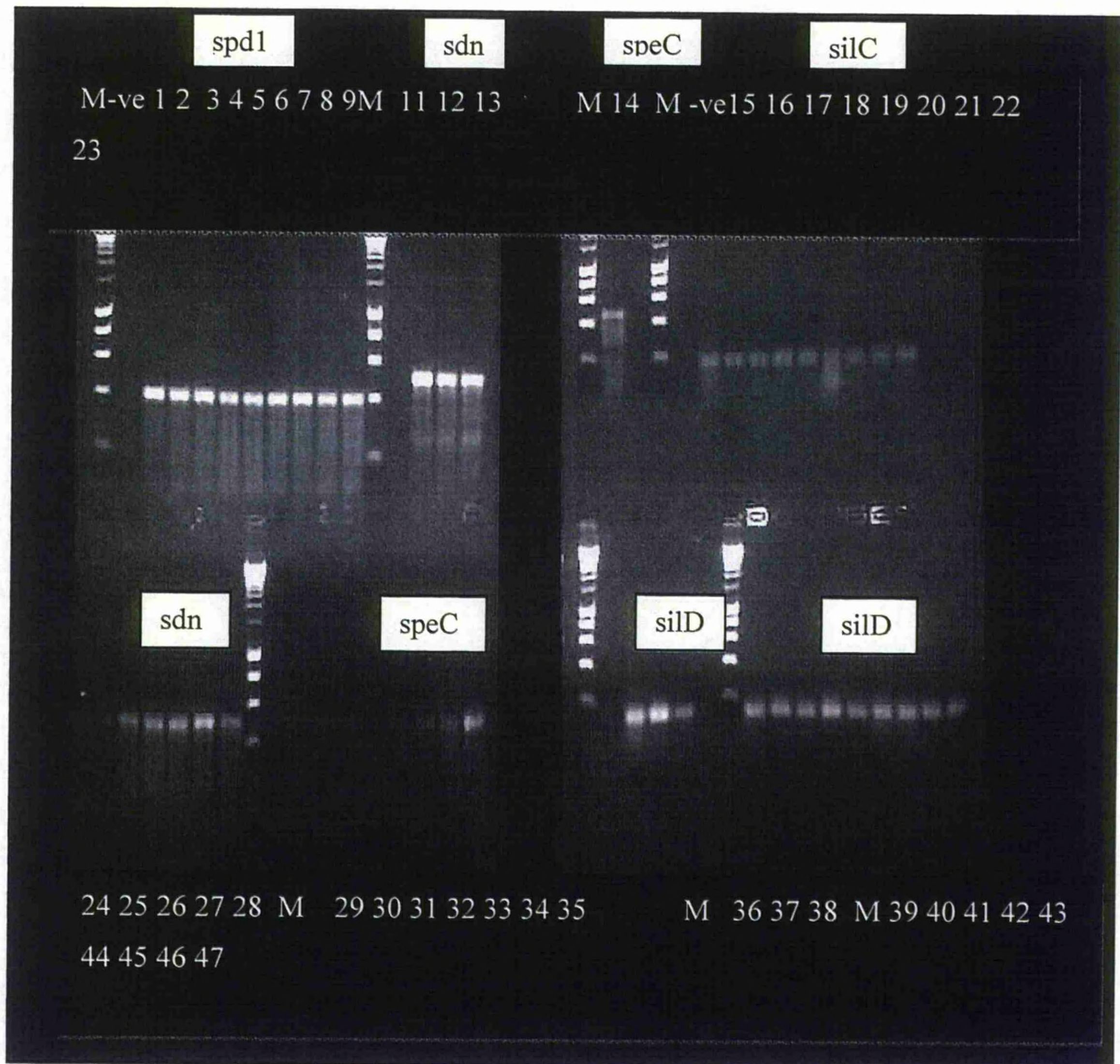


Figure 6. 9 PCR of GAS *emm*5, 12, 28 and 87 with the prophage pyrogenic exotoxin superantigen genes *spdl, sdn,spec .silC and silD*

PCR with prophage pyrogenic exotoxin superantigen – related virulence factors

Lanes marked M are the molecular weight markers

Lanes 1 to 9 *spdl* positive for GAS *emm*5, 12, 28 and 87 genotypes

Lanes 11-13 and lanes 24-28 *sdn* positive for GAS *emm*5 and 87

Lane 14and lanes 29 – 35 are *speC* positive for *emm*12, 28 and 87

Lanes 15 – 23 *silC* positive for *emm*5,12, 28 and87

Lanes 36 – 47 *silD* positive for *emm*12 and 87

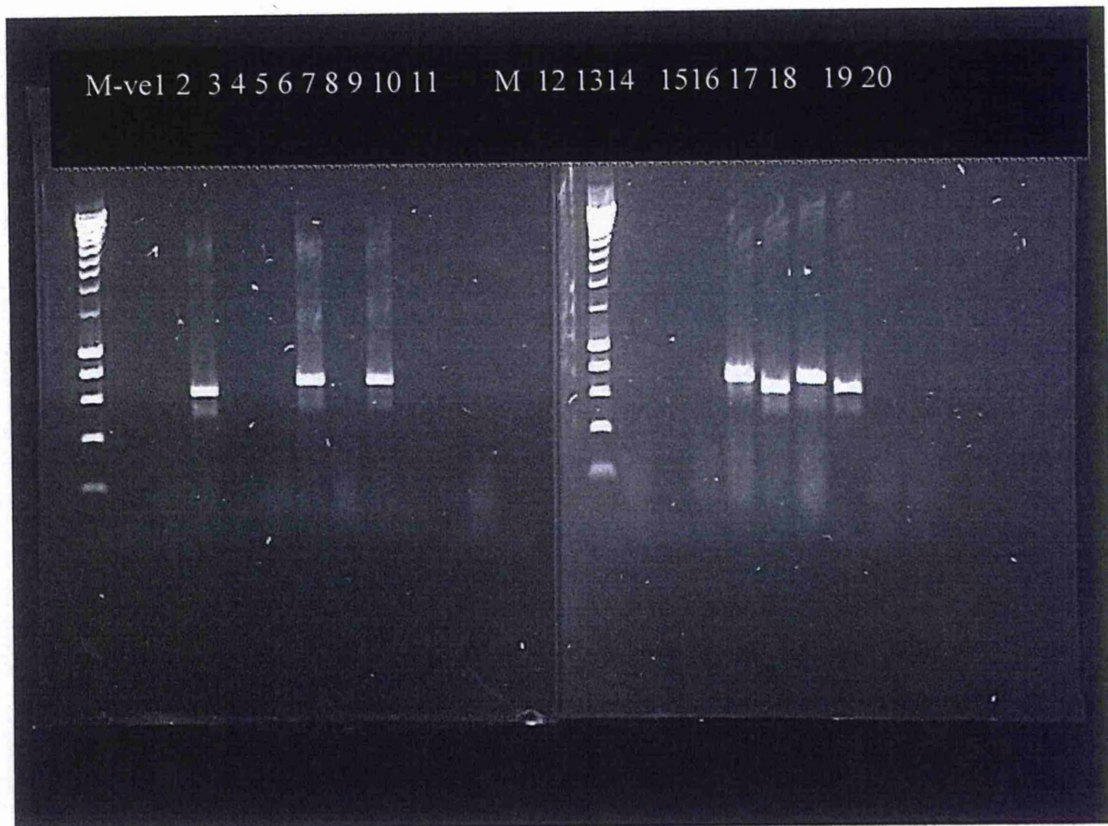


Figure 6. 10PCR of SNA isolates with prophage pyrogenic exotoxin superantigen *sof* genes

Lane M represents the molecular reference weight marker

Lane 1 and lane 18 are *sof* 12 positive for *emm*12 GAS

Lane 5 and lane 7 are *sof* 87 positive for *emm*87 GAS

Lane 15 and lane 17 are *sof* 28 for *emm*28 GAS

Lane 16 is *sof* 5 positve for *emm*5GAS

6.7 Discussion

6.7.1 Emm genotype among GAS isolates with pharyngotonsillitis

This study describes the characteristics of 34 isolates of GAS and GCS, GGS and GBS strains among pharyngotonsillar cultures from patients in Yemen. The isolates included known and new *emm* sequences and were characterized through genotype tests detection of the *sof* and *sfbX* genes, the streptococcal pyrogenic exotoxin genes, *sil* and PFGE analysis of genomic DNA restriction profiles. The most frequent GAS isolates detected among patients with history of ARF and RHD were *emm*87, 12, 28 and 5

More than 200 GAS genotypes have been documented at the CDC *emm* database laboratories (Shulman, *et al* 2004). It is known that some GAS serotypes are more 'rheumatogenic' than others (WHO 2004) The GAS *emm*/M protein serotypes *emm*1, 3, 5, 6, 14, 18, 19, 24, 27 and 29 have been linked to ARF/RHD (Kaplan, *et al* 2001, Stollerman 1991). The rheumatogenic types 3, 5, 6, 14, 18, 19, and 29 made up 49.7% of pharyngeal isolates during the 1960s but only 10.6% of isolates 40 years later (Shulman, *et al* 2009, Steer, *et al* 2009d).

The most common ARF associated *emm* GAS strains were *emm*5 and *emm*18 in the United States, *emm*5 and *emm*1 in Chile (Cresti, *et al* 2002) and *emm*11 and *emm*41 in Trinidad. *emm*/M18 in Utah was associated with ARF (Veasy, *et al* 2004), while the GAS *emm* types isolated from patients with ARF in Hawaii were 65, 69, 71, 92, 93, 98, 103, 122 (Erdem, *et al* 2009) and *emm*25 in Thailand (Pruksakorn, *et al* 2000).

In the Aboriginal territory in Australia *emm*14 and *emm*st3765 were the frequent rheumatogenic strains (Bessen, *et al* 2000) while in Auckland, New Zealand *emm*6, 53, 55 and 66 were associated with ARF (Martin, *et al* 1994). The predominant strains reported in Kuwait were *emm*1, 9, 12 and 33 (Majeed, *et al* 1992b) and in Tunisia *emm*2, 9, 11, 33, and 49 (Mzoughi R2004) while *emm*5 and 6 were found in Iran (Jasir 2000) (Table 6.8).

Table 6. 8Distribution of *emm* GAS rheumatogenic strains on throat swabs in different countries

Country	*Emm* GAS rheumatogenic strains	Reference
Kuwait	1,9,12,33	Mageed et al 1992
New Zealand	6, 53, 55,66	Martin et al 1994
Iran	5, 6	Jasir et al 2000
Australia	14 and st3765	Bessen et al 2000
Thailand	25	Proksakron et al 2000
Chile	1,5	Cresti et al 2002
Tunisia	2, 9, 11, 33,49	Mzoughi et al 2004
Utah	18	Veasy et al 2004
Trinidad	11, 41	Veasy et al 2004
United States	3, 5, 6, 14, 18, 19, 29	Shulman et al 2009
Hawaii	65, 69, 71, 92, 93, 98, 103, 122	Erdem et al 2009
Yemen	5,12,28,87	Current study

The most frequent GAS types among Yemeni patients with acute pharyngotonsillitis having a history of ARF and RHD in this study were *emm*87, 12, 28 and 5 in order of frequency. GAS *emm* 87 was the most frequent genotype detected in more than half ARF and RHD patients 11/24 (52.4%) with acute pharyngotonsillitis. This is the first report of GAS *emm*87 to be detected among patients with ARF/RHD in the literature and it may be potentially rheumatogeinc.

Emm 12 and 28 detected among patients with ARF and RHD may also have a possibility of being rheumatogenic. Even though *emm*12 and 28 are among the relatively common causes of acute pharyngotonsillitis they have not yet been attributed to cause ARF (Abdissa, *et al* 2006, Kaplan 1996) with the exception of two reports. One report from Kuwait where *emm*12 was isolated in 7% of rheumatic children and

their families and the other from Pennsylvania where 9 isolates were recovered from patients with ARF (Majeed, *et al* 1992b, Martin and Green 2006). GAS *emm5* genotype is a global rheumatogenic strain reported in different countries. Very different spectra of GAS genotypes have been associated with ARF in some developing countries, with some being M-nontypable or representing newly identified types (Majeed, *et al* 1992b, Martin, *et al* 2004, Shulman, *et al* 2009). Mc Donald described one emm 87 in throat and skin in populations with high rated of ARF and many GAS skin infections but it is rather difficult to comment on comparisons to Yemen since we have no studies on skin infections and neither skin isolates

Uncommon *emm* GAS serotypes could have a role in the epidemiology of ARF/RHD in Yemen. However we cannot be certain as we do not know the *emm* strains that are not associated with ARF/RHD in the same geographical area for comparison.On the other hand, it might be explained on the basis of the small sample size of isolates evaluated for *emm* type.

6.7.2 Serum opacity factor (*sof*) gene

Rheumatogenic GAS have distinct biologic characteristics. They belong to Class I strains, do not bind fibronectin and are predominantly SOF-negative. Although rheumatogenic strains are less likely to produce SOF, there are no studies to indicate that they cannot produce SOF (Martin, *et al* 1994).

All the GAS isolates which were Group A β haemolyitc Streptococci representing genotypes of *emm* 87, *emm* 28, *emm* 12 and *emm* 5 were both positive for the *sof* geneand *sfbX* genes. The *sof* and *sfbX* genes were located on the same DNA bands of the same chromosome in all the 21 isolates of GAS Group A genotypes by PCR analysis. These findings may further support the fact that *sof* geneis likely to be linked invariably to a second gene (*sfbX*) encoding a new streptococcal fibronectin-binding protein (Johnson, *et al* 2006). The presence of the two genes, *sof* gene and *sfbX* gene encoding fibronectin-binding functions may have an effect on the spectrum of tissue colonization and disease manifestations in specific GAS strains (Jeng, *et al* 2003, Peacock, *et al* 2000, Peacock, *et al* 1999).

The presence of these two genes together could influence the spectrum of tissue colonized by GAS strains (Jeng *et al,* 2003) .The 11 GAS isolates with the same *emm*87 type were positive for the *sof*and *sfbX* geneand contained the *sof* 87 gene sequence. This is in contrast to the literature describing *emm* 87 to be *sof* negative. This may indicate that there is a possibility that *emm*87 may possess a *sof* gene that is only detectable by PCR and is not expressed. Isolates shared a common toxin gene profile with the presence of the five toxin genes *spec, spd1, sdn, silC and silD.* There were no striking differences between the PFGE types of *emm* 87 GAS strains. This is the first report describing the profile of these five exotoxin genes among GAS *emm*87.

The *emm* 12 isolates of GAS were all *sof*and *sfbX* gene positive and contained the *sof* 12 gene sequence among the pharyngeal cultures in this study. This is in contrast to the Italian, Iranian and German studies where the *emm12* isolates were invariably *sof* gene negative (Brandt, *et al* 2001). Although some studies indicate that *emm12* isolates are phenotypically SOF negative but the *sof* gene is detecTable by PCR (Lintges, *et al* 2007). To date, *emm12* represents the only known *emm/emm*-like gene patterns associated with the presence of a *sof* gene (Dicuonzo, *et al* 2001, MA, *et al* 2009).

The *emm*28GAS strains were positive for *sof* and *sfbX* gene which is comparable to a study conducted in Germany (Brandt, *et al* 2001). These *sof* types and their corresponding *emm* sequence types in several combinations of GAS isolates may appear to be highly predictive of some genetically related strain sets (Maripuu, *et al* 2008). The ability to detect the combinations of the *sof* gene and *emm* sequence type in different GAS isolates may lead to a revision of the previous association of *sof* and *emm* type.

6.7.3 Prophage exotoxin superantigen genes

Pyrogenic exotoxin C (*speC*) is bacteriophage-mediated and its expression is likewise highly variable. Recently, mild cases of scarlet fever in England and the United States have been associated with *speC*-positive strains (Vitali, *et al* 2009). In this study, the streptococcus pyrogenic exotoxin C (*speC*) was identified in 11/11 of the *emm*87, 6/6 of *emm*12 and 3/3 of *emm*28 GAS strains. Rivera et al in Spain found positive *speC* in only 1/7 isolates with *emm*12, 7/9 isolates with *emm*28 and 3/3 isolates with *emm*87 (Rivera, *et al* 2006). Bingen et al in France reported *speC* in 2 isolates of *emm*12, 5 strains of

*emm*28 and one strain of *emm*87. This is the highest proportion of the *speC* toxin gene reported among *emm* 87 isolates (Bingen, *et al* 2004).

GAS *emm*28 strains are highly diverse in prophage-encoded virulence gene content and integration site and are contributors to GAS genetic diversity and population biology (Green, *et al* 2005). Three GAS *emm* 28 strains were positive for *speC* and *spd1* genes encoding streptococcal pyrogenic exotoxin C and a DNase, respectively. This is similar to a study by Green et al who observed that GAS *emm*28 genotype had the *speC* and *spd1*genes in 84% of the isolates and was reported to be the major profile for this type. In this study the presence of streptodornase (DNase) *sdn* was not identified among the *emm*28GAS strains in contrast to the presence in 34% of isolates in Israel (Green, *et al* 2005, Moses, *et al* 2003).

6.7.4 Streptococcus invasive locus among GAS strains

PCR was positive for *silC* in 21 (100%) and *silD* in 17 (81%) GAS pharyngotonsillitis isolates compared to a study in French children with invasive GAS infections but not ARF, where both *silC* and *silD* were positive in 12 GAS isolates (16%). The *silC* and *silD* loci were positive in all the *emm*87 and *emm*12 GAS pharyngotonsillitis isolates among Yemeni children with ARF and RHD. In this study, the *emm*5 and *emm*28 GAS strains were only positive for *silC* but not for the *silD* loci. This is in contrast to a study among French children where only one isolate of *emm*87 was positive for both *silC* and *silD* while the *emm*12 and *emm*28 were neither positive for *silC* nor *silD* among their invasive GAS isolates (Bidet, *et al* 2007). The *silC* was found in the three isolates of the *emm*28 and *emm*5 GAS genotype which has not been previously reported in literature.

6.7.5 *Emm* genotype among SNA isolates with pharyngotonsillitis

The reported prevalence of GCS/GGS carriage and disease incidence varies globally. Most studies originate from temperate regions of the northern hemisphere and limited data are available from tropical regions (McDonald, *et al* 2007b, Teixeira, *et al* 2001).

In this study the *emm*/M virulence genotypes of GGS in 6 patients with ARF/RHD and pharyngotonsillitis were genetically heterogeneous having 5 different genotypes each of *emm* st2917, *emm* st7406, *emm* stG652.5, stKNB7 and two isolates of *emm* st7882.2.

The *emm*/M virulence genotypes of 6 GCS patients with acute pharyngotonsillitis were of st2917 in 4 isolates and *emm* stCK249 in two strains.

These GGS and GCS *emm* sequence strains are different from those reported in India, Jerusalem, Portugal, Hungary and Argentina (Cohen 2004, Hashikawa, *et al* 2004, Lopardo, *et al* 2005, McMillan, *et al* 2004, Pinho, *et al* 2006). One *emm* sequence type detected among GGS strain, stKNB 7 was originally recovered among GGS pharyngeal isolates in Chennai, India (Menon, *et al* 2008). These variations might be attributed to geographic differences. The new seven *emm* sequences among GCS, GGS and GBS pharyngeal isolates in this study are the first reports in the literature.

6.7.6 Prophage exotoxin superanitgen and (*sof*) genes among SNA strains

SNA particularly GCS/GGS are known to possess class I M proteins similar to rheumatogenic GAS strains. A high proportion of these strains appear to be *sof* negative, albeit few *sof* positive *emm* types have been reported including stG166b and stG 480.0 (McDonald, *et al* 2007a). The prophage toxin genes *spdl* was positive in all the SNA, GBS, GCS and GGS pharyngotonsillitis isolates. *sof* and *speC* genes were negative in all the SNA, GBS, GCS and GGS different genotypic strains were consistent with the *sof*-negative phenotype described by Bisno & Collins in 2003. There was only one report of GCS strain *st* 4974 positive for speC in the US (Kalia) Interestingly, strain *st* 4974 was recovered from the throat of a patient with ARF but the evidence was not conclusive (Kalai et al 2003).The exotoxin superantigen *sdn* was positive in GCS strains but the GBS and GGS were all negative for this *sdn* superantigen that has not yet been identified elsewhere.

It is noteworthy that all the *emm* genotypes of GCS strains were positive for *spdl*, *sdn*, *silC* and *silD* prophage exotoxin superantigens. The GCS strains shared the presence of these virulence genes with GAS isolates. These GCS strains with particular genotypes in susceptible hosts and their association with the presence of these virulence genes traits may have a greater likelihood to be associated with ARF. This is the first report to highlight the prophage gene toxin sequence of specific GCS strains.

Moreover, the *emm*4 genotype among four GCS and one GGS Yemeni pharyngotonsillitis strains were positive for both *silC* and *silD* toxin gene. Bidet et al reported 50% of GAS isolates harbouring the *silC* and *silD* loci which belonged to *emm*4 genotype (Bidet, *et al* 2007).

6.7.7 PFGE interpretation

Tenover's guidelines were used to determine the relatedness of GAS isolates.The ten throat isolates of GAS *emm*87 were from different schools from different districts of Aden city. PFGE profiles within the ten isolates of individual GAS *emm*87 did not show differences within the chromosomal DNA bands. This suggests that these PFGE profiles were closely related and each individual *emm* type of 87 shared a high degree of genetic relatedness indicating a possible outbreak pattern.

GAS strains belonging to *emm*12 exhibited similar and highly related PFGE profiles. GAS *emm*28 also shared similar DNA patterns within the same *emm* PFGE profiles. Other characteristics varied from isolate to isolate, although some associations were consistently found within some *emm* types. Most strains belonging to a given *emm* type had similar or highly related PFGE profiles that were distinct from profiles of strains of another type. This information may provide baseline data on the epidemiological aspects of GAS pharyngotonsillitis infection.

In some instances, PFGE profiles within a given GCS and GGS *emm* type were not related, but were distinct from the strain profiles of another GCS and GGS *emm* types. These data indicate that the majority of isolates within each individual *emm* type shared a high degree of genetic relatedness among the SNA GCS and GGS genotypes. This high degree of relatedness within certain *emm* types might hold true only within this particular geographical area. However we did not compare PFGE with profiles from other countries.

6.8 Conclusions

The most frequent *emm* GAS isolates among patients with acute pharyngotonsillitis with a history of ARF and RHD were *emm*87, 12, 28 and 5. GAS genotype *emm*5 and

emm 12 were previously reported to be rheumatogenic. The two other *emm* genotypes 87 and 28 have not been previously reported among rheumatogenic GAS strains.

The 11 isolates of *emm*87 GAS strain were positive for the *sof and sfbX* gene and contained the unique *sof* 87 sequence. Isolates shared a common toxin gene profile with the presence of the five toxin genes *speC, spd1, sdn, silC* and *silD*. There were no striking differences of sequence types in the PFGE of *emm* 87 GAS strains.

The *emm*12 isolates of GAS were all *sof*and *sfbX* gene positive and contained the s*of* 12 gene sequence. The *emm*28 GAS were all *sof*and *sfbX* gene positive for the *sof* 28 gene sequence among the pharyngeal cultures. emm5 GAS was *sof*and *sfbX* gene positive

There is a continuing worldwide problem of disease mediated by GAS isolates. Monitoring of GAS isolates and the study of their *emm* genetic variability, should eventually lead to a better understanding of the epidemiology of specific GAS strains. The variations in the serotypes and *emm* genotyping may reflect differences in the circulating strains due to geographical separation or different proportions of submissions by site of isolation and the different period examined.

GAS infections can be appropriately treated with antibiotics. It is essential to assess the burden of the disease with an epidemiologic data on the isolates causing disease through active and continuous surveillance. This will guide public health action policy in for preventive and therapeutic strategies. The understanding of the genetic regulation of GAS, its structure and function will form the basis to the development of effective and safe vaccines.

The high and alarming prevalence of ARF/RHD in underdeveloped and developing countries and the advancement of techniques in molecular biology will facilitate the development of a vaccine that is safe, economical and efficacious to combat GAS infection and its serious life-threatening complications. Knowledge of the genetic diversity among bacterial strains provides an insight into pathogen evolution and facilitates the study of the relationships between strain genotypes and pathogeneicity.

6.9 General implication

This study demonstrates variations in the *emm* genotype sequence among GAS and the SNA GGS and GCS isolates. The pyrogenic exotoxin superantigen genes *spd1* was detected in all the GAS, GCS and GGS strains and the *silC* and *silD* was positive in more than half of the GAS and SNA GCS and GGS isolates. These different strains possessed similar pyrogenic exotoxin superantigen virulence genes that may support the speculation of them sharing distinct structural features with GAS genotypes. Further comparison of the structures of these different genotypes of GAS with GCS and GGS may cast light on the configuration of the virulence protein molecules which could be critical for the expression of rheumatogenicity.

This study presents the phenotypic and genotypic characteristics of GCS and GGS and GBS isolates belonging to seven new *emm* sequence types with a variety of characteristics which are the first to be detected among Yemeni children with acute pharyngotonsillitis and a history of ARF and RHD. The data suggests that strains circulating in Yemen, an area not previously surveyed, may be different from those known to circulate in other geographical areas. Such information can contribute to a better understanding of the local and global dynamics and epidemiologic aspects of GAS and GGS, GCS and GBS infections occurring in various regions.

This study possibly represents the first genotypic and exotoxin virulence gene survey of GAS isolates recovered among children in the Middle-East. Although the study sample was small and from a convenience sample.(A convenience sample describes a sample in which patients were selected at the convenience of the researcher and which related to the inability to assess other isolates due principally to cost constraints),rather than from community-based studies, GAS isolates from patients with pharyngotonsillitis having history of ARF and RHD appeared to be associated with *emm* types not commonly observed in ARF and RHD patients. Although *emm* types are good predictors of clonal types within a given geographic location, there is considerable genetic diversity within certain *emm* types, especially when comparing strains from different countries.

In this part of the study there were no controls that makes it somewhat difficult to determine if the genotype and phenotype of strains associated with GAS and SNA pharyngotonsillitis and a history of RHD reflect the general bacterial population circulating in the Yemen or a specific subset of this and further studies should include a control group.

Chapter 7

7 Antimicrobial Patterns among GAS and SNA pharyngotonsillitis with a history of ARF and RHD

7.1 Introduction

Antimicrobial resistance is a major global problem that challenges physicians with the management of infections (Hawkey and Jones 2009, Jensen, *et al* 2009, Bronzwaer, *et al*2002). Antimicrobial drugs are used in GAS infections to control and to minimize the occurrence of suppurative complications, prevent ARF and reduce person to person transmission (Leung and Kellner 2004, Reynolds 2009). There are many studies documenting the inappropriate use of antibiotics in acute pharyngtonsillitis in children (Deasy 2009, Nash, *et al* 2002, Weber 2005).

GAS remains universally susceptible to penicillin and this continues to be the first-line therapy for GAS pharyngotonsillitis (Bisno 2001, Weber 2005). There are no reports documenting clinical isolates of GAS pharyngotonsillitis resistant to penicillin or cephalosporins *in vitro*(Sauermann, *et al* 2003). Erythromycin is the macrolide antibiotic of choice in patients allergic to penicillin (Betriu, *et al* 1993, Bingen, *et al* 2000, Krause 2002). There are however many countries reporting increasing rates of erythromycin resistance due to excessive usage of macrolides (Bingen, *et al* 2004)

Patients with GAS pharyngotonsillitis can be treated with antibiotics with the use of a clinical scoring system, RADT or positive culture tests (Leung and Kellner 2004). Rapid treatment of GAS infections is important as inappropriate treatment leads to changes in the characteristics of the bacteria enhancing the process of bacterial resistance to antibiotics (Kim and Lee 2004b, Linder, *et al* 2005, Liu, *et al* 2009, Szczypa, *et al* 2004).

Information on bacterial susceptibility to commonly used drugs has not been reported from Yemen, where the use of antibiotics is less regulated and is available over the counter. The aim of this study thus was to analyse antimicrobial susceptibility patterns and genetic diversity among patients with GAS and SNA pharyngotonsillitis.

7.2 Objective

To analyze the susceptibility pattern of antibiotics of GAS and SNA isolates from patients with pharyngotonsillitis

7.3 Literature review

Antibiotic resistance is the ability of bacteria to oppose the inhibitory (bacteriostatic) or killing (bactericidal) effect of antibiotics (Deasy 2009, Szczypa, *et al* 2004). Antibiotic resistance has increased during the past 15 years creating problems in treating bacterial infections (Alos, *et al* 2003, Barker 1999, Woodford, *et al* 2009). Antimicrobial resistance increases morbidity, mortality, and length of hospital stay and health care costs due to delay in therapy (Hawkey and Jones 2009). These adverse outcomes may be the result of the unsuccessful use of antibiotics or a delay in therapy (Borg, *et al* 2008).

Antimicrobial resistance is a growing problem worldwide, requiring international approaches to continuously focus on the importance of the emergence and determinants of resistance and the need for control strategies (Dryden, *et al* 2009, Green, *et al* 2004, Reynolds 2009). The major factors leading to the emergence and transmission of drug-resistant bacterial strains are directly related to the antimicrobial consumption of drugs (Al-Najjar and Uduman 2008, Dryden, *et al* 2009, Jensen, *et al* 2009).

Despite the extensive use of penicillin for more than 50 years, GAS has not acquired resistance to penicillin and still remains the recommended drug of choice for the treatment of GAS pharyngotonsillitis (Bandak, *et al* 2000, Brook and Dohar 2006, Descheemaeker, *et al* 2000, Martin, *et al* 2004). Penicillin exhibits proven efficacy with a narrow spectrum and is cheap (Holm 2000, Linder, *et al* 2005, Palavecino, *et al* 2001). However, reports of failure of penicillin in eradication GAS from the oropharynx of healthy carriers are now causing some concern possibly due to penicillin tolerance

(Adam, *et al* 2000, Beekmann, *et al* 2005, Gerber, *et al* 2009). Amoxicillin is another medication in common use, particularly among children, because of its greater compliance and increased palatability (Barzilai, *et al* 2001, Leelarasamee, *et al* 2000, Shulman, *et al* 2006).

Erythromycin is recommended only for GAS patients allergic to penicillin (Alos, *et al* 2003, Bae, *et al* 2007, Green, *et al* 2004). In contrast to penicillin, erythromycin was introduced in the late 1950s and was immediately followed by the emergence of drug resistance. Lowry & Hurst (1952) in Birmingham reported the first erythromycin resistant GAS (Lowbury and Hurst 1959). Then in the 1970s the first widespread erythromycin resistance was reported in Japan (Miyamoto, *et al* 1978) followed by an increase in erythromycin resistance from Finland (Canton, *et al* 2002, Seppala,*et al*1995). Macrolide resistance is increasing in many countries possibly due to the use and overuse of macrolides (Alos, *et al* 2003, Borg, *et al* 2008, Descheemaeker, *et al* 2000). Studies indicate a nationwide macrolide resistance from as low as 1.3% – 5% to > 45% in several countries (Alos, *et al* 2003, Gagliotti, *et al* 2006, Green, *et al* 2004, Jensen, *et al* 2009).

First-generation cephalosporins are also used for patients allergic to penicillin, but they have a broader spectrum and are more expensive (Adam, *et al* 2000, Palavecino, *et al* 2001). Clindamycin can be used if other antibiotics are not an option (Beekmann, *et al* 2005, Choby 2009). Tetracyclines and sulfonamides are ineffective in eradicating GAS and tetracycline is contraindicated in young children (Beekmann, *et al* 2005, Weber 2005).

Clinical treatment failure of GAS pharyngotonsillitis has rarely been reported (Brahmadathan and Gladstone 2006, Manyemba and Mayosi 2003). A patient with recurrent symptoms of GAS pharyngotonsillitis with positive throat culture following a few weeks of treatment should be considered as having a chronic pharyngeal carriage state with superimposed viral infection, non compliance with antibiotics, or an infection with a different new GAS strain (Oran, *et al* 2000). Recurrent pharyngitis caused by the same GAS strain in a patient who has received proper antibiotic treatment is uncommon (Shulman, *et al* 2009)

7.4 **Patients and methods**

Antibiotic sensitivity patterns were determined in 24 representative culture isolates from patients with acute pharyngotonsillitis of whom 11 were GAS isolates from patients described in chapter 4 and 13 SNA isolates were patients described in chapter 5.A small proportion of isolates were tested for antimicrobial susceptibility patterns due to the lack of funding and this amount of money was generously donated by the Department of Medical Microbiology at the University of Liverpool.

Antibiogram tests were conducted using penicillin G, amoxicillin, erythromycin, , tetracycline and chloramphenicol on the 24 isolates on 5% defibrinated horse blood using the disc diffusion method (Andrews 2001b). The diameter of the zone of inhibition was measured to the nearest millimetre and bacteria were classified as susceptible or resistant according to the standard Table by the BSAC system.

The minimum inhibitory concentration (MIC) was defined as the lowest concentration of the drug that inhibited visible growth after overnight incubation (Andrews 2001a). The breakpoint refers to the point at which GAS is considered to be resistant to a particular antibiotic. MIC $\geq$ breakpoint concentration defines an organism as resistant and the MIC $\leq$ breakpoint concentration defines the organism as susceptible (Scavizzi, *et al* 2002). The zone diameter breakpoints for each antibiotic are derived from the respective MIC breakpoint (MacGowan and Wise 2001). The interpretation of zone diameters was done according to the BSAC standard for penicillin G, amoxicillin, erythromycin, tetracycline and chloramphenicol (Andrews 2007) . The data were analyzed by SPSS17.0 software and the level of significance was determined as $p<0.05$.

7.4 Results

7.4.1 General antimicrobial susceptibility patterns among GAS and SNA strains

The 24 patients recruited in this analysis had a mean (SD) age of 13(1.4) years, with a range of 10- 15 years. There were 15 males and 9 females. Eleven patients had GAS isolates and thirteen SNA isolates.

All GAS strains were sensitive to penicillin and amoxicillin. All SNA strains were sensitive to penicillin and amoxicillin. Erythromycin resistance was encountered among four (36%) GAS strains and seven (54%) SNA isolates. GAS and SNA isolates were resistant to chloramphenicol in 10 (91%) and 12 (92%) patients, respectively. Resistance to tetracycline among GAS isolates was observed in six (54.5%) cases and four (30.8%) SNA isolates. The difference of sensitivity and resistance patterns varied between GAS and SNA isolates but was not statistically significant (Table 7.1).

Table 7. 1 Antibiotic sensitivity and resistance pattern among GAS and SNA pharyngotonsillitis

Antibiotic	GAS n=11(%)		SNA n=13(%)		p
	Sensitive	Resistant	Sensitive	Resistant	
Penicillin	11 (100%)		13 (100%)		
Amoxicillin	11 (100%)		13 (100%)		
Erythromycin	7 (64%)	4 (36%)	6 (46%)	7 (54%)	0.4
Chloramphenicol	1 (9%)	10 (91%)	1 (7.7%)	12 (92.3%)	1.0
Tetracycline	5 (45.4%)	6 (54.5%)	9 (69.2%)	4 (30.8%)	0.4

7.4.2 Anibiotic susceptibility patterns among GBS, GCS and GGS isolates

Erythromycin resistance was observed with equal proportions among the three (50%) GCS and three (50%) GGS isolates. Chloramphenicol and tetracycline were both resistant in all (100%) GCS and GBS isolates. Among the GGS isolates chloramphenicol and tetracycline were both resistant in five (83%) of the patients respectively. The antimicrobial resistance patterns of the five antibiotics are shown in Table 7.2. The patterns differed among GBS, GCS and GGS isolates.

Table 7. 2 Antibiotic sensitivity and resistance in GBS, GCS and GGS pharyngotonsillitis

Antibiotics	GBS n=1(%)		GCS n=6 (%)		GGS n=6 (%)	
	Sensitive	Resistant	Sensitive	Resistant	Sensitive	Resistant
Penicillin	1(100%)		6 (100%)		6 (100%)	
Amoxicillin	1(100%)		6 (100%)		6 (100%)	
Erythromycin		1	3 (50%)	3 (50%)	3 (50%)	3 (50%)
Chloramphenicol		1 (100%)		6 (100%)	1 (17%)	5 (83%)
Tetracycline		1 (100%)		6 (100%)	1 (17%)	5(83%)

7.4.3 Antibiotic susceptibility patterns among GAS *emm* genotypes

The antimicrobial patterns of GAS varied within the six strains of *emm*12 genotype, of which three (50%) were erythromycin resistant, five (83%) chloramphenicol resistant and one(17%) tetracycline resistant. The three GAS *emm* 28 genotype expressed no sensitivity to either chloramphenicol or tetracycline and only one was erythromycin resistant. One GAS *emm* 5 and one *emm*87 were neither sensitive to chloramphenicol nor tetracycline.A statistically significant pattern of antimicrobial sensitivity and resistance pattern was observed with tetracycline within the different GAS *emm* genotypes (Table 7.3 $p<0.05$).

7.4.4 Antibiotic susceptibility patterns among SNA *emm* genotypes

One strain of GBS *emm* stG4974 genotype was resistant to both erythromycin and chloramphenicol (Table7.4).

Table 7. 3 Antibiogram tests in patients with GAS *emm* genotype and *sof* gene based on disc antibiotic sensitivity and resistance pattern

Antibiotic	*Emm* genotype	sof	Sensitive n (%)	Resistant n (%)	*P*
Penicillin	5	+	1 (100%)		
	12	+	6 (100%)		
	28	+	3 (100%)		
	87	+	1 (100%)		
Amoxicillin	5	+	1 (100%)		
	12	+	6 (100%)		
	28	+	3 (100%)		
	87	+	1 (100%)		
Erythromycin	5	+	1 (100%)		
	12	+	3 (50%)	3 (50%)	
	28	+	2 (67%)	1 (33%)	
	87	+	1 (100%)		
Chloramphenicol	5	+		1 (100%)	
	12	+	1 (17%)	5 (83%)	
	28	+		3 (100%)	
	87	+		1 (100%)	
Tetracycline*	5	+		1 (100%)	0.05
	12	+	5 (83%)	1 (17%)	
	28	+		3 (100%)	

*p=<0.05

Table 7. 4 Antibiogram tests in patients with GBS *emm* genotype and *sof* gene based on disc antibiotic sensitivity and resistance pattern

Antibiotic	Bacteria Group	*Emm* genotype	sof	Sensitive n (%)	Resistant n (%)
	GBS	stG4974	Negative		
Penicillin				1 (100%)	
Amoxicillin				1 (100%)	
Erythromycin					1 (100%)
Chloramphenicol					1 (100%)
Tetracycline				1 (100%)	

All the four strains of GCS *emm* genotype st2917 were chloramphenicol resistant, three (75%) tetracycline resistant and two (50%) were erythromycin resistant. Two strains GCS *emm* stCK249 were chloramphenicol resistant while one strain was erythromycin resistant (Table 7.5).

Table 7. 5 Antibiogram tests in patients with GCS *emm* genotype and *sof* based on disc antibiotic sensitivity and resistance patterns

Antibiotic	Bacteria Group	*Emm* genotype	sof	Sensitive n (%)	Resistance n (%)
Penicillin	GCS	st2917	–	4 (100%)	
		stCK249	–	2 (100%)	
Amoxicillin		st2917	–	4 (100%)	
		stCK249	–	2 (100%)	
Erythromycin		st2917	–	2 (50%)	2 (50%)
		stCK249	–	1(50%)	1(50%)
Chloramphenicol		st2917	–		4 (100%)
		stCK249	–		2 (100%)
Tetracycline		st2917	–	1 (25%)	3 (75%)
		stCK249	–	2 (100%)	

Two *emm GGS stG*7882.2 were sensitive to erythromycin. GGS *emm* st2917 and *emm stG*652.5 were resistant to erythromycin and chloramphenicol. One strain of GGS *emmst*7406 was resistant to both chloramphenciol and tetracycline (Table 7.6).

Table 7. 6 Antibiogram tests in patients with GGS *emm* genotype and *sof* based on disc antibiotic sensitivity and resistance pattern

Antibiotic	Bacteria Group	*Emm* genotype	sof	Sensitive n (%)	Resistance n (%)
Penicillin	GGS	*st*2917		1 (100%)	
		*stG*7882.2	–	2 (100%)	
		*st*7406	–	1 (100%)	
		*stG*652.5	–	1 (100%)	
Amoxicillin		*st*2917	–	1 (100%)	
		*stG*7882.2	–	2 (100%)	
		*st*7406	–	1 (100%)	
Erythromycin		*st*2917	–		1 (100%)
		stKNB7	–		1 (100%)
		*st*7406	–	1 (100%)	
		*stG*7882.2	–	2 (100%)	
		*stG*652.5	–		1 (100%)
Chloramphenicol		st2917	–		1(100%)
		stKNB7	–		1 (100%)
		*st*7406	–		1 (100%)
		*stG*7882.2	–	1 (50%)	1 (50%)
		*stG*652.5	–		1 (100%)
Tetracycline		*st*2917	–	1 (100%)	
		*st*7406	–		1 (100%)
		*stG*7882.2	–	2 (100%)	
		*stG*652.5	–	1 (100%)	

7.5 Discussion

7.5.1 Antibiotic sensitivity and resistance patterns among GAS isolates

All GAS strains were sensitive to the β-lactam antimicrobials, penicillin and amoxicillin and which is consistent with the report in different developing and developed countries (Adam, *et al* 2000, Brook and Dohar 2006, Danchin, *et al* 2007, Jain, *et al* 2008, Leung and Kellner 2004, Lloyd, *et al* 2007, Palavecino, *et al* 2001, Rimoin, *et al* 2005).

Table 7. 7GAS resistance pattern to erythromycin in different countries

Country	Erythromycin resistance to GAS	Reference
Germany	18.9% (11/59)	Arvand,*et al* 2000
Spain	19% (19/100)	Granizo,*et al* 2000
Belgium	6.5% (131/2014)	Descheemaeker,*et al* 2000
Italy	43.5% (40/92)	Savoia,*et al* 2000
Iran	0.2% (3/1335)	Jasir,*et al* 2000
France	9.6% (29/303)	Weber,*et al* 2001
Chile	5.4% (32/594)	Palvecino,*et al* 2001
Brazil	0.5% (1/211)	De Melo,*et al* 2003
Canada	14.4% (72/500)	Low,*et al* 2003
Hungary	3.7% (7/191)	Gattringer,*et al* 2004
Poland	12% (98/816)	Szczypa,*et al* 2004
Hawaii	2.9% (16/546)	Erdem,*et al* 2005
India	10.2 (5/49)	Lloyd,*et al* 2007
India	15.6 (5/32)	Jain,*et al* 2008
China	96.8% (182/188)	Liu,*et al* 2009
Yemen	36% (4/11)	Current study

Increased resistance to macrolides by GAS is directly related to the use of antibiotics in pharyngeal infections which is reported in several countries (Gerber 1996, Jensen, *et*

*al*2009, Seppala, *et al* 1997, Tarlow 1997, Weber, *et al* 2001, Yan, *et al* 2000). In this study, GAS was resistant to erythromycin in 36% (4/11) comparably higher to studies in India 10.2% (5/49) and 15.6% (5/32) (Jain, *et al* 2008, Lloyd, *et al* 2007), Iran 0.2% (3/1335) (Jasir, *et al* 2000), France 9.6% (29/303) (Weber, *et al* 2001), Germany 18.9% (11/59) (Arvand, *et al* 2000), Spain 19% (19/100) (Granizo, *et al* 2000), Austria 4.7% (7/150) and 3.7% (7/191), Hungary and Hawaii 2.9% (16/546) (Erdem, *et al* 2005). (Gattringer, *et al* 2004) but lower than that in Italy 43.5% (40/92) (Savoia, *et al* 2000) .

Tetracycline resistance among Yemeni GAS isolates was observed in 54.5% (5/11) which was similar to the GAS pattern in Brazil with 50% tetracycline resistance (De Melo, *et al* 2003). On the contrary it was higher than reports of tetracycline resistance in Iran 42.8% (214/500) and India 25% (8/32) (Jasir, *et al* 2000, Lloyd, *et al* 2007) but lower than that in China 96.6% (140/145) (Liang, *et al* 2008). A higher proportion of the GAS isolates 91% (10/11) were resistant to chloramphenicol in this study higher than the reports from Japan (22%) and India (4.3%) 2/32 (Lloyd, *et al* 2007, Nakae, *et al* 1977).

7.5.2 Antibiotic sensitivity and resistance patterns among SNA isolates

The antimicrobial agent of choice for Group C and Group G streptococci, is penicillin G with reports indicating no resistance to the β lactams (Zaoutis, *et al* 1999). All the thirteen SNA GBS, GCS and GGS strains were sensitive to penicillin and amoxicillin comparable to similar reports in different countries (Palavecino, *et al* 2001, Zaoutis, *et al* 2001).

GCS and GGS strains were each 50% (3/6) resistant to erythromycin which was comparably higher than a report from India ((37.5% (3/8) GCS and 46% (6/13) GGS)), Turkey ((1.5% (1/68) GCS and 16.2% (6/37) GGS)), China ((41.7% (5/12) GCS and 23.5% (23/34) GGS)) and Spain ((5.3% (1/19) GCS and 33.3% (6/18) GGS)) were erythromycin resistant (Di'az, *et al* 2008, Ergin, *et al* 2003, Lloyd, *et al* 2007, Wu, *et al* 1997). This high resistance pattern among GAS, GCS and GGS strains may be explained by the selection of antibiotics used frequently for throat infections and the

free availability of drugs and antibiotics over the counter in Yemen and other countries. All the (6/6) GCS throat isolates in this study did not demonstrate any sensitivity to both tetracycline and chloramphenicol whereas in India, GCS isolates were 62.5% (5/8) tetracycline resistant and 75% (6/8) chloramphenicol resistant (Lloyd, *et al* 2007) wheras in China GCS were all sensitive to tetracycline (12/12) and chloramphenicol sensitivity was detected in 58.3% (7/12) of isolates (Wu, *et al* 1997).

In this study GGS strains were 83% (5/6) resistant to tetracycline which is higher than GGS strains reported in India 23% (3/13), China 73.5% (25/34) and Italy (26.7%). GGS was chloramphenicol resistant in 83% (5/6) which is different from a report in India with all (13/13) GGS isolates chloramphenicol sensitive and in China 2.9% (1/34) chloramphenicol resistant (Lloyd, *et al* 2007, Rondini, *et al* 2001, Wu, *et al* 1997) .

7.5.3 Antibiotic susceptibility patterns among GAS strains positive for the *sof* gene

In this study macrolide resistance was widespread among *sof* gene positive GAS strains including the erythromycin resistant *emm*12 *sof* positive (50%) and *emm*28 *sof* positive (33%) genotypes (Beekmann, *et al* 2005, Sauermann, *et al* 2003, Yan, *et al* 2000). GAS *emm*5, *emm*87 were *sof* positive strains and did not show any erythromycin resistance pattern which is contrary to other reports (Creti, *et al* 2007, Gagliotti, *et al* 2006, Green, *et al* 2004). GAS *emm*5, 28 and 87 were all chloramphenicol and tetracycline resistant, while GAS *emm*12 was chloramphenicol resistant in 5/6 strains (87%) with one strain tetracycline resistant. There are no reports to compare these results in the literature.

GBS *emm stG*4974 genotype was resistant to chloramphenicol and tetracycline. The six GCS genotypes were chloramphenicol resistant and 50% of each genotype was erythromycin resistant. GGS were all chloramphenicol resistant except one emm *stG*7882.2 strain. Erythromycin resistance was detected in one GGS *stKNB*7and one *stG*652.5 strains (Table7.6). The antimicrobial patterns of the GBS, GCS and GGS *emm* genotypes described in this study have not been studied elsewhere and are reported for the first time.

Antibiotics are still seen as miracle drugs and patients expect a rapid cure. There is an increasing trend for antibiotic resistance in clinical practice which creates challenges for the general healthcare provider (Reynolds 2009, Schito 2003). The emergence of antibiotic resistance must be taken into consideration cautiously by clinicians to limit its spread though effective treatments of infections by narrow spectrum antibiotics with appropriate duration (Hawkey and Jones 2009, Jensen, *et al* 2009, Nash, *et al* 2002). It is important to use accurate diagnostic methods for the identification of the causative agent and to educate patients and doctors on use and misuse of antibiotics (Dicuonzo, *et al* 2002, Dryden, *et al* 2009). It is also necessary to continue research to detect any change in the susceptibility pattern of GAS isolates to antimicrobials and to assess the best-practice for antimicrobial regimens (Shet and Kaplan 2004).

7.6 Conclusions

GAS and SNA isolates from Yemen still remain susceptible to the β-lactam antimicrobials used for treating pharyngotonsillits. The level of resistance to erythromycin was moderately high. In GAS infections, a specific genetic background, the phenotype, and host-parasite interaction could favour acquisition of a particular antibiotic resistance. GAS infections may be treated with several therapeutic options but penicillin still remains the drug of choice and continued monitoring of susceptibility of this pathogen are critical for the future.

Chapter 8

8 Cytokines and chemokines in acute rheumatic fever and recurrent rheumatic fever

8.1 Introduction

ARF is a multisystem inflammatory disease of childhood which occurs as a delayed sequelae to GAS (Ralph, *et al* 2006, Wang and Yang 2009). ARF is mediated by an autoimmune response in 3–4% of susceptible and untreated children and adolescents (aged 5–18 years) with GAS infections (Guilherme, *et al* 2006). Arthritis is the earliest presentation of the disease in 60-80% of the patients . Carditis affects 30–45% of ARF patients and is the most serious manifestation of the disease, leading to valvular lesions mainly in the mitral and aortic valves (Fae, *et al* 2006).

The pathogenic mechanisms involved in the development of ARF and progression to RHD are still not fully understood (Karthikeyan and Mayosi 2009, Schafranski, *et al* 2008). The pathogenesis involves the triad of a genetically susceptible individual with a rheumatogenic GAS infection developing an abnormal host immune response(Bryant, *et al* 2009). It is believed that the molecular mimicry mechanism is responsible for the cross-reactions between GAS antigens, mainly M protein epitopes, and human heart tissue proteins leading to autoimmunity in patients with genetic susceptibility(Fae, *et al* 2006) . It is now clear that the disease is mediated by both humoral and cellular immune responses and that the cellular branch of the immune response is more involved in the development of RHD (Guilherme and Kalil 2007).

Cytokine analysis in various body fluids has become a major emerging field of research interest that has enhanced the knowledge of many immunologic and inflammatory disorders (Martins, *et al* 2004). Recently, some cytokines have been implicated as being key coordinators in the development or progression of ARF (Corwin 2000).

The purpose of this study was to analyze the concentrations of a wider range of cytokines and chemokines as potential markers in the immune activation of patients with ARF and RRF. Some of these cytokines and chemokines have not been previously studied in ARF. The results may help further understanding of the mechanism of autoimmunity in ARF and new modalities for treatment to reduce the development and progression of cardiac involvement.

8.2 Objective

To compare the concentrations of selected cytokines and chemokines in Yemeni children with ARF and RRF.

8.3 Literature review

8.3.1 Molecular pathogenesis of ARF and RHD

Molecular mimicry is proposed as a potential autoimmune mechanism that mediates the cross-reactions between GAS antigens and human proteins leading to ARF and RHD (Kaplan 2005, Ramasawmy, *et al* 2007, Veasy and Hill 1997). The presence of molecular mimicry between GAS antigens and human tissue proteins is mediated by the B and T cell responses of peripheral blood cells and cells infiltrating heart lesions (Guilherme and Kalil 2007). Cytokines are produced from these peripheral and heart-infiltrating mononuclear cells. It is suggested that T helper 1 (Th1)-type cytokines are the mediators of RHD heart lesions (Guilherme, *et al* 2005b).

8.3.2 Molecular mimcry and autoimmune reaction following GAS infections

Molecular mimicry is the sharing of epitopes between human and GAS antigens. Molecular mimicry for T cells is mediated by antigen presenting cells such as macrophages, dendritic cells and B lymphocytes. The HLA class II molcelues are expressed on the surface of these antigen presenting cells. Epitope spreading is the mechanism that mediates the recognition of self proteins by other cells where an inintial immune response produces autoantigen recognition that tends to become more diverse as the response persists (Guilherme et al 2009). The molecular basis of T-cell recognition is assessed through heart-cross-reactive antigens expressing some homology with GAS antigens, ie, the GAS antigen epitopes share structural similarity to self

epitopes. This activates the autoreactive T lymphocytes that were not exposed to the immune tolerance by the molecular mimicry which in turn can also activate B cell to produce antigen specific antibodies. The M protein which is the most virulent structure of GAS shares similar structure with α-helical coiled human proteins mainly cardiac myosin, laminin and vimentin. The crossreactive streptococcal epitope involves *N*-acetyl β-Dglucosamine, a polysaccharide present in both GAS cell wall and heart valvular tissue.

Human antibodies against the *N*-acetylβ-D-glucosamine show crossreactivity against the protein laminin that surrounds the heart and its valves . An epitope of the N-terminal M5 and M6 proteins with a five amino acid residue was demonstrated as cross-reactive with cardiac myosin by Cunningham (Cunningham 2000). These GAS and human protein cross-reactive antibodies also bind to the endothelial surface and upregulate the adhesion molecule VCAM-1 leading to inflammation, cellular infiltration and valve scarring (Roberts, *et al* 2001).This cross-reactive immune attack occurs as a result of the immune response polarisation towards either B cells/T cells and causes rheumatic carditis and Syndenham chorea. Since molecular mimicry between GAS and host antigens is present in almost all individuals, the question is why only 3% of patients with GAS infection develop ARF (Carapetis, *et al* 2005a, Guilherme, *et al* 2005b).

8.3.2.1 Humoral immune response

The humoral phase predominates during ARF whereas the cellular response is initated during the acute phase and leaves traces in the chronic phase (Guilherme, *et al* 2007, Olivier 2000). Cardiac myosin and vimentin are the major cross-reactive antigens among the human proteins (Ellis, *et al* 2005). Cunningham discovered that cross-reactive antibodies interact with N-acetyl glucosamine, a polysaccharide present in both GAS and heart valve tissues which results in cytotoxic activity against heart cells that bind to the endothelial surface and lead to inflammation, cellular infiltration and scarring of valves in RHD (Guilherme and Kalil 2007). An exaggerated antibody response to GAS carbohydrate was noted in ARF patients with increased titres in residual MV disease (Carapetis et al 2005). This supports the hypothesis that these antibodies cause valve damage.

8.3.2.2 Cellular immune response

A cellular immune response based on T cell reactivity has been described in ARF patients (Carrion, *et al* 2003, Hilario and Terreri 2002). CD4+ cells were found to be increased in tonsils and peripheral blood of ARF and RHD patients (Cunningham 2003, El-Demellawy, *et al* 1997). Carditis is initially mediated by a humoral immune response, but evidence suggests it is the T-cell-mediated response which leads to RHD (Figueroa, *et al* 2002). Studies on acute ARF/RHD patients demonstrated the presence of CD4+ T cells and macrophages in mitral valve heart lesions (Cunningham 2003, Guilherme, *et al* 2004, Guilherme and Kalil 2004). Zabriskie et al reported the presence of CD4+ and CD8+ T cells in RHD valves (Guilherme, *et al* 2004, Zabriskie 1995).

The Aschoff body, a granulomatous lesion contains T and B cells, mononuclear and multinucleated cells, macrophages and PML is the pathognomonic sign of acute RHD (Fraser, *et al* 1997). It is found in the myocardium and endocardium due to cellular infiltration that occurs through the endothelium (Necil Kütükçüler 2008). The protein M5 (81–103) region was described as one of the GAS trigger of autoimmune reactions in RHD (Cohen 2001). It is not clear whether the initial valvular insult is due to antibody or cell-mediated immunological damage, but subsequent damage seems to be caused by T-cell and macrophage infiltration (Carapetis, *et al* 2005a). This valvular disease is then responsible for cardiac morbidity and mortality of ARF (Lee, *et al* 2009, Roberts, *et al* 2001).

8.3.3 Epitope spreading

Epitope spreading is the recognition process of several self-antigens after an initial response against a pathogen antigen which then become the target of immune response (Cohen 2001, Kivity, *et al* 2009). GAS recognition leads to antibody formation and T-cell epitopes that are crossreactive with human proteins (Cunningham 2004). This crossreactivity might occur first through mimicry that leads to other human valvlular proteins recognition and eventually through an epitope spread (Hilario and Terreri 2002). The structural and immunological similarities between GAS M protein and human myosin, both α-helical coiled molecules seem essential to rheumatic carditis and probably contribute to the progression of tissue damage in ARF/RHD (Cunningham 2001, Ellis, *et al* 2005).

8.3.4 General review of cytokines and chemokines

Cytokines are soluble hormone-like proteins or glycoproteins that act in a network in combination with specific cytokine inhibitors and receptors to regulate the human immune response. They have functions in inflammation and hematopoiesis. Stanley Cohen first introduced the term ''cytokine'' (Cohen et al., 1974, 2004) and the term encompasses interleukins, chemokines, monokines, lymphokines, interferons, Colony Stimulating Factors and Tumour Necrosis Factor (Leonard, 1999). Cytokines are secreted by WBCs, fibroblasts and endothelial cells in response to inducing stimuli; but the predominant producers are Th-cells and macrophages (Vandana et al 2008(Tayal and Kalra 2008).

Basal and cell-stimulated cytokine levels vary between individuals due to both genetic and environmental influences (Smith and Humphries 2009) .The therapeutic use of cytokines is a rapidly progressing area of research. Purified recombinant cytokines and their antagonists are increasingly used in various inflammatory disorders (Dayer 2004, Feldmann 2008, Strom and Koulmanda 2008).

8.3.4.1 8.4.3.1 Classification of Cytokines, Chemokines and Growth Factors

8.3.4.2 Cytokine Families

8.3.4.2.1 Type I Cytokine Family

Type I cytokines and receptors share a similar structure of an extracellular region containing four α helices, a characteristic motif of type I receptors. Members include interleukin IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-12, G-CSF and GM-CSF (Kourilsky and Truffa-Bachi 2001). T cell growth factors include IL-2, IL-3, IL-4 and IL-7 (Rook 2001). Proinflammatory cytokines include IL-1, IL-2 and IL-6. Haematopoietic cytokines are G-CSF and GM-CSF. IL-2 is a Th1 cytokine but IL-4 and IL-5 are Th2 cytokines (Jankovic, *et al* 2001).

8.4.3.1.1.1 Interleukin -1 (IL-1) Family

IL-1 family is called the immunoglobulin superfamily that encodes three peptides, IL-1α, IL-1β, and IL-1 receptor antagonist (IL-1ra). IL-1R family includes four primary members: IL-1α, IL-1β, IL-18, and IL-1Ra (Dinarello 1994). IL-1Ra is the specific

receptor antagonist for IL-1α and IL-1β but not for IL-18. IL-1 ligands (IL-1α and IL-1β, referred as IL-1) induce genes associated with infections, autoimmune disease, solid tumors, asthma, leukemia, Alzheimer's disease and trauma (Smith and Humphries 2009). IL-1 is strongly implicated in rheumatoid arthritis causing cartilage degradation (Szekanecz, *et al* 2003). Il-1β may also be activated in patients with CCF. A biologically active product of IL-1ra, Anakinra I is used in rheumatoid arthritis that blocks IL-1 activity by competitively inhibiting IL-1 binding IL-1RI (Genovese, *et al* 2004)

8.4.3.1.1.2 Interleukin - 6 (IL-6)

IL 6 is a pleiotropic cytokine produced by macrophages and endothelial cells and is involved in B cell stimulation and antibody production (Dayer 2004). IL 6 is likely to be elevated in infective endocarditis because bacteria display surface molecules which stimulate the release of IL 6 from monocytes and endothelial cells (Watkin et al 2007). Increased levels of IL-6 were found in dilated cardiomyopathy, compared to controls (Tentolouris, *et al* 2004). IL-6 was identified in patients with congestive cardiac failure as the most powerful independent predictor of new heart failure episodes, death, or need for heart transplantation (Mocelin, *et al* 2005, Orús, *et al* 2000, Sharma, *et al* 2000).

8.4.3.1.1.3 Interleukin 12p70

Interleukin-12p70 (IL-12p70), the bioactive form of IL-12, is the principal immunoregulatory cytokine that govern Th1 immune response polarization. It is produced rapidly by the activated antigen-presenting cells in response to bacterial antigens and intracellular pathogens. Interleukin-12 p70 (IL-12p70) is a key cytokine produced by dendritic cells which is able to drive the development of Th1 lymphocytes (Dobreva, *et al* 2008). It induces the release of IFN-γ. IL-12p70 is reported to be highly correlated with IL-2 (Gu, *et al* 1997).

8.3.4.2.2 Type II Cytokine Family

Type II cytokines include IL-10, IL-19, IL-20, IL-22 and IFN (IFN-α, -β, -ε, -κ, -ω, -δ, -τ and -γ) (Spellberg, *et al* 2001). They induce cellular antiviral states, inhibit/stimulate cell growth and affect many immune mechanisms (Dumoutier, *et al* 2003, Hu 1998).

IL-10 is an anti-inflammatory cytokine. The IFN system is a major contributor to innate immunity with IFN-γ serving as a potent pro-inflammatory cytokine. The type II cytokine family is comprised of both Th1 and Th2 cytokines; IFN-γ is a Th1 cytokine while IL-10 is a Th2 cytokine (Jankovic, *et al* 2001, Pestka, *et al* 2004).

8.4.3.1.2.1 Interleukin -10 (IL-10)

IL-10 is an important immunoregulatory cytokine which controls Th1 and Th2 balance and is secreted by macrophages, Th2 cells and mast cells. It inhibits some cytokine synthesis such as IL-1, IL-2, IL-3, IL-6, IL-12, IL-18, GM-CSF, TNF-α and IFN-γ (Moore 2001). It stimulates NK cells and augments the ability of IL-18 to stimulate NK cells (Yadav and Sarvetnick 2003). Increased levels of IL-10 are associated with SLE, asthma and influence the inflammatory activation of monocytes and limits thrombotic complications (Patel, *et al* 2009). Patients treated with IL-10 showed some improvement in chronic hepatitis C, Crohn's disease and rheumatoid arthritis (Li, *et al* 2009, Zhou, *et al* 2009).

8.4.3.1.2.2 Interferon-gamma (IFN-γ)

Interferons (IFN) are natural glycoproteins induced in response to viral infections and involved in innate and adaptive immune responses (Baron, *et al* 1991). Type I IFN includes over 25 subtypes of interferon-α, β, omega and tau. Only one type II interferon exists, IFN-γ, distinct from IFN-α and IFN-β (Loza, *et al* 2002). Type I IFNs induce anti-proliferative and anti-viral activity whereas type II (IFN-γ) has weaker anti-viral activity but more potent immunomodulatory properties, and binds to different receptor than type I IFNs (Jonasch and Haluska 2001). IFN-γ is a pleiotropic cytokine, produced by effector T and NK cells and is vital for host defence. IFN-γ is the major proatherogenic Th1 cytokine which promotes macrophage and endothelial cell activation (Pestka 2007). It leads to expression of specific chemokines such as Mig/CXCL9 and IP-10/CXCL10 and their receptor CXCR3 in atheroma (Oliveira, *et al* 2009). IFNs approved for clinical use include: IFN-α-2a, 2b, pegylated IFN-α-2a, 2b, IFN-α-n3, IFN-α con-1, IFN-β-1a, IFN-β-1b and IFN-γ-1b (Hozumi, *et al* 2008, Tayal and Kalra 2008).

8.3.4.2.3 Tumor necrosis factor (TNF) Family

The TNF family is primarily involved in the regulation of cell proliferation and apoptosis with pro-inflammatroy properties (Yadav and Sarvetnick 2003). Members include TNF-α, TNF-β, Fas ligand (FasL), CD40 ligand (CD40L), and TNF-related apoptosis-inducing ligand (TRAIL) (Wong, *et al* 2008). TNF-α is an inflammatory Th1 cytokine released during infection which activates platelets, and has a role in the genesis of fever, anaemia and cachexia (O'Dell, 1999). Elevated TNF- α levels were first documented in congestive cardiac failure and hypertrophic cardiomyopathy (Oral, *et al* 2003, Torre, *et al* 1996). TNF-α has a central role in rheumatoid arthritis and this has led to the emergence of TNF blocking strategies. The first neutralizing monoclonal anti-TNF antibody, infliximab, now has an established role in treating rheumatoid disease (Heiko, *et al* 2009, Szekanecz, *et al* 2003, Wong, *et al* 2008).

8.3.4.2.4 Growth Factor Families

Growth factors have unique functions that include vascular endothelial growth factor (VEGF) and Platelet-derived growth factor (PDGF), potent mitogenic and angiogenic factors (Ferrara and Davis-Smyth 1997). Insulin-like growth factor (IGF)-I and IGF-II belong to the family of IGF that are structurally homologous to proinsulin. The Fibroblast growth factors (FGF)family is another group of cytokines involved in cell growth and differentiation (Jiang, *et al* 2000).

8.3.5 Structure and Classification of Chemokines and their Receptors

Chemokines include a group of secreted proteins within the cytokine family. They are chemoattractants for neutrophils, leukocytes and monocytes that direct white blood cell migration to site of injury/infection (Baggiolini, *et al* 1997, Gouwy, *et al* 2005). Chemokines are important regulators of the immune system through their actions on T and B cell development (Min, *et al* 2002) and are involved in angiogenesis and oncogenesis (Horuk 2009). Chemokine activity is mediated by binding to a family of seven transmembrane G-protein coupled receptors (GPCR) (Sallusto, *et al* 2000). Numerous disease states manifest particular patterns of chemokine expression (Deshmane, *et al* 2009, Luster 1998, Szekanecz, *et al* 2003). The coordinated release of

chemokines into tissues is the most likely mechanism for white cell infiltration seen in these diseases (Gouwy, *et al* 2005, Shin, *et al* 2002).

Chemokine receptors are classified into four families based on number and position of N-terminal-conserved cysteine residues within the receptor binding domain (Esche, *et al* 2005, Sallusto, *et al* 2000). Receptors with two cysteine residues separated by one amino acid are categorized as α-chemokine receptors (CXCR2 and CXCR4); whereas those with two cysteines next to each other are β-chemokine receptors (CCR5, CCR4, CCR3 and CCR2) (Campbell, *et al* 2001, Luster 1998, Moser and Willimann 2004).

8.3.5.1 CC Chemokines

CC chemokines are the largest group of chemokines named *beta/* CC, that target primarlily mononuclear cells and serve in homeostasis and inflammation (Zlotnik and Yoshie 2000). They are divided into allergenic, pro-inflammatory, developmental and homeostatic groups. Inflammatory chemokine are inducible and can amplify inflammation through the recruitment of effector cells (Szekanecz, *et al* 2003).

8.3.5.1.1 CCL2 ((Monocyte chemoattractant protein-1 (MCP-1))

MCP-1, a CC chemokine is potent for monocytes in inflammation sites with an effect on neutrophils similar to IL-8 (Shin, *et al* 2002). It is an important component of a hierarchical, differential pattern of chemokine expression that results in monocyte extravasations through vascular endothelium (Mukaida, *et al* 1998). It has been documented in atherosclerotic lesions. MCP-1 release is stimulated by IL-1, IL-6 and IL-8(Deshmane, *et al* 2009) .

8.3.5.1.2 CCL3 (Macrophage inflammatory protein -1 α (MIP-1 α))

MIP-1 α and MIP-1β are highly related members of the CC chemokine subfamily. Only low levels of MIP-1 mRNA were detected in resting monocytes and macrophages. Significant secretion was observed on stimulation of human blood monocytes with IL-1β and IFN-γ (Rot and Von-Andrian 2004). MIP-1α was detected during inflammation, malaria, tuberculosis and *H. pylori*-associated gastritis (Djoba Siawaya, *et al* 2009, Kutscher, *et al* 2008). HIV-1infected macrophages and T cells have been shown to secrete MIP-1α (Carrol, *et al* 2007, Paximadis, *et al* 2009). A weak inhibition of MIP-1α secretion by T lymphocytes was detected after addition of IL-4, IL-10, IL-18 or IFN-

γ (Menten, *et al* 2002) . IL-10 suppressed the release of MIP-1α. Eosinophils secrete MIP-1α in RSV infections. Pulmonary vascular smooth muscle cells, stimulated with IL-4, IFN-γ, IL-10, IL-1β or TNF-α were also shown to release MIP-1α. Furthermore, bone marrow CD34+ cells and epithelial cells were reported to secrete MIP-1α. (Van Damme 2002).

8.3.5.1.3 CCL4 ((Macrophage inflammatory protein -1 β (MIP-1β))

MIP-1β was effective in augmenting adhesion of T lymphocytes to VCAM-1. High amounts of MIP-1β are produced by monocytes when stimulated with LPS or IL-7. MIP-1β production is counteracted by addition of IL-4 (Menten, *et al* 2002) . IFN-γ up regulates MIP-1β induction, whereas IL-10 down regulates it. Pulmonary vascular smooth muscle cells secreted MIP-1β after addition of IL-1β, TNF, IL-4, IFN-γ and IL-10. It participates in recruitment of monocytes and T cells into the synovium in rheumatoid arthritis (McInnes and Schett 2007, Menten, *et al* 2002). MIP-1α and MIP-1β have been implicated in the pathology of rheumatoid arthritis, glomerulonephritis, pneumococcal meningitis, human sepsis and atherosclerotic lesions (Carrol 2005, Carrol, *et al* 2007, De Jager, *et al* 2007, McInnes and Schett 2007).

8.3.5.1.4 CCL5 ((Regulated upon activation, normal T cell expressed and secreted (RANTES))

RANTES is a chemotactic factor with selective chemoattractant properties on monocytes, macrophages and eosinophils. RANTES causes selective migration of blood monocytes and T lymphocytes expressing the cell surface antigens CD4 involved in memory T cell function (Schall, *et al* 1990, Song, *et al* 2003). Th1 cells preferentially secrete CC-chemokines with selective properties as MIP-1$\alpha$and RANTES as well as MIP-1$\beta$and lymphotactin, but Th2 cells express high levels of MCP-1. INF-γ in synergy with TNF-α produces RANTES from endothelial cells or airway smooth muscle cells, inhibited by Th2 cell (Rossi and Zlotnik 2000). RANTES was also found in higher levels in survivors with HIV infection than in non-survivors suggesting an impaired immune respone (Carrol, *et al* 2007). In rheumatoid arthritis, synovial fibroblasts produce RANTES/CCL5 mRNA upon stimulation with TNF-α or IL-1β. It was shown to serve as a useful marker for monitoring disease activity in rheumatoid arthritis (Yang, *et al* 2009).

8.3.5.2 CXC Chemokines

The second largest group of chemokines named *alpha* or CXC group with a variable amino acid that lays between two N-terminal cysteines, hence CXC. CXC chemokines are divided into two subgroups based upon the presence/absence of a specific 3 amino acid sequence, the Glu-Leu-Arg residues found adjacent to CXC and constitute the ELR motif. ELR(+) chemokines induces chemotaxis and promote angiogenesis. Chemokines in this group include CXCL1, CXCL2, CXCL3, CXCL5, CXCL6, CXCL7, CXCL8, and CXCL15 and interact with neutrophils via CXCR1 and CXCR2 receptors (Strieter, *et al* 2007). ELR(−) chemokines attract lymphocytes and monocyte with little affinity for neutrophils and include CXCL4, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14 and CXCL16. They have angiostatic properties and induce chemotaxis.

8.3.5.2.1 CXCL8 ((Interleukin-8 (IL-8))

IL-8 is an ELR protypic CXC chemokine secreted by human endothelial cells in hypox(ia and infection (Ley, *et al* 2007). It acts as a potent neutrophil chemoattractant, and is secreted by infected airway epithelial cells. CXCL8 mRNA was detected in human atherosclerotic lesions (Charo and Taubman 2004). TNF-α and IL-1β are potent stimuli of endothelial IL-8 secretion. Histamine triggers production of IL-8 enhanced by stimulation with TNF- α (Smith and Humphries 2009).

8.3.5.2.2 CXCL10 ((Interferon-inducible protein-10 (IP-10))

IP-10 is a highly inducible, primary response protein which belongs to the CXC chemokine superfamily (Gouwy, *et al* 2005, Neville, *et al* 1997). IFN-γ in human cells on stimulation was able to induce a transcript encoding a protein named gIP-10 with sequence homology to platelet factor 4 (PF4). IP-10 is capable of stimulating monocytes, NK and T-cell migration, regulation of T-cells, bone marrow progenitor maturation and inhibition of angiogenesis (Campbell, *et al* 2001). More interest is now likely to be focused on IP-10 due to recent cloning of an IP-10 receptor (White, *et al* 2005).

8.3.5.3 XCL1

This is the *gamma* or XC chemokine group with a single cysteine molecule present at the N-terminus. XCL1 (lymphotactin) is the only representative of the C family and targets CD4 + and CD8 + lymphocytes, but does not act on monocytes, and binds a unique receptor, XCR1 (David and Mortari 2000). It has some homology to ligands CCL3 and CCL8, XCL1 but lacks the first and third cysteines characteristic of CC and CXC chemokines (Greaves, *et al* 2001).

8.3.5.4 CX3CL1

This is the *delta* or CX3C group with 3 intervening residues between the first two cysteines. CX3CL1 (Fractalkine, FKN) is the lone member of the CX3C subfamily (Cotter, *et al* 2002). FKN, the ligand for CX3CR1, is found in liver, intestine, kidney, and brain. FKN targets monocytes and T cells (Cook, *et al* 2001)

8.3.6 Chemokine receptors

Chemokine receptor is based on the chemokine group (CC, CXC, C or CX3C) to which its ligand(s) belong and are mainly found on WBCs (Koenen and Von-Hundelshausen 2008). Chemokine and their receptors function in haematopoiesis, cell differentiation, viral pathogenesis and immunity (Esche, *et al* 2005).

8.3.7 Anticytokines as therapeutic agents

Anticytokine therapy has and will be successful when disease causing mechanisms are relatively "simple" and cytokine driven. Future direction in complex disorders may be the simultaneous inactivation/activation of multiple cytokines. Cytokine gene therapy for cancer treatment is the latest development in the field of cytokines (Podhajcer et al., 2007). Cytokine-based drugs and anti-cytokines play a crucial role in the understanding of pathogenesis and the management of several diseases. Research is continuing to develop new therapies, refine those already in use and establish the safest and most effective dosage levels.

8.3.8 Cytokines and Chemokines in ARF/RHD

Cytokines can be strong stimuli for influencing cytokine patterns, as quantitative differences in their expression leads to ARF (Dayer 2004, Necil Kütükçüler 2008). In a susceptible host the pathogenic response in ARF includes the production of

crossreactive antibodies and microbial epitopes recognized by MHC proteins that present these epitopes to inflammatory (Th1, CD4+) and cytotoxic (CD8+) T cell subsets (Cunningham 2004). CD4+ T cells (Th1) produce tissue inflammation and scarring while cytotoxic CD8+ T cell subsets cause destruction of target tissues (Shevach 2000). CD4+ Th1 lymphocytes produce IFN-γ that may influence the CD8+ T cells to become more effective cytotoxic lymphocytes (Cunningham 2003, Pestka 2007). Cross-reactive antibodies and influential cytokines also affect the endothelium and VCAM-1 expression, a sign of inflammation and cellular infiltration (Springer1994).

Thus lesions within ARF valves contain both CD4p and CD8p lymphocytes (Roberts, *et al* 2001) .CD4þ lymphocytes have the subsets Th1and Th2 which secrete a known lymphokine profile. Th1 cells secrete IL-2, IFN-γ, TNF-α and IL12. Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and IL-13. CD8þ T cells have subsets which exhibit Th1-like (Tc1) and Th2-like (Tc2) profile (McInnes and Schett 2007, Rochman, *et al* 2009). Arthritis, chorea and RHD are partly due to a reaction probably mediated by Th2-type cytokines, leading to an exaggerated humoral response (Guilherme, *et al* 2007). Increased levels of CD4+ and CD25+ cells are due to activated T cells in peripheral blood during ARF (Cunningham 2003).

8.3.8.1 Cytokines in the peripheral blood

The kinetics of cytokines shows an imbalance in the production of cytokines due to abnormality in the T-cell response in ARF. Antigen-activated $CD4^+$ T cells polarize to T-helper (Th1 or Th2) based on the pattern of cytokines they secrete .Th1 cells are involved in the cellular immune response and produce TNF-α, IFN-γ, IL-2, IL-12. Th2 cells mediate humoral immune responses and produce IL-4, IL-5,IL-10 and IL-13 (Guilherme, *et al* 2011). The evidence for an increased cellular immune response in ARF is the increased percentages and absolute counts of $CD4^+$ and $CD25^+$ cells, the CD4/CD8 ratio, and the increased plasma concentration of cytokines and chemokines .

TNF-α is the initiator of inflammatory response in ARF and usually produced earlier than all other cytokines during the acute phase of inflammation. TNF-α is produced prior to IL-β that is produced earlier than IL-10, whereas IL-6 is produced later and followed by IL-12 (Murthy, *et al* 2000). TNF-α is also associated with the severity of

ARF and leads to IL-6 and IL-8 production (Kütükçüler, *et al* 2008). Moreover, IL-6 and TNF-α are considered to be inducers of acute phase of ARF and are strongly correlated with ESR and C-reactive protein (Yegin, *et al* 1997).IL-8 is increased in the active phase of ARF and RHD patients with CCF; it is involved in the activation of chemotaxis and accumulation of WBC at the inflammation site (Kutukculer and Narin 1995). IL-1 and IL-2 production may persist in ARF patients for 48 weeks. Production of IL-1, IL-2, TNF-α, IFN-γ by monocytes /macrophages and T cells correlates with the Aschoff nodule progression in the heart lesions and this is a major mechanism in mounting an immune response in ARF/ARHD (Guilherme and Kalil 2010).

Thus GAS antigens are able to stimulate peripheral mononuclear cells to produce increased amounts of proinflammatory cytokines and inflammatory cytokines in patients with ARF or RRF(Guilherme and Kalil 2010) . The release of cytokines depends on the function of monocytes/macrophages and T cells which are usually functional in a highly active state during the acute period of the disease . Further studies are needed to clarify the kinetics of cytokines in ARF mainly during the early days of the active period and data is required on the the differences in cytokine levels between the time of onset of symptoms and hospital admission.

Increased levels of TNF-α, IL-1, IL-2, IL-6 and IL-8 in peripheral blood/plasma were reported in ARF and active CRHD patients (Morris, *et al* 1993, Narin, *et al* 1995). IL-1α and IL-1β were increased in ARF but with no statistical significance. TNF-α was reported to be the major cytokine in ARF leading to IL-6 and IL-8 production (Miller, *et al* 1989, Yegin, *et al* 1997). Oral reported patients with chronic MR to have high production of TNF-α in the plasma (Oral, *et al* 2003). Increased IL-8 plasma levels by

cellular infiltrates were reported in ARF and decreased levels during remission (Kutukculer and Narin 1995). Miller reported that blood mononuclear cell cultures from ARF produced more TNF-α and IL-2 than controls (Miller, *et al* 1989). IL-7 concentrations remained unchanged during the acute and chronic stages of ARF (Kutukculer and Narin 1995). In ARF/ RHD patients, increase in TNF-α, IL-6 and IL-8 concentrations were correlated with CRP and ESR levels (Miller, *et al* 1989, Samsonov, *et al* 1995, Yegin, *et al* 1997). Increased plasma concentrations of IL-1, IL-2 and TNF-α

with alterations in lymphocyte subsets have been reported as evidence of increased cellular immune response in ARF(Guilherme and Kalil 2010).

8.3.8.2 Cytokines in the heart valve lesions and myocardium

In ARF and CRHD patients, mononuclear cells from heart lesions predominantly secrete IFN-γ and TNF-α (Guilherme and Kalil 2010). Cytokine profile studies demonstrated TNF-α, IL-1 and IL-2 secretion by macrophages in Aschoff's bodies (Fraser, *et al* 1995). IFN-γ secreted by Th1cells also mediate the granuloma formation in Aschoff nodules in ARF valves (Guilherme, *et al* 2006). Cells derived from myocardium produced IL-4 and IL-10 that play a protective role during regression of inflammation (Fraser, *et al* 1997). Cells derived from the valves produced small amounts of IL-10 and did not secrete IL-4 which may contribute to permanent valve damage . Oral *et al* showed increased expression of TNF-α in the myocardium in CRHD (Oral, *et al* 2003). IL-1 and TNF-α stimulate the production of IL-8 by tissue macrophages and synovial cells (Narin, *et al* 1995). The predominance of inflammatory cytokines in the heart lesions confirms that RHD is mediated by inflammatory immune responses .

Mononuclear cell in the tonsils secreted small amounts TNF-α, IL-1 and IL-2(Miller, *et al* 1989). Increased IL-8 levels by cellular infiltrates were reported in ARF joints and decreased levels during remission (Kutukculer and Narin 1995). Antigen-specific crossreactive infiltrating T cell clones displayed the same cytokine pattern, as observed *in situ*, thus reinforcing the proinflammatory cytokines' role in the progression of RHD (Guilherme, *et al* 2006).

8.3.9 Genetic influence of Cytokines and Chemokines

Recently cytokine and chemokine genes have been studied to try and understand the ARF/RHD pathogenesis. IL-1 is one of these gene cluster located on chromosome 2 (Smith and Humphries 2009). It includes the genes expressing IL-1α and IL-1β and their inhibitor IL-1RA. A study from Taiwan reported variations in IL-1β and IL-1RA not associated with RHD but future studies are required before excluding IL-1 as a susceptible or protective factor (Chou, *et al* 2005). A molecular study of TNF-α documented its association with a gene having inflammatory function located in the

MHC class II regions (Ramasawmy, *et al* 2007). TNF increases the production of proinflammatory cytokines which activates the transcription of proinflammatory genes (Hernández-Pacheco, *et al* 2003).

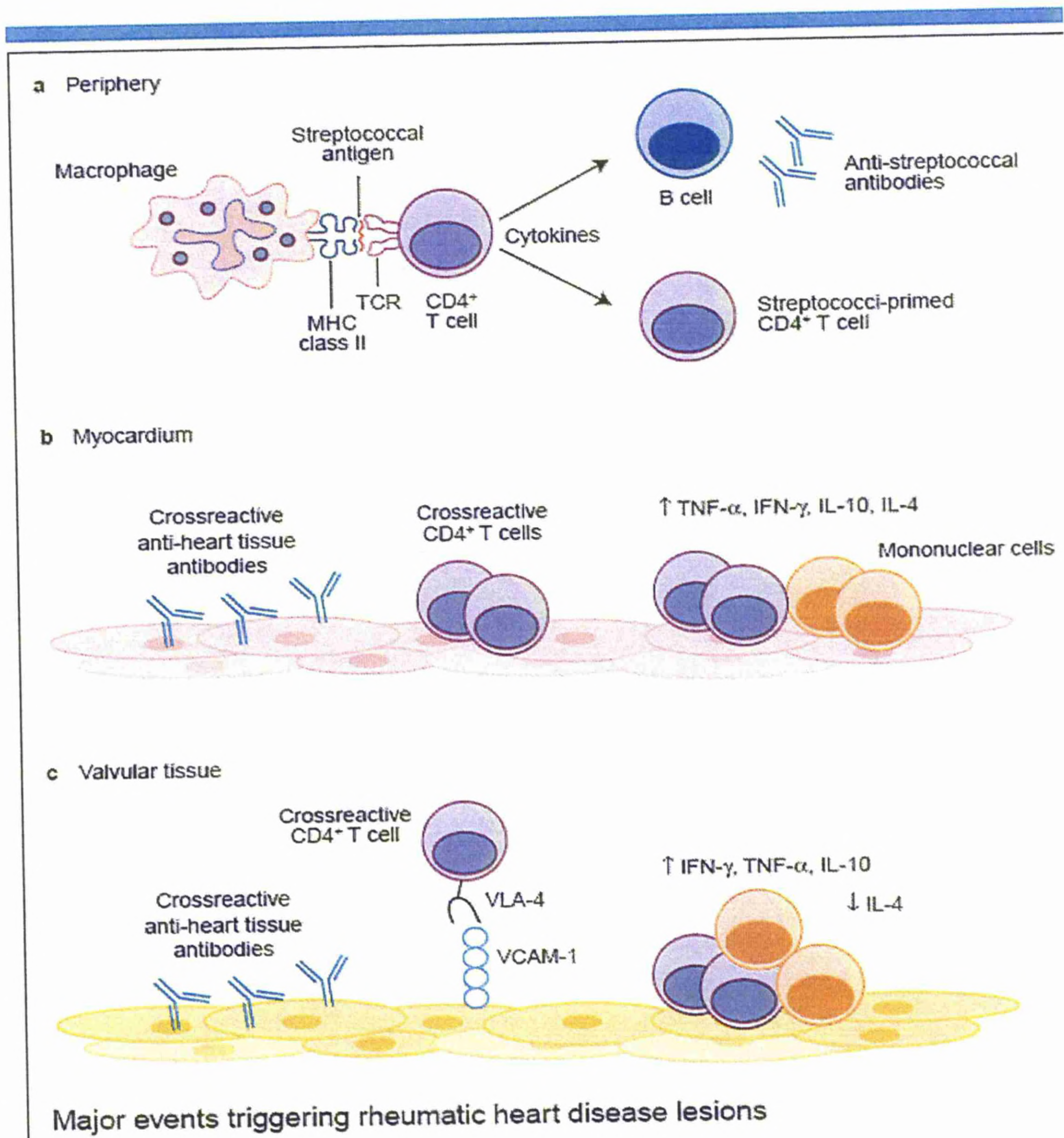


Figure 8. 1Cytokine and chemokine expression in RHD lesions(Guilherme, *et al* 2005a)

A key role for TNF- α in the immunopathogenesis of ARF/RHD has been proposed (Guilherme, *et al* 2007). In a Brazilian study either one of two TNF-α alleles 308A or 238A was associated with ARF/RHD (Ramasawmy, *et al* 2007). In Mexico and Turkey the TNF-α allele 308 was associated with ARF/RHD (Hernández-Pacheco, *et al* 2003, Sallakci, *et al* 2005). Another Turkish study did not confirm the presence of any of the TNF-α allele in ARF/RHD(Berdeli, *et al* 2006). It is probable that one TNF-α allele variant is capable of acting in synergy with other genetic and environmental factors in ARF/RHD (Guilherme and Kalil 2007). Cytokine gene mutations can lead to a degree of difference in their expression and cause further damage (Sallusto and Baggiolini 2008).

8.3.10 Role of Genetics in ARF and RHD

The determination of a genetic pattern of susceptibility to ARF and RHD has been sought for more than a century and has often been debated (Wilson and Morton 1937). A familial incidence and a single recessive gene suggested that genetic factors play a role in ARF susceptibility (Wilson, *et al* 1943). Some have assumed an autosomal recessive model and a Mendelian inheritance (Davis 1970, Winter 1972).

Recent studies have uncovered specific markers such as B-cell alloantigen D8/17 (Kaur, *et al* 1998, Rodriguez, *et al* 1990). Subsequent studies of HLA class II antigens have disclosed an association with different HLA-DR alleles according to the population analyzed (Ayoub, *et al* 1986, Guilherme, *et al* 2005a).

Anastasiou et al (1986) in Utah reported a negative association between RHD and DR6 (Anastasiou-Nana, *et al* 1986). Maharaj et al (1987) found a high frequency of DR6 in South Africans (60%) with MVD (Maharaj, *et al* 1987). Kayanagi et al (1996) reported a high frequency of DRB1*1405 –DQA1*0104 – DQB1*0503 in Japanese RHD patients with MV lesions. Kaur et al (1998) suggested ethnic differences in susceptibility marker(s) in ARF/RHD patients using different monoclonal antibodies (Kaur, *et al* 1998). Guedez et al (1999) found the HLA class II associations with RHD were more evident and consistent among patients with similar clinical findings (Guedez, *et al* 1999). HLA A10, DRw11haplotypes and DRB1*0701 allele in RHD patients have been detected (Olmez, *et al* 1993). Carreno et al (2000) documented an increased frequency of HLA-DR4 and HLA-DR2 with RHD white and black patients,

respectively (Carreno-Manjarrez, *et al* 2000). Others have implicated DR1 and DRw6. DR7 and Dw53and DR4 in different populations (Bhat, *et al* 1997, Guilherme and Kalil 2004, Guilherme, *et al* 1991, Rajapakse, *et al* 1987, Visentainer, *et al* 2000). Certain class II MHC molecules and/or the D8/17 alloantigens were reported with an increased risk of ARF (Cunningham 2004, Guilherme, *et al* 2004, Hernandez-Pacheco, *et al* 2003).

These conflicting results have raised speculations between different haplotypes of class II HLA among ARF and RHD and that the observed associations might be because there are class II genes close to an ARF-susceptibility gene (Carreno-Manjarrez, *et al* 2000) (Table 8.1).

Table 8. 1HLA markers of ARF and RHD in different countries

Author (year of study)	Country	HLA markers
Cheadle and Goodhart (1889)	UK	First familial predispostion of ARF
Wilson (1943)	New York	Autosomal recessive trait
Taranta (1959)	Australia	Low concordance in monozygotic twins
Patarryo (1979)	New York	Novel B-cell alloantigen marker 883
Zabriskie (1985)	South Africa	Monoclonal antibodies
Ayoub (1986)	Florida	HLA-DR2 and HLA-DR4
Monplaisir (1986)	Martinique	HLA-DR1
Maharaj (1987)	South Africa	DR1 and DRW6 markers of RHD
Rajapakse (1987)	Saudi Arabia	HLA-DR4
Khanna et al (1989)	West Indies	Third monoclonal antibody D8/17
Arnold (1989)	Australia	HLA-DR4 and HLA-DR2
Guilherme (1991)	Brazil	HLA DR7 and DW 53
Ozkan (1993)	Turkey	HLA-DR3 and HLA-DR5
Koyanagi (1996)	Japan	DRB1*1405-DQA1*0104-DQB1*0503
Goldberg (1997)	India	HLA-Dr4 and HLA-DR7
Gue´dez (1999)	Egypt	DRB1*0701, DR6 and DQB1*0201
Donadi (2000)	Brazil	HLA-B49 and HLA-DR1
Carreno (2000)	Unites States	HLA-DR4 and HLA-DR2
Harel (2002)	Israel	B-cell alloantigen D8/17
Stanvechia (2003)	Latvia	DRB1*07-DQB1*0401-2 and DRB1*07-DQB1*0302
Hernandez-Pacheco (2003)	Mexico	HLA-DR16 (DRB1*1602) allele and DR16-DQA1*0501-DQB1*0301
Sadia (2007)	Pakistan	HLA-DRB1*07
Chou (2008)	China	DR11-05-DQ7

Factor H (FH) and factor H-like protein 1 (FHL-1) regulate complement activation via the alternative pathway (AP). AP attacks any surface not specifically protected against

it. Host cells need down regulation on their surfaces by plasma protein complement factor H (CFH) that provides a first line defence against invading GAS. GAS protect themselves from AP attack by obtaining host CFH on their surfaces via domains 6–7 and this permits domains 1–4 to regulate C activation on GAS (Kotarsky, *et al* 2001). M protein binds to FH and FHL-1 that potentially minimize complement C3 derived opsonin deposition on their surface contributing to GAS phagocytosis resistance. Several GAS strains bind host CFH via its domain 7 and avoid complement attack and C3b-mediated opsonophagocytosis (Haapasalo, *et al* 2008).

It has been documented that CFH binding by GAS is essential for bacterial survival in human blood. A recombinant fragment of CFH lacking regulatory functions can be used to inhibit CFH binding on the GAS surface and impair its blood survival. One CFH variant 402H was found to impair CFH binding to.GAS and was associated with decreased susceptibility to recurrent tonsillitis (Haapasalo, *et al* 2012). CFH binding was shown to be important for GAS survival in vivo by studying the association between the polymorphism Y402H of the CFH domain 7 and clinical GAS infections. It was reported that host CFH was important for immune evasion of GAS in blood. It could also be blocked by using CFH5–7 that causes impaired GAS survival. It was reported that impaired GAS–CFH interaction mediating allele 402H (1277C) may be associated with protection from human recurrent tonsillitis (Haapasalo, *et al* 2012).

Mannan-binding lectin (MBL) is a collectin, an acute phase inflammatory protein that binds to *N*-acetylglucosamine, a target molecule present on the GAS cell wall and human heart valves. MBL functions as a soluble pathogen recognition receptor in innate immunity due to its ability to opsonize pathogens. MBL then activates the complement system through the lectin pathway leading to production of C3 convertase (C4b2b) and the membrane-attack complex releasing many opsonic and inflammatory fragments causing phagocytosis and several immune-mediated reactions.

MBL is encoded by the gene MBL2 located on the chromosome 10q11.1-q21 region. Mutations in exon 1 of the MBL2 gene correlate with deficient MBL in the plasma and have been shown to be associated with recurrent infections in children (Schafranski, *et al* 2008).

It has been reported that high levels of MBL and *MBL2* associated genotypes were detected in patients with CRHD. The association between *MBL2* polymorphisms, *YA/YA* and *YA/XA* genotypes in ARF was reported with acute carditis associated with the high production of MBL (Messias Reason, *et al* 2006).Although *MBL2* genotypes related to low production of MBL were commonly seen in patients with rheumatic arthritis.

Increased serum levels of immune complexes and complement activation products (C3d) were seen in patients with ARF and CRHD, suggesting that the complement system might be involved in their pathogenesis (Schafranski, *et al* 2008) (Gibofsky and Zabriskie, 2001). (Ramasawmy, *et al* 2008). Future studies on immune regulation will contribute to the understanding of new genetic markers and how these genes act in the complex network of autoimmune reactions that occur in ARF/RHD(Guilherme, *et al* 2011).

In summary ARF appears to be a post-infectious autoimmune disease triggered by specific GAS characteristics and some undefined genetic human predisposition. RHD is considered to be an organ-specific T1-type autoimmune disease . Molecular mimicry between GAS antigens and heart proteins is considered as the mechanism leading to ARF/RHD (Cunningham 2004, Stollerman 1991). Intralesional CD4qT cells may cross react with GAS M proteins and heart proteins and play a direct role in the development of ARF (Guilherme and Kalil 2004). A greater understanding of the pattern of cytokines and chemokines in acute and recurrent rheumatic fever may help to shed some more light on the pathogenic process.

8.4 Objective: 6

Compare the concentrations of selected cytokines and chemokines in Yemeni children with ARF and RRF.

8.5 Patients and methods

Fourteen serum cytokine and chemokine concentrations determined in Yemeni children with ARF and RRF are described in this chapter. These include IL-1β, IL-6, -7, IL-8, IL-9, IL-10, IL-12p70, TNF-α, IFN-γ, MCP-1, MIP-1α, MIP-1β, IP-10 and RANTES.

These were analyzed by the BD FACS Array Bioanalyzer and using FCAP Array Software.

Review of the literature on different studies which investigated the levels of cytokines and chemokines in healthy controls was done. The values for the detection levels of the studied cytokines and chemokines in healthy controls are summarized in Table 8.2. The interpretation of the results in this study was based on these detection levels.

8.5.1 Data analysis

Data analysis was conducted using SPSS version 17 with descriptive statistics using median and inter-quartile ranges. The Mann-Whitney test was used to compare medians between ARF and RRF groups. Medians and interquartile ranges were used to compare the levels of cytokines and chemokines between the two groups. Spearman correlation coefficient was conducted on the study of correlations between the different cytokines and chemokines and the level of significance was $p = \leq 0.05$.

The multiple comparisons were not corrected for in the statistical analysis using the Bonferroni correction since statistical advice cautioned on its relevance for this type of analysis. The Bonferroni correction is a conservative correction leading to *P* values that may be too high and confidence intervals that are too wide (Thomas 1998). The correction thus may lead to insufficient power to detect significant differences and may miss the real differences. It is currently not recommended to apply the correction, even if a large number of tests are performed, since few, if any tests, will be significant after the correction has been applied (McHugh 2011)

Table 8. 2 Lowest detection levels of cytokines and chemokines in healthy controls

Cytokine/chemokine	Lowest detection level in healthy controls pg/ml	Reference
IL-1β	1.2 – 15	Gu et al 2009 Orus et al 2000
IL-6	3 – 6	Orus et al 2000
IL-7	8.6-29.3	Darcissa et al 2001 Kutukculer 1995
IL-8	1.9- 21.7	Arican et al 2005 Yegin et al 1997
IL-9	7.8- 8.9	Gu et al 2009
IL-10	0.3 – 5	Shimada et al 2001
IL-12p70	2.9	Arican et al 2005
TNF-α	7.5-20	Gu et al 2009 Yegin et al 1997
IFN-γ	15	Fujii et al 2004
MCP-1	20	Gu et al 2009
MIP-1α	9-25	Breemen et al 2007
MIP-1β	17	Breemen 2007
IP-10	0 - 41.5	Shimada et al 2001 Fujii et al 2004
RANTES	6.3 – 26	Bai et al 2005. Gu et al 2009

8.6 Results

8.6.1 Cytokine and chemokine concentrations

Although hypothetically 96 well plates could test 40 in duplicate and we had 2 plates equal to 80, we could only test 49 samples due to the need to retest some specimens and technical problems with the machine. Forty nine patients, 24 with ARF and 25 with RRF were included in this analysis. One serum sample of an ARF patient was discarded due to an interpretation error. Patients with ARF had a mean (SD) age of 12.3 (2.3) years and 11 were male while patients with RRF had a mean (SD) age of 14.8 (1.5) years and 9 were male.

All patients fulfilled the Duckett Jones' criteria with one major manifestation or arthritis or carditis and two minor manifestations (Ferrieri 2002). The major manifestations included carditis in 29 (59%) and polyarthritis in 21 (41%) patients. None of the patients had congestive cardiac failure. There were no patients with chorea, erythema marginatum or subcutaneous nodules. Patients with RRF were echo-proven to have RHD and had established valvulopathies. The mitral valve lesions were MR diagnosed in 18, MR with AR in four and MR with MVP in six patients. ASO titres were > 200 Todd units, CRP was positive and ESR was raised in all patients. A positive family history was obtained in 19 patients and 18% reported a family history of ARF with echo-proven RHD in 10 (20%) family members.

8.6.2 Cytokines and chemokines in children with ARF and RRF

The cytokine and chemokine levels are in Table 8.3 and Figure 8.2. All the cytokines and chemokines concentrations were elevated above healthy control levels as determined by the levels in different studies shown in Table 8.2.

Overall, children with RRF had higher median concentrations of cytokines and chemokines than children with ARF. Chemokines, IL-1β, IL-8 and RANTES were only significantly higher among RRF when compared to ARF patients.

IL-8 had higher median concentrations in patients with RRF (383.6pg/ml) than patiens with ARF (224.5pg/ml) ($p = 0.04$). IP-10 concentrations were high in both RRF and

ARF patients with medians of 167.8pg/ml and 162.4pg/ml, respectively (p=0.4). Patients with RRF had higher concentrations of IL-12p70 and IFN-γ (65pg/ml versus 67.1pg/ml) than children with ARF (55pg/ml versus 56.5pg/ml) respectively (p=0.001) (Table 8.2).

Similarlily median IL-6 and IL-10 concentrations were higher in RRF than ARF patients (106.5 versus 90.2pg/ml and 89pg/ml versus 73pg/ml) respectively, (p=0.001). IL-1β, IFN-γ and TNF-α values were also significantly higher in patients with RRF than patients with ARF (p= 0.001).

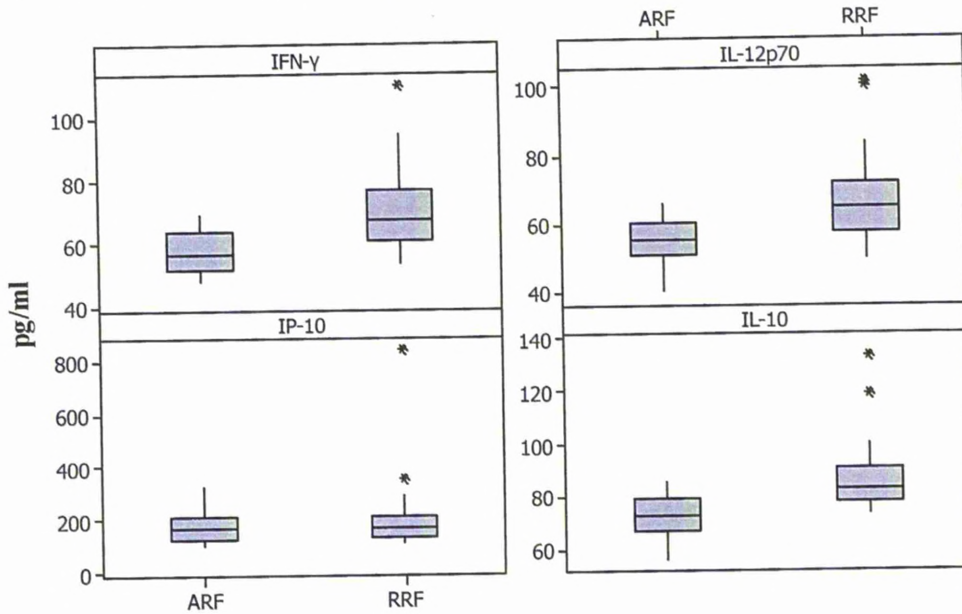
MIP-1α levels did not vary between ARF and RRF patients (p=0.9). MIP-1β had higher median values in patients with RRF (123pg/ml) than patients with ARF (95.7pg/ml) (p = 0.02). MCP-1 had a higher median value in RRF (85pg/ml) than ARF (77pg/ml) patients (p=0.1). The median concentration of RANTES was statistically higher in patients with RRF (91pg/ml) than in patients with ARF (73pg/ml) (p=0.004).

Table 8. 3 Median cytokine and chemokine concentrations in patients with ARF/RRF

Cytokines And chemokines	ARF n=24 Median pg/ml	IQR	RRF n=25 Median Pg/ml	IQR	p
IL-1β	66.3	62.8 – 71.1	76	72.4 – 91.3	0.001
IL-6	90.2	83.7 – 92.6	106.5	101.2 – 115.1	0.001
IL-7	91.7	81.2 – 107.5	100.7	90.8 – 140.8	0.001
IL-8	224.5	166.6 – 373.8	383.6	196.6 – 831.6	0.04
IL-9	81.2	65.9 – 94.3	89.2	77.4 – 123.6	0.001
IL-10	73	66.8 – 79.3	89.2	82.1 – 100.7	0.001
IL-12p70	54.9	50.9 – 60.2	64.6	57.1 – 71.9	0.001
TNF-α	71.9	67.5 – 76.3	80.4	74.5 – 93.4	0.001
IFN-γ	56.5	51.4 – 63.6	67.1	60.4 – 77.1	0.001
MCP-1	76.9	62.8 – 93.0	85.1	76 – 115.3	0.1
MIP-1α	92.6	85.5 -112.6	111.6	101.2 – 134.1	0.9
MIP-1β	95.7	80.4 – 113.3	123.1	101.2 – 155.1	0.02
IP-10	162.4	121.8 – 209.5	167.8	131.3 – 209.3	0.4
RANTES	72.8	67.1 -84.4	90.8	79.3 – 117.5	0.004

IQR = interquartile range

a. IFN-γ †† , IL12p70 †† , IP-10 and IL-10 ††



b. MIP-1a, MIP-1ß † , MCP-1 and RANTES ††

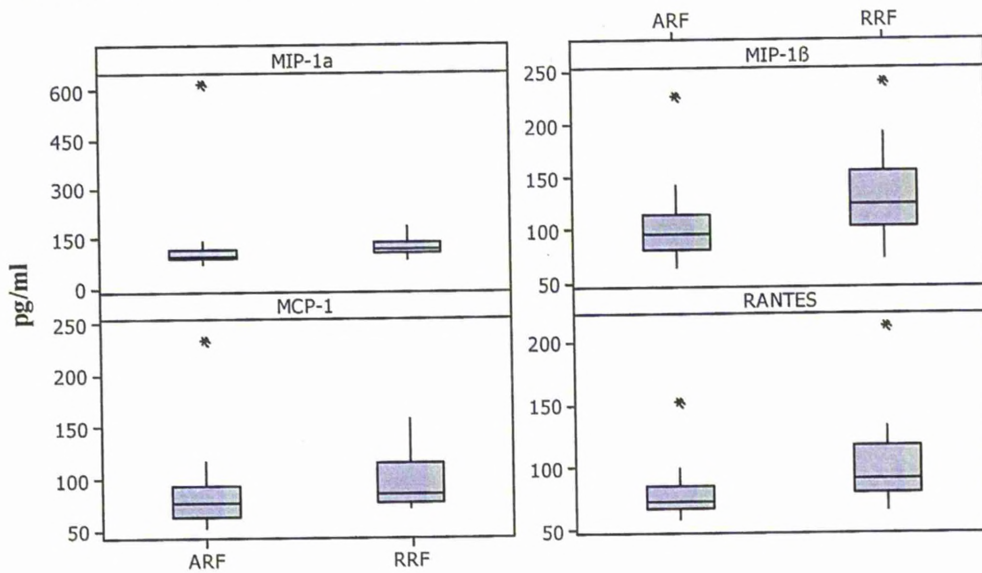
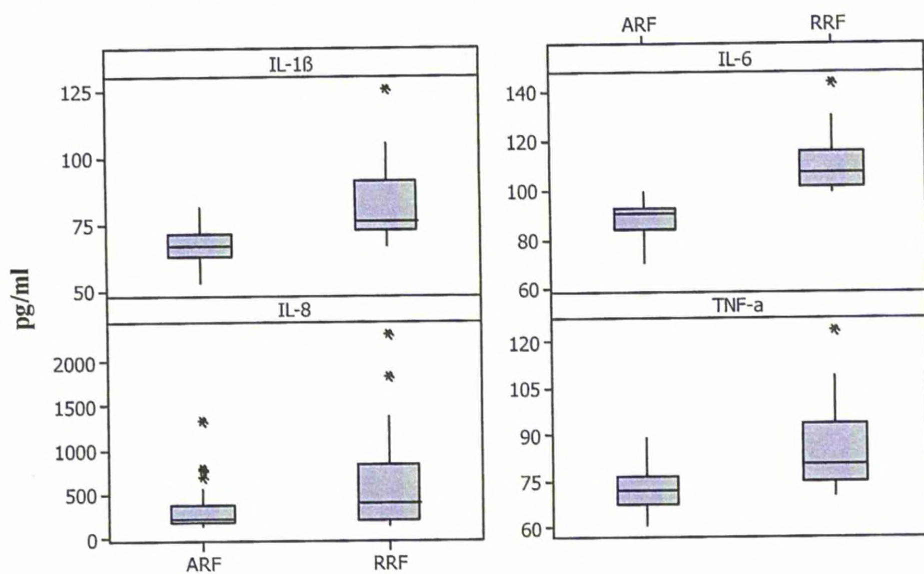


Figure 8. 2 (a-b) Cytokine and chemokine concentrations in patients with ARF andRRF Boxplots describe medians, interquartile values and range. Asterix depict outliers, and † denotes a p value <0.01 and †† a p value <0.001

c. IL-1ß †† , IL-6 †† , IL-8 † and TNF-a ††



d. IL-7 †† and IL-9 ††

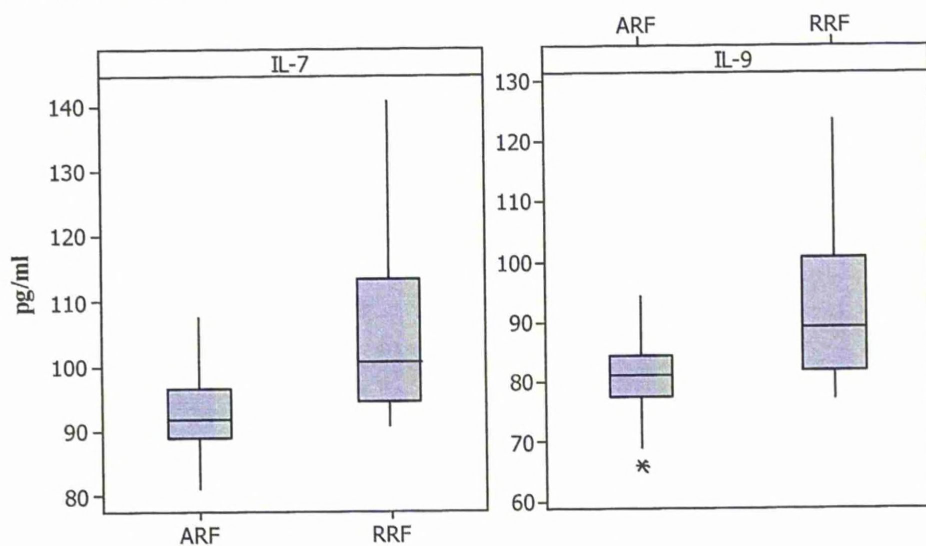


Figure 8. 3 (c-d) Cytokine and chemokine concentrations in patients with ARF and RRF

Boxplots describe medians, interquartile values and range. Asterix depict outliers, and † denotes a p value <0.01 and †† a p value <0.001

8.6.3 Correlations between cytokines and chemokines concentrations in patients with ARF

The correlations of the cytokines and chemokines tested in patients with ARF are shown in Table 8.3. A large number of correlations were observed. IL-1β was positively associated with IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, TNF-α and IFN-γ (p=0.01).

TNF-α and IFN-γ had significant positive correlations (correlation coefficient 0.9, p=0.001). TNF-α and IFN-γ had strong positive correlations with IL-1β, IL-6, IL-7, IL-9, IL-10, IL-12p70 and MIP-1β (p=0.001). TNF-α and IFN-γ were not correlated with IL-8, MCP-1 and RANTES (p >0.1).

In addition to IL-1β, TNF-α and IFN-γ, IL-6 was correlated with IL-7, IL-9, IL-10 and IL-12p70. IL-8 was significantly correlated with IL1β, and MIP1-β (p=0.01). IL-7 and IL-9 were significantly correlated with IL1β, IL-6, IL-9, IL-10, IL-12p70, MIP1-β, IP-10 TNF-α and IFN-γ. IL-10 was positively correlated with IL1β, IL-6, IL-7, IL-10, IL-12p70, MIP1-β, TNF-α and IFN-γ (p=0.01).

IL-12p70 was associated with IL1β, IL-6, IL-7, IL-9, IL-10, MIP1-β, TNF-α and IFN-γ.

MIP1-β was correlated with IL-7, IL-8, IL-9, IL-10, IL-12p70, IP-10, TNF-α and IFN-γ (p=0.01). IP-10 was significantly correlated with IL-7,IL-8 and MIP-1 β (p=0.05).

IP-10 was associated with IL-7 and MIP-1β. RANTES was only positively correlated with its subgroup MCP-1 (p=0.01). MIP-1-α was not correlated with other cytokines nor chemokines.

Table 8. 4 Correlations of Cytokines and Chemokines in patients with ARF

	IL-1 β	IL-6	IL-8	IL-10	IL-12p70	MCP-1	MIP-1 α	MIP-1 β	IP-10	TNF-α	IFN-γ	RANTES
IL1β		0.7**	0.8**	0.8**	0.9**	0.02	-0.04	0.4	0.2	0.8**	0.7**	0.2
IL6	0.7**		0.1	0.5*	0.7*	0.2	0.01	0.3	0.3	0.7**	0.7**	0.01
IL7	0.8**	0.6**	0.3	0.7**	0.8**	0.01	0.01	0.6**	0.5*	0.8**	0.7**	0.3
IL8	0.8**	0.1		0.3	0.2	-0.1	-0.01	0.5*	0.1	0.2	0.1	0.1
IL9	0.9**	0.6**	0.2	0.8**	0.9**	0.2	-0.1	0.5*	0.2	0.9**	0.8**	0.3
IL10	0.8**	0.5*	0.3		0.8**	0.3	0.02	0.5*	0.1	0.8**	0.8**	0.3
IL12p70	0.9**	0.7**	0.2	0.8**		0.2	0.03	0.4*	0.2	0.9**	0.8**	0.2
MCP-1	0.02	0.2	-0.1	0.3	0.2		-0.1	0.1	0.1	0.2	0.4	0.5**
MIP-1α	-0.04	-0.01	-0.01	0.02	0.03	-0.1		0.02	-0.1	-0.1	-0.01	-0.1
MIP-1β	0.4	0.3	0.5*	0.5*	0.4*	0.1	0.02		0.4*	0.6**	0.5**	0.03
IP-10	0.2	0.3	0.1*	0.1	0.2	0.1	-0.1	0.4*		0.3	0.3	0.3
TNF-α	0.8**	0.7**	0.2	0.8**	0.9**	0.2	-0.1	0.6**	0.3		0.9**	0.3
IFN-γ	0.7**	0.7**	0.1	0.8**	0.8**	0.4	-0.01	0.5**	0.3	0.9**		0.3
RANTES	0.2	0.01	0.1	0.3	0.2	0.5**	-0.1	0.03	0.3	0.3	0.3	

** p = <0.01   *p = <0.05

8.6.4 Correlations of cytokines and chemokines in patients with RRF

Similarly to patients with ARF, patients with RRF had a large number of correlations as shown in Table 8.4. Proinflammatory cytokines TNF-α and IFN-γ were correlated with each other (Spearman rho coefficient of 0.9, p=0.01) and these in turn had strong correlations with IL-1β, IL-6, IL-7, IL-9, IL-10, IL-12p70 and MCP-1 (p=0.01). TNF-α and IFN-γ were not correlated with IL-8, MIP-1 α, MIP-1β, IP-10 and RANTES.

IL-1β was correlated with IL-6, IL-7, IL-9, IL-10, IL-12p70, MCP-1, TNF-α and IFN-γ (p=0.01). IL-6 was correlated with IL-1β, IL-7, IL-9, IL-10, IL-12p70, TNF-α, IFN-γ (p=0.01) and MCP-1 (p=0.05). IL-8 was negatively correlated with IL-9 and IL-12p70 (p=0.05) but had no significant correlations with MCP-1, MIP-1α, MIP-1β and RANTES.

IL-10 was positively correlated with IL-1β, IL-6, IL-7, IL-9, IL12p70, MCP-1, TNF-α and IFN-γ (p=0.01).IL12p70 was positively correlated with IL-1β, IL-6, IL-7, IL-9, IL-10, MCP-1, TNF-α and IFN-γ, and was negatively correlated with IL-8.

MCP-1 was positively correlated with IL-1β, IL-6, IL-7, IL-9, IL-10, IL-12p70, TNF-α, IFN-γ and RANTES (p=0.01).IP-10 was correlated with MIP-1α (p=0.01) and MIP-1β (p=0.05). RANTES was positively correlated with MCP-1 (p=0.01).

Table 8. 5 Correlations of Cytokines and Chemokines in patients with RRF

	IL-1β	IL-6	IL-8	IL-10	IL-12p70	MCP-1	MIP-1α	MIP-1β	IP-10	TNF-α	IFN-γ	RANTES
IL1β		0.9**	-0.3	0.9**	0.9**	0.7**	0.2	0.2	-0.1	0.9**	0.9**	0.4
IL6	0.9**		-0.2	0.9**	0.8**	0.5*	0.3	0.2	0.2	0.8**	0.9**	0.3
IL7	0.9**	0.8**	-0.4	0.9**	0.9**	0.7**	0.1	0.2	-0.1	0.9**	0.9**	0.4
IL8	-0.3	-0.2		-0.4	-0.5*	0.01	0.4	0.1	0.2	-0.3	-0.4	0.3
IL9	0.9**	0.8**	-0.5*	0.9**	1.0**	0.6**	0.1	0.1	-0.3	0.9**	0.9**	0.3
IL10	0.9**	0.9**	-0.4		0.9**	0.6**	0.1	0.1	-0.1	0.9**	0.9**	0.3
IL12p70	0.9**	0.8**	-0.5*	0.9**		0.6**	0.1	0.1	-0.2	0.9**	0.9**	0.3
MCP-1	0.7**	0.5*	0.01	0.6**	0.6**		0.2	0.3	-0.1	0.7**	0.6**	0.7**
MIP-1α	0.2	0.3	0.4	0.1	0.1	0.2		0.6**	0.7**	0.2	0.2	0.3
MIP-1β	0.2	0.2	0.1	0.1	0.1	0.3	0.6**		0.5*	0.2	0.1	0.2
IP-10	-0.1	0.2	0.2	-0.1	-0.2	-0.1	0.7**	0.5*		-0.1	0.02	-0.03
TNF-α	0.9**	0.8**	-0.3	0.9**	0.9**	0.7**	0.2	0.2	-0.1		0.9**	0.4
IFN-γ	0.9**	0.9**	-0.4	0.9**	0.9**	0.6**	0.2	0.1	0.02	0.9**		0.3
RANTES	0.4	0.3	0.3	0.3	0.3	0.7**	0.3	0.2	-0.03	0.4	0.3	

** $p = <0.01$ *$p = <0.05$

8.6.5 Difference in the correlation of cytokines and chemokines in ARF compared with RRF

IL-1β was significantly correlated with IL-8 in patients with ARF but not in patients with RRF. IL-1β was significantly correlated with MCP-1 in patients with RRF patients but not in patients with ARF.

IL-6 was significantly correlated with MCP-1 in patiens with RRF but not in patients with ARF. IL-7 was associated with MIP-1β and IP-10 in patients with ARF but was not present in RRF, IL-7 was associated with MCP-1 in patients with RRF but was absent in patients with ARF.

IL-8 was significantly correlated with IL-1β and MIP-1β in patients with ARF but was not evident in patients with RRF. In patients with RRF, IL-8 was significantly negatively correlated with IL-9 and IL12p70 but was not present in ARF.

IL-9 was correlated with MIp-1β in patients with ARF but not in RRF while it was positively correlated with MCP-1and negatively correlated with IL-8 in patients with RRF. IL-10 was signifcantly correlated with MIP-1β in patients with ARF but not in RRF. IL-10 was correlated with MCP-1 in patients with RRF but not in ARF.

MCP-1 was significantly correlated with IL-1β, IL-6, IL-7, IL-9, IL-10, I L-12p70, TNF-α and IFN-γ in patients with RRF but this correlation was not present in patients with ARF. MIP-1α was associated with MIP-1β and IP-10 in patients with RRF but this correlation was not evident in patients with ARF. MIP-1β was significantly correlated with IL-7, IL-8, IL-9, IL-10, IL-12p70, TNF-α and IFN-γ in patients with ARF. In patients with RRF, MIP-1β was only correlated with MIP-1α but not in patients with ARF. IP-10 was correlated with IL-7 in ARF but not in patients with RRF while it was correlated with MIP-1α in patients with RRF but not in patients with ARF.

TNF-α and IFN-γ were both significantly associated with MIP-1β in patients with ARF but not in patients with RRF. TNF-α and IFN-γ were both significantly associated with MCP-1 in patients with RRF but not in patients with ARF.

Table 8. 6 Correlations of cytokines and chemokines which are present in statistical significant concentrations in patients with ARF but not in patients with RRF

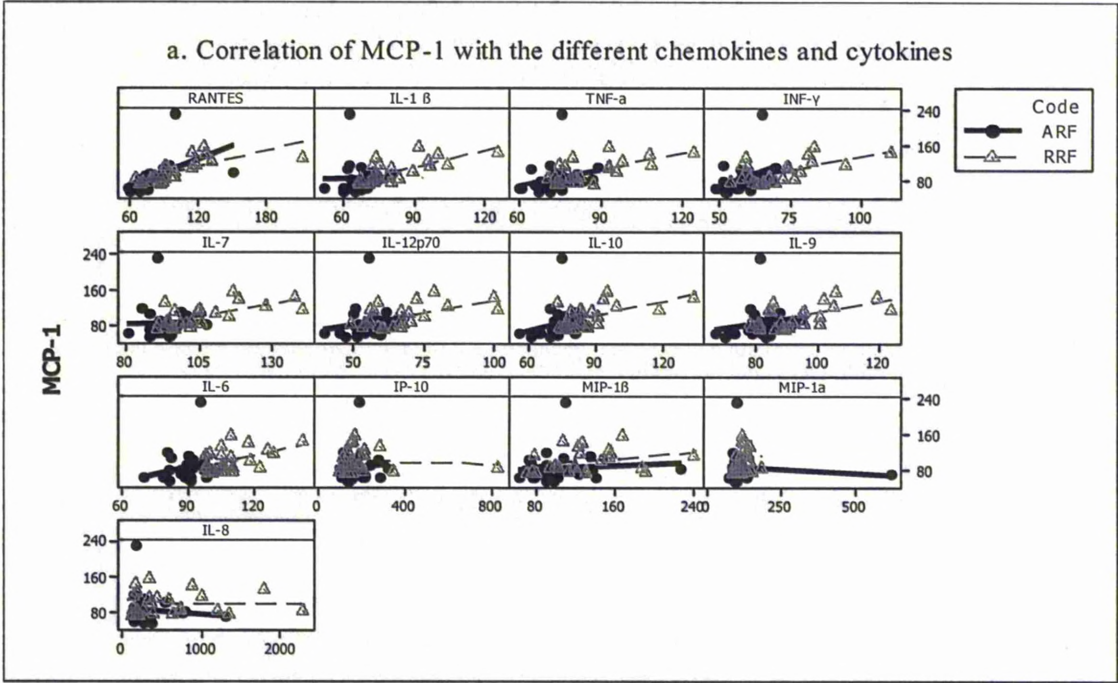
	IL-1β	IL-6	IL-7	IL-8	IL-9	IL-10	IL-12p70	MCP-1	MIP1-α	MIP1-β	IP-10	TNF-α	IFN-γ	RANTES
IL1β				0.8**				0.02						
IL-6								0.2						
IL-7								0.01		0.6**	0.5*			
IL-8	0.8**				0.2		0.2			0.5*				
IL-9				0.2				0.2		0.5*				
IL-10								0.3		0.5*				
IL-12p70				0.2				0.2		0.4*				
MCP-1	0.02	0.2	-0.01		0.2	0.3	0.2					0.2	0.4	
MIP-1α										0.02	-0.1			
MIP-1β			0.6**	0.5**	0.5**	0.5**	0.4**		0.02			0.6**	0.5**	
IP-10			0.5*						-0.1					
TNF-α								0.2		0.6**				
IFN-γ								0.4		0.5**				
RANTES														

** p = <0.01 *p = <0.05

Table 8. 7 Correlations of cytokines and chemokines which are present in statistical significant concentrations in patients with RRF but not in patients with ARF

	IL-1β	IL-6	IL-7	IL-8	IL-9	IL-10	IL-12p70	MCP-1	MIP-1α	MIP-1β	IP-10	TNF-α	IFN-γ	RANTES
IL1β				-0.3				0.7**						
IL6								0.5*						
IL7								0.7**		0.2	-0.1			
IL8	-0.3				-0.5*		-0.5*			0.1				
IL9				-0.5*				0.6**		0.1				
IL10								0.6**		0.1				
IL-12p70				-0.5*				0.6**		0.1				
MCP-1	0.7**	0.5*	0.7**		0.6**	0.6**	0.6**					0.7**	0.6**	
MIP-1α										0.6**	0.7**			
MIP-1β			0.2	0.1	0.1	0.1	0.1		0.6**			0.2	0.1	
IP-10			-0.1						0.7**					
TNF-α								0.7**		0.2				
IFN-γ								0.6**		0.1				
RANTES														

** p = <0.01 *p = <0.05



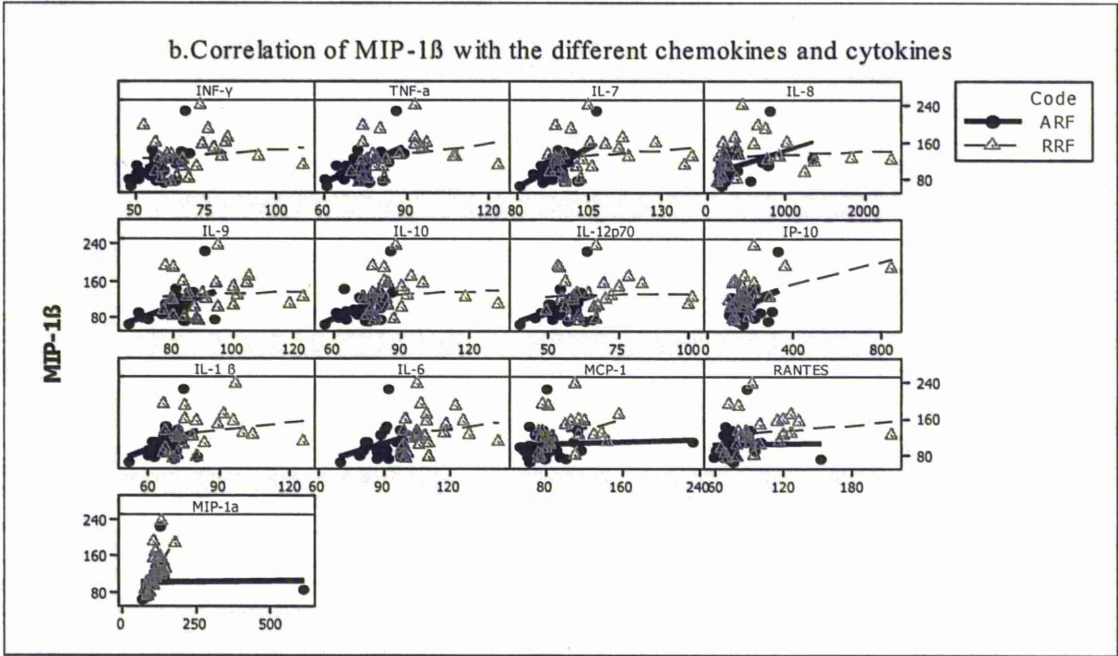


Figure 8. 4(a-b) Scatterplots of cytokine and chemokine concentrations

IL-1β, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p20, TNF-α, IFN-γ, MCP-1, MIP-1α, MIP-1β, IP-10 and RANTES in patients with ARF and RRF (See Appendex c-i) for the correlations of the other chemokines and cytokines)

8.7 Discussion

8.7.1 General characteristics of cytokines and chemokines

ARF and RHD pathogenesis is a complex autoimmune process involving genetic and environmental factors (Guilherme and Kalil 2010). The differential cytokine polarization in RHD is probably related to immigrant autoreactive T cells, local chemokines and adhesion molecules.

In this study the concentrations of all the cytokines were observed to be significantly higher in patients with RRF than ARF. Selected chemokines IL-8, MIP-1β and RANTES were only significantly higher in patients with RRF than ARF patients.The proinflammatory cytokines TNF-α and IFN-γ was significantly correlated with IL-1β, IL-6, IL-10, IL-12p70 in ARF and RRF patients. The variation observed in chemoattractant chemokines correlation profile was interesting in ARF and RRF patients. MIP-1β was significantly correlated with IL-7, IL-8, IL-9, IL-10, IL-1p270, IP-10, TNF-α and IFN-γ only in ARF patients. MCP-1 was significantly correlated with IL-1β, IL-6, IL-7, IL-9, IL-10, IL-12p270, TNF-α and IFN-γ only in RRF patients. MIP-1β may play a significant role in the induction of acute inflammatory reactions and may possibly activate macrophages to facilitate the autoimmune process in patients with rheumatic fever. MIP-1β may probably serve as an inflammatory biomarker in patients with ARF. MCP-1 does not appear to have a role in acute inflammatory reaction. MCP-1 may probably serve as an inflammatory biomarker in patients with RRF.

Chemokines direct the migration of leukocytes throughout the body and play an important role in the coordination of the inflammatory response. Serum chemokines levels may be clinically useful as an objective indicator of activity, and may have the potential to improve disease management. Patients with high serum chemokine levels may be more likely to develop early recurrence of ARF with possible cardiac valve involvement and end up with RHD. Recurrences of ARF may have a greater likelihood to occur in patients with marked elevation of some serum chemokines. Monitoring frequent chemokine levels in rheumatic patients may provide a tool to evaluate disease activity and identify patient's likelihood of future recurrences and RHD development. Further prospective studies with a larger number of patients and matched control group are needed to evaluate this.

Guilhereme (2006) reported that heart lesions secrete IFN-γ and TNF-α in ARF and CRHD patients (Guilherme, *et al* 2006). Recently, Oral documented that patients with MR had a high production of TNF-α in the serum and the myocardium (Oral, *et al* 2003). These results are in line with this study where patients with ARF and RRF showed elevations of both TNF-α (72pg/ml versus 80.4pg/ml) and IFN-γ (56.5pg/ml versus 67.1pg/ml) compared to normal healthy controls (TNF-α <20pg/ml and IFN-γ <15pg/ml) as reported in different studies (Fujii, *et al* 2004, Gu, *et al* 2009, Yegin, *et al* 1997) . These values of TNF-α and IFN-γ were significantly higher in patients with

RRF than ARF (p=0.001). The significant elevations of TNF-α and IFN-γ in ARF and RRF patients in this study may favour the suggestion that they act by augmenting the inflammatory response triggered by an autoimmune reaction indicating that Th1-type cytokines (TNF-α and IFN-γ) could mediate RHD (Guilherme and Kalil 2004).

Increased proinflammatory cytokines such as IL-1, IL-6, and TNF-α have been described in patients with congestive cardiac failure and rheumatoid arthritis (Armstrong, *et al* 2006, Necil Kütükçüler 2008). Macrophages secrete the IL-1 and TNF-α required for B and T cell activation and aggregation, suggesting that macrophages arrive at rheumatic injury before the lymphocytes to produce an early inflammatory response (De Jager, *et al* 2007, Fraser, *et al* 1997). Monocyte-macrophages can produce TNF-α while a number of endogenous mediators such as IL-1 are active inducers of TNF-α (Dayer 2004). In this study the median concentrations of cytokines IL-1β and TNF-α were similar but significantly higher in RRF (IL-1 β = 76pg/ml versus TNF-α = 80.4pg/ml) than ARF (IL-1 β = 66.3pg/ml versus TNF-α = 80.4pg/ml) patients. These changes are consistent with reports from different countries (Morris, *et al* 1993, Narin, *et al* 1995, Samsonov, *et al* 1995).

Infiltrating mononuclear cells produce cytokines which correlate with activity of Aschoff nodules during the acute active phase of ARF and RHD and may serve as indicators of disease progression and severity (Guilherme and Kalil 2007). The high concentration of T cells which infiltrate the heart in RHD may be related to the differential secretion of specific chemokines (Cunningham 2004). These cells may have been activated by IL-5, a Th2-type cytokine, which contributes to local inflammation

through several proinflammatory cytokines e.g. IL-6, IL-8, TNF- α, IL-1 and chemokines (Spellberg, *et al* 2001). In this study IL-6 cytokine was elevated with higher values in patients with RRF than ARF who all clinically had high ESR and positive CRP levels. IL-6 has been reported to have a direct effect on the regulation of the release of the acute-phase proteins from hepatocytes and Kuppffer cells (Nishimoto and Kishimoto 2006)

IL-8 (normal value < 21.7pg/ml) had the highest magnitude among all the cytokines in both ARF and RRF (224.5pg/ml versus 383.6pg/ml) and was twice as high in children with RRF as ARF. Yegin (1997) found a significant increase in TNF-α, IL-6 and IL-8 in ARF and significant IL-6 and TNF-α correlation with CRP and ESR levels in ARF /RHD patients. In this study a significant correlation was demonstrated between IL-6 and TNF- α in ARF and RRF groups and all the patients had increased levels of ESR and CRP. Kutukculer & Narin (1995) reported that IL-8 concentrations were significantly elevated in the acute stage and decreased in remission. It is possible that the excessive production of IL-8 could be caused by the cellular infiltration in the joints during the ARF period.

IL-10 is a cytokine with both an anti-inflammatory and immune- regulatory activities. It is mainly produced by Th2 subset of CD 4 and some activated B cells(Beebe, *et al* 2002). It inhibits the synthesis of pro-inflammatory cytokines and chemokine by monocyte, macrophages, neutrophils and eosinophils (Bryant, *et al* 2009). It is also an important immunoregulatory cytokine that inhibits T-cell function, inhibits antigen-presenting cells and promotes B-cell-mediated functions (Sonderegger, *et al* 2012).

IL-10 is a cytokine with both an anti-inflammatory and immune- regulatory activities. It is mainly produced by Th2 subset of CD 4 cells and some activated B cells(Beebe, *et al* 2002),(Armstrong, *et al* 2006, Diveu, *et al* 2008). It inhibits the synthesis of pro-inflammatory cytokines and chemokines by monocytes, macrophages, neutrophils and eosinophils (Bryant, *et al* 2009). It is also an important immune regulatory cytokine that inhibits T-cell function, inhibits antigen-presenting cells and promotes B-cell-mediated functions (Sonderegger, *et al* 2012). Increased levels of IL-10 and IFN-γ were reported in patients with acute malaria (Angulo and Fresno 2002). In this study IL-10 and IFN-γ

were observed to be significantly higher among RRF than ARF patients. This is different to a report from India where higher levels of IL-10 were observed in patients with CRHD with no active disease process than active ARF (Bhatnagar, *et al* 1999).

It relates to the clinical presentation of the disease either with arthralgia, arthritis or carditis with or without CCF and ESR and CRP to determine the activity of the disease (Col-Araz, *et al* 2012). IL-10 was reported to be synthesized later than other immuno regulatory cytokines by activated T cells or monocytes, demonstrating its regulatory role in later phases of the immune response (Settin, *et al* 2007) . It is a prominent regulatory cytokine secreted by large numbers of cells in both valve and myocardial tissues (Col-Araz, *et al* 2012) . Its production in the valvular lesions of ARF patients was correlated with Aschoff nodule progression (Guilherme, *et al* 2011). This could be explained by the time and site variation when the sample was taken during the disease process. Children with active process of RHD are more likely to have extensive inflammatory disease response.

The profile of chemokines RANTES, MCP-1, MIP-1α and MIP-1β have not been described in ARF and this is the first report to assess these chemokines among these patients. MCP-1was reported to be elevated in patients with congestive cardiac failure (Bidzhekov, *et al* 2006). TNF-α induces the production of RANTES(Deshmane, *et al* 2009) and the latter RANTES is reported to be upregulated in the heart (Guilherme, *et al* 2004, Schober 2008). In this study RANTES was detected with significantly higher values in RRF than ARF group ($p=0.004$). MCP-1 and MIP-1α values did not significantly vary between either group. MIP-1β concentration was significantly elevated in RRF than ARF patients. MIP-1 and RANTES have been studied in an animal model of destructive autoimmune myocarditis(Cunningham 2001). The significant elevations of the above chemokines RANTES, MCP-1, MIP-1α and MIP-1β in RRF and ARF may be explained by their possible recruitment from macrophages and activated T cells which could contribute to the inflammatory response and damage to the tissues (Guilherme and Kalil 2010). They may provide to act as useful markers of immune activation and may help to provide information on prognosis of this utoimmune disease.

IP-10 is produced by inflammatory cells such as macrophages and monocyte which attract activated T lymphocytes (Cassatella, *et al* 1993). IP-10 is reported to be manily induced by IFN-γ (Chuang, *et al* 2005). It could particate in the activation of some cells during the autoimmune response in some infectious diseases but not in ARF (Nicoletti, *et al* 2002, Salomon, *et al* 2002, Shimada, *et al* 2001). In this study patients with ARF and RRF had elevated IP-10 values. This is the first report to describe IP-10 levels in patients with ARF/RRF which may suggest its role in T cell-mediated autoimmunity. It may also indicate that disease reccurence, the target autoanitgens become more activated during the immunoinflammatory processes. Thus IP-10 may act as a new inflammatory biomarker to assess the susceptible patients to the disease severity.

IL-12p70 was expressed with a low concentration similar to IFN-γ in both groups. However, IL-12p70 had significantly higher values within RRF than ARF patients. This is the first study to describe the cytokine IL-12p70 in patients with ARF and supports the suggestion that IL-12p70 regulates Th1 response by inducing IFN-γ.

Significantly higher concentration of the profiles of 14 cytokines and chemokines investigated in this study was demonstrated among RRF than ARF patients. The report by Guilherme (2001) found that mitral valve T cells in ARF displayed lower inflammatory levels of IL-10, IFN-γ and TNF-α than in RHD patients (Guilherme, *et al* 2001). It could be explained by the different sites ie.heart tissue as opposed to serum from where the samples were taken and to different activity of the disease

8.7.2 Correlations of Cytokines and Chemokines in ARF and RRF patients

Recently cytokines have been implicated as key coordinators in several inflammatory and autoimmune diseases including ARF. The results in this study support the role of cytokines and chemokines in the initiation of the inflammatory response during the active phase of ARF and RHD. Proinflammatory cytokines TNF-α and IFN-γ were significantly correlated with IL-1β, IL-6, IL-10, IL-12p70 in both ARF and RRF patients. Previous reports have demonstrated the role of TNF-α, IFN- γ, IL-1β and IL-6 in the production of acute phase reactants (Armstrong, *et al* 2006). Yegin (1997) reported TNF-α to be a major cytokine in ARF leading to production of IL-6 and IL-8 while Kapadia (1996) described these cytokines in congestive cardiac failure. Angulo et al (2002) reported the levels of IL-6, IL-10, TNF-α and IFN-γ were found in higher values among patients who died of malaria that those who survived (Angulo and Fresno 2002).TNF-α was reported to have additive effects with IL-1 in ARF (Necil Kütükçüler 2008). This is the first report to describe the correlation of IL-12p70 in ARF and RRF with TNF-α and IFN-γ. In previous reports IFN-γ was found in valves of patients with carditis involved in Th-1 granuloma forming Aschoff nodules (Pestka 2007). In this study the strong colocalization of TNF-α and IFN-γ in both ARF and RRF patients may be due to active inflammatory response associated with carditis, a major feature in both groups.

IFN- γ has immunoregulatory properties and preferentially inhibits Th2 proliferation responsible for IL-10 production (Pestka 2007). In experimental studies IL-10 exhibited a proinflammatory action but in patients with acute coronary syndrome it had a protective effect against proinflammatory mediators (Armstrong, *et al* 2006). These findings are different to this study where IFN-γ was significantly correlated with IL-10 in both ARF and RRF patients. This difference in the pattern of cytokines may reflect the variation in the response to the pattern of inflammatory cells in different tissues and needs to be clarified in future studies.

IL-8 an inflammatory cytokine produced by TNF-α, plays a role in the increased neutrophil chemotaxis during ARF (Kutukculer and Narin 1995). In this study, IL-8 in

patients with ARF was significantly correlated with MIP-1α and IP-10 while in patients with RRF, IL-8 was negatively correlated with IL-12p70. RANTES was only positively correlated with its subgroup MCP-1 in both patients with ARF and RRF. Further studies with larger sample size are required to explain these significant correlations.

The significant correlations of IL-1β and IL-6 with cytokines IL-10 and IL-12p70 was higher in patients with RRF than ARF. IP-10 was significantly correlated with MIP-1α only in patients with RRF but with MIP-1β in both ARF and RRF patients. IL-10 shared similar significant correlations with IL-1β, IL-6 and IL12p70 in patients with ARF and RRF but the difference was with MIP-1β in patients with ARF and MCP-1 in patients with RRF. These differences express the variations in response of inflammatory cells by different tissues during acute and recurrent disease activity

The variation observed in chemoattractant chemokines profile was interesting in patients with ARF and RRF. MIP-1β was significantly correlated with IL-8, IL-10, IL-12p70, IP-10, TNF-α and IFN-γ only in patients with ARF. MCP-1 was significantly correlated with IL-1β, IL-6, IL-10, IL-12p70, TNF-α andIFN-γ in RRF patients which was not evident in patients with ARF . The chemoattractant chemokine MIP-1β probably serves as an immunological biomarker in patients with ARF and chemoattractant cytokine MCP-1 probably serves as an immunological biomarker in patients with RRF. These differences in overall pattern of chemokines and cytokines may result from a variation of balance in the inflammatory cells in the lesions and the severity of the damage in patients with ARF and RRF.

The non correlation of IL-1β with IL-8 and IP-10 with IL-7 in patients with RRF could possibly serve as potential markers to differentiate ARF from RRF. The significant negative correlation of IL-8 with IL-12p70 and IL-9 in patients with RRF not evident in ARF could be a potential marker to differentiate between the two groups.MIP-1α was not associated with MIP-1β and IP-10 in patients with ARF but this correlation was evident in patients with RRF making it a possible point of differentiation between them. The association of IP-10 with IL-7 in patients with ARF but not in RRF was also significant. TNF-α and IFN-γ were not correlated with MIP-1β in patients with RRF and

also not associated with MCP-1 in patients with ARF and this serves a potential point to differentiate between the two groups.

The studied chemokines and cytokines could play an important role in the development of the inflammatory response in patients with ARF and RRF. These results will provide new clues for the potential roles of these chemokines and cytokines in the exacerbation of this disease and shed light on the development of ARF and RRF disease markers.The identification of specific chemokines involved in T-cell polarization and/or differential migration contribute toward a better understanding of immunopathogenesis and treatment of ARF and RHD patients (Guilherme, *et al* 2004). This information may open new possibilities for immunotherapy such as T-cell vaccination for patients with severe RHD. Molecular knowledge of the autoimmune reactions will aid in the choice of GAS protective epitopes for an effective and safe vaccine (Guilherme, *et al* 2005a).

Future direction in complex disorders may be the simultaneous inactivation/activation of multiple cytokines (Kourilsky and Truffa-Bachi 2001). Cytokine gene therapy for cancer treatment is the latest development in the field of cytokines (Feldmann 2008) Podhajcer et al., 2007). Cytokine-based drugs and anti-cytokines play a crucial role in understanding pathogenesis and management of several diseases (Feldmann 2008). Research is continuing to develop new therapies, refine those already in use and establish the safest and most effective dosage levels (Mellado and Carrasco 2008).

It is essential for clinicians to stay abreast with the latest information on the ongoing studies for the benefit of their patients. Since GAS vaccine development is progressing and hopefully will eventually lead to prevention of ARF and RHD in the near future (Guzman-Cottrill, *et al* 2004).

8.8 Conclusions

In this study, in addition to the role of IL-1β, IL-6, IL-8, IL-10, TNF-α and IFN-γ involved in the inflammatory response of ARF, a further six new cytokines and chemokines RANTES, MCP-1, MIP-1α , MIP-1β, IP-10 and IL12-p70 were for the first time analyzed in patients with ARF.

MCP-1 was significantly correlated with cytokines, IL-1β, IL-6, IL-10, IL-1p270, TNF-α, IFN-γ and RANTES in patients with RRF. This suggests that MCP-1 could serve as a potential inflammatory biomarker for patients with RRF with underlying RHD. MIP-1β had significant correlations with IL-8, IL-10, IL-1p270, IP-10, TNF-α and IFN-γ in patients with ARF not apparent in patients with RRF and this MIP-1β may serve as a potential inflammatory biomarker in patients with ARF.

8.9 Recommendations

These interesting results require further comprehensive studies on cytokine and chemokine profilė among patients with ARF and RRF in developing countries to lead to a better understanding on the immunopathogenesis of the disease. This will facilitate in the development of a vaccine for the prevention of ARF and RHD.

8.10 Clinical implications

Knowledge of the humoral and cellular cross-reactive epitopes is important for the production of a safe vaccine to prevent GASinfections. Some cytokines and chemokines such as TNF-αalleles and other immunoregualted genes which are located in the same chromosomal region as HLA genes are currently under investigation. It will contribute to the understanding of new genetic markers and how these genes act in the complex autoimmune network in ARF/RHD.

8.11 Limitations

Although hypothetically 96 well plates could test 40 in duplicate and we had 2 plates equal to 80, we could only test 49 samples due to the need to retest some specimens and logistic problems with the machine.The small sample size of patients investigated for cytokines and chemokines in this study (Sadiq, *et al* 2008) requires a future study with a larger sample size to confirm their role as immunological biomarkers for ARF. It might be interesting to correlate the type of inflammatory cells ie. macrophages, monocytes and endothelial cells present with the pattern of chemokine and cytokine expression.The association of ARF with a genetic susceptibility in the causation of RHD has not been studied in Yemen. One of the original aims of this study was to determine the distribution of HLA class II system genes among Yemeni patients with RHD. However,

due to the sad death of Professor Tony Hart, my principal supervisor and limitations in the funding this objective has been postponed.

9 General Discussion

9.1 General overview discussion

In this chapter the work contribution to the knowledge on immunopathogenesis of ARF and RHD due to GAS and SNA pathogens is reviewed according to the objectives outlined in this study.

9.2 RHD among Yemeni school children at Aden city

9.2.1 9.2.1 Prevalence of RHD among Yemeni school children

This study has demonstrated that RHD is a major contributor to cardiovascular disease in both children and adults in Yemen. This will lead to recurrent hospital admissions and premature disability. The school echocardiography survey for RHD in this study found a prevalence of 36.5/1000 which is alarmingly high.This is the highest figure for the prevalence of echo-proven RHD amongst school children in any country. The highest prevalence of RHD was between 11 -1 6 years. However, this high prevalence could still be an underestimate of the true prevalence as students with poor health may experience frequent school absenteeism. This high RHD prevalence requires urgent regular school screening surveys and RHD prophylactic programs to try to control the problem ARF/RHD in Yemen.

9.2.2 9.2.2 Socio economic factors

Overcrowding, poverty and poor housing conditions in children was associated with RHD but this observation did not differ from reports in other countries (Lennon 2004; Kurahara, Grandinetti et al. 2006).

9.2.3 9.2.3 Echocardiogaphic findings

Of 219 children with RHD, 51% had isolated MR involvement, 26.6% had MR with MVP and 17.8% combined MR and AR. There was with no sex predilection which is consistent with previous reports (Carapetis 2008b, McDonald,*et al.* 2005). Mitral stenosis was detected in one female and four males even though it is more commonly

reported among females (Andy & Soomro 2001). More than one third of the children had complex lesions of MR and MVP involving the anterior leaflet. Further consideration should be given to include MVP as a common cardiac valve lesion in ARF/RHD and specifically correlate its primary or secondary origin. Normal values may vary from one laboratory to another from CRP 0–1.0 milligrams per deciliter (mg/dL) or less than 10 mg/L (SI units). The values for CRP in the tables are <5mg/dL and >5mg/dL. The highest values for CRP were up to 10mg/dL equivalent to 100mg/L.

It is essential to monitor CRP during the follow up of RHD patients since a higher proportion of children with multiple valve involvement presented with increased CRP. Continually raised CRP levels may be a marker of on-going inflammation of severely affected valves in RHD (Gölbasi, Uçar et al. 2002).

9.2.4 9.2.4 Familial pattern of ARF/RHD

RHD was diagnosed in more than one family member in 53 children with ARF/RHD including 20 affected siblings. Further studies are required to document the familial patterns of ARF/ RHD and undertake research on possible specific genetic modes of inheritance. However, studies so far have found a wide range of gentic markers associated with ARF/RHD with no particular marker demonstrating a strong correlation.

9.2.5 9.2.5 Penicillin prophylaxis

Health professionals should provide continuous education and reassurance to families on RHD prevention since only 16.4% of parents were aware of a RHD problem in their children prior to the survey. It is important to identify children with ARF/RHD early and to start penicillin prophylaxis to prevent ARF recurrences and progression of RHD. Only 18 students were on secondary prophylaxis and in many it was irregular. 24 parents refused prophylaxis due to fear of penicillin hypersensitivity.

9.3 GAS among patients with acute pharyngotonsillitis

9.3.1 Prevalence of GAS among patients with acute pharyngotonsillitis

This is the first Yemeni report of GAS prevalence in children with acute pharyngotonsillitis and the 41.5% prevalence is higher than other neighbouring countries (Menon, *et al* 2004, Rimoin, *et al* 2008) The prevalence was 68.3% in the 11-

15 years age group. A low GAS frequency was found in children <5 years of age which may be due to absence of classical symptoms at this age. A high occurrence of GAS pharyngotonsillitis 85% during the winter months was consistent with other countries (Rimion et al 2008). GAS pharyngotonsillitis requires early diagnosis with prompt appropriate antibiotic therapy to reduce the risk of ARF and RHD.

9.3.2 9.3.2 Diagnostic clinical criteria

The clinical characteristics of fever, sore throat, tonsillar erythema and exudates with enlarged anterior tender lymph nodes were noticed in a higher frequency in Yemeni children with GAS pharyngotonsillitis than in other reports (Lazar R. 2004, Mzoughi R2004) Cough, hoarseness and rhinorrhea favoured a viral infection as reported by others (Lindbaek, Hoiby et al. 2005). In this study many patients with GAS pharyngotonsillitis presented with petechial lesions on the soft palate and a red erythematous uvula which was three times higher than other reports(Bisno, *et al* 2002) Kaplan et al 2006). Including these two particular signs into the MacIssac score will improve the specificity of GAS diagnosis. In poor countries with limited laboratory resources, the clinical diagnosis of GAS pharyngotonsillitis is an essential part in the effort to control ARF.

9.3.3 9.3.3 Rapid Antigen Detection Test for GAS

In this study a McIssac score with a cut off point of 4 had a higher sensitivity (93%) and specificity (82%) which could remove the need of a backup culture to confirm GAS diagnosis. However, throat cultures are still the gold standard for the diagnosis of GAS pharyngotonsillitis. Although RADT can overcome the time delay in diagnosis associated with GAS throat cultures, they are expensive. Current RADT are expensive. However when used on a large scale such as with malaria and HIV testing these become more accessible. Some RADT for the latter diseases cost about £1each. These reduced costs may still be considered high for a disease that is less common and not widely perceived to be a serious problem by some authorities.Further studies on cost benefit are required before routine application of RADT in poor countries. A school education program is essential to increase awareness of ARF and its sequelae among parents having children with recurrent GAS sore throats.

9.4 Non-group A beta-haemolytic streptococci, Lancefield groups C, G and B

9.4.1 Prevalence of SNA pharyngotonsillitis

This is the first study to document SNA pathogens, GCS, GGS and GBS in children with acute pharyngotonsillitis in Yemen. The prevalence of 4.3% was similar to other reports (Wong and Chung 2002, Zaoutis 2004) Also similar gender distribution was observed within 11 – 15 years age group.

9.4.2 Diagnostic criteria of patients with SNA pharyngotonsillitis

All patients with SNA pharyngotonsillitis presented with five positive criteria of McIssac scoring system. RADT was negative in all SNA patients. Six patients had GCS, six GGS and one had GBS. Their clinical manifestations were consistent with GAS pharnygotonsillitis as in other studies (WHO 2004; Gerber et al 2004). An interesting finding was that all SNA patients presented with pain on swallowing which was not identified in other reports (Lindbaek, *et al* 2005).

9.4.3 History of ARF and RHD in patients with SNA pharyngotonsillitis

There are no reports to confirm that SNA are implicated in the pathogenesis of ARF/RHD. However, all patients with GCS, GGS and GBS pharyngotonsillitis in this study had a positive ARF history and were echocardiography-proven cases of RHD. Also all had a positive family history of ARF and RHD which suggests a possible clinical association of SNA with ARF and RHD. Stronger evidence for rheumatogenic potential of SNA could be obtained if these patients had regular follow up for any reccurrence of ARF and throat swabs taken. Further studies on the possible association between SNA and ARF are required.

9.5 Emm serotypes, exotoxin genes and serum opacity factor of GAS

9.5.1 emm genotypes among GAS pharyngotonsillitis

Thrity four GAS and SNA strains obtained from pharyngealtonsillar isolates in patients with ARF/RHD included some known and new *emm* sequences characterized by using

genotypic tests, streptococcal pyrogenic exotoxin genes and genomic DNA restriction profiles by PFGE. *emm*87, 12, 28 and 5 were the most frequent GAS isolates. *emm*87 was detected in 11 (52.4%) of throat swabs of GAS. This is the first report of GAS *emm*87 to be potentially associated with ARF/RHD patients and suggests that it is likely to be rheumatogenic. Also GAS *emm*28 detected in 3 (14.3%) has not been identified as being rheumatogenic. These results suggest that uncommon *emm* GAS genotypes may have a significant role in the epidemiology of ARF/RHD in Yemen. Further studies with larger sample size are required.

9.5.2 Pyrogenic exotoxin superantigen and sof gene among GAS isolates

GAS *emm*87 strains were all *sof* gene positive for the unique *sof* 87 sequence which suggests that the *sof* gene is detecTable by PCR only. This is the first report to describe the profile of the five prophage gene exotoxin sequences among GAS *emm*87 with the presence of *speC*, *spd1*, *sdn*, *silC* and *silD* and no striking sequence differences in their PFGE. *emm*12 GAS isolates were all *sof* positive for *sof* 12 gene sequence. Pyrogenic exotoxin C (*speC*) was identified in all *emm*87, 12 and 28 GAS pharyngotonsillitis strains. This is the first report of *speC* toxin gene among *emm* 87 GAS throat isolates. GAS *emm*28 strains revealed positive results for exotoxin genes for *spec* and *spd1* genes but not for *sdn*.

9.5.3 Streptococcal invasive locus (sil) among GAS pharyngotonsillitis isolates

The *silC* and *silD* loci were PCR positive in all *emm*87 and *emm*12 GAS pharyngotonsillitis isolates. *SilC* was positive in *emm*28 and *emm*5 isolates which has not been described among these strains before. This is the first report to describe the *sil* exotoxin gene pattern of *emm*87, 12, 28 and 5 isolates on throat swabs from GAS patients with a history of ARF/RHD.

9.5.4 Emm serotypes, exotoxin genes and serum opacity factor gene of SNA

The *emm* virulence genotypes of GGS pharyngotonsillitis in patients with a history of ARF/RHD were genetically heterogeneous. They comprise 5 different genotypes, two isolates of *emm* st7882.2, and one isolate each of *emm* st2917, *emm* st7406, *emm*

stG652.5 and stKNB7. The *emm* virulence genotypes of GCS pharyngotonsillitis were *emm* st2917 in four isolates and two isolates of *st*CK249. One GBS genotype was stG4974. These GGS,GCS and GBS *emm* genotypes sequence strains from pharyngeal isolates are totally different from that found in the literature (Horii et al., 2006). stKNB 7 in the GGS strain was the only gene sequence previously reported in India (Menon, *et al* 2008). These new *emm* sequences among GCS, GGS and GBS pharyngeal isolates were detected for the first time and possibly related to study in a different geographical site, Yemen. *sof* gene was negative in all SNA GBS, GCS and GGS different genotypic strains with no available reports to compare.

9.5.5 Pyrogenic exotoxin superantigen in patients with SNA pharyngotonsillitis

The prophage toxin gene *spd1* was positive and *speC* was negative in all SNA, GBS, GCS and GGS isolates. The superantigen gene *sdnN* was positive in GCS strains but negative in the GBS and GGS isolates. It is noteworthy that all *emm* genotypes of GCS strains were positive for *spd1*, *sdn*, *silC* and *silD* prophage exotoxin superantigens. The GCS strains shared the presence of these virulence genes with the GAS isolates. The presence of these GCS strains with particular genotypes in a susceptible host and their association with these virulence genes traits supports the likelihood of an association with ARF. This is the first report to highlight the prophage gene toxin sequence of specific GCS strains. There are no current reports of these pyrogenic exotoxin superantigens in patients with SNA pharyngotonsillitis available for comparison in the literature.

9.5.6 Streptococcal invasive locus among SNA pharyngotonsillitis

The presenc of the *emm*4 genotype among four GCS and one GGS pharyngotonsillitis demonstrated positive results for both *silC* and *silD* toxin genes which have been similarly reported in invasive streptococcal isolates in France (Bingen et al 2007). Streptococcus invasive locus including *silC* and *silD* in some of these genotypes of GGS and GCS has not been described in the literature.

9.5.7 PFGE pattern interpretation

PFGE profiles of *emm*87 GAS isolates were similar and highly related. GAS *emm*12 and *emm*28 did not differ among their related genotypes in their DNA PFGE profiles fragments. PFGE profiles within a given GCS and GGS *emm* type were not related, but were distinct from other *emm* GCS and GGS profile strains. These results may indicate that each individual *emm* type shared a high degree of genetic relatedness among SNA GCS and GGS genotypes.

9.6 Antimicrobial patterns among GAS and SNA pharyngotonsillitis

9.6.1 Overall antimicrobial patterns among GAS and SNA pharyngotonsillitis

GAS strains were all sensitive to the β- lacatm antimicrobials penicillin and amoxicillin consistent with findings in different countries (Adam, *et al* 2000, Brook and Dohar 2006, Lloyd, *et al* 2007, Palavecino, *et al* 2001). GAS isolates among Yemeni patients exhibited tetracycline and chloramphenicol resistance with rates comparably higher than in studies from Iran and India (Jasir, *et al* 2000, Lloyd, *et al* 2007). All the SNA GBS, GCS and GGS strains were sensitive to penicillin and amoxicillin similar to other reports (Di'az, *et al* 2008, Ergin, *et al* 2003, Zaoutis, *et al* 2001).

GAS developed with increasing resistance to erythromycin to GAS in several countries directly related to antibiotics use in throat infections (Weber 2005). GAS was erythromycin resistant in 36% of the strains and GCS and GGS strains were each 50% resistant to erythromycin which is comparably higher than some reports (Di'az, *et al* 2008). Macrolide resistance is also reported to be widespread among *sof* positive GAS strains consistent with *emm*12 *sof* positive (50%) and *emm*28 *sof* positive (33%) genotypes in this study. GAS *emm*87 and *emm*5 were *sof* positive and erythromycin resistant but it differed from other reports (Bernard Beall et al 2002). The antimicrobial patterns of GBS, GCS and GGS *emm* genotypes varied among their sensitivity and resistance patterns which is reported for the first time. This high macrolide resistance

pattern among GAS, GCS and GGS strains may be explained by the free availability of drugs over the counter in Yemen.

It is necessary to continue further careful research to detect any change in the susceptibility pattern of GAS isolates to antimicrobials and to assess the best-practice for antimicrobial regimens (JoAnn Deasy et al 2009). It is difficult to create and follow a strict single prediction rule for all countries with the changing epidemiology of GAS infections but it is essential to minimize the inappropriate use of antibiotics and avoid antimicrobial resistance.

9.7 Cytokines in acute rheumatic fever and recurrent rheumatic fever

9.7.1 General aspects of Cytokines in ARF and RRF

There were significantly higher concentrations of all the 14 profiles of cytokines and chemokines in patients with RRF than in ARF patients. The significantly higher elevation of TNF-α and IFN-γ in RRF than ARF supports the possibility of their secretion by heart mononuclear cells associated with an autoimmune reaction and suggests that Th1-type cytokines could mediate RHD (Guilherem et al 2004). Inflammatory cytokines IL-1β and TNF-α which were significantly elevated in RRF than in ARF patients correlates with the progression of Aschoff nodule activity during the acute active phase of ARF and RHD and may serve as useful indicators of disease activity. IL-8 was twice as high in children with RRF compared to ARF probably caused by cellular infiltrates in the joints during the ARF period. IL-10 secreted by CD4þ T cells was higher among RRF than ARF patients in contrast to a study in India where higher IL-10 levels were observed in CRHD than ARF patients.

The profile of RANTES, MCP-1, MIP-1α, MIP-1β, IL-9, IP-10 and IL12p70 has not been previously studied in patients with ARF and this is the first study to assess these cheomokines and cytokinesly among patients with ARF and RRF. IL-12p70 was comparable to IFN-γ levels with significantly lower values in patients with ARF than RRF supporting the fact that IL-12p70 regulates Th1 response by inducing IFN-γ. This

was the first study to describe elevated concentrations of IP-10 and IL12p70 in patients with ARF.

9.7.2 Correlations of cytokines and chemokines in patiens ARF and RRF patients

The results in this study support the role of cytokines in the initiation of inflammatory response during the active phase of ARF and RHD. Proinflammatory cytokines TNF-α and IFN-γ showed strong positive correlations with cytokines IL-1β, IL-6, IL-10 and IL-12p70 in both patients with ARF and RRF. Previous reports demonstrated TNF-α, IFG- γ, IL-1β and IL-6 in the production of acute phase reactants. However, this is the first report to describe the correlations of TNF-α and IFN-γ with IL-10 and IL-12p70 among patients with ARF.

The results suggest that the strong colocalization of TNF-α and IFG-γ in patients with ARF and RRF may be associated with carditis, a major feature in both groups. It is well established that the lymphokine IFN- γ preferentially inhibits Th2 proliferation responsible for anti-inflammatory cytokine IL-10 production. This fact does not coincide with this study where IFN-γ was strongly correlated with IL-10 (Th-1 response) among patients with ARF and RRF and needs to be clarified in future studies.

In patients with ARF IL-8 was positively correlated with MIP-1α and IP-10 while among patients with RRF. IL-8 was negatively correlated with IL-12p70. RANTES was only positively correlated with its subgroup MCP-1 in patients with ARF and RRF. The expression of positive correlations between IL-1β and IL-6 with cytokines IL-10 and IL-12p70 was stronger in patients with RRF than ARF. IP-10 was associated with MIP-1β in both patients with ARF and RRF. IP-10 was correlated with MIP-1α in patients with RRF. IL-10 shared strong positive correlations with IL-1 β, IL-6, and IL12p70 in patients with ARF and RRF. IL-10 was only positively correlated with MIP-1β in patients with ARF but with MCP-1 in patients with RRF.

Patients with ARF expressed chemokine MIP-1β with strong positive significant correlations with cytokines IL-8, IL-10, IL-1p270, IP-10, TNF-α and IFG-γ that was not apparent in patients with RRF. RRF patients expressed chemoattractant chemokine

MCP-1with strong positive significant correlations with cytokines, IL-1 β, IL-6, IL-10, IL-1p270, TNF-α and IFG-γ and RANTES which was not evident in patients with ARF.

TNF-α and IFN-γ were not correlated with MIP-1β in patients with RRF and also were not associated with MCP-1 in patients with ARF which serves a potential point to differentiate between the two groups. The chemoattractant chemokine MIP-1β may probably serve as a biological marker in patients with ARF and the chemoattractant cytokine MCP-1 may probably serve as a biological marker in patients with RRF. These differences in the overall pattern of chemokines and cytokines may result from a variation of balance in the inflammatory cells in the lesions and the severity of their damage in patients with ARF and RRF. Further future analysis with a large sample size are required to study these correlations with different cytokines and chemokines that have not been reported in patients with ARF to explain their pathogenic importance.

The identification of these specific chemokines and cytokines and their significant correlations may contribute towards a better understanding on immunopathogenesis of ARF and RHD. This information will introduce novel possibilities for immunotherapy such as T-cell vaccination for patients with severe RHD. The molecular knowledge of the autoimmune reactions mediated by intralesional T cells will aid in the choice of GAS protective epitopes for an effective and safe vaccine (Guilherme et al 2005)

9.8 Conclusions

The prevalence of RHD among Yemeni school children was 36.5/1000. This is higher than in other Middle Eastern countries and demonstrates that RHD continues to be a major and serious health problem in Yemen.

In the primary health care and school survey a high prevalence of GAS pharyngotonsillitis was detected particularly in the 11 – 15 years age groups. The most frequent genotypes of GAS isolated from children with a history of ARF and RHD were *emm*87, 12, 28 and 5. The genotypes *emm* 87 and *emm*28 were possible new rheumatogenic GAS strains not previously reported. The existence of a significant proportion of newly recognized *emm* types with a variety of characteristics, suggests

that strains circulating in Yemen, an area not previously surveyed, may be different from those known to circulate in other geographic areas. Such information can contribute to a better understanding of the local and global dynamics and epidemiologic aspects of GAS and GGS, GCS and GBS infections occurring in different regions.

SNA strains, GCS, GGS and GSB were infrequent causes of pharyngotonsillitis compared with GAS. This study presents the genotypic characteristics of GCS, GGS and GBS isolates belonging to seven new *emm* sequence types first detected among Yemeni children with acute pharyngotonsillitis having a history of ARF and RHD.

This is the first report to describe the pattern of the exotoxin prophage superantigen genes *spec, spd1, sdn, silC, silD* and *sof* of *emm* 87, 12, 28 and 5 GAS and GCS, GGS and GBS isolates in patients with pharyngotonsillits and a history of ARF and RHD. The PFGE profiles of *emm*87, 12 and 28 GAS isolates did not differ within their related genotypes. GAS and SNA isolates still remain susceptible to the majority of antimicrobials tested including the β-lactams antibiotics penicillin and amoxicillin. There were some erythromycin resistant strains.

Seven new cytokines and chemokines RANTES, MCP-1, MIP-1α, MIP-1β, IP-10 and IL12-p70 were investigated for the first time in patients with ARF. They were elevated in patients with ARF and RRF. The chemokine MIP-1β may be a potential immunological biomarker in patients with ARF due to its strong positive correlation with cytokines IL-8, IL-10, IL-12p70, IP-10,TNF-α and IFG-γ. MCP-1 may probably be a potential immunological biomarker in patients with RRF with RHD due to its strong positive correlation with cytokines IL-1β, IL-6, IL-10, IL-1p270, TNF-α and IFG-γ and RANTES.Chemokines direct the migration of leukocytes throughout the body and play an important role in the inflammatory response. Serum chemokines levels may be clinically useful as an objective indicator of activity and may have the potential to improve disease management. Patients with high serum chemokine levels may be more likely to develop early recurrence with possible cardiac valve involvement and end up with RHD. Monitoring frequent chemokine levels in rheumatic patients could provide a tool to possibly evaluate the disease activity and identify patients with a likelihood of

future recurrences and RHD development. Further prospective studies with larger number of patients are needed to make firm conclusions.

9.9 Recommendations

It is important to use schools as a means to educate and motivate teachers, parents and children about the importance of treatment of sore throats. Primary prevention of a first attack of ARF in children with oral penicillin for GAS pharyngotonsillitis requires strong evidence (Lennon, *et al* 2009). Penicillin may fail to prevent an acute attack of ARF. Guidelines for diagnosis and management of GAS pharyngotonsilitis with cheap and available rapid tests will aid prevention of ARF(Lennon and Craig 2009). Primary health care delivery will be more effective when focused on a wider community of individuals including siblings and schools to reduce GAS load on the population

It is time for the public health authorities to understand the burden of this hazardous preventable heart disease ARF/RHD and provide necessary financial and manpower resources to promote a successful program of control with support of policy makers.

Future studies are required to assess the diagnosis of pharyngotonsillitis in children and should include the two clinical signs of petechie in the soft palate and red erythematous uvulawhich in this study were predictive clinical criteria of GAS pharyngotonsillitis. Further research is required to investigate the role of SNA in the causation of ARF/RHD.

It is important to monitor GAS isolates and their *emm* genetic variability through active and continuous surveillance to lead to a better understanding of the epidemiology of specific GAS strains in different countries.Further large sample size studies of are required to confirm the role of these chemokines as biomarkers for ARF and increase our understanding of how they act in the complex network of autoimmune reactions that occur in ARF/RHD. A cohort longitudinal study or cross-sectional study of patients with ARF, RRF and RHD with a control healthy group adjusted for age sex and race. The patients should be followed with frequent visits (4 to 5 visits per year) during active and inactive stages of the disease. Chemokine levels could then be compared at consecutive visits, either before and during active disease or before and during inactive

stage. The development and use of the advanced techniques in molecular biology will enhance understanding of the immunology of ARF/RHD which is required for the production of a vaccine.

9.10 Limitations of the study

The presence of a portable echocardiography in the field work could have minimized the loss of participants who were clinically diagnosed as RHD. Transport problems caused a delay in taking some of the throat isolates to the laboratories which might have led to some false negative cultures in positive RADT specimens. The absence of LancefieldStreptococci Grouping kits during the field work may have caused an underestimate of the SNA contribution to pharyngotonsillits. Detailed study of the streptococci obtained from throat swabs was only undertaken on a subgroup of the total (n=34) due to limited funding. The study of genotype and prophage exotoxin superantigen genes on a larger sample size of GAS and SNA isolates would have provided more comprehensive results on the epidemiologic aspects of these infections.

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11 Appendices

11.1 Appendix (A): Questionnaire (I)

11.1.1 Questionnaire for children with ARF and RHD

Serial number [ ] Code [ ] Date [ ]

Name [ ] Age [Years] Gender: [M] [F]

Address			
Permanent Address	House number:	Street	Telephone number
	District:	Governorate:	
Temporary Address	House number:	Street	Telephone number
	District:	Governorate:	

Name of School: [ ] District: [ ]

Primary class level [ ] No of students in school [ ]

Epidemiological background:

Family History			
	First degree relative	[ ]	Family income:

Consanguinity

Second degree relative

Third degree relative

Riyals per month:

Family size

No of siblings

Sibling's order

No of bedrooms

Persons per bedroom

Housing Conditions

Good

Satisfactory

Bad

Occupation:

Father

Mother

Education level

Father

Mother

Access to Health Service

Primary

Secondary

Tertiary

Family history

Relatives affected

Relation to patient

Relation to patient

Sore throat

Scarlet fever

URTI

RF

RHD

Clinical Background

Weight | Height | BMI | MAC.

Nutritional status | Grade I ☐ | Grade II ☐ | Grade III ☐

Temp ☐ | P.R. ☐ | R.R. ☐ | B.P. ☐

Major Manifestations

Carditis

Apical systolic murmur ☐ Apical mid-diastolic murmur ☐

Change in murmur ☐ Cardiac enlargement ☐

Pericarditis ☐ Congestive cardiac failure ☐ Arrhythmias ☐

Cardiac lesion

MR ☐ MS ☐ AR ☐ TR ☐ AS ☐ Combined lesions ☐ ☐

Arthritis

Single /Multiple Ankle ☐ Knee ☐ Hip ☐

Wrist ☐ Hand ☐ Elbow ☐ Shoulder ☐

Chorea

Subcutaneous nodule Site ☐ Number ☐

Erythema marginatum Site ☐ Number ☐

Minor Manifestations

Fever ☐ Arthralgia ☐ Previous ARF ☐ Previous RHD ☐

Recent sore throat ☐ Recent scarlet fever ☐

Others: Abdominal symptoms ☐ Pleural and pulmonary manifestaions ☐

Epiataxis ☐ Anemia ☐ Vomiting ☐ Weight loss ☐ Malaise ☐

Para clinical Investigations

Hb ☐ gm% WBC Total ☐ P ☐ L ☐ E ☐ B ☐

ESR ☐ ASO ☐ CRP ☐ MPS ☐ Others ☐

Chest x-Ray

ECG

Throat swab ☐ ☐ Gram stain ☐ Culture ☐ Sensitivity ☐

Echocardiography findings

Drugs	Yes	No		Yes	No
Penicillin	☐	☐	Aspirin	☐	☐
Steroids	☐	☐	Digoxin	☐	☐

Furosemide			Vitamins Tonics		

Prophylaxis

Primary Drug [ ] Dose [ ] Frequency [ ] Duration [ ]

Secondary Drug [ ] Dose [ ] Frequency [ ] Duration [ ]

Secondary Prophylaxis Regular [ ] Irregular [ ] Duration [ ]

Complications	Infect Endocarditis	CCF	Arrhythmias	Others
Rheumatic fever Recurrence	Frequency	Interval	Major criteria	Minor criteria

Hospital admission [ ] Duration of stay [ ] Number [ ] Interval [ ]

Surgical Intervention if any [ ] Dental [ ] Tonsillectomy [ ] Others [ ]

Outcome: Full recovery [ ] Recovered but with valvulopathy [ ]

Others [ ] Cause of Death [ ]

11.1.2 Questionnaire for children with Sore Throat

Serial number [ ] Code [ ] Date [ ]

Name [ ] Age [ ] Sex [ ]

Polyclinic		Area of Residence	
School		Name of district	

Permanent Address	House number:	Street	Telephone No
	District:	Governorate:	

Clinical Manifestations of Sore Throat

Group A Beta Hemolytic Streptococcal Infection

Symptoms	Yes	No	Signs	Yes	No
Sudden onset			Erythema on tonsil		
Sore throat			Erythema on pharynx		
Headache			Exudate on tonsil		
Fever 38 – 40'C			Exudate on pharynx		
Pain on Swallowing			Swollen tender anterior cervical lymph nodes		
Nausea			*Soft* palate petechiae		
Vomiting			Red swollen uvula		
Abdominal pain			Skin Rash		

McIsaac Scoring System

Criteria	Point

Temperature > 38'C	1
No cough	1
Tender cervical lymphnodes	1
Tonsillar swelling or exudates	1
Age 3 – 15 years	1

No of attacks per year ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐ ☐

No of attacks per 6 months ☐ ☐ ☐ ☐ ☐ ☐

No of attacks per month ☐ ☐ ☐ ☐

Family history	No of Relativesaffected	Relation to patient	Relation to patient
Sore throat			
URTI			
RF			
RHD			

Para clinical Investigations

WBC Total		P		L		E		B		ESR	
ASO titer				CRP							

	Positive	Negative
Gram stain		
Culture		
Catalase test		
Bacitracin Sensitivity		

Rapid Antigen Detection Test: Positive ☐

Negative ☐

Doubtful ☐

ASOT Positive ☐ Value ☐

Negative ☐

CRP Positive ☐ Value ☐

Negative ☐

Complications of GABHSI			
Suppurative		Non-Suppurative	
Peritonsillar abscess(quinsy)	☐	Rheumatic fever	☐
Para-pharyngeal abscess	☐	Acute GNephritis	☐
Retro-pharyngeal abscess	☐	Reactive arthritis	☐

Treatment

Symptomatic: Adequate Fluid intake ☐ Warm water gargle ☐

Analgesics: Paracetamol ☐ Ibuprofen ☐

Others ☐ Herbal medicine ☐

Antibiotics

Type	Dose	Frequency	Route	Duration
Oral Penicillin				
Amoxicillin				
Benzathine Penicillin				
Procaine Penicillin				
Erythromycin				
Clindamycin				
Others				

Surgery

Tonsillectomy ☐ Age done ☐ Date ☐

What were the Indications for Tonsillectomy?

Sore throat due to tonsil inflammation ☐

3 episodes in 6 months ☐

4 episodes in 12 months ☐

>4 episodes in 12 months

Peritonsillar abscess ☐ Attacks interfere with patients function ☐

Patient at risk for Rheumatic fever ☐ or RHD ☐

Relative at risk for Rheumatic fever ☐ or RHD ☐

11.2 Appendix (C) Consent Forms

11.2.1 Minor Child Consent Form No. 1

Primary Investigator: Dr. Iman Ali Ba-Saddik

Title of Project: Rheumatic Fever and Rheumatic Heart Disease: Prevalence Among Yemeni School Children and Role of Genetics in Predisposition

I acknowledge that on ________________(date) I was informed by:

Dr.Iman Ali Ba-Saddik, Pediatric Specialist at the department of Pediatrics and Child Health, Faculty of Medicine and Health Sciences, Aden University; of a project having to do with the following :

Screening for normal school children will be undertaken by simply listening to their chest with a stethoscope to find out if any of them may have a heart problem even though if they are asymptomatic; If any abnormal heart sound is heard by simple auscultation, they will be referred to a pediatric cardiologist to perform an echocardiography to diagnose the type of cardiac defect and then given the recommended treatment.

I have understood the verbal communication to parents providing the essentials of the proposed project AND AM HAPPY FOR MY CHILD TO TAKE PART. I am fully aware of the nature and extent of my child's participation in this project and agree with full knowledge to all details to allow my child to participate. I understand that I may

withdraw my child's participation from the research at any time without any penalty of any kind; and that the investigator will take into consideration my child's feeling and protect my child's privacy.

Child's name:______________________________

Relation to child: __________________________

Date: ____________________

I do give my consent for my child to participate: ___________________

11.2.2 Minor Child Consent Form No.2

Primary Investigator: Dr. Iman Ali Ba-Saddik

Title of Project: Rheumatic Fever and Rheumatic Heart Disease: Prevalence Among Yemeni School Children and Role of Genetics in Predisposition

I acknowledge that on __________________(date) I was informed by:

Dr.Iman Ali Ba-Saddik, Pediatric Specialist at the Department of Pediatrics and Child Health, Faculty of Medicine and Health Sciences, Aden University; of a project having to do with the following :

The common bacterial causes of throat infections among our children which may lead to a heart problem are group A βeta hemolytic streptococci. We would like to undergo this survey to identify the prevalence of this infection among patients with sore throat. The test will be done by taking two throat swabs from the patients at the pediatric polyclinic. One throat swab will be immediately tested on a diagnostic slide to check for the presence of these bacteria. If this test turns to be positive within 10 to 15 minutes, then the second throat swab will be taken to specialized laboratory. In this diagnostic laboratory a medical microbiologist will grow these bacteria on special plates by culture methods to detect the specific family strains of these bacteria. The positive culture test and the sensitivity of the antibiotic to the specific bacteria will determine the treatment to be given to your child to avoid the future heart complications on your child.

I have understood the verbal communication to parents providing the essentials of the proposed project AND I AM HAPPY FOR MY CHILD TO TAKE PART. I am fully aware of the nature and extent of my child's participation in this project and agree with full knowledge to all details to allow my child to participate. I understand that I may withdraw my child's participation from the research at any time without any penalty of any kind; and that the investigator will take into consideration my child's feeling and protect my child's privacy.

Child's name:________________________________

Relation to child: ____________________________

Date: ______________________

I do give my consent for my child to participate: ____________________

11.2.3 Minor Child Consent Form No.3

Primary Investigator: Dr. Iman Ali Ba-Saddik

Title of Project: Rheumatic Fever and Rheumatic Heart Disease: Prevalence Among Yemeni School Children and Role of Genetics in Predisposition

I acknowledge that on ________________(date) I was informed by:

Dr.Iman Ali Ba-Saddik, Pediatric Specialist at the department of Pediatrics and Child Health, Faculty of Medicine and Health Sciences, Aden University; of a project having to do with the following :

Your child has been diagnosed having Rheumatic Heart Disease by Echocardiography at the cardiac clinic. A simple blood test through venipuncture

will be taken to investigate the possible role of immunogenesis in this heart problem. This special genetic test will help in the determination of the autoimmune pattern of this disease in risky children.

I have understood the verbal communication to parents providing the essentials of the proposed project AND AM HAPPY FOR MY CHILD TO TAKE PART. I am fully aware of the nature and extent of my child's participation in this project and agree with full knowledge to all details to allow my child to participate. I understand that I may withdraw my child's participation from the research at any time without any penalty of any kind; and that the investigator will take into consideration my child's feeling and protect my child's privacy.

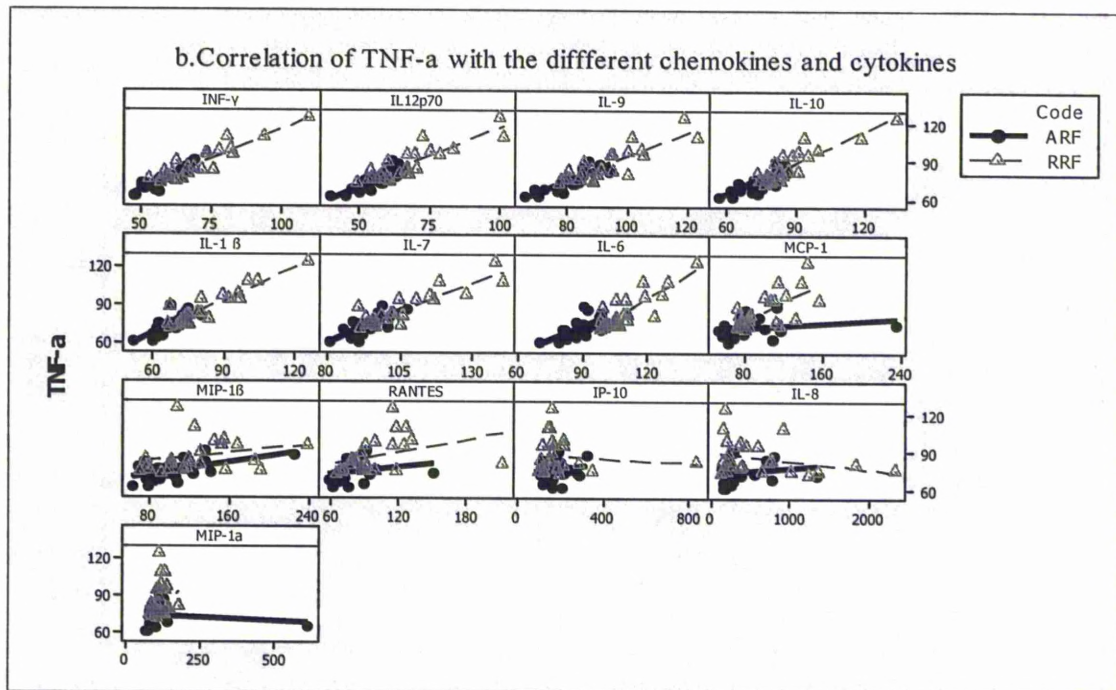
Child's name: ______________________________

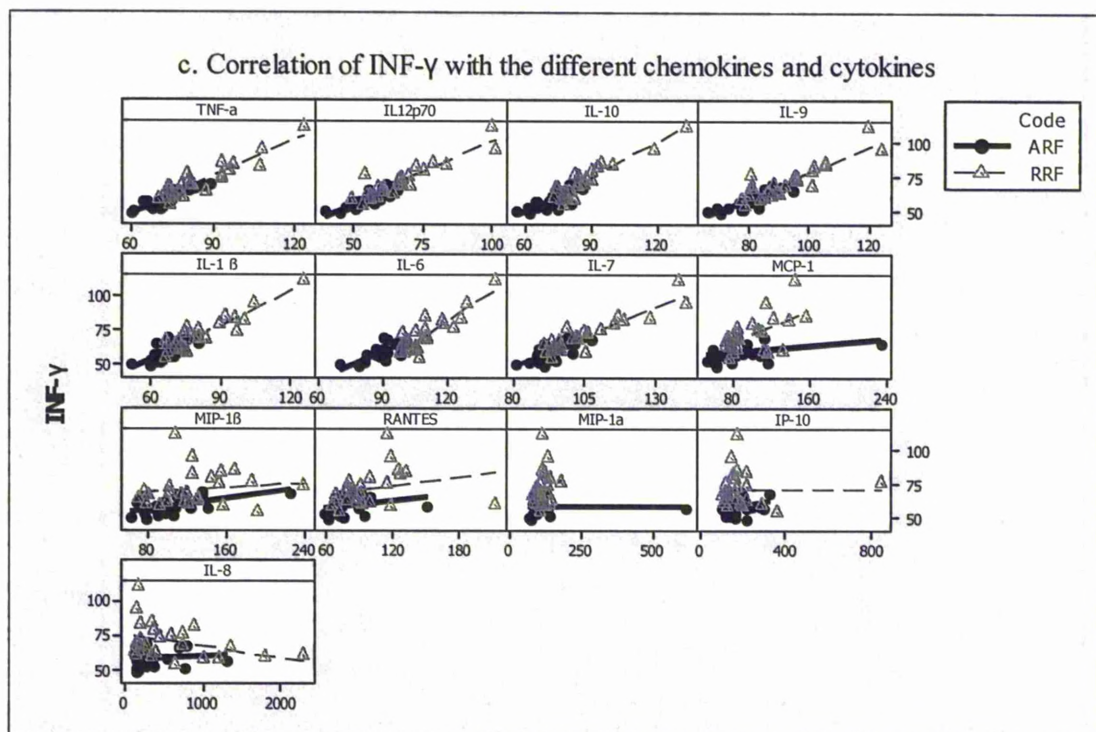
Relation to child: __________________________

Date: ____________________

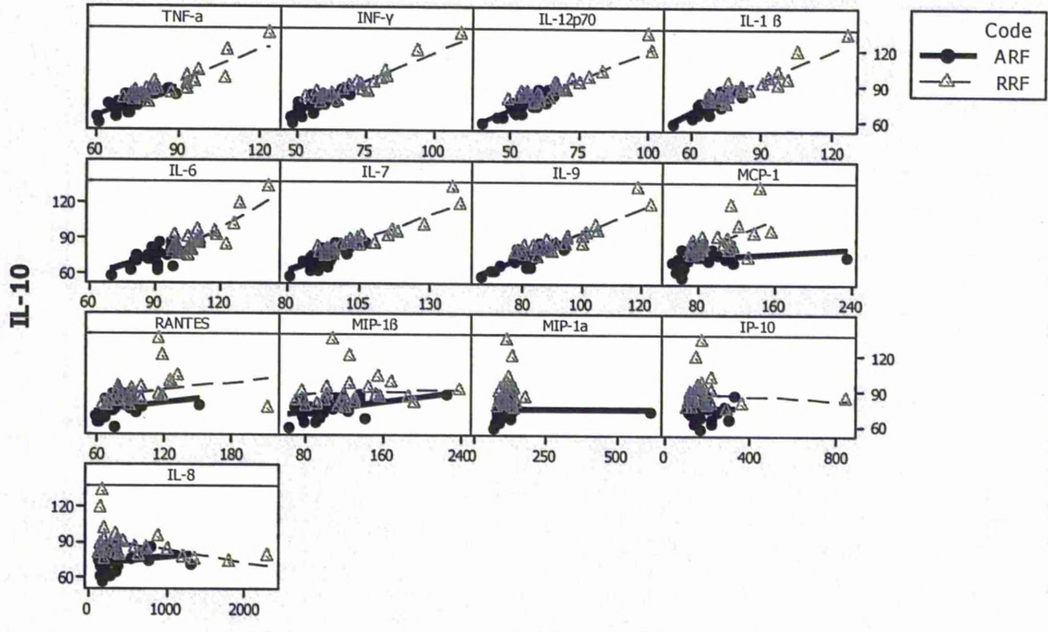
I do give my consent for my child to participate: ___________________

11.2.4 Appendex D Correlations of the different chemokines and cytokines in patients with ARF and RRF

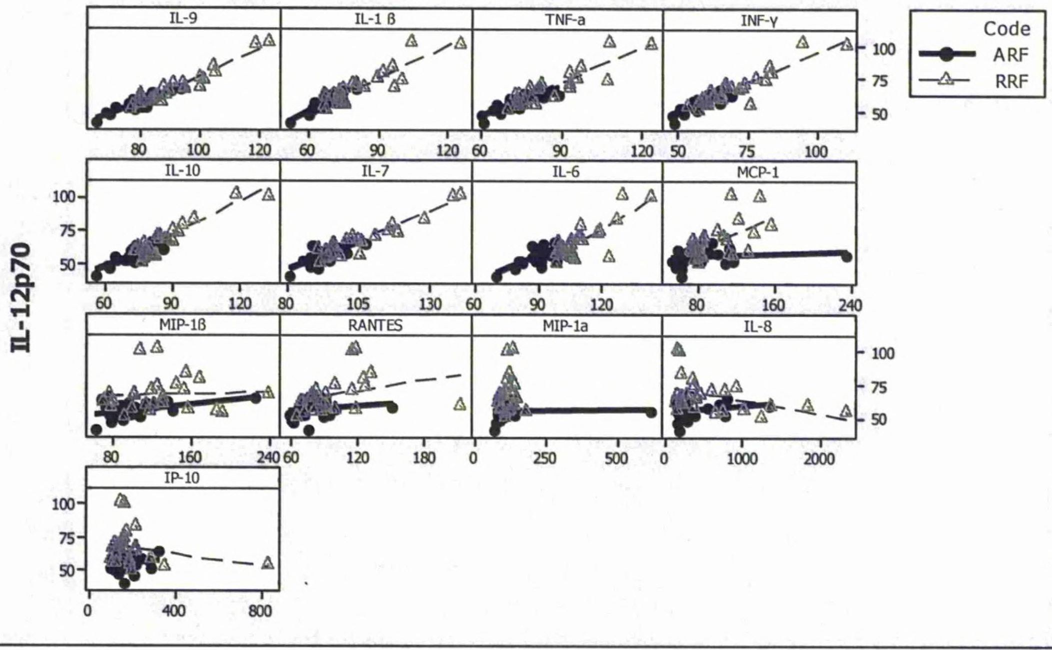


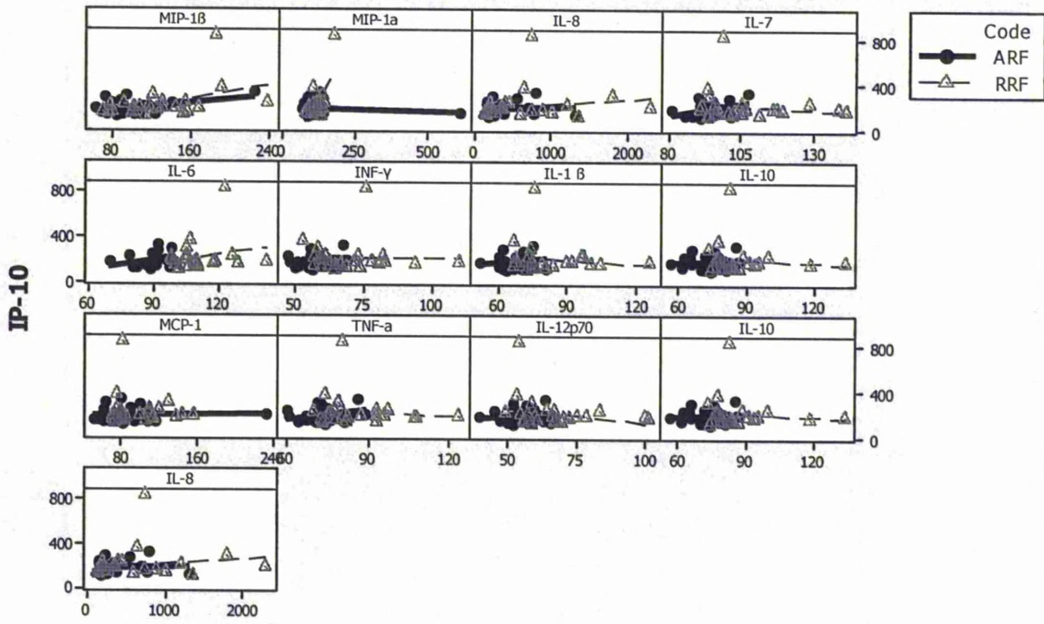


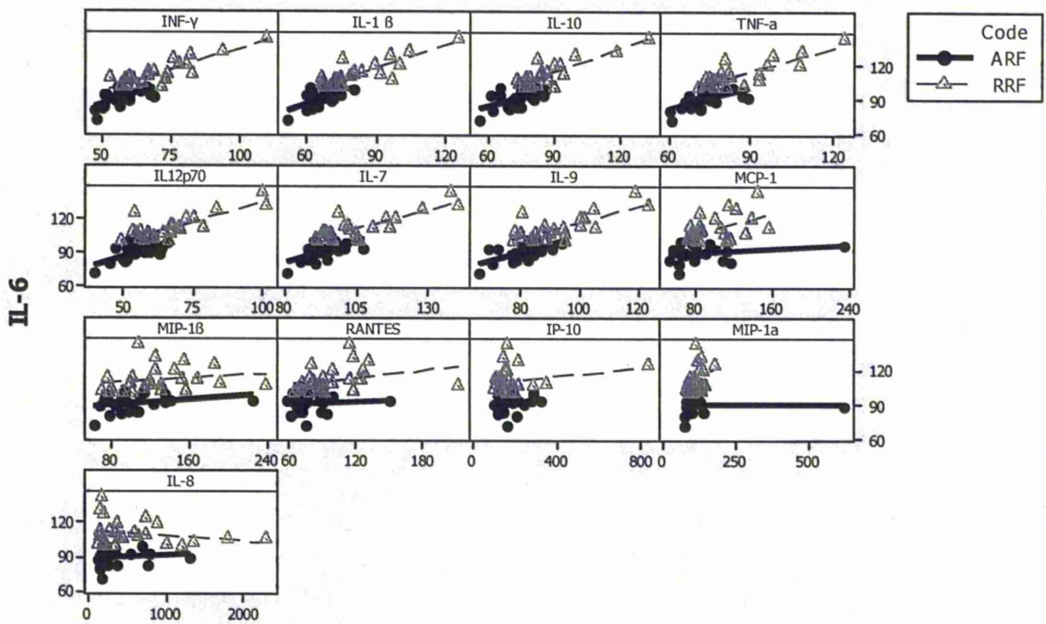
h. Correlation of IL-10 with the different chemokines and cytokines

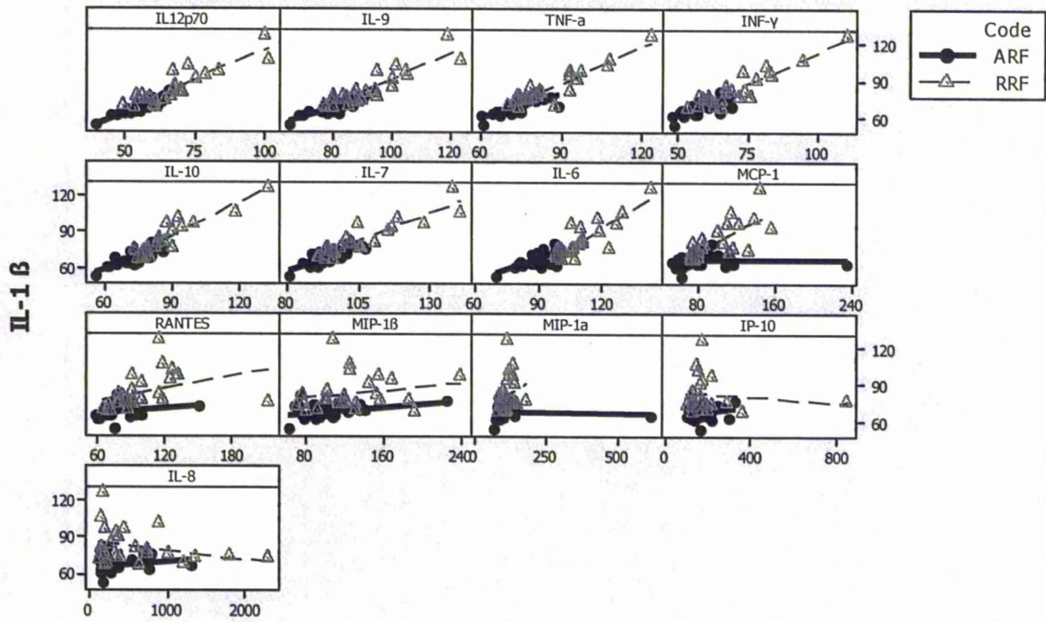


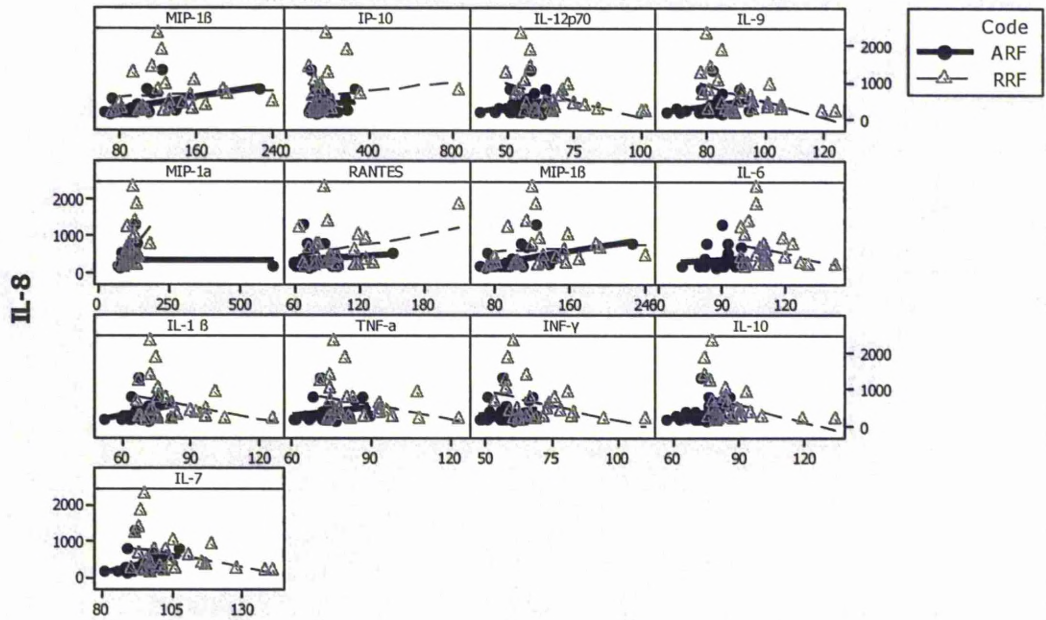
e. Correlation of IL-12p70 with the different chemokines and cytokines

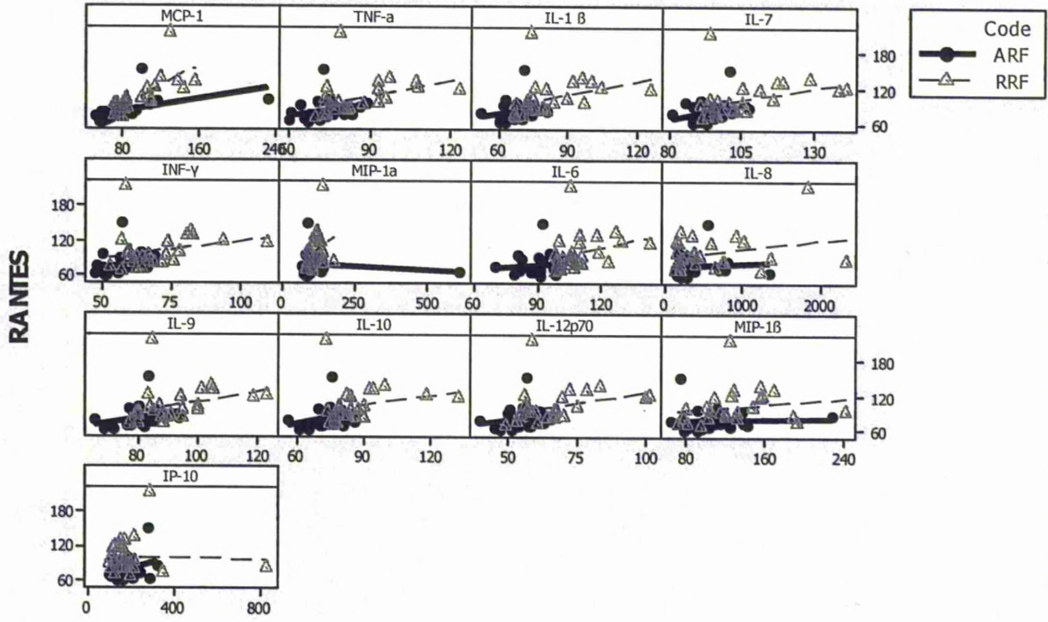


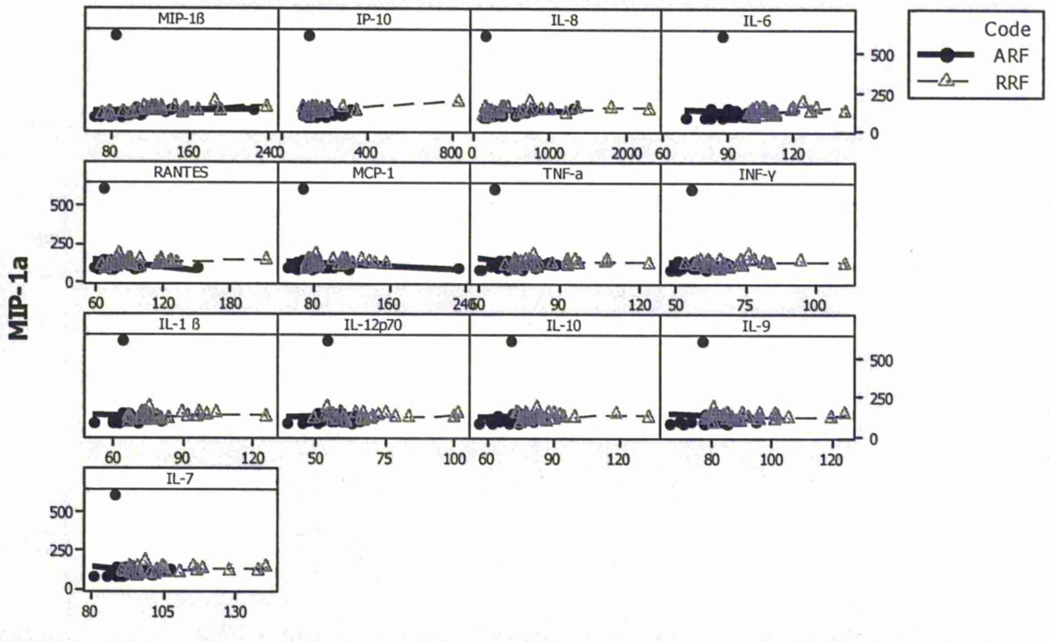

i. Correlation of IP-10 with the different chemokines and cytokines
IP-10
MIP-1β
MIP-1a
IL-8
IL-7
IL-6
INF-γ
IL-1 β
IL-10
MCP-1
TNF-a
IL-12p70
IL-10
IL-8
Code
ARF
RRF


d. Correlation of IL-6 with the different cytokines and chemokines
IL-6
INF-γ
IL-1 β
IL-10
TNF-a
IL12p70
IL-7
IL-9
MCP-1
MIP-1β
RANTES
IP-10
MIP-1a
IL-8
Code
ARF
RRF


a. Correlation of IL-1 ß with the different chemokines and cytokines
IL12p70
IL-9
TNF-a
INF-γ
IL-10
IL-7
IL-6
MCP-1
RANTES
MIP-1ß
MIP-1a
IP-10
IL-8
IL-1 ß
Code
ARF
RRF


j. Correlation of IL-8 with the different cheokines and cytokines
MIP-1ß
IP-10
IL-12p70
IL-9
MIP-1a
RANTES
MIP-1ß
IL-6
IL-1 ß
TNF-a
INF-γ
IL-10
IL-7
IL-8
Code
ARF
RRF


k. Correlation of RANTES with the different chemokines and cytokines
MCP-1
TNF-a
IL-1 ß
IL-7
INF-γ
MIP-1a
IL-6
IL-8
IL-9
IL-10
IL-12p70
MIP-1ß
IP-10
RANTES
Code
ARF
RRF


l. Correlation of MIP-1a with the different chemokines and cytokines
MIP-1ß
IP-10
IL-8
IL-6
RANTES
MCP-1
TNF-a
INF-γ
IL-1 ß
IL-12p70
IL-10
IL-9
IL-7
MIP-1a
Code
ARF
RRF