STUDY OF β-HAEMOLYTIC STREPTOCOCCI AMONG PHARYNGITIS CASES VISITING BIR HOSPITAL

A

DISSERTATION

SUBMITTED TO THE CENTRAL DEPARTMENT OF MICROBIOLOGY

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BY

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RECOMMENDATION

This is to certify that Mr. Atmaz Kumar Shrestha has completed this dissertation work entitled **"Study of β-haemolytic streptococci among pharyngitis cases visiting Bir hospital"** as a partial fulfilment of Master of Science degree in Microbiology (Medical) under our supervision. To our knowledge this work done has not been submitted for any other degree.

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ABSTRACT

Pharyngitis is commonly encountered upper respiratory tract illnesses for which patients seek medical advice. The purpose of this study was to analyze the underlying bacterial pathogens in pharyngitis cases. During study period February 2010 to August, 2010, a total of 134 patients visiting E.N.T. OPD of Bir hospital complaining sore throat were studied. Throat swabs collected were cultured to 5.0% blood agar plate and incubated at 37^0C of 24-48 hrs, clear transparent β-haemolytic colonies were selected and identified. Group A streptococcus (GAS) was identified by β-haemolytic colonies with Bacitracin and Penicillin sensitivity, Gram staining, catalase negativity and growth on crystal violet blood agar (CVBA). In this study 23 (17.2%, 23/134) of patients were positive for β-haemolytic streptococci (BHS), among them 12 (9%) were identified as GAS. The prevalence of culture positive for BHS was highest (56.5%, 13/23) among the age group of 21-40 years. Antibiotic susceptibility test for isolates was done by using modified Kirby-Bauer disk diffusion method. Pathogens other than BHS were isolated from 9 patients, of which 4 cases showed mixed bacterial growth i.e. 14 bacterial isolates from 9 cases. Other bacterial isolates reported in the study were *Staphylococcus aureus* 6 (4.5%), *Klebsiella pneumoniae* 5 (3.7%), *Pseudomonas aeruginosa* 2 (1.5%), *Citrobacter freundii* 1 (0.7%), and yeast cells 1 (0.7%). It was found that infections caused by β-haemolytic streptococci is significantly higher P=0.0084 i.e. (P<0.05) than infections caused by other bacteria. This study demonstrated that young adults of age group 21-40 were affected by streptococcal pharyngitis. The isolates of GAS were 100% sensitive to Amoxycillin, Cephalexin, Cefotaxime, Ciprofloxacin and Azithromycin followed by Cotrimoxazole (91.7%) and Erythromycin (91.7%).

Key words: β-haemolytic streptococci, BHS, Bir hospital, GAS, Pharyngitis, Sore throat

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LIST OF ABBREVIATION

APT	Acute pharyngo-tonsillitis
ARF	Acute Rheumatic Fever
ARI	Acute Respiratory Infection
ASM	American Society of Microbiology
AST	Antibiotic Susceptibility Test
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BA	Blood Agar
BHS	β-Haemolytic Streptococci
CA	Chocolate Agar
CAMP	Christie, Atkins and Munch Peterson
CDC	Center for disease control
CHF	Congestive heart failure
CLSI	Clinical and Laboratory Standard Institute
D	Dalton
DNA	Deoxy-ribonucleic acid
FDA	Food and Drug Administration
FDP	Fibrin Degradation Products
GAS	Group A Streptococcus
GTP	Guanosine triphosphate
ICE	Integrative and conjugative element
IL-1	Interleukin-1
LPS	Lipopolysaccharide
MA	MacConkey agar
MDR	Multi Drug Resistant
MF	Mitogenic factor
MFS	Major Facilitator Superfamily
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
MLS	Macrolide, Lincosamide, and Streptogramin
MR	Methyl Red
NA	Nutrient Agar
NADase	Nicotine-Adenine-Dinucleotidase
NCCLS	National Committee for Clinical Laboratory Standards
NK	Natural killer
PCR	Polymerase Chain Reaction
PMNL	Polymorphonuclear Leukocytes
RADT	Rapid Antigen Detection Test
RBC	Red Blood Cells
RF	Rheumatic fever
RHD	Rheumatic Heart Disease
RNA	Ribonucleic acid
RT	Recurrent Tonsillitis
SBA-SXT	Sheep Blood Agar Containing Trimethoprim – Sulfamethoxazole
SIM	Sulfide indole motility
SLO	Streptolysin O

SPE	Streptococcal pyrogenic exotoxins
StrepTSS	Streptococcal Toxic Shock Syndrome
STs	Sequence Types
SXT	Sulfomethoxazole
TLRs	Toll-like receptors
TMP-SXT	Trimethoprim-Sulfomethoxazole
TSI	Triple sugar iron
TSS	Toxic shock syndrome
URT	Upper Respiratory Tract
URTI	Upper Respiratory Tract Infections
VP	Voges Proskauer
WHO	World Health Organization

CHAPTER-I

INTRODUCTION AND OBJECTIVES

1.1 Background

Diseases of upper respiratory tract (URT) are named according to the anatomic sites involved. Most of these infections are self-limiting and most are caused by viruses. The disease of upper respiratory tract includes laryngitis, laryngotracheobronchitis, epiglottitis, pharyngitis, tonsillitis, peritonsilar abscesses, rhinitis, stomatitis, thrush, peridontal infections and salivary gland infections (Forbes, *et al*., 2007). Viral infections account for approximately 70% of all pharyngitis and bacteria are responsible for rest of 20-30 % (Stillerman and Bernstein, 1961; Somro *et al*., 2011).

Streptococcus pyogenes (group A streptococcus, GAS) is an important species of Gram-positive pathogen which colonizes the throat or skin and is responsible for a broad spectrum of diseases that range from simple and uncomplicated pharyngitis and skin infections (impetigo, erysipelas, and cellulitis) to scarlet fever and life-threatening invasive illnesses including pneumonia, bacteremia, necrotizing fasciitis, streptococcal toxic shock syndrome (TSS), and nonsuppurative sequelae such as acute rheumatic fever, reactive arthritis and glomerulonephritis (Cunningham, 2000; Pfoh *et al*., 2008).

Streptococcal pharyngitis continues to be one of the most common childhood illnesses throughout the world, with an estimated 7.3 million outpatient physician visits each year among children in the USA, 15% to 36% of which are due to GAS (Bisno, 1996; Linder *et al*., 2005). Rheumatic fever (RF) is a delayed sequel to GAS pharyngitis (Dale and Beachey, 1986; Adderson *et al*., 1998).

Group A streptococcal infection is endemic throughout the world, but sporadic epidemics are common, particularly among school children, in residential facilities for the elderly, and in other unique populations such as military personnel. Although group A streptococcal colonization and infection of the URT is common and can occur in people of any age, streptococcal pharyngitis/tonsillitis primarily affects children between the ages of 5–15 years. In 1994, it was estimated that 12 million individuals suffered from RF and RHD worldwide (WHO, 1994), and at least 3 million had

congestive heart failure (CHF) that required repeated hospitalization. The epidemiological association between group A β-haemolytic streptococcal infections and the subsequent development of acute rheumatic fever (RF) have been well established. RF is a delayed autoimmune response to Group A streptococcal pharyngitis, and the clinical manifestation of the response and its severity in an individual is determined by host genetic susceptibility, the virulence of the infecting organism, and a conducive environment (WHO, 2004).

It is estimated that over 600 million cases of symptomatic GAS pharyngitis occur annually among people aged over 4 years, and that over 550 million of these occur in less developed countries. Additional cases occur in children aged under 5 years, although GAS pharyngitis becomes less common in younger children. Therefore, it seems reasonable to conclude that approximately 15% of school-age children will suffer a symptomatic episode of GAS culture-positive pharyngitis each year in more developed countries, and that 4–10% of adults will be similarly affected. Based on the data from India, Kuwait, and the New Zealand, Maori and Pacific Islander populations, the incidence in less developed countries may be five to ten times greater (Carapetis *et al*., 2005).

Few studies have been conducted to ascertain the incidence and prevalence of GAS in developing countries. However, the studies were non-standardized with regards to the laboratory investigation methods for throat culture diagnosis (Steinhoff and Rimoin, 2004). The reported incidence of culture-proven GAS pharyngitis in prospective studies in a few sites appears to be much higher than that reported from the United States in the 1950s: up to 900 versus approximately 200 per 1,000 child-years of observation (Somro *et al*., 2011).

In the developing countries pharyngo-tonsilitis caused by GAS remains an endemic disease with annual incidence range from 100-200 per 1,00,000 school children is major cause of cardiovascular mortality (Oliver, 2000). RF is reported occur in 1-3% of streptococcal throat infections of children living in underprivileged conditions (Shrestha *et al*., 2001).

Several programmes for the control of streptococcal infections are being carried out in developed countries like USA and in developing countries like India. WHO also

recommends very large treatments coverage of streptococcal infections as well as having adequate diagnostic facilities for identifying streptococcal throat infections for developing countries within the infrastructures of existing health care delivery in neighbouring countries like India, Bangladesh, Pakistan, Burma, Thailand, Srilanka. Some part of survey, control and/or prevention programmes for streptococcal infections are being carried out (WHO, 2005).

Nepal is a developing country and has poor health indicators. It ranks 138th of the 169 countries on the Human Development Index of the United Nations of 2010 (UNDP, 2011). Ministry of Health and Population, Government of Nepal has recently decided to launch a National Program for Prevention and Control of RF/RHD in Nepal. This program is being launched with technical and Organizational support from Nepal Heart foundation. This is the 1st project launched by Government of Nepal for the Prevention and control of heart diseases. Nepal Heart Foundation is the main authorized organization for implementation of this program (Nepal Heart Foundation, 2010). There is very little information about epidemiological association between pharyngitis caused by GAS infections and the subsequent development of RF/RHD in Nepal. Therefore, the problem of RF/RHD in our context is quite great due to seriousness of this disease leading to disability in young adults, high mortality and morbidity and high costs of surgical treatment (Yadav *et al*., 2010). There is an urgent need to launch a National Programme for the control of RF/RHD in Nepal with emphasis on primary and secondary prophylaxis of the disease (Regmi and Pandey, 1997).To initiate control programme of RF/RHD data on the prevalence and incidence of β-haemolyic streptococci especially GAS pharyngitis are required. However, only scant information is available in Nepal regarding the magnitude of this problem (Prashad and Bhattarai *et al*., 1990). There is importance of regular surveillance programs to monitor the rate of GAS carriage and the antibiotic susceptibility of GAS isolates in the community.

This study is expected to explore the current situation about prevalence of streptococcal carriage in the throat of symptomatic patients in Bir Hospital. Moreover, antibiotic sensitivity pattern is important for treatment of streptococcal infection.

1.2 Objectives

1.2.1 General Objective

To isolate bacterial pathogens from pharyngitis cases and study antibiotic susceptibility pattern of the isolates.

1.2.3 Specific Objectives

I. To find out β-haemolytic streptococci in pharyngitis cases.
II. To isolate other possible pathogens.
III. To perform antibiotic susceptibility of isolates.
IV. To correlate direct Gram staining in relation to culture of β-haemolytic streptococci.

CHAPTER-II

LITERATURE REVIEW

2.1 Respiratory Tract Infections Introduction

Among the many infections that confront clinicians every day, there is probably no more common and yet controversial one than pharyngitis/tonsillitis caused by group A β haemolytic streptococcus (*Streptococcus pyogenes*). This illness concerns clinicians because not only is there an acute illness for an individual patient, but the potential spread of the organism and the resulting public health implications cannot be ignored either (Salazar, 2004). *S. pyogenes* may be carried in upper respiratory tract of humans it is rarely considered as normal flora and should be deemed clinically important whenever it is encountered (Forbes *et al*., 2007). The ultimate source of group A streptococci is a person harbouring these organisms. The individual may have a clinical or subclinical infection or may be a carrier distributing streptococci directly to other persons via nasal discharges and droplets from the respiratory tract or skin (Brooks *et al*., 2004).

In temperate regions GAS pharyngitis generally peaks in the late winter and early spring months. Studies conducted in North America have shown that carriage rates tend to increase during the early winter and peak in the spring months (Cornfeld and Hubbard, 1961; Majeed *et al*., 1992). GAS pharyngitis generally peak during the cooler seasons of tropical countries as well (El-Kholy *et al*., 1974; Carapetis *et al*., 1999; Nandei *et al*., 2001). A study by Sarkar *et al*. (1988) in Varanasi, India, suggested a significant increase in cases of GAS pharyngitis during the cooler months, similar observation is reported from Nandei *et al*. (2001) from northern India and from Koshi and Benjamin, (1977) in Vellore, India. Carriage also appears to be seasonal, El Kholy *et al*. (1974) reported from Egypt a pattern of highest monthly GAS carrier rate during the late autumn and early winter and the minimal rate in the summer (Steinhoff and Rimoin, 2004). Streptococcal tonsillitis is rare in infants and among pre-school children it is not very frequent (Powers and Boisve, 1944). In high income countries GAS pharyngitis is common in children aged 3–15 years. In low-income countries, there is limited data on the age distribution of GAS pharyngitis in children. A recent study in India indicated that the peak age for GAS pharyngitis was at 11 years (Nandi *et al*., 2001).

2.2 Upper Respiratory Tract Infections

The infections in upper respiratory tract are nasopharyngitis, otitis media, sinustitis and epiglottitis. Among these mentioned infections, pharyngitis is the most frequent one in addition, the untreated infection may have serious sequelae (Vandepitte *et al*., 2003). The typical patient with acute streptococcal pharyngitis is a school-age child with sudden onset of fever and sore throat in the late winter or spring. Headache, malaise, abdominal pain, nausea, and vomiting occur frequently. Cough, rhinorrhea, stridor, hoarseness, conjunctivitis, and diarrhoea are distinctly unusual. The pharynx is erythematous. Petechiae may be seen on the soft palate. Tonsils are enlarged and red, with patchy exudates. The papillae of the tongue may be red and swollen (strawberry tongue). Tender, enlarged anterior cervical nodes are common. Any of these 'classic' symptoms and signs may be absent in a particular patient; reliable diagnosis requires swabbing the throat for culture or a group A carbohydrate antigen detection test. Fever is documented in more than 90% of patients with streptococcal pharyngitis (Stillerman and Bernstein, 1961). The fever may be low-grade, 37–38^{o}C, or as high as 40^{o}C (Siegel *et al*., 1961).

Pharyngitis may occur in different grades of severity. Milder infections present with discomfort in the throat, some malaise and low grade fever. Pharynx in these cases is congested but there is no lymphadenopathy. Moderate and severe infections present with pain in throat, dysphagia, headache, malaise and high fever. Pharynx in these cases shows erythema, exudate and enlargement of tonsils and lymphoid follicles on the posterior pharyngeal wall. Very severe cases show oedema of soft palate and uvula with enlargement of cervical nodes. It is not possible, on clinical examination, to differentiate viral from bacterial infections but, viral infections are generally mild and are accompanied by rhinorrhoea and hoarseness while the bacterial ones are severe (Dingara, 2008).

2.3 Bacterial Agents of pharyngitis

Patients with bacterial pharyngitis generally do not have rhinorrhea, cough or conjunctivitis. The most common agents involved in bacterial pharyngitis are group A haemolytic Streptococci. Its importance lies not only in its frequency of occurrence, but due to its two serious sequelae, that is, acute rheumatic fever and post-streptococcal glomerulonephritis. β-haemolytic Streptococci other than group A (group C and G) produce symptoms similar

to, but milder than those of group A strains. They are not associated with rheumatic fever as a non-infectious sequel (Middleton, 1991). *Streptococcus pyogenes* is by far the most frequent cause of bacterial pharyngitis and tonsillitis. When streptococcal pharyngitis is associated with a characteristic skin rash, the patient is said to have scarlet fever. In infants, a streptococcal throat infection may often involve the nasopharynx and be accompanied by a purulent nasal discharge (Vandepitte *et al*., 2003).

Non-group-A, β-haemolytic streptococci (e.g. groups B, C and G) are uncommon causes of bacterial pharyngitis and if detected should be reported (Vandepitte *et al*., 2003). Several studies have demonstrated that group C streptococci are a relatively common cause of acute pharyngitis among college students and among young adults seeking care (Meier *et al*., 1990; Turner *et al*., 1997). Group C streptococcus has a prevalence of less than 5% in adult pharyngitis patients (Zwart *et al*., 2000). Although not as common as group C streptococcal pharyngitis, group G can have a similar presentation (Lindboek *et al*., 2005).

Corynebacterium diphtheriae causes a typical form of infection, characterized by a greyish-white membrane at the site of infection (pharynx, tonsils, nose, or larynx) (Vandepitte *et al*., 2003). There is much local inflammation of the nasopharynx, with a characteristic 'bull neck' appearance from enlarged lymph nodes. Toxigenic strains of *C. diphtheriae* produce a polypeptide that causes local destruction of epithelial cells and spreads systemically to cause myocarditis and polyneuritis (Myint *et al*., 1999). Before artificial immunization, diphtheria was mainly a disease of small children. The infection occurred either clinically or sub clinically at an early age and resulted in the widespread production of antitoxin in the population. An asymptomatic infection during adolescence and adult life serves as a stimulus for maintenance of high antitoxin levels (Brooks *et al*., 2004).

Gonococcal pharyngitis has been recognized with increasing frequency in some countries, with rates that parallel the incidence of cervical and urethral gonorrhoea (Vandepitte *et al*., 2003). Gonococcal pharyngitis is mild and may even be asymptomatic (Dingara, 2008).

Borrelia vincenti in association with Fusobacterium species and other Gram negative anaerobes causes Vincent's angina (Cheesbrough, 2006). Necrotizing ulcerative pharyngitis (Vincent angina) is a rare condition characterized by a necrotic ulceration of the pharynx with or without formation of a pseudomembrane. Although both species belong to the normal mouth flora, their presence in large numbers in a Gram-stained smear of ulcerated

lesions should be reported as a "fusospirochaetal complex" (Vandepitte *et al*., 2003).

Candida albicans or other *Candida* spp. may be part of the normal oral flora, oral candidiasis results when the number of organisms increases considerably in certain pathological conditions (Vandepitte *et al*., 2003). Debilitated and immune suppressed individuals, such as persons with diabetes mellitus, persons receiving corticosteroids, recipients of haematopoietic stem cell transplants and persons with human immunodeficiency virus infection, are more susceptible to oropharyngeal candidosis. The affected area—tongue, tonsils, throat or buccal mucosa —may be extremely red, or covered with white patches or a confluent grey-white membrane (thrush) (Richardson and Warnock, 2003).

The following pathogens can give rise to a carrier state in the upper respiratory tract:

Staphylococcus aureus. Intracellular residing *S. aureus* is the most common cause of recurrent tonsillitis (RT) and indicate that *S. aureus* uses this location to survive the effects of antibiotics and the host immune response (Zautner *et al*., 2010).

Neisseria meningitidis. Carriage of meningococci may be very prevalent (20% or more) even at non-epidemic times. Identification of pharyngeal carriers of meningococci is rarely needed, and need not be performed prior to the administration of antibiotics to family or other close contacts of patients with meningococcal disease (Vandepitte *et al*., 2003).

Streptococcus pyogenes. Carriage of this organism in low numbers may be prevalent, especially among school children (20–30%) (Vandepitte *et al*., 2003).

Corynebacterium diphtheriae. The carrier rate of the diphtheria bacillus is high in non-vaccinated populations. In such communities, it may be justified to identify and treat carriers among the close contacts of a patient with proven diphtheria (Vandepitte *et al*., 2003).

2.4 Normal Flora of Upper Respiratory Tract

The normal flora of the pharynx includes a large number of species that should be neither fully identified nor reported when observed in throat cultures:

1. viridans (α-haemolytic) streptococci and pneumococci.
2. nonpathogenic *Neisseria* spp.
3. *Moraxella catarrhalis*
4. staphylococci (*S. aureus*, *S. epidermidis*)

5. diphtheroids (with the exception of *C. diphtheriae*)
6. *Haemophilus* spp.
7. Yeasts (*Candida* spp.) in limited quantity
8. various strictly anaerobic Gram-positive cocci and Gram-negative rods, spirochaetes and filamentous forms.

More than 700 bacterial species, of which over 50% have not been cultivated, have been detected in the oral cavity (Aas *et al.*, 2005). Adult human saliva is reported to contain approximately 6 billion ($6x10^9$) micro-organisms per millilitre. The major source of salivary bacteria appears to be the tongue (Gibbons and Houte, 1975).

There are no regions of the body which normally support a more abundant and varied population of microbes than the mouth, nose and throat (Aas et al., 2005).The anatomy of oral cavity and upper pharynx provides a living place for numerous types of aerobic as well as anaerobic bacteria (Dey and Dey, 1990). It can be presumed that when the immune status of the patient becomes low, some microorganisms which were cateroiged as normal flora can become virulent and cause the disease (Bista *et al.,* 2005). Bacteria enter the upper respiratory tract from the air during breathing and most of them are trapped in nasal passages and expelled, again with the nasal secretions. Potentially harmful bacteria, such as *Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus pyogenes,* and *Corynebacterium diptheriae* are often pan of the normal flora of the nasopharynx of healthy individuals. These individuals are carriers of the pathogens but do not normally acquire disease because the other resident microorganisms compete successfully for resources and limit pathogen growth. The local immune system is particularly active at mucosal surface and may also inhibit the growth of pathogens (Madigan and Martinko, 1997).

Brook and Gober (2006) associated the isolation of increased recovery of *Moraxella catarrhalis* and *Haemophilus influenzae* in conjunction with the recovery of GAS. The increased isolation of *H. influenzae* (in APT only) and *M. catarrhalis* in association with GAS may be due to a synergistic relationship between these organisms (Brook and Gillmore, 1996; Lafontaine *et al*., 2004). The ability of *H. influenzae* and *M. catarrhalis* to produce the enzyme β-lactamase may also make them, as well as GAS, more resistant to eradication by Penicillin and contribute to failure of Penicillin therapy (Brook, 1984).

2.6 Epidemiology of Upper Respiratory Tract Infections

2.6.1 Global Epidemiology of Upper Respiratory Tract Infections

There were very few recent population based studies documenting the incidence of GAS pharyngitis. The most comprehensive studies came from the USA during the 1950s and 1960s, documenting incidences of serologically-proven symptomatic GAS pharyngitis ranging from 0.15 to 0.22 per person-year in children and approximately 0.06 per person-year in adults (Cornfeld *et al*., 1961; Dingle *et al*., 1964; Breese, 1966). A further prospective study of 678 people in Egypt during 1967 to 1969 documented an incidence of serologically proven GAS pharyngitis of 0.42 per person year in children aged 2 to 4 years, 0.31 in children aged 6 to 12 years, and 0.10 in adults aged >25 years (El-Kholy *et al*., 1974).

However, most of these infections were asymptomatic; the authors commented that the occurrence of typical exudative pharyngitis was rare. Other data suggest that acute pharyngitis is one of the most common illnesses for which patients seek medical advice in more developed countries, accounting for nearly 18 million office visits in 1996 and over 7 million visits to paediatricians each year in the United States (Woodwell, 1992; Woodwell, 1996). In Australia, 4% of all symptomatic presentations to general practitioners are for throat complaints, second only to cough at 7%, and sore throat consultations result in an antibiotic prescription in 89% of cases (Anonymous, 2002; Britt *et al*., 2001). GAS is the most common bacterial cause and is estimated to account for 15-30% of cases of pharyngitis in children and 5-10% of cases in adults (Komaroff *et al*., 1986; Bisno, 1996).

Only four population-based studies after 1980 were found. Three of these took place in populations where acute rheumatic fever (ARF) is also common; in a predominantly Maori and Pacific Islander region of Auckland, New Zealand, in Kuwait and in northern India (Majeed *et al*., 1993; Lennon, 2000; Nandi *et al*., 2001). In these populations, sore throat with a positive throat swab for GAS occurred once every one to two years in each child. These infections were not confirmed using serology, so the true incidence will be somewhat lower. Only one study came from an affluent population; a study from Melbourne, Australia, which found an incidence of serologically proven symptomatic GAS pharyngitis of 0.14 per child-year (Danchin *et al*., 2004). This suggests that the incidence

has not changed dramatically in similar settings since the studies half a century earlier in the USA (WHO, 2005).Therefore, it seems reasonable to conclude that approximately 15% of school-age children will suffer a symptomatic episode of GAS pharyngitis each year in more developed countries, and that 4-10% of adults will be similarly affected. The incidence in less developed countries may be five to ten times greater (WHO, 2005).

In higher income regions rheumatic fever was first described in 1898 and its association with GAS noted in the 1930s. Reports of rheumatic carditis as an important tropical problem first appeared in the medical literature only in the 1930s to 1950s. It has since become clear that post-streptococcal rheumatic disease occurs in low income regions at rates similar to or greater than the rates reported in the early 20th century from countries with high incomes. For example, in the late 1980s reports from Ethiopia suggest that the prevalence of rheumatic heart disease among children was essentially identical to rheumatic heart disease prevalence rates from the US four decades previously (Tewodros and Kronvall, 1993). It is also known that some low-income regions with increasing economic resources have shown a remarkable decline in rheumatic disease (Arguedas and Mohs, 1992), similar to the historical patterns reported in high-income regions (Steinhoff and Rimoin, 2004).

2.6.2 High Income Countries

In high-income countries pharyngitis is common in children ages 3–15years. On average in the USA, each child has approximately one GAS pharyngitis infection by age 5, with a mean of 3 episodes (range 1–8) by 13 years of age, (Wannamaker, 1972). Before the availability of Penicillin in the 1940s, ARF rates declined by 50% from 1900. From 1935 to 1960, the incidence of ARF in the USA ranged between 40 and 65/100,000 in all age groups. From the late 1960s, there was a marked decline in ARF; since 1970s, incidence of rheumatic fever has ranged from 0.023 to 1.88 per 100,000 population (Holmberg and Faich, 1983; Schwartz *et al*., 1983; Odio, 1986). Localized outbreaks of ARF were reported in parts of the USA in the 1980s (Steinhoff and Rimoin, 2004).

2.6.3 Low Income Countries

Few studies have been conducted to ascertain the incidence and prevalence of GAS in developing countries. The studies used varying and non-standardized methods for throat cultures, ASO titers or rapid antigen tests for diagnosis (Steinhoff and Rimoin, 2004).

2.6.4 Incidence of GAS pharyngitis

Few precise estimates of the incidence of GAS pharyngitis in low-income countries are available. The reported incidence of culture-proven GAS pharyngitis in prospective studies in a few sites appears to be much higher than that reported from the United States in the 1950s: up to 900 versus. approximately 200 per 1,000 child-years of observation (Steinhoff and Rimoin, 2004). These apparently elevated rates of infection suggest high rates of carriage and transmission. Since streptococci are spread through the respiratory route, and low income regions are characterized by crowded housing, these data are not surprising (Steinhoff and Rimoin, 2004).

2.6.4 Prevalence in Clinic Settings

Interpretation of studies of streptococcal pharyngitis carried out in clinics and hospitals can be difficult because of the conflation of categories of 'infection', 'carriage' and 'streptococcal pharyngitis'. Clinical studies in Kuwait, Egypt, Ethiopia, and India have shown that prevalence rates of GAS pharyngitis in clinics range from 0.3 to 37%. However, as it has been suggested that in some countries patients with symptoms of pharyngitis may not seek care at health facilities or complain of sore throat due to economic, cultural or other factors (Carapetis *et al*., 1999). Reviews of data from some developing countries suggest that a lower percentage of children presenting with pharyngitis have positive GAS throat cultures than in the USA (Quinn, 1989; Carapetis *et al*., 1999). In addition, clinical experience suggests that classic textbook cases of streptococcal pharyngitis with enlarged tonsils, exudates and large, tender nodes are infrequently seen in clinics in some regions (Steinhoff and Rimoin, 2004). A study in India found that of 53 children with GAS isolated from the pharynx, 54% had serological evidence of infection, yet none of these patients had complaints of symptoms typically associated with streptococcal pharyngitis (Gupta *et al*., 1992). One cannot distinguish if this apparent low proportion of classical clinical presentation of GAS pharyngitis is related to regional differences in the organism, variation

in the host responses, or to patterns of care seeking and referral, although the latter is the least well studied factor (Salazar, 2004).

2.7 *Streptococcus*

The genus Streptococcus is named according to its typical morphology (Greek-streptus=flexible or pliant; Latin-coccus=a sphere) Streptococci are spherical or ovoid cells, arranged in chains or pairs. The individual cocci are 0.5 to 1 μm in diameter and are arranged in chains. Some members show a diplococcal appearance, and rod-like forms are occasionally seen. The lengths of chain vary widely with cultural conditions (Chakraborty, 2003). All species are non motile, Gram positive, Non-sporing, Catalase negative, with complex nutrition. All are facultative anaerobic; most will grow in air but some require the addition of CO_2 for growth. All species fail to reduce nitrate. Ferment glucose with the production mainly of lactic acid never of gas. Many species are commensal microflora in mucosal of human or animals and some are highly pathogenic (AppendixVI). The G+C content of DNA 30-46 mol% (Collier *et al*., 2007). Some strains of *S. pyogenes* form a capsule composed of hyaluronic acid, which results in their forming mucoid colonies on blood agar. Most strains belonging to group A and C possess capsules composed of hyaluronic acid. While members of groups B and D show polysaccharide capsules (Chakraborty, 2003).

2.7.1 Classification of Streptococci

The classification of streptococci into major categories has been based on a series of observations over many years:

(1) Colony morphology and haemolytic reactions on blood agar.

(2) Serologic specificity of the cell wall group-specific substance or capsular antigens.

(3) Biochemical reactions and resistance to physical and chemical factors.

(4) Ecological features.

Molecular genetics have also been used to study the streptococci. Combinations of the above methods have permitted the classification of streptococci for purposes of clinical and epidemiologic convenience, but as the knowledge evolved, new methods have been introduced with the result that several classification systems have been described. In some cases, different species names have been used to describe the same organisms; in other instances, some members of the same species have been included in another species or

classified separately. The genus enterococcus, for example, now includes some species previously classified as group D streptococci (Brooks *et al*., 2004).

The haemolytic classification of streptococci are described in the following paragraphs:

2.7.2 Haemolytic Classification

Many streptococci are able to haemolyze red blood cells in vitro in varying degrees. Complete disruption of erythrocytes with clearing of the blood around the bacterial growth is called β-haemolysis. Incomplete lysis of erythrocytes with reduction of hemoglobin and the formation of green pigment is called α haemolysis. Other streptococci are non-haemolytic (sometimes called gamma haemolysis). The haemolysis patterns of the streptococci of medical importance to humans are shown in Appendix VII. The haemolytic patterns is used primarily with the streptococci and not with other bacteria that cause disease and typically produce a variety of hemolysins (Brooks *et al*., 2004).

Three types of haemolytic reactions are observed in blood agar after 24 hours incubation:

Alpha (α) haemolytic streptococci : They produce a partial haemolysis with an ill-defined greenish discolouration around the colony. The zone of lysis is small (1-2 mm wide) with persistence of unhaemolysed RBCs detectable by microscopic examination. Greenish discolouration is due to formation of reductant haemoglobin. Alpha haemolysis is seen in viridans streptococci and pneumococcus (Chakraborty, 2003).

Beta (β) haemolytic streptococci : They produce a wide (2-4 mm wide) clear zone of complete haemolysis around the colony in which no red cells are visible on microscopic examination. Two types of beta haemolysis are released. Streptolysin O (inactivated by atmospheric oxygen) is demonstrable only in deep colonies while streptolysin S (oxygen stable) is responsible for surface colony haemolysis (Chakraborty, 2003).

Gama (γ) haemolytic streptococci : They produce no haemolysis and *S. faecalis* is a typical non-haemolytic species (Chakraborty, 2003).

2.7.3 Serological Classification

1. Group specific polysaccharide antigen of cell wall

The carbohydrate contained in the cell wall of many streptococci and forms the basis of serologic grouping into Lancefield groups A–H and K–V. The serologic specificity of the group-specific carbohydrate is determined by an amino sugar (Brooks *et al*., 2004). The β-haemolylic streptococci also called haemolytic streptococci are classified by Lancefield, (1933) serologically into a number of broad groups based on the differences in the structure of group specific polysaccharide antigen in the cell wall. So far 20 Lancefield groups have been identified numbered A-V (without I and J) by precipitation reaction performed with appropriate sera (produce in immunized rabbits) (Brooks *et al*., 2004).

Cell wall carbohydrate specific for Lancefield's groups of streptococci

Aminosugar	Lancefield's Group
Rhamnose-N-actyl glucosamine	A
Rhamnose-glucosamine polysaccharide	B
Rhamnose-N-acetylgalactosamine	C
Glycerol teichoic acid containing D-alanine	D
Glucopyranosyl-N-acetylgalactosamine	F

Source: (Brooks *et al*., 2004)

Extracts of group-specific antigen for grouping streptococci are prepared by a variety of methods: extraction of centrifuged culture treated with hot hydrochloric acid, nitrous acid, or formamide; by enzymatic lysis of streptococcal cells (e.g., with pepsin or trypsin); or by autoclaving of cell suspensions. These extracts contain the carbohydrate group–specific substance that yields precipitin reactions specific antisera. Typing is generally done only for groups A, B, C, F, and G which cause disease in humans and for which there are reagents that allow typing using simple agglutination or colour reactions (Brooks *et al*., 2004).

2. Type Specific Antigen

The strains of *S. pyogenes* is further sub-divided by type specific antisera into approximately 130 serotypes (type 1, type 2 etc.) according to their specific surface M proteins (Fischetti, 1989). Other type specific antigen is T substance which has no relationship to virulence of

streptococci. Unlike M protein, T substance permits differentiation of certain types of streptococci by agglutination with specific antisera, while other types share the same T substance. Yet another surface antigen has been called R protein (Brooks *et al*., 2004).

3. Capsular Polysaccharides

The antigenic specificity of the capsular polysaccharides is used to classify *S. pneumoniae* into over 90 types and to type the group B streptococci (*S. agalactiae*) (Brooks *et al*., 2004).

2.7.4 Biochemical Reactions

Biochemical tests are most often used to classify streptococci after the colony growth and haemolytic characteristics have been observed. Biochemical tests are used for species that typically do not react with the commonly used antibody preparations for the group-specific substances, groups A, B, C, and G (Table1). For example, the viridans streptococci do not react with the antibodies commonly used for the Lancefield classification. Speciation of the viridans streptococci requires a battery of biochemical tests (Brooks *et al*., 2004). *S. pyogenes* like other streptococci, is catalase negative and insoluble in bile. It differs in some reactions from other haemolytic streptococci. Fermentation tests should be done in sugar serum peptone waters. *S. pyogenes* is positive in the PYRase test which distinguishes it from non-group A haemolytic streptococci (Collee *et al*., 2006).

Table 1: *Biochemical reactions of principal β–haemolytic streptococci

Key ± some strains positive, some negative

Reaction	**Lancefield Group**			
	A	**B**	**C**	**G**
Acid Produced from:				
Lactose	+	±	±	±
Mannitol	±	-	-	-
Raffinose	-	-	-	-
Ribose	-	+	±	+
Sorbitol	-	-	±	-
Hydrolysis of :				
Aesculin	-	-	±	±
Hippurate	-	+	-	-
Acetoin produced	-	+	-	-
Pyrrolidonyl peptidase (PYRase) produced	+	-	-	-

Source:*(Collee *et al*., 2006)

Many species of streptococci, including *S. pyogenes* (group A), *S. agalactiae* (group B), and the enterococci (group D), are characterized by combinations of features: colony growth characteristics, haemolysis patterns on blood agar (β-haemolysis, γ-haemolysis, or no haemolysis), antigenic composition of group-specific cell wall substances, and biochemical reactions. *S pneumoniae* (pneumococcus) types are further classified by the antigenic composition of the capsular polysaccharides. The viridans streptococci can be γ-haemolytic or non haemolytic and are generally speciated by biochemical reactions (Brooks *et al*., 2004).

2.8 *Streptococcus pyogenes*

2.8.1 Virulence factors of *Streptococcus pyogenes*

The M1 strain of *S. pyogenes* contains 1,852,442-bp sequences 1,752 predicted protein-encoding genes. Approximately one- third of these genes have no identifiable function, with the remainder falling into previously characterized categories of known microbial function. In *S. pyogenes* more than 40 putative virulence- associated genes have been identified. Additional genes have been identified that encode proteins likely associated with microbial ‘‘molecular mimicry’’ of host characteristics and involved in rheumatic fever or acute glomerulonephritis (Ferretti *et al*., 2001).

Capsular hyaluronic acid

Group A streptococci are gram-positive bacteria covered with an outer hyaluronic acid capsule and a layer of group A carbohydrate, a polymer of rhamnose with N-acetylglucosamine side chains. Some strains of *S. pyogenes* possess thick capsules of hyaluronic acid resulting in large mucoid colonies on blood agar (Stevens, 1994). Utilizing an isogenic mutant of an M-18 strain, Wessels *et al*. (1991) showed that the strains producing hyaluronic acid capsule were resistant to phagocytosis.

Cell Wall

Lipoteichoic acid present in cell wall GAS of may play a role in pathogenesis by facilitating the adherence of GAS to pharyngeal epithelial cells (Hasty *et al*., 1992). Peptidoglycan, like endotoxin from gram-negative bacteria, is capable of activating the alternative complement pathway (Greenblatt *et al*. 1978; Bisno, 1979; Verhoef and Kalter, 1985).

M-Proteins

The ability of *S. pyogenes* to persist in infected tissues is due to primarily due to cell surface exposed M proteins (Lancefield, 1928). Over 130 different M-protein types of GAS are currently described (Fischetti, 1989). The protein is a coiled-coil consisting of four regions of repeating amino acids (A–D), a proline/glycine-rich region which serves to intercalate the protein into the bacterial cell wall, and a hydrophobic region which acts as a membrane anchor (Fischetti, 1991). Region A near the N-terminus is highly variable, and antibodies to this region confer type-specific protection. Within the more conserved B–D regions lies an area which binds one of the complement regulatory proteins (factor H), stearically inhibiting antibody binding and complement-derived opsonin deposition, and effectively camouflaging the organism against humoral immune surveillance (Fischetti *et al*., 1995). M-protein also protects the organism against phagocytosis by polymorphonuclear leukocytes (Peterson *et al*., 1979).

Immunoglobulin Binding Proteins (M-Like Proteins)

The M protein is one of family of proteins which share overall structure homologues sequences, and the ability to interact with various host proteins including immunoglobin (Kehoe, 1994). Group A streptococcus produces a family of proteins which share structural similarities to M-proteins and also bind immunoglobulins including IgG, IgM and IgA. Unlike M-protein, these molecules do not inhibit phagocytosis in the absence of type specific antibody. Nonetheless, these molecules, like M-protein, may play a role in pathogenesis by interfering with complement activation (Stevens, 2004).

Streptococcal superantigens

Superantigens are a unique group of glycoproteins synthesized by bacteria and viruses that can bridge Class II major histocompatibility complex molecules to nonpolymorphic V β-chains of the T-cell receptors, simulating antigen binding. The T-cells bearing the appropriate V β-chain are activated (to release cytokines or become cytotoxic), regardless of their antigenic specificity. Some T-cells activated in this manner can have autoreactive specificities, since previously anergized T-cell subsets are susceptible to superantigenic stimulation. In the case of streptococci, much work has focused on the role of the superantigen-like activity of M-protein fragments (PeP M5, in particular), as well as the

streptococcal pyrogenic exotoxin, in the pathogenesis of RF. Superantigenic activation is not limited to the T-cell compartment alone. Streptococcal erythrogenic toxin may behave like a super-antigen for the B-cell, leading to the production of autoreactive antibodies, but as noted above, much of the evidence is still indirect (WHO, 2004).

Fibronectin Binding Protein (Protein F)

Interaction with host fibronectin has long considered the mechanism by which *S. pyogenes* adhere to epthilial cells (Collier *et al*., 2007). The binding of bacterial surface ligands to host surface receptors is the most crucial event in the colonization of the host, and it is initiated by fibronectin and by streptococcal fibronectin-binding proteins (Simpson *et al*., 1987).

Protein SIC (Streptococcal Complement Inhibitory Protein)

Akesson *et al*. (1996) described a novel, extracellular protein of 305 amino acid residues which inactivates the membrane attack complex of complement. SIC was found only in M-types 1 and 57. In addition, the SIC gene is located in the mga regulon of M-type 1 GAS, directly adjacent to the emm gene (Akesson *et al*., 1996). Through this mechanism the organism could evade destruction by the membrane attack complex (C5–C9) generated by either the alternative or classical complement pathway (Stevens, 2004).

Opacity Factor

Group A streptococci can be subdivided into more than 130 distinct types, based upon a characterization of the M protein of the cell wall, opacity factors antigens produced by the organism, and by molecular sequencing of the emm gene that codes for M protein (WHO, 2004). The opacity factor OF is a type-specific lipoprotein lipase whose role in pathogenesis is unknown. Evidence suggests a relationship with the presence of OF and arrangement of specific emm genes. Specifically, OF is associated with M-types which are largely skin strains (Bessen *et al*., 1996).

Hemolysins

The β-haemolytic group A *S. pyogenes* elaborates two hemolysins (streptolysins) (Brooks *et al*., 2004). Streptolysin O belongs to a family of oxygen-labile, thiol-activated cytolysins, and causes the broad zone of haemolysis surrounding colonies of *S. pyogenes* on blood agar plates (Alouf and Geoffroy, 1984). Streptolysin O is a protein (MW 60,000) that is haemolytically

active in the reduced state (available–SH groups) but rapidly inactivated in the presence of oxygen (Brooks *et al*., 2004). Thiol-activated cytolysins bind to cholesterol on eukaryotic cell membranes creating toxin-cholesterol aggregates that contribute to cell lysis via a colloid-osmotic mechanism (Tweten, 1988).

Streptolysin O contributes to pathogenesis of streptococcal infections in several ways. First, in high concentrations, it is cytotoxic. In lower concentrations, it activates a variety of cells such as human polymorphonuclear leukocytes (PMNL) and endothelial cells, and acts synergistically with other streptococcal factors (e.g. pyrogenic exotoxins) to induce cytokine production (Stevens, 2004). It also stimulates the production of antistreptolysin O antibody (ASO) (Cheesbrough, 2006).

Streptolysin S is a cell-associated haemolysin which does not diffuse into the agar media. The role of Streptolysin S in pathogenesis may be through direct, contact dependent, cytotoxicity (Nizet *et al*., 2000).

Deoxyribonucleases (DNases)

DNases are produced by streptococci of group A, C and G. These are four antigenically distinct enzymes, desiginated A,B,C and D (Collee *et al*., 2006). DNase B, elicits production of anti-DNase antibody following either pharyngeal or skin infection (Stevens, 2004).

Hyaluronidase

Hyaluronidase splits hyaluronic acid, an important component of the ground substance of connective tissue. Thus, hyaluronidase aids in spreading infecting microorganisms (spreading factor) (Brooks *et al*., 2004). This extracellular enzyme hydrolyzes hyaluronic acid in deeper tissues and may facilitate the spread of infection along fascial planes. Anti-hyaluronidase titres rise following *S. pyogenes* infections, especially those infections involving the skin (Stevens, 2004).

Nicotine-Adenine-Dinucleotidase (NADase)

This extracellular enzyme also called NAD glycohydrolase has been produced by many strains of GAS (Lutticken *et al*., 1976). It is not know what function this enzyme has for the organism per se and it is also unclear what role NADase may play in pathogenesis of any GAS infection. Recently, it was shown that NADase is expressed in M-1 and M-3 strains of

GAS, but not in non-invasive strains (Stevens *et al*., 2000). NADase likely contributes to pathogenesis by directly attenuating leukocyte functions such as chemotaxis and phagocytosis (Stevens *et al*., 2000).

Streptokinase

Streptokinase is produced by all strains of GAS and is located in the extra cellular milieu. In contrast, a plasminogen binding site is found on the surface of strains of GAS (Lottenberg *et al*., 1987). Once plasminogen is bound, streptokinase proteolytically converts bound plasminogen to active plasmin (Lottenberg *et al*., 1987). Plasmin then cleaves fibrin into fragments known as fibrin degradation products, or FDPs. The role of streptokinase infections is unclear, though it may play a role in post-streptococcal glomerulonephritis (Stevens, 2004).

Pyrogenic Exotoxins

The Streptococcal pyrogenic exotoxins (SPE) are members of a family of bacterial pyrogenic toxin, which share biological and biochemical properties (Collier *et al*., 2007). Streptococcal pyrogenic exotoxins type A, B, and C, also called scarlatina or erythrogenic toxins, induce lymphocyte blastogenesis, potentiate endotoxin-induced shock, induce fever, suppress antibody synthesis, and act as superantigens (Barsumian *et al*., 1978). The gene for pyrogenic exotoxin A (SPEA) is transmitted by bacteriophage, and stable production depends upon lysogenic conversion in a manner analogous to toxin production by *Corynebacterium diphtheriae* (Nida and Ferretti, 1982). Control of SPEA production is not yet understood, though this is likely an important mechanism since it is well established that the quantity of SPEA produced by strains varies dramatically from decade to decade. In addition, point mutations in the SPEA gene result in dramatic changes in the potency of this toxin (Kline and Collins, 1996). Historically, streptococcal pyrogenic exotoxin A and B producing strains have been associated with severe cases of scarlet fever and more recently, with streptococcal toxic shock syndrome (StrepTSS) (Stevens *et al*., 1989; Hauser *et al*., 1991). Although all strains of GAS are endowed with the gene for streptococcal pyrogenic exotoxin B (SPEB), not all strains produce SPEB and even among those strains which produce this toxin, the quantity produced varies greatly from strain to strain (Nida and Ferretti, 1982; Hallas, 1985; Kohler *et al*., 1987; Hauser *et al*., 1991). Pyrogenic exotoxin C (SPEC), like SPEA, is bacteriophage-mediated and expression is likewise highly variable. Mild cases of scarlet fever in England and the United States have been associated with SPEC-positive strains (Hallas, 1985).

2.8.2 Pathogenesis

The most common route of entry of *S. pyogenes* is the upper respiratory tract, where the primary infection is established (Ross, 1998). *S. pyogenes* adhere to the pharyngeal epithelium by means of lipoteichoic acid-covered surface pili. The glycoprotein fibronectin (MW 440,000) on epithelial cells probably serves as lipoteichoic acid ligand. In infants and small children, the sore throat occurs as a subacute nasopharyngitis with a thin serous discharge and little fever but with a tendency of the infection to extend to the middle ear and the mastoid. The cervical lymph nodes are usually enlarged. The illness may persist for weeks. In older children and adults, the disease is more acute and is characterized by intense nasopharyngitis, tonsillitis, and intense redness and edema of the mucous membranes, with purulent exudate, enlarged, tender cervical lymph nodes, and (usually) a high fever. Twenty percent of infections are asymptomatic (Brooks *et al*., 2004). Only a proportion of infected individuals develop tonsillitis or pharyngitis. The others may have mild atypical infections of become symptomless carriers (Ross, 1998).

S. pyogenes owes its major success as a pathogen to its ability to colonize and rapidly multiply and spread in its host while evading phagocytosis and confusing the immune system (Frey and Paul, 2003). .*S. pyogenes* is responsible for a broad spectrum of diseases that range from simple and uncomplicated pharyngitis and skin infections (impetigo, erysipelas, and cellulites) to scarlet fever and life-threatening invasive illnesses including pneumonia, bacteremia, necrotizing fasciitis, streptococcal toxic shock syndrome (TSS), and nonsuppurative sequelae such as acute rheumatic fever, reactive arthritis and glomerulonephritis (Cunningham, 2000; Pfoh *et al*., 2008).

2.8.3 General Pathogenic Mechanisms

Anti-Phagocytic properties of M-protein contributes to invasiveness through its ability to impede phagocytosis of streptococci by human polymorphonuclear leukocytes (PMNL). Conversely, type specific antibody against the M-protein enhances phagocytosis. Following infection with a particular M-type, specific antibody confers resistance to challenge with viable GAS of that M-type (Lancefield, 1962). While strains of M-types 1 and 3 have accounted for the vast majority of strains isolated from cases of StrepTSS, many other M-types, including some non-typeable strains, have also been isolated from such cases. M-types 1 and 3 are also commonly isolated from asymptomatic carriers, and patients with pharyngitis

or mild scarlet fever (Kohler *et al*., 1987; Johnson *et al*., 1992). There may be major differences in susceptibility to opsonization by anti-M-type antibody among strains of a given M-type (Villasenor-Sierra *et al*., 1991; de Malmanche and Martin, 1994).

Cytokine Induction

There is strong evidence suggesting that SPEA, SPEB and SPEC activity as superantigens and stimulate T cell responses through their ability to bind to both the class II MHC complex of antigen presenting cells and the V_β region of the T cell receptor (Mollick and Rich, 1991). The net effect is induction of T cell proliferation (via an IL-2 mechanism) with concomitant production of cytokines (e.g. IL-1, TNFα, TNFβ, IL-6, IFNγ) that mediate fever, shock, and tissue injury. SPEA induces both TNFα and TNFβ from mixed cultures of monocytes and lymphocytes supporting the role of lymphokines (TNFβ) in shock associated with strains producing SPEA (Hackett and Stevens, 1992). Kotb *et al*. (1990) have shown that a digest of M-protein type 6 can also stimulate T cell responses by this mechanism.

2.8.4 Laboratory Diagnosis

Culture of throat swab is helpful for checking the continuing presence of *S. pyogenes* in throat. Serologically tests provide evidence of recent streptococcal infection. A rising titre of antibodies to group A streptococcal antigens are usually detectable. ASO test is routinely done in diagnostic laboratory. A.S.O titre of 200 units or more is significant in rheumatic fever. A.S.O titer is usually found in high levels in respiratory disease and rheumatic fever; but in streptococcal skin infections and acute glomerulonephritis, ASO titre tends to be low and DNAase-B test is more reliable. Complement (C3) level is also reduced in serum in acute glomerulonephritis (Chakraborty, 2003)

The principal reservoirs for the spread of *S. pyogenes* to susceptible hosts are infected persons and carriers. For the accurate diagnosis of pharyngitis reliable bacteriological methods are needed to establish if group A streptococcus is the cause and to enable appropriate antibiotic treatment. Prompt treatment is necessary to prevent RF particularly in populations at high risk, to prevent suppurative complications, shorten duration of symptoms and to reduce the risk of transmission (Denny *et al*., 1950; Cantanzaro *et al*., 1954; Dajani *et al*., 1995; Bisno *et al*., 1997). Until 9 days from onset of streptococcal pharyngitis, Penicillin therapy could prevent RF (Cantanzaro *et al*., 1954). Using clinical signs and symptoms alone correctly

predicted only 372 of 536 (69.6%) positive throat cultures (Breese and Disney, 1954). Although a number of clinical scoring algorithms exist, accurate diagnosis still depends on culture of a group A streptococcus or a positive rapid antigen detection test (RADT). Neither throat culture nor rapid testing can reliably distinguish between the acute streptococcal infection or the streptococcal carrier with a concomitant viral infection (Bisno *et al*., 1997). In school-aged populations, at highest risk for RF, 70–80% sore throats are most likely to be of non-bacterial etiologies (Pichichero, 1998). An informed decision as to which diagnostic tests should be used will take account of the high or low risk category for RF of the person under test, their age, the epidemiology of streptococcal infection in the local population, and the physical presenting symptoms (Martin, 2004).

2.8.5 Diagnostic Sensitivity of the Throat Swab

The optimal and traditional method for establishing the actual presence of a group A streptococcus in the throat is the throat swab. Diagnostic sensitivity is dependent on a number of variables including the adequacy of the swabbing technique, conditions operating for transportation of the swab, the methodology employed to detect a group A streptococcus and the interpretation of the results obtained including consideration of any recent antimicrobial exposure. The recommended procedure for a throat swab is direct visualization of the tonsillar-pharyngeal area and vigorous swabbing of the tonsils or tonsillar crypts and of the posterior pharyngeal wall (Bisno *et al*., 1997). The type of swab (cotton or synthetic fibre) used for specimen collection is usually determined by the choice of swab available at the point of collection. Any delay between collection of a specimen and its examination in the laboratory increases the likelihood of false-negative results (Johnston *et al*., 1996). If transportation to the laboratory is prolonged, streptococci are more likely to remain viable when held in a dry state. Survival of streptococci has been shown to be less than optimal on swabs that remain moist for a long time during transit, particularly in higher temperatures (Rubbo and Benjamin, 1951; Redys *et al*., 1968;). Transport media present in the tubes of commercial swabs provide holding environments suitable for retaining viable streptococci for at least 24h (Facklam, 1976). Some commercial swab systems use a liquid medium, Modified Stuart's transport medium, contained on a pad at the base of the tube. Systems that use an agarose butt of Amies semi-solid transport medium allow better recovery of streptococci as the agar plug surrounding the swab protects streptococci from changes in humidity within the tube. Swabs can be successfully transported by just replacing them in their tube but higher

recovery rates may be obtained by placing the swab in contact with sterile desiccant (silica gel removes moisture from around the swab) (Redys *et al*., 1968) or by smearing on to sterile filter paper strips housed in foil (Hollinger *et al*., 1960). When a dry method of transportation is used care has to be taken that swabs are not left exposed to ultraviolet light or extremes of temperature. Culturing of dried specimens can be postponed for up to 7 days without significant decrease in streptococcal counts (Martin, 2004).

2.8.6 Laboratory Culture

The gold standard test for detection of a group A streptococcus in the throat is culture on blood agar. The agar medium used must be enriched to support growth as streptococci are fastidious organisms and must also contain blood to allow observation of β-hemolysis (Facklam, 1976). Specimen should be inoculated immediately or sent to the laboratory in Spike's transport medium (blood agar containing 1 in 1,000,000 crystal violet and 1 in 16,000 sodium azide). Specimen is inoculated in blood agar medium and incubated at 37°C for overnight. Haemolysis develops better under anaerobic conditions or under 5-10% carbon dioxide. The bacterial colonies are small. Typically matt or dry and surrounded by β-haemolysis. After Gram staining to verify that they are Gram-positive cocci, the colonies should be submitted to specific identification tests for *S. pyogenes.* For clinical purposes presumptive identification of *S. pyogenes* is based on its susceptibility to a low concentration of Bacitracin. For this purpose: a special differential disc is used containing 0.02-0.05 IU of Bacitracin. The ordinary discs in the susceptibility test, with content of 10 units, are not suitable for identification. A β-haemolytic streptococcus showing any zone of inhibition around the disc should be reported as *S. pyogenes*. If the haemolytic colonies are sufficiently numerous, the presence of absence of an inhibition zone may be read directly from the primary blood agar plate. If the colonies are less numerous, one of two should be picked from the primary plate, streaked on another plate and each inoculated area covered with a Bacitracin disc. After overnight incubation, the subcultures should be read for inhibition zones (Vandepitte *et al*., 2003). .*S. pyogenes* is always sensitive to benzyl penicillin and therefore placing a 1 μg disc of the antibiotic on a primary culture plate (well area) can also help to presumptively identify *S. pyogenes* (Cheesbrough, 2006). Crystal violet (1 in 500,000) blood agar is a useful inexpensive selective medium for isolating *S. pyogenes* where *S. aureus* may be present with *S. pyogenes*. Crystal violet will inhibit the growth of *S. aureus*.

Alternatively, use a 30 µg neomycin disc on the heavy part of the inoculum (Cheesbrough, 2006).

Blood is used at a concentration of 5% most commonly in a base of columbia blood agar or trypticase soy agar. To view hemolysis, swabs are both rolled over the complete plate or over half the plate and then further streaked to separate colonies. Haemolysis can vary with the animal source for the blood, or the type of basal medium used. In some parts of the world, particularly where there is difficulty with supply of sheep or horse blood, outdated human blood is often used. This is not recommended as there is a lack of consistency in the product and the blood may contain antibodies or anti- microbial agents which can prevent growth of streptococci. A broth-enhanced culture method whereby the swab is grown in a suitable broth either prior to, or additional to, plating on blood agar has been shown to be valuable for increasing the yield of streptococci (Facklam, 1976). Todd Hewitt broth cultures are incubated in a waterbath to ensure an even growth temperature. To improve recovery of group A streptococci from throat swabs Blood agar with colistin (10µg/ml) and nalidixic acid (15µg/ml) inhibits gram-negative organisms but not staphylococci nor corynebacteria (Ellner *et al*., 1966). The use of oxolinic acid (5µg/ml) in place of nalidixic acid is inhibitive to staphylococci, corynebacterium and gram-negative organisms (Petts, 1984). SBA-SXT, that is 5% sheep blood agar containing trimethoprim (1.25µg/ml)-sulfamethoxazole (23.75µg/ml), has been shown to inhibit α-haemolytic streptococci and pneumococci while supporting the growth of group A streptococci (Gunn *et al*., 1977).

Streptococci are aerobic or facultatively anaerobic. Growth is stimulated by an atmosphere of 5–10% CO_2 or by anaerobic conditions. Aerobic incubation reduces the numbers of β-haemolytic streptococci other than group A streptococci. Aerobic conditions are quite satisfactory and reduction in oxygen tension to facilitate recognition of subsurface haemolysis can be achieved if cuts are made in the surface of the agar at the time of streaking. Anaerobic incubation is favoured by many because it enhances haemolysis but is also more costly and time consuming. Anaerobic conditions are also favourable to many facultative anaerobes that then must be differentiated from group A streptococci. The optimum temperature of incubation is 35–37^{0}C. Cultures negative for *S. pyogenes* following overnight incubation are incubated for another 24h. This increases in the recovery of streptococci of up to 46% have been reported (Kellogg, 1990).

Identification of Group A Streptococci

Any colonies on blood agar plates showing β-haemolysis require to be identified as to their serogroup, or by using Bacitracin discs presumptively identified as a group A streptococcus. Most β-haemolytic group A streptococci will be apparent on blood agar within 24 hours. Colonies vary in shape and size and may be mucoid. Under aerobic conditions some may appear β-haemolytic (Pinney *et al*., 1977). Non-haemolytic group A streptococci have also been reported (James and McFarland, 1971). Colonies of group A streptococci are often indistinguishable from other β-haemolytic streptococci, especially serogroups C and G. The number of colonies on a plate is not a good indicator for differentiating true infection from the carrier state (Martin, 2004). Patients with streptococcal pharyngitis generally show massive growth of *S. pyogenes*, with colonies over the entire surface of the plate. Plates of carriers generally show fewer than 20 colonies per plate. Even rare colonies of β-haemolytic streptococci should be colonies confirmed and reported (Vandepitte *et al*., 2003).

In sub-culture group A streptococci can be presumptively identified using a 0.04 unit Bacitracin differential disc. Inappropriate results will be achieved if the higher dose antibiotic sensitivity discs are used in error. Bacitracin is strongly inhibitory to *S. pyogenes* but not usually to other β-haemolytic streptococci, although it has been shown that streptococci of groups B, C, and G, can be as sensitive as group A's (Maxted, 1953). Erroneous results may be obtained if Bacitracin discs are placed on primary cultures, rather than pure cultures, and it has been suggested that up to 50% of *S. pyogenes* may be missed by this practice. An alternative screening test used is the PYR test which relies on the ability of organisms to hydrolyze L-pyrrolidonyl β-naphthylamide or L-pyroglutamic acid β-naphthylamide (PYR) (Facklam *et al*., 1982). Most group A streptococci are PYR-positive. A few group C and G isolates have also been found PYR- positive (Johnston *et al*., 1996). A number of different methods are available for extraction of the group-specific carbohydrate including extraction with hydrochloric acid, formamide, nitrous acid, or enzymes (Johnston *et al*., 1996). Commercially available streptococcal grouping kits, equivalent to the RADTs, are marketed for laboratory use. Most use group-specific rabbit antibody bound either to protein A or to latex beads. Visual specific agglutination is the end point (Martin, 2004).

Streptococcal Antibody Tests

Antibody tests have no value in the diagnosis of a streptococcal sore throat. However, they are very important in providing evidence for antecedent streptococcal infection in support of the major and minor manifestations described in the Jones criteria (American Heart Association, 1992). Streptococcal antibody titres take around 10–14 days to elevate. Thus, a sequential rise in titers will confirm recent streptococcal infection. The serological tests most commonly performed are the antistreptolyin O test (ASO), and anti-deoxyribonuclease B (anti-Dnase B). True group A streptococcal infections involve a specific immunologic response as measured by a significant increase in the titer of antibodies to at least one of the extracellular antigens, streptolysin O, deoxyribonuclease B, hyaluronidase, streptokinase and nicotinamide adenine dinucleotidase. The neutralization of enzyme by specific antibodies present in a patient's serum is the basis of each streptococcal antibody test used. Antibody raised against extracellular enzyme antigens reaches a peak 2–3 weeks after acute infection. The level of antibody reached is generally governed by idiosyncratic differences within individuals and will be determined by the existing level of that antibody from previous infections. Antibody is maintained for 2–3 months before declining (Ayoub, 1982). This test is mainly used in the diagnosis of acute rheumatic fever, acute glomerulonephritis and other post-streptococcal diseases (Vandepitte *et al*., 2003).

There are two types of commercial antistreptolysin O test kits:

I. The ASO latex slide agglutination test is used to screen sera to identify those with raised ASO titres (200IU or higher).
II. The ASO tube test is a haemolysis inhibition test that is used to determine ASO antibody titre in serum samples that are positive in the ASO latex slide agglutination test. A titre of less than 50IU does not confirm the diagnosis of acute rheumatic fever (Vandepitte *et al*., 2003).

Streptolysin O titers are often reported as Todd Units which represent the highest dilution of serum showing complete inhibition of haemolysis (Todd, 1932). About 20% of infected individuals will not respond with an increase in anti-streptolysin O antibody (Ayoub and Wannamaker, 1962). Thus, a negative anti-streptolysin O titer alone cannot be used to rule out preceding streptococcal infection (Martin, 2004).

Group A streptococci produce four deoxyribonucleases (DNases) designated A, B, C and D. During infection greatest response is against DNase B. The presence of antibodies against DNase B provides evidence of recent infection with *S. pyogenes* (Ayoub and Wannamaker, 1962) although some isolates of groups C and G streptococci also produce DNase B. The anti-DNase B test is usually used in parallel with the ASO test. Peak levels of anti-DNase B may be slower to occur, taking 6–8 weeks to peak, but may remain elevated for longer periods than ASO (Dawson and Martin, 1982).

Anti-nicotinamide adenine dinucleotidase (NADase) and anti-streptokinase (ASK) tests currently are uncommonly used for determination of streptococcal antibodies (Martin, 2004).

2.8.7 Treatment

Most people with sore throat manage the condition successfully without seeing a doctor. Paracetamol is an effective analgesic, with less risk of adverse effects than non-steroidal anti-inflammatory drugs. Aspirin should be avoided in children because of the risk of Reye's syndrome. The immediate benefits from antimicrobial chemotherapy are actually very meagre. Symptoms usually persist for 5-7 days with or without antibiotics, which only shorten illness by 24h. The same control of symptoms can probably be achieved with paracetamol (Greenwood *et al*., 2007).

Drugs of choice for Streptococcal sore throat are Penicillin V or a macrolide, and these should be given for at least 10 days to eradicate the organism and prevent recurrence. Glandular fever commonly causes symptoms and signs that are indistinguishable from streptococcal throat infection. Ampicillin, Amoxycillin, and Co-amoxiclav should not be used, as they will cause a rash if the sore throat is the herald of glandular fever. Tetracyclines are also inappropriate because of the high incidence of resistance among streptococci (Greenwood *et al*., 2007).

Symptomatic therapy – including warm saline gargles, antipyretics and analgesics (e.g. acetaminophen, aspirin, and nonsteroidal anti-inflammatory agents) – remains an important component of the management of sore throat. In the great majority of cases, GAS pharyngitis is a self-limited illness, even if no antimicrobial therapy is given (Peter and Bisno, 2004)

The main goals of therapy for group A β-haemolytic streptococcus pharyngitis are to avoid suppurative complications (peritonsillar or retropharyngeal abscess, cervical lymphadenitis, mastoiditis, sinusitis and otitis media), prevent rheumatic fever, abort person to person

transmission, and diminish the signs and symptoms associated with this disease. Oral and parenteral Penicillins, first through third generation oral cephalosporins, macrolides, and clindamycin have all been studied and found to be effective in meeting the majority of these goals. Nevertheless, leading authorities continue to recommend Penicillin as the drug of choice for GAS pharyngitis (Bisno *et al*., 2002). This recommendation is based on Penicillin's efficacy, narrow spectrum of antimicrobial activity, the infrequency of associated adverse reactions, and its low cost. In addition Penicillin remains the only antibiotic that has been shown to effectively prevent primary (Denny *et al*., 1950; Wannamaker *et al*., 1951) and secondary attacks of rheumatic fever (Chamovitz *et al*., 1951). Penicillin V remains the 'gold standard' for GAS pharyngitis therapy (Bisno, 1996), and no resistant *S. pyogenes* strains to Penicillin G have been isolated (Macris *et al*., 1998). However, 5–30% of patients harbor GAS in the pharynx after completion of Penicillin oral therapy. In addition, patients can be allergic to β-lactam antibiotics. Macrolides represent an alternative therapy deserving consideration. Newer macrolide compounds offer improved gastro-intestinal tolerance than the initial Erythromycin derivatives (Salazar, 2004).

Newer macrolide such as Azithromycin is characterized by a long apparent elimination half-life, allowing short-term therapy (3–5 days) with one administration per day. Results with Azithromycin therapy are controversial (Bryskiera and Giuseppe, 2004). Some studies concluded to results similar to those obtained with Penicillin V, after 3 days (O'Doherty, 1996) or of 5 days (Hooton, 1991) of Azithromycin therapy. Other studies raised concerns about the rate of bacterial eradication, shown to be inferior with Azithromycin 3 days than with Penicillin V 10 days at the late post-treatment visit (Hamill, 1993; Schaad and Heyneu, 1996; Tarlow, 1997). The same concern was expressed in a study comparing 10 days of clarithromycin and 5 days of Azithromycin (Kaplan *et al*. 2001). Sulfonamides and Tetracyclines are not recommended for treatment because of the higher rates of resistance to these agents and the frequent failure of these agents to eradicate even susceptible organisms from the pharynx (Salazar, 2004).

2.8.8 Mechanisms of antibiotic resistance in *Streptococcus pyogenes*

Penicillin resistance has not been a significant problem in the treatment of GAS (Kaplan *et al*., 1999; Bisno *et al*., 2002). In fact, there has yet to be a single, well-documented report of a clinical isolate resistant to Penicillin. Several investigators have searched for Penicillin

resistance in isolates of GAS and in all these studies the isolates were extremely sensitive to Penicillin G (MIC <0.06 μg/ml) (Salazar, 2004). However, decreased susceptibility of GAS to sulfa drugs and macrolides has been well documented (Seppala *et al*., 1992; Bassetti *et al*., 2000). Inappropriate overuse of Erythromycin in Japan during the 1960s and 1970s resulted in more than half of GAS developing resistance to Erythromycin. More recently, documentation of Erythromycin resistance in northern Europe and fewer than 5% of GAS isolated in the United States have been documented (Coonan and Kaplan, 1994).

There are presently three recognized mechanisms of resistance of macrolide antibiotics: target modification, efflux and inactivation. A target site modification by an rRNA-methylating enzyme encoded by the ermAM and ermTR. The second mechanism of resistance, named the M phenotype, is mediated by an efflux pump encoded by the mefA gene, rendering the bacteria resistant to 14- and 15-membered macrolides, with retained susceptibility to clindamycin and streptogramin B. Resistance to macrolide, lincosamide, and streptogramin (MLS) antibiotics due to inactivation has been described for a number of clinically important organisms including *Staphylococcus aureus*, *Staphylococcus haemolyticus*, and *Escherichia coli*. No form of macrolide inactivation has been described for streptococci (Yamanaka, 2004).

rRNA Methylases

There are currently at least eight classes of rRNA methylase genes (erm) distinguishable by hybridization criteria (Leclercq and Courvalin, 1991; Weisblum, 1995). Erm methylases add either one or two methyl residues to a highly conserved adenine residue in domain V, the peptidyl transferase center, of 23S rRNA (Lai and Weisblum, 1971; Weisblum, 1995). This modification renders the strain resistant to most macrolides, lincosamides and streptogramin B compounds. Phenotypically, this resistance pattern is known as MLS_B resistance (Fernandez-Munoz *et al*., 1971; Lai and Weisblum, 1971) (Fig. 1).

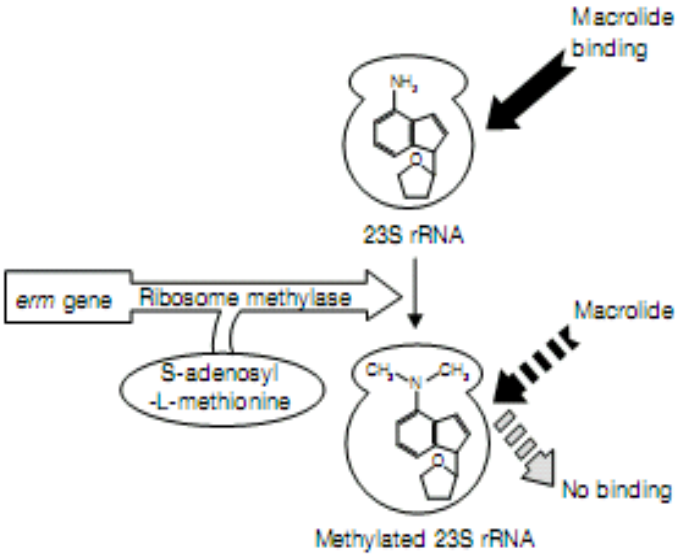


Fig.1. erm-dependent methylation of 23S rRNA

In streptococci MLS_B resistance has commonly been due to genes belonging to the ermAM (ermB) gene class (Horinouchi *et al*., 1983). Thereafter, genes of the same class have been sequenced from *S. pneumoniae*, *S. pyogenes*, and *S. agalactiae*. Seppälä *et al*., 1998 have characterized a novel erm gene, ermTR, from an Erythromycin-resistant clinical strain of *S. pyogenes* (A200) isolated in Finland.

Efflux System

Efflux system, have been found in increasing frequency in certain gram-positive pathogens. Active drug efflux is mediated in *S. pyogenes* by the mefA (macrolide efflux) gene (Clancy *et al*., 1996). It causes resistance to 14- and 15-membered macrolide compounds only; this phenotype is called the M-phenotype (Clancy *et al*., 1996; Kataja *et al*., 1999). Many of these proteins (mefA, mefE, and lmrA) have homology to the major facilitator superfamily (MFS) of efflux proteins. Recently, *Streptococcus pneumoniae* strains which carry both mef and ermB genes and have the MLS_B phenotype have been identified (Johnston *et al*., 1998; Luna *et al*., 1999). The mefA gene was described in *S. pyogenes*, while the mefE gene was found in *S. pneumoniae*. Since the two genes share 90% DNA and 91% amino acid homology, Roberts *et al*. (1999) recommended that these two genes be considered a single class, A: mefA gene and MefA protein.

2.8.9 Literature review on throat infection in Nepal

There are few recent studies documenting BHS pharyngitis in Nepal. Mostly studies were carried out in school children. Data from various researchers has been summarized in Table 2.

Table 2: Research activity in throat infection in Nepal

Reference	Year of Study	Place	Age of Patients	No Screened	No of cases of β-haemolytic Streptococci	Type of Study
Prashad *et al*., 1990	1990	Pharping village Kathmandu	<16	311	31	Pilot survey
Bhattarai and Shrestha, 1995	1995	_	<16	1976	54	School survey
Shakya, 1998	1997 February 1997 to June 1997	TUTH Kathmandu	<25	134	2	Survey
Shrestha *et al*., 2001	2001	Pokhara	3-16	750	190	School survey
Acharya, 2002	March-August 2002	Kathmandu valley	4-14	303	39	School Survey
Bista *et al*., 2005	March 2000 to August 2001	E.N.T. Department of T.U. Teaching Hospital, Kathmandu, Nepal	2-51	50	9	Pediatric Survey
Sakota *et al*., 2006	1998-1999 Nov 2000	8 villages in Kailali Kanchanpur, Seti Mahakali	1-10	399	67	Genetic variation study
Shrestha *et al*., 2006	2006	Kanti Children's Hospital, Kathmandu	<16	192	113	Pediatric Survey
Rijal *et al*., 2009	2009	Pokhara	<16	487	45	School Survey
Dumre *et al*., 2009	February - April 2007	4 schools situated at different locations of Kathmandu valley	5-15	350	38	School Survey
Gurung *et al*., 2010	August 2009 through February 2010	Pediatric OPD, BPKIHS, Dharan	5-15	100	5	Pediatric Survey

Various researchers have worked in the area of throat infection in Nepal and published literatures found are listed below:

Prashad, *et al*. (1990) conducted a pilot survey to find out the prevalence of β- haemolytic streptococci, particularly Group A, from the throat of asymptomatic school children. Altogether 311 children from Pharping, a village in the Kathmandu valley were studied. 31 children (9.9%) were found to be harbouring β-haemolytic streptococci while GAS was found in 16 children (5.1%).

Bhattarai and Shrestha, (1995) conducted a survey to find out prevalence rate of β-haemolytic streptococci from throat of 1018 and 958 children studying indifferent public schools and boarding schools. Of 1018 asymptomatic school children of public school, 49 were found to have β-haemolytic streptococci. The carrier rate of β-haemolytic streptococci was 4.80% in public school in this study. On further serological grouping of β-haemolytic streptococci, group A strain was found in 11 children i.e. 1.08% group C strain in 10 children i.e. 0.9% and group G strain in 28 children i.e. 2.75% and peneumococci was found in 3 children. Of 958 asymptomatic children of boarding school, only 5 were found to have β-haemolytic streptococci. On further grouping, group C strain, group D strain and group F strain was found in 1 children respectively and group A in 2 children.

Shakya, (1998) conducted a study at Department of Community Medicine and Family Health Laboratory of Tribhuvan University Teaching Hospital (TUTH). During the period total 134 swab samples were collected from primary school children, lower secondary school children, high school children, and undergraduate medical student during five months period February 1997 to June 1997. Only two isolates of β-haemolytic streptococci group 'G' was isolated from primary school children and under graduate medical students. However none of cases of GAS was reported in the study.

Shrestha *et al*. (2001) conducted a cross-sectional and observational study on the prevalence of streptococcal pharyngitis among school children of Pokhara valley, Nepal. During seven months period, a total 750 children were examined from six government primary schools. 25.3% were found to have symptoms of clinical pharyngitis and the prevalence of GAS pharyngitis was 7.2% among these school children. GAS pharyngitis found more among male in the age group of 3-10 years.

Acharya, (2002) conducted a study at Department of Community Medicine and Family Health Laboratory of Tribhuvan University teaching hospital (TUTH), from March 2002 to August 2002. Total of 303 throat swab samples were collected from three different schools and examined for presence of β-haemolytic streptococci. Out of 303 samples, 12.9% (39/303) were found to be BHS while GAS constituted 38.5% (15/39). GAS from males (8/5) and 46.7% GAS from females (7/15). The prevalence of BHS and GAS was found to be higher in males than females however the result was statistically insignificant. Highest sensitivity was shown by Penicillin-G 93.3% (14/15), least sensitivity was shown by Cephalexin-53.3% (8/15), and highest resistivity was shown by Erythromycin- 26.7% (4/15).

Bista *et al*. (2005) conducted a study to find out the most common organism affecting the tonsils in recurrent tonsillitis and to compare the microorganisms found in the core and surface of tonsils. A total of 50 patients attending E.N.T. Out patient Department of Tribhuvan University Teaching Hospital (TUTH), Maharajgunj from February 2000 to August 2001. All patients had undergone tonsillectomy under general anaesthesia by dissection method. Among these 39 patients had undergone tonsillectomy for recurrent tonsillitis and 11 had undergone tonsillectomy for reasons other than recurrent tonsillitis. The culture was taken from the surface and the core after cutting the tonsils into half by a sterile blade and with another sterile blade only core was extracted in a wedge shaped manner and was cultured in aerobic (5% sheep blood agar, Chocolate agar and McConkey's agar) and anaerobic media for 24 - 48 hours. The most common microflora seen in recurrent tonsillitis was *Streptococcus viridans* from the surface, as well as the core. Other common micro organisms prevalent on the surface in recurrent tonsillitis were *Staphylococcus aureus*, and *Brahmnella catarrhalis*. Likewise other common micro organisms in the core in recurrent tronsillitis were *Staphylococcus aureus*, *Klebsiella pneumonie*, and *Haemophilus influenzae*. In this study prevalence of GAS was 18% among the paieints.

Sakota *et al*. (2006) conducted a study to assess genetic variation among GAS in western Nepal involving eight villages in the districts of Kailali and Kanchanpur, in the Seti and Mahakali zones in far-western Nepal in November 1998 and also in November 1999. Sixty children aged 1 to 10 years from each village were randomly selected. During November 2000 oropharyngeal swabs were collected from all children and skin swabs were collected from those with clinically diagnosed impetigo lesions. Impetigo lesions were cleaned and left intact for swabbing. Swabs were transported in silica packets at room temperature to the

Centres for Disease Control and Prevention (CDC), where they were cultured and subjected to serologic and phenotypic testing by established methods. GAS were isolated from 67 (17%) of 399 oropharyngeal swabs and 53 (58%) of 92 impetigo lesions. In total, 120 throat and skin lesion GAS isolates were recovered from 111 individuals and included in this analysis. Then 120 noninvasive GAS, collected from eight different villages, were genetically characterized using emm typing, sof sequencing, and multilocus sequence typing (MLST). A high level of genetic diversity was observed among these isolates, with 51 genotypes based upon 51 multilocus sequence types (STs), 45 emm sequence types, and 28 sof sequence types. On the basis of shared ST-emm and sof-emm associations, 40 of the 51 genotypes were identical or highly related to genotypes characterized from locations outside of Nepal, even though most of the emm sequence and clonal types are rare among GAS within the United States.

Shrestha *et al*. (2006) conducted a study with aims to identify the causative bacterial agents of upper respiratory tract infection (URTI) among paediatric patients of Kanti Children's Hospital and to reveal the antibiotic susceptibility pattern of the major pathogen during November 2001 to October 2002. The throat swab samples from 192 children suspected of URTI were obtained from the outpatient department of Kanti Children's Hospital and inoculated in the culture medium. The bacterial infection was confirmed only in 152 patients. The organisms isolated on medium were identified by their cultural, morphological and biochemical characteristics. The infections were found to be due to *S. pyogenes*, *S. aureus* and *S. pneumoniae*. Most of the infections were due to *S. pyogenes*. The information obtained during sample collection showed that the high rate of infection was found to be in male patients, in infants and in urban children. Antibiotic susceptibility pattern for the isolated organisms revealed that Amoxycillin and Cephalexin, Erythromycin and Cephalexin were the most effective for *S. pyogenes* and *S. aureus* respectively whereas both Amoxycillin and Cephalexin were equally and the most effective for *S. pneumoniae*. The effective antibiotic for overall URTI was found to be Amoxycillin.

Rijal *et al*. (2009) conducted study to find out the rate of asymptomatic throat carriage of *S. pyogenes* and antibiotic susceptibility of the isolates in school children of Pokhara, Western Nepal. A total of 487 randomly selected children younger than 16 years were included in the study. Throat swabs collected were subjected to 5.0% Sheep blood agar supplemented with crystal violet (CVBA). GAS was identified by β-haemolytic colonies, Bacitracin sensitivity,

Cotrimoxazole resistivity, catalase negativity and PYR positivity. Antibiotic susceptibility test was performed on Muller Hinton agar containing 5% sheep blood by modified Kirby-Bauer disc diffusion method. Out of total 487 throat swabs, GAS was isolated in 9.2% (n=45). Among the isolates, 46.6% (n=21) were from male children where as 53.4% (n=24) from female children. There was no significant sex difference in colonization of GAS ($p>0.05$). Out of 45 isolates, 100% isolates were sensitive to antibiotic Penicillin-G and Amoxycillin where as 15.6%, 6.6%, and 2.2% isolates were resistant to antibiotic Erythromycin, Tetracycline and Azithromycin respectively.

Dumre *et al*., 2009 carried out this preliminary study to determine the throat carriage rate and antimicrobial resistance trend of *S. pyogenes* among the Nepalese school children. Four schools situated at different locations of Kathmandu valley were included in the study. Throat swabs from 350 students of age group 5-15 years were collected, and were processed for *S. pyogenes* following standard microbiological procedures. Antimicrobial susceptibility testing of the isolates was performed by Kirby Bauer disc diffusion method following CLSI guidelines. *S. pyogenes* was isolated from 10.9% (38/350) of the screened children. The GAS colonization rate was statistically insignificant ($P>0.05$) with sex and age sub-groups, although the rate was slightly higher among girls and age sub-group 9-12 years. No significant difference in carrier rate was observed among different schools ($P>0.05$). All isolates were susceptible to Azithromycin. No resistance was detected for Penicillin and its derivative antibiotic Ampicillin. Highest resistance rate was observed for Cotrimoxazole (71.0%) followed by Chloramphenicol (7.8%), Ciprofloxacin (5.2%) and Erythromycin (5.2%).

Gurung *et al*. (2010) conducted a prevalence of streptococcal pharyngitis in pediatric patients presenting with sore throat. A total of 100 patients aged 5–15 years were enrolled in the study. *S. pyogenes* was identified on the basis of Gram's stain and its sensitivity towards 0.04 units of Bacitracin disc. Antimicrobial susceptibility testing of the isolates was performed using Kirby Bauer disc diffusion method. Out of 100 patients which were included, 54 were male and 46 were female. GAS was isolated from 5 patients, of which 3 were male and 2 were female. Of the 5, *S. pyogenes* strains isolated, 2 (66.6%) were found to be resistant to Cotrimoxazole. Ciprofloxacin resistance was seen in one isolate. No resistance was detected in Gentamycin, Cefalexin, Penicillin, and Erythromycin. The results showed that the prevalence of streptococcal pharyngitis is 5% among OPD cases.

CHAPTER-III

MATERIALS AND METHODS

3.1 Materials

A complete list of equipments, media, reagents and chemicals required for isolation and identification of pathogenic bacteria causing pharyngitis have been listed in Appendix II.

3.2 Methods

3.2.1 Study Design, Site and Population

This descriptive cross sectional study was designed to isolate and identify bacteria causing pharyngitis from throat swab. Throat swab samples were taken from patients with sore throat complain, visiting E.N.T. out-patient department of Bir Hospital during study conducted from February, 2010 to August, 2010. Samples were processed at microbiology laboratory of Bir Hospital Mahabaudha, Kathmandu, Nepal. The patients referred by medical practitioners were the target population in this study.

3.2.2 Data Collection

Data on age and gender of patients were collected.

3.3 Collection and dispatch of specimens

Altogether 134 samples were processed for culture, isolation and identification of causative organism of Pharyngitis/Tonsillitis.

3.4 Specimen collection and transport

Inclusion criteria: Patients presenting with sore throat visiting E.N.T. out-patient department of Bir Hospital, recommended by medical practitioners for throat swab culture.

Exclusion criteria: The following patients were excluded from this study,

i. Patients on antibiotics.
ii. Patients/parents not giving consent to give throat swab.

Sample collection:

The patient was asked to sit facing a light source. The tongue was kept down with a tongue depressor, a sterile cotton-wool swab was rubbed over each tonsil, over the back wall of the pharynx, and over any other inflamed area. Care was taken not to touch the tongue or buccal surfaces. Two swabs from the same areas were taken. One was used to prepare a smear for Gram Staining, while the other for culture.

3.5 Processing of the specimens

Direct microscopy

The throat swab was smeared on a clean glass slide and Gram staining was performed. The standard protocol provided by Brancato and Parker (1966) was used for interpretation of Gram staining. The smear was observed at a magnification of 100 x for the presence of leucocytes. If none was found, it was deemed negative. If leucocytes were present, the areas with the greatest proportion of polymorphonuclear cells or showing 'disruption' (loss of cytoplasmic integrity and cellular outlines) were located. The microbial flora associated with the polymorphonuclear cells in these areas were examined at a magnification of 1000 x. Spherical Gram-positive cocci occurring singly and in pairs (structure typical of *S. pyogenes*) were differentiated from other Gram-positive cocci, which are more elongated or encapsulated (pneumococci), from chains or clumps. If Gram-positive cocci of typical structure were found associated with the polymorphonuclear cells, the slide was deemed positive. Then as shown in Table 3, sensitivity and specificity of Gram stain in relation to culture was calculated (Appendix V)

Table 3: Sensitivity and Specificity Gram stain in relation to culture for BHS

		Gold Standard: Culture positive BHS on Blood Agar Plates	
Gram Staining	Positive	POSITIVES Culture positive cases for BHS and Gram staining positive cases (true positive)	NEGATIVES Culture negative cases for BHS but Gram staining positive cases (false positives)
	Negatives	Culture positive for BHS but Gram staining negative cases (false negatives)	Culture negative cases for BHS and Gram staining negative cases (true negatives)

3.6 Culture

Culture for *Streptococcus pyogenes*

Immediately upon receipt in the laboratory, the swab was rubbed over blood agar plate, and the rest of the plate streaked with a sterile loop. The blood agar was prepared from a basal agar medium without glucose (or with low glucose content).

Isolation and identification

To have better recognition of β-haemolytic colonies all the inoculated plates were incubated at 37^{o}C in CO_2 jar (for overnight and then again re-incubation for 48 hours), the blood agar plates was examined for the presence of small (0.5–2mm) colonies surrounded by a relatively wide zone of clear haemolysis. Gram-staining was done to verify that they are Gram-positive cocci in chains, then colonies were submitted to specific identification tests for *S. pyogenes*. For clinical purposes, presumptive identification of *S. pyogenes* was based on its susceptibility to a low concentration of Bacitracin and Penicillin discs. For this purpose, a special differential disc was used containing 0.04 IU of Bacitracin and 10 IU disc of benzyl penicillin on crystal violet blood agar plate (CVBA). A β-haemolytic streptococci showing any zone of inhibition around the Bacitracin disc and sensitive to benzyl penicillin was reported as *S. pyogenes* i.e., GAS was identified on the basis of β-haemolytic colonies, Bacitracin sensitivity, catalase negativity, Gram-staining and growth on CVBA. Standard protocol provided by Vandepitte *et al*. (2003) and Cheesbrough (2006) was used for identification of *S. pyogenes*. The non beta haemolytic colonies were further cultured on nutrient agar and after incubation, the plates were observed then the colonies were further identified by various biochemical tests.

Culture for aerobic organisms other than BHS: Throat swab was rubbed over MacConkey agar (MA) and incubated at 37ºC for overnight. After incubation, the plates were observed then the colonies were further identified by various biochemical tests.

Isolation and identification: The aerobic culture plates were examined after over night incubation. The visual growths of the organisms were observed on the agar medium. For aerobic organisms, the identification was done using standard bacteriological chart. In every

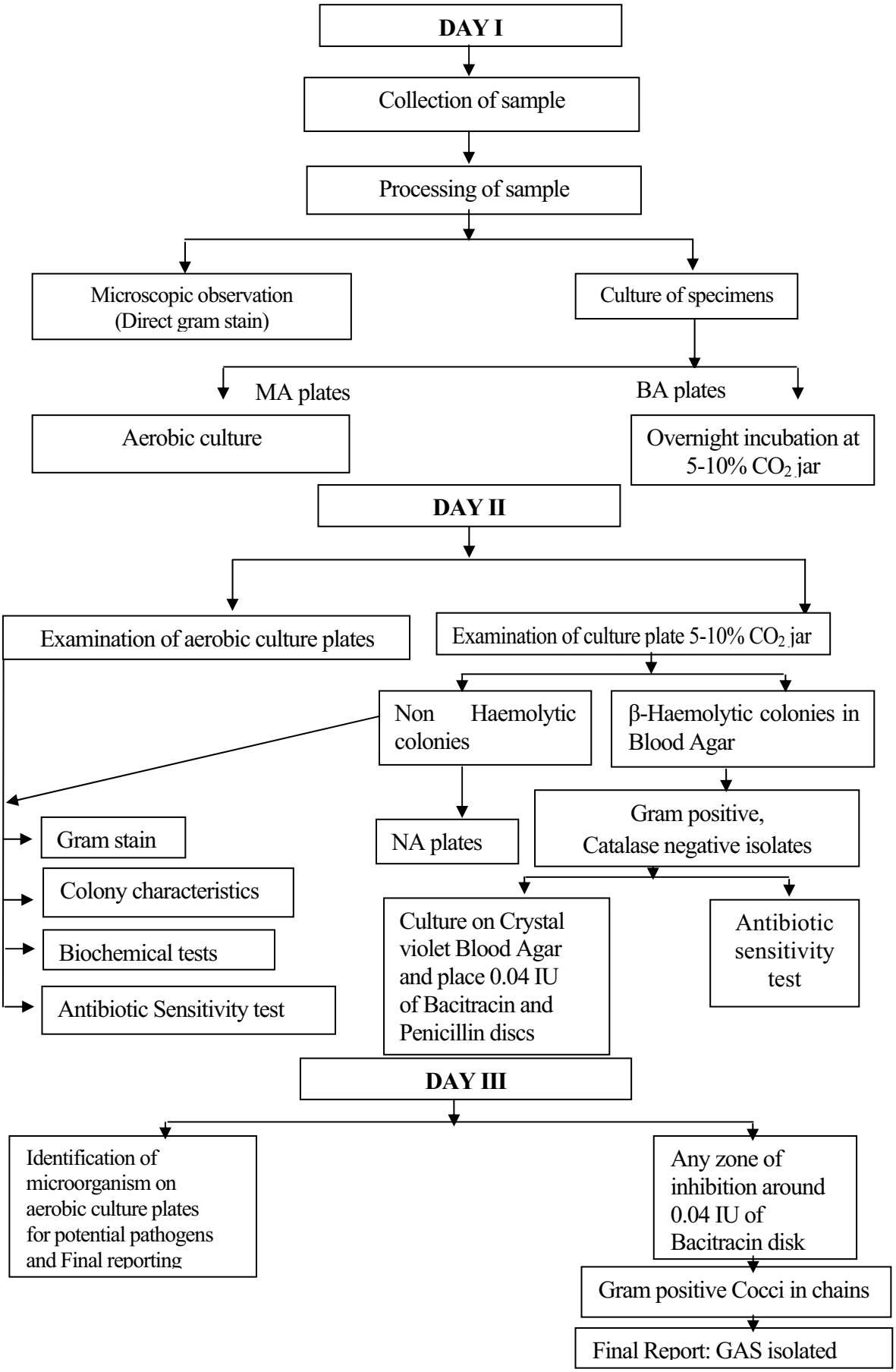


Fig: 2 Protocol for laboratory examination of throat swab

case, colony morphology was studied; Gram-stained smear was prepared and observed under the microscope. Biochemical tests were then followed for identification of bacteria. Different biochemical tests performed were catalase test, oxidase test, coagulase test, oxidative-fermentative (OF) test, methyl-red (MR) test, voges-proskauer (VP) test, indole test, motility test, hydrogen sulphide (H_2S) production test, triple sugar iron (TSI) reactions, citrate utilization test, and urease test. The composition of media and reagents used for different biochemical tests and their procedures are given in appendix III.

3.7 Antimicrobial Susceptibility Testing

All the pathogenic microorganisms isolated from throat swab samples were subjected for antimicrobial susceptibility test by Kirby-Bauer disk diffusion method as recommended by CLSI (formerly NCCLS). The disc diffusion method (modified Kirby-Baur method), originally described in 1966 is well standardized and has been widely evaluated. Official agencies have recommended it, with minor modifications, as a reference method which could be used as a routine technique in the clinical laboratory as recommended by NCCLS (Vandepitte *et al*., 2003). In this technique the antimicrobial agent diffuses from the disc to the medium. Following overnight incubation, the culture was examined for areas of no growth around the disc. Bacterial strains sensitive to the antimicrobial are inhibited from the disc where as resistant strain grows up to the edge of the disc. The different antibiotics used and the test procedures are given in the appendix (IV C). In case of β-haemolytic streptococci bacterial susceptibility to antimicrobial agent was done in vitro by modified Kirby-Bauer method in Muller Hinton Agar Medium (containing 5% blood).

3.8 Quality control

For the confirmation of accuracy of the test results, quality control was maintained throughout the period of the study. Strict aseptic condition and techniques were followed by the batch test of the culture medium and monitoring the antibiotic discs by using standard ATCC culture as recommended by CLSI.

All the necessary quality controls of media and culture plates were performed during the experiment. The media lot no. and expiry date were also checked. *S. pyogenes* ATCC 19615 was taken as control for susceptibility test of low concentration of

Bacitracin for identification of GAS. For the standardization of Kirby-Bauer test and for performance testing of antibiotics and MHA, control strains of *E. coli* (ATCC25922) and *S. aureus* (ATCC 25923) were tested primarily. Quality of sensitivity tests was maintained by maintaining the thickness of Mueller-Hinton agar at 4 mm and the pH at 7.2-7.4. All antibiotics disc and were checked for their expiry date.

3.9 Purity plate culture

Purity plate indicates whether the further procedure was performed with pure/single bacteria or not. For the purpose it was done during each biochemical test. The growth of the organism in pure form in both pre and post inoculation area indicated that the experiment was performed with pure culture.

3.10 Data analysis

All the results were entered in the worksheet of Statistical Package for Social Science (SPSS) software (Version 17.0). Chi-square test was used to determine significant association of dependable variables like age group to number of BHS positive cases. The detailed of data analysis is shown in Appendix V.

CHAPTER-IV

RESULTS

During the study period, a total of 134 throat swab samples were processed for the isolation of pathogenic microorganisms. In this study β-haemolytic streptococci were isolated in17.2% cases and Group A β-haemolytic streptococcus were isolated in 9% cases.

4.1 Age and gender wise distribution of patients

The sample obtained were categorized into different age groups <15years, 16-20 years, 21-40 years, 41-65 years and >66 years respectively. Maximum number 56.7% (n=76) of samples were received from age group 21-40 years, 20.1% (n=27) samples were from age group 16-20 and similarly 13.5% (n=18) samples were from age group 41-65. (Table 4)

Table 4: Age and gender wise distribution of the patients

Age group	Male		Female		Total	
	Number	%	Number	%	Number	%
< 15	10	83.3	2	16.7	12	9.0
16-20	20	74.1	7	25.9	27	20.1
21-40	42	55.3	34	44.7	76	56.7
41-65	6	33.3	12	66.7	18	13.5
> 65	0	0	1	100	1	0.7
Total	78		56		134	100

Out of the 134 throat swab samples, 58.2% (n=78) samples were received from the male patient whereas 41.7% (n=56). samples from female patients. Out of 78 samples from male patients, 15.3% (12/78) showed growth of β-haemolytic streptococci. Similarly, of the 56 samples from female, 19.6% (11/56) showed growth of β-haemolytic streptococci. (Table 5)

Table 5: Gender wise distribution of the samples

Clinical Sample	Positive Cases for BHS %	Total Samples	Male	%	Female	%
Throat Swab	17.2	134	78	58.3	56	41.7

4.2 Pattern of growth in Throat swab specimens

Out of 134 samples, 17.2% (23/134) were found to be β-haemolytic streptococci (BHS), Bacterial pathogens other than BHS accounted for 6.7% (9/134) cases and no pathogenic organisms were reported in 76.1% (102/134) cases. Group A β-haemolytic streptococcus (GAS) constituted 52.2% (12/23). (Table 6)

On the basis of age wise distribution of the β-haemolytic streptococci, 56.5% (13/23) BHS were isolated from the age group 21-40 and 21.8% (5/23) BHS were isolated from age group 16-20 similarly 13% (3/23) BHS from age group <15 and 8.7% (2/23) BHS from age group 41-65 as shown in Table 7. There was no significant association between β-haemolytic streptococci cases and the age group of the patients (P>0.05).

Table 6: Age and gender wise distribution of the patients with positive cases of β-haemolytic streptococci

Age group	Male		Female		Total BHS	
	Number	%	Number	%	Number	%
< 15	3	100	0	0	3	13.0
16-20	4	80	1	20	5	21.8
21-40	5	38.5	8	61.5	13	56.5
41-65	0	0	2	100	2	8.7
Total	12		11		23	100

Age wise distribution of the GAS showed that 16.7% (2/12) GAS isolated from the age group <15, 8.3% (1/12) from age group 16-20 and 75% (9/12) from age group 21-40.However no GAS was isolated from age group greater then 41 years. (Table 7)

Table 7: Age and gender wise distribution of the patients with positive cases of Group A β-haemolytic streptococcus

Age group	Male		Female		Total GAS	
	Number	%	Number	%	Number	%
< 15	2	100	0	0.0	2	16.7
16-20	0	0	1	100	1	8.3
21-40	4	44.4	5	55.6	9	75.0
Total	6		6		12	100

4.3 Bacterial isolates other than β-haemolytic streptococci from throat samples

Out of 134 throat swabs that were cultured, 23 BHS were isolated and 9 cases showed pathogenic microorganisms other than BHS. In 9 cases 4 cases had mixed growth of other pathogenic bacteria as shown in Table 16. The other pathogenic microorganisms isolated were *S. aureus* 6 (4.5%), *K. pneumoniae* 5 (3.7%), *P. aeruginosa* 2 (1.5%), *Citrobacter freundii* 1 (0.7%), and Yeast Cells.1 (0.7%). (Table 9)

Table 8: Microbiological profile of throat isolates and their genderwise distribution

Organisms		Male		Female		Total %	Total no. of isolates
		No. of isolates	(%)	No. of isolates	(%)		
β Haemolytic streptococci	β Haemolytic streptococci Other than GAS	3	2.2	8	6.0	8.2	11
	Group A β Haemolytic streptococcus	5	3.7	7	5.2	9.0	12
Staphylococcus aureus		4	3.0	2	1.5	4.5	6
Klebsiella pneumoniae		2	1.5	3	2.2	3.7	5
Pseudomaonas aeruginosa		2	1.5	0	0	1.5	2
Citrobacter freundii		0	0	1	0.7	0.7	1
Yeasts		1	0.7	0	0	0.7	1

Among samples examined in 6.7% (9/134) cases pathogenic bacteria other than BHS were isolated and out of 9 cases 4 cases of mixed growth of other pathogenic bacteria. The association between culture result and isolation of β-haemolytic streptococci was statistically significant (P=0.0084). This data clealy showed that β-haemolytic streptococci was major pathogen in pharyngitis patients.

Table 9: Age wise distribution of pathogens other than β-haemolytic streptococci

Pathogenic Microorganisms other than BHS	Age groups					
	<15 years	16-20 years	21-40 years	41-65 years	>65 years	Count
Staphylococcus aureus	-	2	4	-	-	6
Klebsiella pneumoniae	-	-	3	2	-	5
Pseudomaonas aeruginosa	1	-	1	-	-	2
Citrobacter freundii	-	-	1	-	-	1
Yeast Cells spp.	1	-	-	-	-	1

In cases pathogenic bacteria other than BHS most of the isolates were from age groups 21-40 years and 41-65 years. *S. aureus*, *K. pneumoniae*, *Citrobacter freundii* and *Escherichia coli* were predominant in adults. In two cases of *P. aeruginosa* one was from school aged children (<15) and another from young adult (21-40 years). (Table 9)

4.4 Antibiotic susceptibility pattern of the β-haemolytic streptococci isolates

All the isolated microorganisms were tested with Kirby-Bauer method of disk diffusion on Mueller-Hinton agar containing 5% blood. The sensitivity and resistance pattern shown by BHS isolates towards different antibiotics are given in Table 10 and 11 From the data, it was observed that most sensitive drugs for GAS were Amoxycillin, Cephalexin, Cefotaxime, Ciprofloxacin and Azithromycin which were 100% sensitive followed by Cotrimoxazole (91.7%) and Erythromycin (91.7%). Most sensitive drugs for BHS were Amoxycillin, Cefotaxime, Ciprofloxacin and Azithromycin were 100% sensitive followed by Cotrimoxazole (90.9%), Erythromycin (72.7%) and Cephalexin (63.6%). (Table 11)

Table 10: Antibiotic susceptibility pattern of β-haemolytic streptococci isolates

Antibiotics	BHS Other than Group A					GAS				
	Sensitive		Intermediate	Resistant		Sensitive		Intermediate	Resistant	
	No.	%	No.	No.	%	No.	%	No.	No.	%
Amoxycillin	11	100	0	0	0	12	100	0	0	0
Cephalexin	7	63.6	0	4	36.4	12	100	0	0	0
Cefotaxime	11	100	0	0	0	12	100	0	0	0
Cotrimoxazole	10	90.9	0	1	9.1	11	91.7	0	1	8.3
Ciprofloxacin	11	100	0	0	0	12	100	0	0	0
Erythromycin	8	72.7	0	3	27.3	11	91.7	0	1	8.3
Azithromycin	11	100	0	0	0	12	100	0	0	0

Table 11: Antibiotic susceptibility pattern for isolated Group A β-haemolytic streptococcus

S.N.	Sample code	Organism Isolated	Antibiotics used						
			AM	CP	CE	CF	CO	E	AT
1	**001ENT**	GAS	S	S	S	S	S	S	S
2	**002ENT**	GAS	S	S	S	S	S	S	S
3	**003ENT**	GAS	S	S	S	S	R	R	S
4	**004ENT**	GAS	S	S	S	S	S	S	S
5	**005ENT**	GAS	S	S	S	S	S	S	S
6	**006ENT**	GAS	S	S	S	S	S	S	S
7	**007ENT**	GAS	S	S	S	S	S	S	S
8	**008ENT**	GAS	S	S	S	S	S	S	S
9	**009ENT**	GAS	S	S	S	S	S	S	S
10	**010ENT**	GAS	S	S	S	S	S	S	S
11	**011ENT**	GAS	S	S	S	S	S	S	S
12	**012ENT**	GAS	S	S	S	S	S	S	S

Note: AM= Amoxycillin, CP= Cephalexin, CE= Cefotaxime, CO= Cotrimoxazole, E= Erythromycin, AT= Azithromycin, POL= Polymixin-B, S= Sensitive, R= Resistant.

Table 12: Antibiotic susceptibility pattern of β-haemolytic streptococci other than Group A β-haemolytic streptococcus

S.N.	Sample code	Organism Isolated	Antibiotics used						
			AM	CP	CE	CF	CO	E	AT
1	**013ENT**	BHS	S	S	S	S	R	R	S
2	**014ENT**	BHS	S	S	S	S	S	S	S
3	**015ENT**	BHS	S	S	S	S	S	S	S
4	**016ENT**	BHS	S	R	S	S	S	R	S
5	**017ENT**	BHS	S	S	S	S	S	S	S
6	**018ENT**	BHS	S	R	S	S	S	S	S
7	**019ENT**	BHS	S	S	S	S	S	S	S
8	**020ENT**	BHS	S	R	S	S	S	S	S
9	**021ENT**	BHS	S	S	S	S	S	R	S
10	**022ENT**	BHS	S	S	S	S	S	S	S
11	**023ENT**	BHS	S	R	S	S	S	S	S

Note: AM= Amoxycillin, CP= Cephalexin, CE= Cefotaxime, CO= Cotrimoxazole, E= Erythromycin, AT= Azithromycin, POL= Polymixin-B, S= Sensitive, R= Resistant.

4.5 Antibiotic susceptibility pattern of bacteria other than BHS

The antibiotic sensitivity pattern of bacterial isolates other than BHS showed highest sensitivity to Ciprofloxacin (78.6%), Gentamycin (75%) and Amikacin (75%), followed by Chloramphenicol (71.4%), Azithromycin (66.7%), Cefotaxime (57.1%), Penicillin (33.3%) and Cephalexin (42.9%) and least sensitivity to Amoxycillin (28.6%) and Cotrimoxazole (21.4%). Polymyxin B was used only for *P. aeruginosa*.The two isolates of *P. aeruginosa* showed sensitivity to Polymyxin B. (Table 13, 16)

Table 13: Antibiotic susceptibility pattern of bacteria other than BHS

Microorganisms		***S.aureus***	***K.pneumoniae***	***P.aeruginosa***	***C. ferundii***	**Total**	**%**
Antibiotics		No	No	No	No	No	
Ciprofloxacin	S	5	5	0	1	11	78.6
	I	0	0	0	0	0	0
	R	1	0	2	0	3	21.4
Amoxycillin	S	3	1	0	0	4	28.6
	I	0	1	0	0	1	7.1
	R	3	3	2	1	9	64.3
Cephalexin	S	3	2	0	1	6	42.9
	I	1	0	0	0	1	7.1
	R	2	3	2	0	7	50.0
Cefotaxime	S	4	3	0	1	8	57.1
	I	0	0	0	0	0	0
	R	2	2	2	0	6	42.9
Gentamicin	S	-	5	0	1	6	75.0
	I	-	0	0	0	0	0
	R	-	0	2	0	2	25.0
Amikacin	S	-	5	0	1	6	75.0
	I	-	0	0	0	0	0
	R	-	0	2	0	2	25.0
Chloramphenicol	S	4	5	0	1	10	71.4
	I	0	0	0	0	0	0
	R	2	0	2	0	4	28.6
Cotrimoxazole	S	2	1	0	0	3	21.4
	I	0	0	0	0	0	0
	R	4	4	2	1	11	76.6
Polymxin B	S	-	-	2	-	2	100
	I	-	-	0	-	0	0
	R	-	-	0	-	0	0
Azithromycin	S	4	-	-	-	4	66.7
	I	0	-	-	-	0	0
	R	2	-	-	-	2	33.3
Penicillin	S	2	-	-	-	2	33.3
	I	0	-	-	-	0	0
	R	4	-	-	-	4	66.7

Note: S= Sensitive, I=Intermediate and R= Resistant.

4.5.1 Antibiotic sensitivity pattern of *Staphylococcus aureus*

S. aureus showed highest sensitivity to Ciprofloxacin (83.3%) followed by Cefotaxime (66.7%), Azithromycin (66.7%), Chloramphenicol (66.7%) and Cephalexin (50.0%). It was least susceptible to Penicillin (33.3%) and Cotrimoxazole (33.3%). (Table 14)

Table 14: Antibiotic sensitivity pattern of *Staphylococcus aureus* (n=6)

Antibiotics	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ciprofloxacin	5	83.3	0	0	1	16.7
Amoxycillin	3	50.0	0	0	3	50.0
Penicillin	2	33.3	0	0	4	66.7
Cephalexin	3	50.0	1	16.7	2	33.3
Cefotaxime	4	66.7	0	0	2	33.3
Azithromycin	4	66.7	0	0	2	33.3
Chloramphenicol	4	66.7	0	0	2	33.3
Cotrimoxazole	2	33.3	0	0	4	66.7

4.5.2 Antibiotic sensitivity pattern of *Klebsiella pneumoniae*

K. pneumoniae showed highest 100% sensitivity towards Ciprofloxacin, Chloramphenicol, Amikacin, Gentamicin and 60% sensitivity towards Cefotaxime. It was least susceptible towards Cephalexin (40%), Amoxicillin (20%) and Cotrimoxazole (20%). (Table 15)

Table 15: Antibiotic sensitivity pattern of *Klebsiella pneumoniae* (n=5)

Antibiotics	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Ciprofloxacin	5	100	0	0	0	0
Amoxycillin	1	20.0	1	20.0	3	60.0
Cephalexin	2	40.0	0	0	3	60.0
Cefotaxime	3	60.0	0	0	2	40.0
Gentamicin	5	100	0	0	0	0
Amikacin	5	100	0	0	0	0
Chloramphenicol	5	100	0	0	0	0
Cotrimoxazole	1	20.0	0	0	4	80.0

4.5.3 Antibiotic sensitivity pattern of *Citrobacter freundii*

Single isolate of *Citrobacter freundii* was sensitive towards Ciprofloxacin, Cephalexin, Gentamicin, Amikacin and Cefotaxime whereas it was resistant against Amoxycillin and Cotrimoxazole.

4.5.4 Antibiotic sensitivity pattern of *Pseudomonas aeruginosa*

The two isolates of *P. aeruginosa* were sensitive towards Polymyxin B (100%). Both isolates showed resistance against Ciprofloxacin, Amoxycillin, Cephalexin, Gentamycin, Cotrimoxazole, Cefotaxime, Chloramphenicol and Cotrimoxazole.

Table 16: 4 Antibiotic sensitivity pattern for isolated microorganisms other than β-haemolytic streptococci

S.N.	Sample code	Organism Isolated	Antibiotics used										
			CF	AM	CP	CE	AK	G	C	CO	AT	POL	P
1	S001A	*K. pneumoniae*	S	I	S	S	S	S	S	R	-	-	-
	S001B	*S. aureus*	R	S	I	R	S	S	S	S	S	-	R
	S001C	*C. freundii*	S	R	S	S	S	S	S	R	-	-	-
2	S002A	*K. pneumoniae*	S	S	R	S	S	S	S	S	-	-	-
	S002B	*S. aureus*	S	S	S	S	S	S	R	R	R	-	S
3	S003A	*S. aureus*	S	R	R	S	S	S	S	R	S	-	R
	S003B	*K. pneumoniae*	S	S	R	R	S	S	S	R	-	-	-
4	S004A	*P. aeruginosa*	R	R	R	R	R	R	R	R	-	S	-
	S004B	*K. pneumoniae*	S	R	R	R	S	S	S	R	-	-	-
5	S005A	*P. aeruginosa*	R	R	R	R	R	R	R	R	-	S	-
6	S006	*S. aureus*	S	R	R	S	S	S	S	R	S	-	R
7	S007	*S. aureus*	S	S	S	S	S	S	R	R	R	-	S
8	S008	*K. pneumoniae*	S	S	S	S	S	S	S	R	-	-	-
9	S009	*S. aureus*	S	R	R	R	S	S	S	S	S		R

Note: CF= Ciprofloxacin, AM= Amoxycillin, CP= Cephalexin, CE= Cefotaxime, AK= Amikacin, G= Gentamicin, C= Chloramphenicol, CO= Cotrimoxazole,, AT= Azithromycin, POL= Polymixin-B, P= Penicillin, S= Sensitive, I=Intermediate and R= Resistant.

4.6 Validity of direct Gram staining of throat swab in relation to culture

In this study direct Gram stain smear was performed for 134 samples. Out of which 13.5% (18) cases showed positive correlation with culture result. No β-haemolytic streptococci in culture, however, Gram positive cocci observed only in gram staining was reported in 70.9% (95) cases. Gram positive cocci and pus cells observed in Gram stain and β-haemolytic streptococci isolated in culture was reported in 13.5% (18) cases. Gram positive cocci and pus cells were observed in gram staining and no pathogenic organisms were isolated in among 11.9% (16) cases. No Gram positive cocci in chain but pus cells observed in Gram stain smear but β-haemolytic streptococci isolated in culture in 3.7% (5) cases. (Table 17)

Table 17: Co-relation of Gram stain in relation to culture

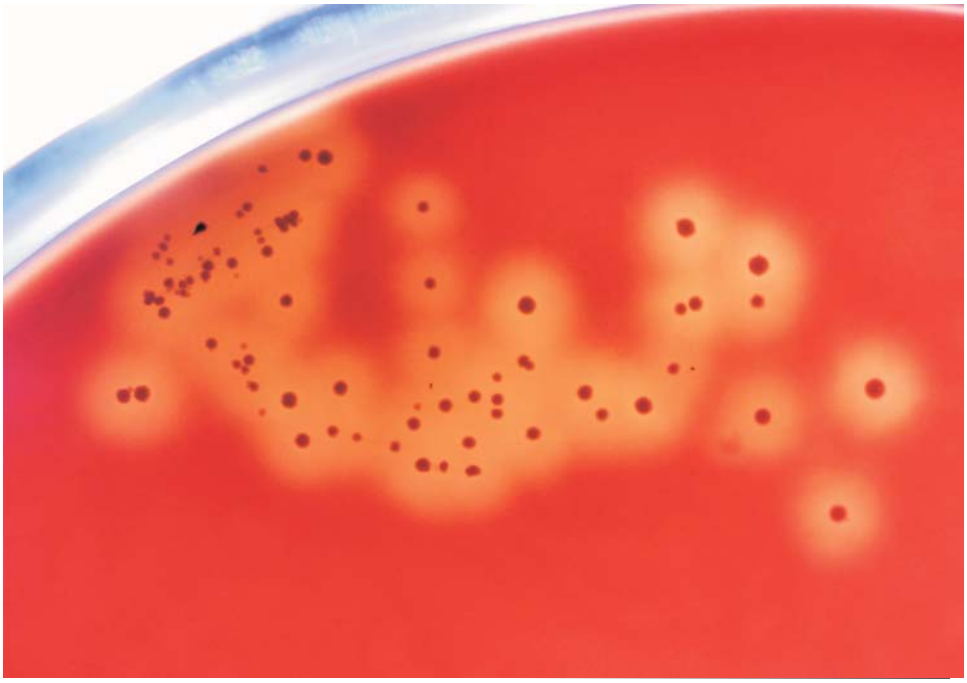
Nature of result	Throat specimen	
	Number	%
1. Direct smear : Gram positive cocci in chain and Pus cells observed Culture result: No β-haemolytic streptococci isolated	95	70.9
2. Direct smear : Gram positive cocci in chain and Pus cells observed Culture result: β-haemolytic streptococci isolated	18	13.5
3. Direct smear : No Gram positive cocci in chain and No Pus cells observed Culture result: No β-haemolytic streptococci isolated	16	11.9
4. Direct smear : No Gram positive cocci in chain but Pus cells observed Culture result: β-haemolytic streptococci isolated	5	3.7
Total	**134**	**100**

In this study sensitivity (positive culture and positive Gram stain) and specificity (negative culture negative Gram staining) of direct Gram-stained smear was calculated using culture as gold standard. The mean sensitivity, specificity and predictive value positive of direct Gram-stained smear as 78.3%, 14.4 % and 15.9%. (Table 18 and Appendix V)

Table 18: Sensitivity, specificity of Gram stain for detecting streptococci in relation to culture

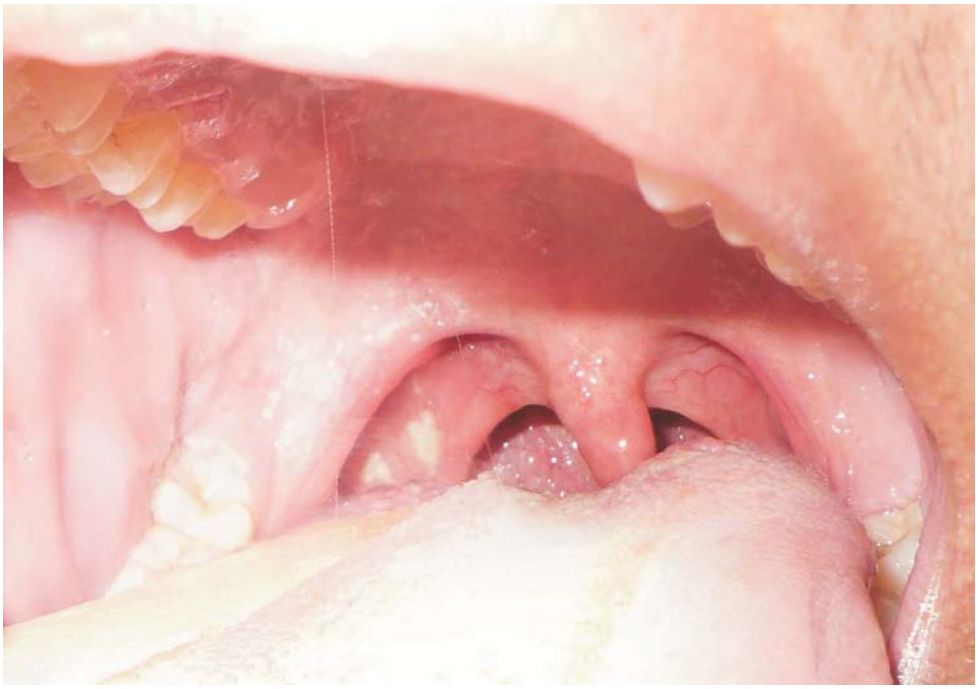
Direct Gram Staining	Culture for β-haemolytic streptococci		Sensitivity %	Specificity %	Predictive value +ve of test %
	+ve	-ve			
Positive	18	95	78.3	14.4	15.9
Negative	5	16			

Photograph 1: Culture plate of Group A β-haemolytic streptococcus (*S. pyogenes*) on Blood agar 0.04IU Bacitracin (BA)-Sensitive, 10 IU Penicillin-Sensitive [Sample code: 003ENT].



Photograph 2: β-haemolytic colonies of *Streptococcus pyogenes* enlarged [Sample code: 005ENT].

Photograph 3: Antibiotic susceptibility pattern of *Streptococcus pyogenes* in Blood agar. AM-Sensitive, CE-Sensitive, CP-Sensitive, CF-Sensitive, AT-Sensitive, CO- Resistant, E-Resistant [Sample code: 003ENT].



Photograph 4: Case of sore throat with red swollen uvula and with whitish spots on tonsils [Sample code: 003ENT].

CHAPTER-V

DISCUSSION

It is estimated that Bangladesh, Bhutan India, Indonesia, Myanmar and Nepal together account for 40% of the global acute respiratory infection (ARI) (WHO, 2002). The incidence of URTI in developing countries has been difficult to determine accurately (Campbell, 1995). It is estimate GAS pharyngitis has over 616 million incident cases per year in the world (Carapetis et al. 2005).

In this study, 134 throat swab samples were processed for detection of bacterial pathogens causing pharyngitis. Out of 23 (17.2%) cases that were found to be positive to BHS and 12 cases were accounted for GAS. This study showed that the prevalence of GAS was 9% in patients, studies conducted by Bista *et al*. (2005), Shrestha *et al*. (2006), Rijal *et al*. (2009), Gurung *et al*. (2010), showed the prevalence of GAS being 18%, 58.8%, 9.2% and 5% respectively. However in present study more samples were obtained from adults of age >15 years, only Bista *et al*. (2005) in above mentioned studies included adult population. In this study among 23 positive β-haemolytic cases 88.46% (20/23) patients were greater than 15 years. Data obtained in this study correlates with studies done by Poses *et al*. (1985); Komaroff *et al*. (1986); Ebell *et al*. (2000) that the prevalence of GAS is reported to be around 5% to 10% in adults. There are an estimated 6.7 million visits to primary care providers by adults who complain of sore throat each year in the United States, and antibiotics are prescribed at 73% of these visits (Linder and Stafford, 2001). The high prevalence of BHS in adult population in patients in Bir hospital may be due to hygienic and socioeconomic condition or be due to adult population serve as carrier of BHS. In this study, the frequency of BHS was not similar in all age groups, but it was slightly higher in adults aged 21-40 years however it was statically insignificant (P>0.05). In this study it has been seen that the age of the patients coming for treatment shifted from school aged children to young adults 21-40 years of age. This is more likely because Bir Hospital entraps more of adult population and children usually visit children's hospital located near by, thus eluding attention.

During this study, 76.1% cases culture result showed growth of non pathogenic microorganisms of which may be either due to the viral infection in patients or allergic reactions which were not taken into consideration. According to study done by Modrzyński *et*

al. (2003) Immunohistochemical findings supports the hypothesis that allergic sensitization takes place in the adenoid and tonsils. The study confirms that tonsil dendritic cells, macrophages, eosinophils, and mast cells are important in allergic tonsillitis (Modrzyński *et al*. 2005).

In this study highest number of pathogenic organism in throat swab was represented by BHS, then followed by *S. aureus* 6 (4.5%), *K. pneumoniae* 5 (3.7%), *P. aeruginosa* 2 (1.5%), *Citrobacter freundii* 1 (0.7%) and Yeast 1 (0.7%) respectively. According to Vandepitte *et al*. (2003) these microorganisms do not cause pharyngitis, except in association with granulocytopenia, it is advisable to report such isolates to the clinician, as they occasionally indicate the existence of (or may sometimes give rise to) a lower respiratory tract infection (e.g. pneumonia) or bacteraemia. Isolation of these organisms in throat of may be due to transient colonisation of these organisms in adults secondary to repeated use of antibiotics (Longanathan *et al*., 2006). In this study it was found that infections caused by BHS in tonsillitis/pharyngitis patients is significantly higher than infections caused by other pathogenic bacteria $P=0.0084$ i.e. ($P<0.05$). Thus, according to data obtained it can be said that BHS was the major pathogen in pharyngitis cases.

In present study, *S. aureus* and *K. pneumoniae* were reported in 4.5% and 3.7% cases respectively. Bista *et al*. (2005) in comparative study of core and surface culture of tonsillectomy cases done in T.U.T.H. reported *Staphylococcus aureus* in 21% of cases. Likewise *K. pneumoniae* on 10% cases, both organisms were more significantly seen in >20 age group than others. According to Longanathan *et al*. (2006) *S. aureus* was reported in 45.5% of cases and was the most common organism in adults with sore throat followed by *K. pneumonia* in 26.0% of cases and was the second most common organism. The low prevalence of these organisms in present study is may be due to pharyngeal swab culture do not reliably reflect presence of pathogen in tonsilar core as shown in study carried out by Al-roosan *et al.* (2008).

In this study *Haemophlius* spp. were not isolated from throat swabs. However in studies conducted by Brook and Gober (2006); Zautner *et al*. (2010) showed that *Haemophilius* spp. also causes pharyngitis and should not be ignored as commensal organism. *H. influenzae* produces β-lactamase and protects other bacteria from Penicillin, which is an important factor in mixed infections (Jeong *et al*., 2007). *H. influenzae* and *M. catarrhalis* have been recovered

in 10–20% of acutely (Brook, 1985), recurrently (Stjernquist-Desatnik *et al*., 1990) and chronically (Brook *et al*., 1995) inflamed tonsils. However, Brook and Gober, (2006) associated their isolation in conjunction with the recovery of GAS. The increased isolation of *H. influenzae* (in APT only) and *M. catarrhalis* in association with GAS may be due to a synergistic relationship between these organisms (Brook and Gillmore, 1996; Lafontaine *et al*., 2004). The ability of *H. influenzae* and *M. catarrhalis* to produce the enzyme β-lactamase may also make them, as well as GAS, more resistant to eradication by Penicillin and contribute to failure of Penicillin therapy (Brook and Gober, 2006). This may be due to the ability of β-lactamase to inactivate the Penicillin ivn the tonsillar tissues (Brook, 1984).

In this study, GAS was identified using Bacitracin inhibition test, since serotyping was not available. The low concentration Bacitracin disks of 0.04 unit i.e. MAST ID ^{TM} Bacitracin disc (D 40) was used to identify GAS. According to the manufactures of above mentioned Bacitracin disks, presence of any zone of inhibition around disks is considered positive presumptive identification of Lancefield Group A streptococci. Out of 23 BHS isolates 12 isolates showed zone of inhibition around low concentration Bacitracin disks. Maxted, (1953) originally used the low concentration of Bacitracin disks for presence or absence of a zone of inhibition to identify group A streptococci. Washington *et al*. (1974) using a disc content of between 0.02 and 0.04 unit, successfully employed a specific zone size of 11 mm to differentiate group A from non-group A strains. On the other hand, Ederer *et al*. (1972), using discs containing 0.04 unit of Bacitracin and the manufacturer's recommendations of a specific zone of inhibition of 10 mm, found that 6% of group A strains were not identified and 13% of non-group A streptococci were wrongly identified as group A, and Arvilommi, (1976) falsely identified 43% of non-group A strains as group A using 0.2 unit discs and a cut-off zone size of 10 mm recommended by the manufacturer. According to Coleman *et al*. (1977) accurate measurement of the 12 mm cut-off point is required for the 0.04 unit disc with a zone or no zone reading would appear to be a better choice for routine use**.** The Bacitracin inhibition test was found to identify 99.9% strains of Lancefield group A streptococci (Coleman *et al*., 1977).

Routinely laboratories use Bacitracin inhibition test to differentiate group A streptococci from strains belonging to the other groups. In this study 11 BHS isolates other than GAS could not be identified to its respective Lancefield groups as these isolates did not show zone of inhibition around low concentration Bacitracin disks. These isolates may belong to groups C

or G. It is well known that some streptococci of groups B, C, and G can be as sensitive to Bacitracin as group A strains, and MICs for Bacitracin of group A and non-group A streptococci overlap (Coleman *et al*., 1977).

In this study it was observed that most sensitive drugs for GAS were Amoxycillin, Cephalexin and Cefotaxime, Ciprofloxacin and Azithromycin which were 100% sensitive, followed by Cotrimoxazole (91.7%) and Erythromycin (91.7%). The most sensitive drugs for BHS other than Group A are Amoxycillin, Ciprofloxacin and Azithromycin which were 100% sensitive. The result of sensitivity pattern of macrolide used in present study correlates with study carried out by Rijal *et al*. (2009) which showed 97.8% isolates were sensitive to Azithromycin and 84.4% isolates were sensitive to Erythromycin. Dumre *et al*. (2009) reported 94.8% isolates were sensitive to Erythromycin. Gurung *et al*. (2010) reported Erythromycin showed 100% sensitivity. The present study showed that macrolide antibiotics are sensitive towards GAS. Hence, Azithromycin is drug of choice for pharyngitis patients who are allergic to Penicillin.

In present study, GAS isolates showed 100% sensitivity to Ciprofloxacin and Cotrimoxazole showed 91.7% sensitivity. This finding is in agreement with finding of Dumre *et al*. (2009) which reported 94.8% isolates sensitive to Ciprofloxacin but only 29.0% isolates were sensitive to Cotrimoxazole. Most sensitive drugs for BHS other than GAS in this study were Amoxycillin, Cefotaxime, Ciprofloxacin and Azithromycin were 100% sensitive followed by Cotrimoxazole (90.9%), Erythromycin (72.7%) and Cephalexin (63.6%).

In present study Amoxycillin inhibited 50% of *S. aureus*, Cotrimoxazole 33.3% of isolates where as Azithromycin and Penicillin inhibited 66.7% and 33.3% of isolates. In study carried out by Shrestha *et al*. (2006) Amoxycillin and Cotrimoxazole inhibited 76.3% of isolated *S. aureus*, Erythromycin and Penicillin inhibited 65.8% and 44.7% isolates.

In this study most of antibiotics used were ineffective against *P. aeruginosa*, in the two cases concerning *P. aeruginosa* showed resistance against Cephalexin, Ampicillin, Cotrimoxazole, Chloramphenicol, Cefotaxime, Amikacin, and Gentamicin. According to study carried out by Danielides *et al*. (2001) *P. aeruginosa* is an occasional finding in tonsil smears as part of normal microbial flora, but it rarely produces suppurative tonsil infection. However, study carried out by Longanathan *et al*. (2006) on tonsillar core culture of tonsillectomy patients *P. aeruginosa* was reported in 3.7% of cases and regarded as causative organism in recurrent

tonsillitis. *P. aeruginosa* is a major causative microorganism of nosocomial respiratory infections. Importantly, immunocompromised patients are at increased risk for *P. aeruginosa* infection (Burns *et al*., 2001; Emerson *et al*., 2002; West *et al*., 2002). Multi-Drug Resistant *P. aeruginosa* (MDRPA) strains are the most challenging organisms for the clinicians to be overcome (Karki, 2011).

In this study all 134 samples of which direct Gram stain smear was performed, only 13.5% (18/134) showed positive correlation with culture result. Throat culture results showed the mean sensitivity, specificity and positive predictive value of direct Gram-stained smear of throat swab as 78.3%, 14.4% and 15.9%. In similar study carried out by Sharma and Subbukrishnan, (1981) showed the mean sensitivity, specificity and predictive value of a positive Gram-stained smear in relation to culture result of pharyngeal secretions as 70%, 89% and 69%. Thus data in this study suggest that direct Gram staining of throat swab is not accurate as culture due to low specificity of Gram staining for diagnosis of streptococcal pharyngitis. According to Vandepitte *et al*. (2003) Gram staining of direct smear of throat swab is not useful for the detection of *Streptococci* or *Neisseria* spp. This must be because normal flora in the oral cavity has similar morphological structure as that of pathogen such as BHS i.e. Gram positive cocci in chains which can be misleading and giving false positive result.

CHAPTER-VI

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

Tonsillo-pharyngitis is one of the major health problems in Nepal that creates complication in management of RF. Present findings demonstrated that antibiotic resistance of *S. pyogenes* is not clinically significant problem. However, over treatment of clinical pharyngitis with Penicillin may pave way to increased Penicillin resistance. Thus scientifically speaking, it is better to manage treatment after confirming a GAS etiology which would overcome the above problems.This study concludes that surveillance of its susceptibility pattern is crucial to monitoring the development of antibiotic resistance in *S. pyogenes*. This study was based solely on aerobic bacteria that exist in the tonsillar surface. Therefore, research based on anaerobic bacteria and viruses along with quantitative analysis of bacterial molecular biology together with allergic reactions in consideration will be necessary in the future. Present findings demonstrate that young adults of age group 21-40 are affected by BHS Tonsillitis/pharyngitis so more detailed long term study is needed in this matter in future.

6.2 RECOMMENDATIONS

1. Study for larger sample from different places and age groups are to be made to delineate the broader situation.
2. There is a need for of high quality reagents for use in diagnosis and management of streptococcal infections. The reagents should include materials for bacteriological and antibody determinations (i.e., typing sera and standard antisera).
3. Further studies should include development of methods to determine the prevalence of groups C and G streptococci in the upper respiratory tract.

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APPENDIX-I

A. CLINICAL PROFILE:

Sample No: Hospital
Name: a) Ward
Address: b) O.P.D.
Sex: Age: c) Cabin
Date: d) Bed No.

Short Clinical History:

Sample site:

Patient on Antibiogram:

Investigation requested for:

Type of sample:

Time of Collection:

Macroscopic examination:

B. MICROBIOLOGICAL PROFILE:

Day 1

Direct microscopic examination

Gram staining:	**Result**
a. Gram positive cocci	
b. Gram positive bacilli	
c. Gram negative bacilli	
d. Gram negative cocci	
e. Pus cells/WBC	
f. Others	

Culture of Specimens:

Media Used:	**Type of Colony**
Nutrient agar (NA)	
MacConkey agar (MA)	
Blood agar (BA)	

Day 2

Biochemical Test Employed **Result**

a. Catalase test
b. Oxidase test
c. Coagulase test
   i. Slide coagulase
   ii. Tube coagulase

d. Methyl Red (MR)
e. Voges Proskauer (VP)
f. Triple Sugar Iron (TSI)
g. Sulphide Indole Motility (SIM)
h. Citrate Utilization
i. Urea hydrolysis
j. Oxidative Fermentative (OF)
k. Bacitracin Sensitivity (0.04 unit) for BHS only
l. Penicillin Sensitivity (10 unit) for BHS only

Day 3

ORGANISM ISOLATED:
Antibiotic Sensitivity Profile

Antibiotics used	**Zone of Inhibition**	**Remarks**
Ciprofloxacin		
Amoxycillin		
Cephalexin		
Cefotaxime		
Gentamicin		
Amikacin		
Chloramphenicol		
Cotrimoxazole		
Polymxin B		
Azithromycin		
Penicillin		

Checked by:

APPENDIX-II

C. LIST OF MATERIALS

1. Equipments

Autoclave
Anaerobic gas jar
Burner
Incubator
Hot air oven
Waterbath
Microscope
Refrigerator
Glass wares: Petri plates, tubes, slides, glass rod etc.

2. Microbiological media (Hi-Media)

Nutrient Agar
Nutrient Broth
Mac Conkey Agar
Blood Agar
Muller Hinton Agar
Hugh and Leifson (OF) Media
Simmon's Citrate Agar
TSI Agar
MRVP Broth
Urease Broth
SIM Media

3. Chemicals/Reagents

Catalase reagent (3% H_2O_2)
Oxidase regent (1% Tetramethyl p-phenylene diamine dihydrochloride)
Kovac's reagent
Barrit's reagent (40% KOH, 5% α-napthol in a ratio 1:3)
Crystal violet
Gram's iodine
Acetone-alcohol
Safranin
Blood plasma
Methyl red

4. Antibiotic discs (Hi-media)

Bacitracin (0.04 units)
Ciprofloxacin(5 mcg)
Cephalexin(30 mcg)
Gentamicin (10 mcg)
Chloramphenicol (5 mcg)
Polymxin B (300 units)
Azithromycin(15 mcg)
Penicillin (10 units)
Amoxycillin (10 mcg)
Cefotaxime(30 mcg)
Amikacin (30 mcg)
Co-Trimoxazole (25 mcg)
Erythromycin (15 mcg)

5. Miscellaneous

Inoculating loops, Straight wires, Cotton swabs, Distilled water, Immersion oil, Lysol, Oil, Dropper, etc.

APPENDIX-III

MEDIA USED (Hi Media):

1) Blood agar 2) Crystal Violet Blood agar 3) MacConkey agar 4) Mueller Hinton agar 5) Nutrient agar 6) Biochemical media: Simmons citrate agar, Hugh and Leifson media, Triple Sugar Iron agar, Sulphur Indole Motility, Urea agar

A. Composition and Preparation of Different Types of Culture Media

1. Blood Agar (Hi-Media)
(Blood agar base infusion agar) + 5% Blood

Composition	**gram/lt**
Beef heart infusion	500
Tryptose	10
Sodium chloride	5
Agar	1.5
Final pH at 25º C	7.3±0.2

Preparation

As directed by the manufacturing company 40 gm of blood agar base was dissolved in 1000 ml distilled water. The medium was then sterilized by autoclaving at 15 lbs pressure (121 ºC) for 15 min. Then the prepared medium was cooled to about 40-50 ºC, to which 50 ml of sterile defibrinated blood was added aseptically. Then the medium was poured into petri plates.

2. Crystal Voilet Blood Agar
1 ml of 0.02% w/v aqueous solution of crystal violet is added to every 100 ml of sterile blood agar. Then the medium is poured into petri plates.

3. MacConkey Agar (Hi-Media)
(With sodium taurocholate, without salt and crystal violet)

Composition	**gram/lt**
Peptone	20
Lactose	10
Sodium taurocholate	5
Agar	20
Neutral red	0.04
Final pH at 25 ºC	7.4±0.2

Preparation

As directed by the manufacturing company, 55 gm of the medium was dissolved in 1000 ml distilled water. It was sterilized by autoclaving at 15 lbs pressure (121 ºC) for 15 minutes.

4. **Nutrient Broth (Hi-Media)**

Composition	**gram/lt**
Peptone	5
Sodium chloride	5
Beef extract	1.5
Yeast extract	1.5
Final pH at 25ºC	7.4±0.2

Preparation

As directed by the manufacturing company, 13 gm of the medium was dissolved in 1000ml distilled water and was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

5. Peptone Water (Hi-Media)

Composition	**gram/lt**
Peptone	10
Sodium chloride	5
Final pH at 25°C	7.2±0.2

Preparation

As directed by the manufacturing company, 15 gm was dissolved in 1000ml distilled water and was sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes.

6. Nutrient agar (Hi-Media)

Composition	**gram/lt**
Beef extract	10
Peptone	10
Sodium chloride	5
Agar	12
Final pH at 25º C	7.4±0.2

Preparation

As directed by the manufacturing company, 39 gm of the medium was dissolved in 1000 ml distilled water. It was sterilized by autoclaving at 15 lbs pressure (121º C) for 15 minutes.

7. Muller Hinton Agar (Hi-Media)

Composition	**gram/lt**
Beef extract	300
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17
Final pH at 25ºC	7.4±0.2

Preparation

As directed by the manufacturing company, 38 gm of the medium was dissolved in 1000 ml distilled water and was sterilized by autoclaving at 15 lbs pressure (121º C) for 15 minutes.

B. Composition and Preparation of Different Types of Biochemical Media and Procedures of tests

1. Sulphide Indole Motility Medium (SIM) (Hi-Media)

Composition	**gram/lt**
Beef extract	3
Peptone	30
Peptonized iron	0.2
Sodium Thiosulphate	0.025
Agar	3
Final pH at 25ºC	7.3±0.2

Preparation

As directed by the manufacturing company, 36 gm of the medium was dissolved in 1000 ml distilled water and distributed into tubes. The medium was sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes.

Procedure for indole test

1. The test organism was inoculated in the 5 ml SIM medium with a sterile straight wire and incubated at 37°C for 48 hours.
2. A few drops of Kovac's reagent was added, shaken gently and observed for development of red colour.

Procedure for motility test

1. The test organism was stabbed in the SIM medium with a sterile straight wire and was incubated for 24 hours at 37°C.
2. The medium was observed for positive growth by the appearance of spreading turbidity from the stab line or turbidity throughout the medium.

Procedure for Hydrogen sulphide production test

1. The test organism inoculated by stabbing in the medium with a sterile straight wire and incubated at 37ºC for 24 hours.
2. Observation was done for black iron-containing precipitate in the medium indicating the production of H_2S gas.

2. Citrate medium (Hi-Media)

Composition	**gram/lt**
Magnessium sulphate	0.2
Mono-ammonium phosphate	1
Dipotassium phosphate	1
Sodium citrate	2
Sodium chloride	5
Agar	15
Bromothymol blue	0.08
Final pH at 25º C	6.8±0.2

Preparation

As directed by the manufacturing company 24.2 gm of the medium was dissolved in 1000 ml distilled water. The medium was then dispensed in test tubes and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes. The sterilized medium in the tubes were allowed to set in slopes.

Procedure for citrate utilization test

1. Using a sterile straight wire, organism was inoculated by streaking on the slope of the medium.
2. It was then incubated at 37ºC for 24 hours.
3. The change in colour the from green to blue was then observed.

3. MR-VP Medium (Hi-Media)

Composition	gram/lt
Buffered peptone	7
Dextrose	5
Dipotassium phosphate	5
Final pH at 25°C	6.9±0.2

Preparation

As directed by the manufacturing company, 17 gm of the medium was dissolved in 1000 ml of distilled water and distributed into test tubes. The medium was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Procedure for Methyl Red (MR) test

1. 2.5 ml of sterile Glucose-Phosphate broth (MR-VP broth) tube was taken and inoculated with the test organism.
2. Overnight incubation at 37°C was done.
3. Few drops of Methyl Red solution were added. Bright red colour was observed in the positive test indicating acidity.

Procedure for Voges-Proskauer (VP) test

1. 2.5 ml of sterile MR-VP (Glucose-phosphate) broth was taken, to which was added the test organism.
2. It was incubated for 24 to 48 hours at 37ºC.
3. After incubation, 0.6 ml α-napthol and 0.2 ml. of KOH was added and gently shaken and allowed to stand for 15 minutes.
4. It was observed for the development of pink colour indicating a positive reaction whereas the negative test appears colorless or yellow.

4. Urea Agar base Medium (Hi-Media)

Composition	gram/lt
Peptone	1
Dextrose	1
Sodium chloride	5
Disodium phosphate	1.2
Monopotassium phosphate	0.8
Phenol red	0.012
Agar	15
Final pH at 25°C	6.9±0.2

Preparation

As directed by the manufacturing company, 24 grams of the medium was dissolved in 950 ml distilled water and sterilized by autoclaving at 10 lbs pressure (115°C) for 20 minutes. The medium was cooled to about 45°C to which 50 ml of sterile 40% urea solution was mixed aseptically and distributed into sterile test tubes. The medium was allowed to set in a slant position.

Procedure for urease test

1. Christensen's urea broth was inoculated with the pure culture of the test organism and the tube was incubated at 37ºC for 24 to 48 hours.
2. The change in the colour was noted. Pink color indicated the positive reaction.

5. Triple Sugar Iron Agar (TSI) (Hi-Media)

Composition	**gram/lt**
Peptone	10
Tryptone	10
Yeast extract	3
Beef extract	3
Lactose	10
Saccharose	10
Dextrose	1
Ferrous sulphate	0.2
Sodium chloride	5
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12
Final pH at 25°C	7.4±0.2

Preparation

As directed by the manufacturing company, 65 gm of the medium was dissolved in 1000 ml distilled water and then distributed into test tubes. The medium was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The medium was allowed to set in a slope form with a butt of about 1 inch.

Procedure:

1. The organism was inoculated by stabbing the butt with straight wire and streaking the surface of the slant.
2. The tubes were then incubated at 37ºC for 24 to 48 hours.
3. The tubes were observed for gas formation, carbohydrate utilization and H_2S production.

Interpretation

1) Production of gas ⟶ indicated by cracking of the media.
2) Production of H_2S ⟶ indicated by the formation of black iron containing precipitate in the butt.
3) Fermentative pattern ⟶ (a) Acid/Acid: - Lactose and sucrose fermented. (Both slant and butt-yellow)
(b) Alk/Acid: - Non lactose fermenter but sucrose fermenter (Slant-red but butt-yellow)
(c) Alk/Alk or Alk/NC: - Non glucose fermenter (Both slant and butt-red)

6. Hugh-Leifson's (OF) Media (Hi-Media)

Composition	**gram/lt**
Peptone	2
Sodium chloride	5
Dipotassium phosphate	0.3
Agar	2
Bromothymol blue	0.08
Final pH	7.1

Preparation

As directed by the manufacturing company, 0.34 gm of the basal medium was dissolved in 100 ml distilled water and was sterilized by autoclaving at 15 lbs pressure (121º C) for 15 minutes. Then 10 ml of sterile 10% dextrose solution was added to the medium and mixed thoroughly. Finally, the medium was dispensed in 5 ml amounts into sterile test tubes.

Procedure for Oxidative-Fermentative (OF) test

1. Two tubes of OF medium were taken. Using a sterile straight wire, the test organism was inoculated in it.
2. The inoculated medium in one of the tubes was covered with layer of paraffin oil to exclude oxygen while other was open to air.
3. Both tubes were then incubated at 37°C for up to 14 days and the tubes were observed for carbohydrate utilization.
4. Fermentative organism utilizes carbohydrate in both tubes changing the medium from green to yellow whereas the oxidative organisms utilize carbohydrate of open tube only.

C. Composition and Preparation of Staining Reagents

Gram stain reagents

1. Crystal Violet Stain

Composition	gram/lt
Crystal violet	20
Ammonium oxalate	9
Ethanol	95
Distilled water	

Preparation

20 gm of crystal violet was weighed and transferred to a clean brown bottle. Then, 95 ml of ethanol was added and mixed until the dye was completely dissolved. 9 gm of ammonium oxalate dissolved in 200 ml of distilled water was then added to the mixture. Finally, distilled water was added to make the volume 1000 ml.

2. Lugol's Iodine Solution

Composition	gram/lt
Potassium iodide	20
Iodine	10
Distilled water	

Preparation

To 250 ml of distilled water, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Finally, the volume was made 1 lt. by adding distilled water.

3. Acetone-alcohol decoloriser

Composition	volume (ml)
Acetone	500
Ethanol (absolute)	475
Distilled water	25

Preparation

To 25 ml distilled water, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

4. Counter stain Solution

Composition	**gram/lt**
Safranin	10
Distilled water	

Preparation

10 gm of safranin was weighed and transferred to a clean bottle. Then 1lt. distilled water was added to the bottle and mixed well until safranin dissolves completely.

5. Normal saline

Sodium Cholride	0.85 g
Distilled Water	100 ml

The sodium chloride was weighed and transferred to a leak-proof bottle premarked to hold 100 ml. Distilled water was added to the 100 ml mark, and mixed until the salt was fully dissolved. The bottle was labeled and stored at room temperature.

D Composition and Preparation of Test Reagents

1. Catalase Reagent (To make 100 ml)

Composition	**volume (ml)**
Hydrogen peroxide solution	3
Distilled water	97

Preparation

To 97 ml distilled water, 3 ml of hydrogen peroxide was added and mixed well.

Procedure:

1. Using a sterile wooden stick or a glass rod, a small amount of colony from pure culture was picked and placed on a clean glass slide.
2. A drop of 3% H_2O_2 was added over the organism on slide.
3. Observation for bubbling was done.
4. Positive catalase reaction showed production of active gas bubbles almost immediately.

2. Oxidase Reagent (To make 10 ml)

Composition	
Tetramethyl P-Phenylenediamine dihydrochloride (TPD)	0.1 gm
Distilled water	10 ml

Preparation

0.1gm TPD was dissolved in 10 ml distilled water. To that solution strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then these stripes were freeze dried and stored in a dark bottle tightly sealed with a cap.

Procedure for oxidase test

1. A piece of filter paper soaked with few drops of oxidase reagent i.e. 1% solution of tetramethyl-p-phenylene diamine dihydrochloride was taken.
2. Using a sterile stick or a glass rod, a colony of test organism was then smeared on the filter paper.
3. It was observed for development of purple color within few seconds

3. Kovac's Indole Reagent (To make 40 ml)

Composition

4-dimethyl aminobenzaldehyde	2gm
Isoamyl alcohol	30 ml
Conc. Hydrochloric acid	10 ml

Preparation

To 30 ml isoamyl alcohol, 2 gm of 4-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. To it, 10 ml conc. HCl was added and mixed well.

4. Methyl Red Solution (To make 50 ml)

Composition

Methyl red	0.05 gm
Ethanol (absolute)	28 ml
Distilled water	22 ml

Preparation

To 28 ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml distilled water was added and mixed well.

5. Voges-Proakauer Reagent (Barrit's Reagent)

i. VP Reagent A (To make 100 ml)

Composition

α-napthol	5 gm
Ethanol absolute	100 ml

Preparation

To 25 ml distilled water, 5 gm α-napthol was dissolved and transferred to a clean brown bottle. Then the final volume was made 100 ml by adding distilled water.

ii. VP Reagent B (To make 100 ml)

Composition

Potassium hydroxide	40 gm
Distilled water	100 gm

Preparation

To 25 ml distilled water, 40 gm of KOH was dissolved and transferred to a clean brown bottle and final volume was made 100 ml by adding distilled water.

6. Procedure of coagulase test

i. Tube coagulase test for free coagulase

1. The plasma was diluted 1 in 10 physiological saline (mixing 0.2 ml of plasma with 1.8 ml of saline).
2. 3 tubes were taken and labeled as: "T" for test organism; "P" for positive control (*S. aureus*); "N" for negative control (sterile broth).
3. 0.5 ml of diluted plasma was pipetted into each test tube.
4. 0.5 ml of an overnight broth culture or an agar culture suspension was added to tube "T", *S aureus* to tube labeled "P" and sterile broth to tube labeled "N".
5. After mixing gently, tubes were incubated at 35°C-39°C. It was then observed for clotting after 3-6 hours by gently tilting the tubes.

ii Slide test for bound coagulase

1. A drop of physiological saline was placed on each end of a slide.
2. A colony of test organism was then emulsified in each of the drops to make two thick suspensions.
3. A drop of plasma was then added to one of the suspension and was mixed gently.
4. The appearance of agglutination or clumping of the organism on the suspension with plasma is the indication of positive slide coagulase test.

APPENDIX-IV

A. Gram-staining Procedure

The following steps are involved in Gram-stain of direct throat swab:

1. Throat swab was rolled on a slide as rolling the swab avoids damaging the pus cells.
2. A thin film of the material to be examined was prepared and dried.
3. The smear on slide was air dried. After that smear was fixed with two drops of absolute ethanol.
4. Then the alcohol on the smear was left until the alcohol evaporates.
5. The slide was flooded with crystal violet stain and allowed to remain without drying for 10-30 seconds.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with iodine solution and allowed to remain on the surface without drying for 1 minute.
8. The slide was rinsed with tap water, shaking off excess.
9. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer.
10. The slide was flooded with counter stain (safranin) for 30 seconds and washed off with tap water.
11. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under low power field at 100X for polymorphonuclear cells and oil immersion at 1000X for gram positive cocci in chains.

The following steps are involved in Gram-stain of colony from culture plate:

1. With the help of inoculating loop, isolated pure colonies from culture plate was touched with the tip and transferred to a clear and grease free slide containing a drop of distilled water.
2. A uniform smear of material to be examined was prepared and dried.
3. The material on the slide was heat fixed and allowed to cool before staining.
4. The slide was flooded with crystal violet stain and allowed to remain without drying for 10-30 seconds.
5. The slide was rinsed with tap water, shaking off excess.
6. The slide was flooded with iodine solution for 1minute after which slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer.
8. The slide was flooded with counter stain (safranin) for 30 seconds and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 1000X.

B. Interpretation charts for different antibiotics

Antibiotic	Symbol	Strength	Diameter of zone of inhibition in mm		
			Resistant (mm or less)	Intermediate (mm)	Sensitive (mm or more)
Penicillin β-haemolytic streptococci Staphylococci	P	10 units	- 28mm	- -	24 mm 29 mm
Amoxycillin Gram –ve enteric organism Staphylococci β-haemolytic streptococci	Am	10 mcg	13 19	14-17 - -	18 20 24
Bacitracin Group A β-haemolytic streptococcus	B	0.05 units	-	-	15
Chloramphenicol	C	30 mcg	12	13-17	18
Ciprofloxacin	CF	5 mcg	15	16-20	21
Co-Trimoxazole	Co	25 mcg	10	11-15	16
Cephalexin	CP	30 mcg	14	15-17	18
Cefotaxime	CE	30 mcg	14	15-22	23
Erythromycin Staphylococci Streptococci	E	15 mcg	 13 15	 14-22 16-20	 23 21
Azithromycin	AT	15 mcg	13	14-17	18
Amikacin	AK	30 mcg	14	15-16	17
Gentamicin	G	10 mcg	12	13-14	15
Polymyxin B	PB	300 units	8	9-11	12

Manufacturers: HiMedia Laboratories Pvt. Limited, Mumbai, India.

C. Procedure of Antibiotic susceptibility test for β-haemolytic streptococci

1. MHA plate containing 5% blood was taken.
2. The inoculum was prepared by transferring 3-4 β-haemolytic pure culture colonies into normal saline. The suspension was standardized to match the turbidity of the 0.5 MacFarland turbidity standards (1.5 X 10 to the power 8 cfu/ml) using direct colony suspension procedure.
3. Inoculation was done by dipping a sterile cotton swab into the inoculum and it was rotated by pressing against the upper inside wall of the tube to remove excess inoculum. Then uniform swabbing was done on the agar surface (carpet culture). Then the plate was allowed to dry for 10 minutes.
4. With the help of flamed forcep, discs were carefully placed on the inoculated plate, at least 15mm away from the edge , at equal distances and sufficiently separated (about 30 mm between two discs) to avoid overlapping of zone of inhibition. The plates were allowed to stand at room temperature for 30 minutes (perfusion time).
5. The plates were incubated at 37ºC for 18 hours.
6. Zone of inhibition are measured from the upper surface of the agar illuminated with reflected light, with the cover of petriplate removed.

D. Bacitracin sensitivity test

Principle: To demonstrate the organism's susceptibility to antibiotic, Baitracin.

1. **Direction:** Disc containing 0.04 units of bacitracin is placed on a blood agar plate inoculated with BHS and incubated at 37^{o}C for 24 hours.
2. The size of zone of inhibition exhibited by *Streptococcus pyogenes* is more than 15 mm.

E. Procedure of Antibiotic susceptibility test

1. MHA plate with medium depth of 4mm was taken.
2. The inoculum was prepared by transferring 3-4 pure culture colonies into nutrient broth (5ml). Moderate turbidity was obtained by incubating at 37ºC for 4 hours. The suspension was standardized to match the turbidity of the 0.5 MacFarland turbidity standards (1.5 X 10 to the power 8 cfu/ml) which is done by adding 0.5ml of 1.175% $BaCl_2.2H_2O$ solution to 99.5 ml of 0.36N H_2SO_4.
3. Inoculation was done by dipping a sterile cotton swab into the inoculum and it was rotated by pressing against the upper inside wall of the tube to remove excess inoculum. Then uniform swabbing was done on the agar surface (carpet culture). Then the plate was allowed to dry for 10 minutes.
4. With the help of flamed forcep, discs were carefully placed on the inoculated plate, at least 15mm away from the edge , at equal distances and sufficiently separated (about 30 mm between two discs) to avoid overlapping of zone of inhibition. The plates were allowed to stand at room temperature for 30 minutes (perfusion time).
5. The plates were incubated at 37ºC for 24 hours.
6. Zone of inhibition was measured.

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MASTDISCS™ ID
Bacitracin Discs

D40/D41. For the identification of Lancefield Group A streptococci (*Streptococcus pyogenes).*

FOR IN VITRO DIAGNOSTIC USE ONLY

Contents: 100 discs in a vial (D40/D41) or a pack of 5 cartridges (D40C/D41C), each cartridge containing 50 discs.

Formulation

Order Code	Material:	Content per disc:
D40/D40C	Bacitracin	0.04 units
D41/D41C	Bacitracin	0.1 unit

Storage and shelf life
Store at 2-8°C in the containers provided until the expiry date shown on the pack label. Allow to equilibrate to room temperature before opening.

Precautions
For *in vitro* diagnostic use only. Observe approved biohazard precautions and aseptic techniques. To be used only by adequately trained and qualified laboratory personnel. Sterilise all biohazard waste before disposal. Refer to Product Safety Data sheet.

Materials required but not provided
Standard microbiological supplies and equipment such as loops, MAST culture media, swabs, applicator sticks, incinerators and incubators, etc., as well as serological and biochemical reagents and additives such as blood.

Procedure
1. Subculture the test beta-haemolytic streptococcus by spreading over the surface of a blood agar plate e.g. MAST Blood Agar Base Special (DM101) supplemented with 5-7% sterile defibrinated horse or sheep blood. Alternatively discs can be applied directly to media inoculated with suitable clinical specimens e.g. throat swabs.
2. Place a MAST ID™ Bacitracin Disc of either strength on the inoculated medium.
3. Incubate for 18-24 hours at 35-37°C.

Interpretation of results
The presence of any zone of inhibition around a 0.04 unit MAST ID™ Bacitracin Disc (D40), or a zone of inhibition of 12mm or greater around a 0.1 unit MAST ID™ Bacitracin Disc (D41) is considered positive for a presumptive identification of Lancefield Group A streptococci.

Any known bacitracin sensitive strain of Lancefield Group A *Streptococcus* may be used as a sensitive control organism.

Quality control
Check for signs of deterioration. Quality control must be performed with at least one organism to demonstrate a positive reaction and at least one organism to demonstrate a negative reaction. Do not use the product if the reactions with the control organisms are incorrect. The list below illustrates a range of performance control strains which the end user can easily obtain.

Test Organisms	Result
Streptococcus pyogenes ATCC® 19615	Appropriate zone of inhibition
Streptococcus agalactiae ATCC® 13813	No zone of inhibition

Limitations
It is recommended that biochemical and/or serological tests are performed on colonies from pure culture to confirm identification e.g.
MASTASTREP™ Enzyme extraction (RST201).
It is important that an inoculum resulting in confluent growth be used. If an excessive inoculum is used, some group A streptococci may not show a zone of inhibition. If the inoculum is too light, this may result in false zones of inhibition. Some group B, C and G strains of streptococci are reported to be bacitracin sensitive.

References
Bibliography available on request.

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APPENDIX V

Calculation of sensitivity and specificity

	Gold Standard: Culture positive BHS on Blood Agar Plates		
Gram Staining	Positive	POSITIVES Culture positive cases for BHS and *Gram staining positive cases (true positive) 18	NEGATIVES Culture negative cases for BHS but *Gram staining positive cases (false positives) 95
	Negatives	Culture positive for BHS but Gram staining negative cases (false negatives) 5	Culture negative cases for BHS and Gram staining negative cases (true negatives) 16

Sensitivity

Sensitivity is the ability of a test to identify correctly those who have the disease or condition. It is the proportion of true positive cases having a disease or condition, sensitivity measures the true positive cases among diseased persons. In this case sensitivity measures number of culture positive cases for BHS and Gram staining positive cases among true positive and false positive cases. It is expressed in percentage. It is calculated as follows :

$$\text{Sensitivity} = \frac{\text{number of true positive cases}}{\text{number of true positive cases + false negatives}}$$

$$= \frac{\text{number of culture positive cases for BHS and Gram staining positive cases}}{X+Y}$$

Where,
X= Culture positive cases for BHS and Gram staining positive cases
Y= Culture positive cases for BHS and Gram staining negative cases

$$= \frac{18}{18+5} X\ 100$$

Sensitivity = 78.3%
*Gram staining positive cases= Gram-positive cocci in chain in association with the polymorphonuclear cells

Specificity

It is ability or a test to identify correctly those who don't have the disease. It is proportion of true negative cases among non disease persons. Specificity is the measure of true negatives among non diseased individuals. It is also expressed in percentage. In this case it is measure of number of culture negative cases and Gram staining negative cases among false positive and true negative cases. It is calculated using the following formula :

$$\text{Specificity} = \frac{\text{number of true negatives}}{\text{false positive} + \text{number of true negative cases}}$$

$$= \frac{\text{number of culture negative cases and Gram staining negative cases}}{U + V}$$

Where,
U= Culture negative for BHS but Gram staining positive cases
V= Culture negative for BHS and Gram staining negative cases

$$= \frac{16}{95+16} X\ 100$$

Specificity = 14.4%

Predictive value positive (PVP)

The predictive value positive or precision rate is the proportion of subjects with positive test results who are correctly diagnosed. It is critical measure of the performance of diagnostic method, as it reflects the probability that a positive test reflects the underlying condition being tested for. Its value does however depend on the prevalence of the outcome of interest, which may be unknown for a particular target population. In this case it is number of culture positive for BHS and Gram staining positive among true positive and false positive cases.

$$\text{PVP} = \frac{\text{number of true positive cases}}{\text{number of true positive cases} + \text{false positive}}$$

$$= \frac{\text{number Culture positive cases for BHS and Gram staining positive cases}}{S + T}$$

S= Culture positive for BHS and Gram staining positive cases
T= Culture negative for BHS and Gram staining positive cases

$$= \frac{18}{18+95} X\ 100$$

Predictive value positive = 15.9%

APPENDIX VI

Characteristics of Medically Important Streptococci

Name	Group-Specific Substance[a]	Haemolysis [b]	Habitat	Important laboratory Criteria	Common and important Disease
Streptococcus pyogenes	A	Beta	Throat, skin	Large colonies (> 0.5 mm), PYR[c] test positive, inhibited by Bacitracin	Pharyngitis, impetigo, rheumatic fever, glomerulonephritis Toxic shock
Streptococcus agalactiae	B	Beta	Female genital tract	Hippurate hydrolysis, CAMP-positive[d]	Neonatal sepsis and meningitis
Streptococcus dysgalactiae subspecies *equisimilis;* others	C, G	Beta (human) infections), alpha, none	Throat	Large (> 0.5 mm) colonies	Pharyngitis, pyogenic infections similar to group A streptococci
Enterococcus faecalis (and other enterococci)	D	None, alpha	Colon	Growth in presence of bile, hydrolyze esculin, growth in 6.5% NaCl, PYR-positive	Abdominal abscess, urinary tract infection, endocarditis
Streptococcus bovis group	D	None	Colon,billary tree	Growth in presence of bile, hydrolyze esculin, no growth in 6.5% NaCl, degrades starch	Endocarditis, common blood isolate in colon cancer, billary disease
Streptococcus anginosus group (*S anginosus, S. intermedius, S. constellatus, S. milleri* group)	F (A, C, G) and untypable	Alpha, beta, none	Throat, colon, female genital tract	Small (< 0.5 mm) colony variants of beta-haemolytic species. Group A are Bacitracin-resistant and PYR-negative. Carbohydrate fermentation patterns	Pyogenic infections, including brain abscesses
Viridans streptococci (many species)	Usually not typed or untypable	Alpha, none	Mouth, throat, colon, female genital tract	Optochin-resistant. Colonies not soluble in bile. Carbohydrate fermentation patterns	Dental caries *(S mutans),* endocarditis, abscesses. Some species, such asstreptococcus mitis have high-level resistance to Penicilline
Streptococcus pneumoniae	None	Alpha	Nasopharynx	Susceptible to optochin. Colonies soluble in bile, quellung reaction-positive	Pneumonia, meningitis, endocarditis, otitis media, sinusitis
Peptostreptococcus (many species)	None	None, alpha	Mouth, colon, female genital tract	Obligate anaerobes	Abscesses (with multiple other bacterial species)

[a]Lancefield classification.
[b]Hemolysis observed on 5% sheep blood agar after overnight incubation.
[c]Hydrolysis of L-pyrrolidonyl-2-naphthylamide ("PYR").
[d]Christie, Atkins, Munch-Peterson test.

Source:*(Brooks *et al*., 2004)

APPENDIX VII

Differentiation of β-haemolytic Streptococci

Species	***S. pyogenes***	***S. agalactiae***	***E. faecalis* var. zymogenes**	**Others**
Lancefield group	A	B	D	C,G,F
Haemolysis	β	β[b]	β	β
Zone around the differential Bacitracin disc	+	0[C]	0[C]	0[d]
Bile–aesculin agar (growth and blackening)	0	0	+	0
CAMP test	0	+	0	0
Cotrimoxazole susceptibility	0	0	0	+
PYR test[f]	+	0	+	0

[a]*E. faecalis* var. zymogenes produces β-haemolysis only on horse-blood agar.
[b]5% are non-haemolytic.
[c]5% are positive.
[d]10% are positive.
[e] Same disc as in the Kirby–Bauer method.
[f]PYR: pyrrolidonyl-b-naphtylamide.

Source:*(Vandepitte *et al*., 2003)