CLINICAL AND MOLECULAR EPIDEMIOLOGY OF BETA-HAEMOLYTIC STREPTOCOCCAL INFECTIONS IN A TERTIARY CARE HOSPITAL

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Certificate

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COIMBATORE

CERTIFICATE

This is to certify that the dissertation work entitled **"Clinical and Molecular Epidemiology of Beta Hemolytic Streptococcal infections in a Tertiary Care Hospital"** submitted by **Dr. B. Leelavathi**, is the work done by her during the period of study in this department from June 2017 to May 2020. This work was done under the guidance of **Dr. S. Parvathi**, Professor, Department of Microbiology, PSGIMS&R.

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Declaration

DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation entitled **"Clinical and Molecular Epidemiology of Beta Hemolytic Streptococcal infections in a Tertiary Care Hospital"** is a bonafide and genuine research work carried out by me under the guidance of Dr. S. Parvathi, Professor, Department of Microbiology, PSG IMS&R, Coimbatore. This dissertation is submitted to The Tamil Nadu Dr. M.G.R Medical University in fulfillment of the university regulations for the award of MD degree in Medical Microbiology. This dissertation has not been submitted for award of any other degree or diploma.

Signature of the candidate

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Introduction

Beta-haemolytic streptococci are characterized by the Lancefield group based on the carbohydrates present in the cell wall. Groups A, B, C, G are generally defined as Beta-haemolytic streptococci. They cause a wide variety of invasive and non-invasive infections ranging from mild throat infections to life threatening infections in the blood or organs. They infect people of all age groups (neonates, small children, adults, and older people). Groups A, B, C and G are the most common organisms which cause bacteremia. They are transmitted usually by direct contact with droplets of saliva or nasal secretions or through skin contact especially by contact with infected skin lesions. Both infected persons and carriers may transmit the disease.

Group A Streptococcus is one of the most aggressive pathogens which is encountered in microbiological laboratories. *S.pyogenes* is also known as GAS cause mild infections such as pharyngitis, impetigo to severe infection such as STSS (Streptococcal Toxic Shock Syndrome) and flesh eating necrotizing fasciitis. If untreated it may lead to suppurative and non-suppurative sequalae. The incidence of GAS infection has been increased in recent years due to increased number of immuno-compromised people with organ transplantation, AIDS, diabetes and other illness.

Group B streptococcal infection usually occurs in neonates who acquire infection before or during birth process and this occurs mostly by colonization in maternal vagina. Most common species among GBS is *S.agalactiae*. It is the most common causative agent of neonatal sepsis and meningitis. It is usually an opportunistic pathogen which colonizes in

GIT & genito urinary tract in most healthy adults. CDC recommends the screening for GBS carriage in pregnant women between 35 and 37 weeks of gestation.

Group C Streptococci (GCS) and Group G Streptococci (GGS) mainly cause infections in animals. In humans, they are the normal commensals of upper respiratory tract and colonizers of skin, GIT & female genital tract. They cause a variety of infections including pharyngitis, skin and soft tissue infections, meningitis, bacteremia and toxic shock syndrome.GCS and GGS of human origin are now considered to constitute a single species *S.dysgalactiae* subspecies *equisimilis*[1]. Cellulitis is the most common clinical manifestation in GGS infection. Streptococci of animal origin also contains G or C antigen and are occasionally associated with human infections. They also cause bacteremia and the probable source is mostly through skin and soft tissue infections.GGS has also emerged as an important cause of Infective endocarditis in patients with pre-existing valvular disease and is often more aggressive than endocarditis caused by viridans group.

Group F streptococcus (GFS) has emerged in recent years as a group of organisms which are mainly associated with purulent infection in man. They are the normal commensals of oropharynx, GIT and perineum[2]. *S.anginosus* is the most common organism in this group causing infection. They are mostly isolated from wound infection and abscess; the common sites affected are dental, cervico facial and intra-abdominal areas. This study is aimed to find the epidemiology of Beta-haemolytic streptococcal infections in a tertiary care hospital.

Aim and Objectives

Aim:

To study the clinical and molecular epidemiology of beta-haemolytic streptococci in our hospital.

Objectives:

Primary:

To isolate and characterize beta-haemolytic streptococcal infections in our hospital

Secondary:

- Confirmation of serogrouping by manual and commercial antigen extraction methods.
- Comparative evaluation of above two methods.
- Molecular characterization of virulence genes

Review of Literature

Beta-haemolytic Streptococci

Streptococci come under the family Streptococcacae. They are gram positive, non-motile, non-sporing, catalase negative, facultative anaerobes which occur in chains or pairs.

Discovery

In 1874, Dr. Theodore Billroth Viennese surgeon first discovered and named STREPTOCOCCUS and identified that this organism causes skin infections. In Greek, "streptos" means twisted or chain and "kokhos" means berry which refers to the globular shaped structures, and hence the name of the genus, Streptococcus, following which individual streptococcal species were named after the disease they caused or sites of infection[3].

Taxonomy

- Phylum: Firmicutes
- Class: Bacilli
- Order: Lactobacillales
- Family: Streptococcacae
- Genes: Streptococcus

Schotmuller in 1903 described blood agar technique for differentiating haemolytic from non-haemolytic streptococci. In 1919, Brown made systemize study of patterns of

hemolysis and introduced the terms α, β, γ hemolysis. They are identified based on their haemolytic properties on sheep blood agar.

- α haemolytic species which does not produce soluble hemolysin causing partial clearing and greenish color on blood agar.
- β haemolytic species causes complete rupture of RBCs, appear as wide areas clear of blood cells surrounding bacterial colonies.
- γ haemolytic species causes no hemolysis.

Beta Hemolysis

It is also called complete hemolysis. It occurs due to the presence of exotoxin streptolysin (an enzyme produced by bacteria). There are two types of streptolysin – Streptolysin O and Streptolysin S. Streptolysin S is an oxygen stable non-immunogenic hemolysin capable of damaging the host cell. Streptolysin O is an immunogenic, capable of lysing erythrocytes, leukocytes and will produce hemolysis only in the absence of room air[4].

Lancefield Classification

Rebecca Lancefield, in 1933, made a classification of beta-haemolytic streptococci into distinct groups based on group specific polysaccharides. Initially, from 106 trains of beta-haemolytic streptococci studied, she recognized five antigenically distinct groups and named them from A to E. Her scheme now includes 20 sero groups designated sequentially with the letters A to H, and K to V. Further sub-division of group A streptococcus by Lancefield was based on M protein which is found on the surface of the

bacterial cell valve. It is the main virulent factor of group A streptococcus and prevents phagocytosis. During 1920s and 1930s, further researchers contributed to the identification of toxins (streptococcal pyrogenic exotoxins) produced by streptococci.

Streptococcus species and their Lancefield grouping[5]

Lancefield group	**Species**	**Infections**
A	*S.pyogenes*	Human infections
B	*S.agalactiae*	Human and bovine infections
A, C, G, L	*S.dysgalactiae subsp. equisimilis*	Human and animal infections
C	*S.equi subsp. zooepidemicus*	Human and animal infections
G	*S.canis*	Infections in dogs and humans
A, C, F, G	*S.anginosus*	Human infections
C	*S.constellatus subsp. pharyngis*	Human infections

Group A Streptococci (GAS)

It was discovered in 1883 by a European scientist, Fehleisen. He observed this has chain like bacteria which causes throat infections. Later in the same year, they linked the bacteria which he discovered with streptococcus. The term *S.pyogenes* was coined by

Friedrich Julius Rosenbach in 1884[6]. *S.pyogenes* produces human infections but it does not cause any infection in animals. It is the most common bacteria causing acute pharyngitis in children. Certain strains express one or more pyrogenic exotoxins (A, B or C). Scarlet fever is caused by one such strain. It was found to be the fatal infection causing epidemics with high mortality. But in this modern era with the availability of antibiotics, it has now become less severe presents as pharyngitis with skin rash, without toxic mediated symptoms. It causes both suppurative and non-suppurative infections.

The suppurative infections produced by *S.pyogenes* include: Impetigo, pharyngitis scarlet fever, erysipelas, postpartum fever, necrotizing fasciitis, septicemia, and toxic shock syndrome. Nonsuppurative infections are mainly due to the molecular mimicry of streptococcal antigens with human antigens. Due to the antigenic cross reactivity, antibodies produced against previous streptococcal infections cross react with human tissues to produce lesions. This causes a number of non-suppurative complications such as: Acute rheumatic fever, post streptococcal glomerulonephritis, guttate psoriasis, reactive arthritis and Paediatric Autoimmune Neuropsychiatric Disorders Associated with *S.pyogenes* (PANDAS).

Carriage

S.pyogenes is carried asymptomatically on the superficial layers of the skin and mucous membranes like oropharyngeal mucosa, nasal mucosa, perianal area and genital tract. A pharyngeal carrier of GAS is an asymptomatic individual with positive throat culture with no active immune or inflammatory response, or an asymptomatic child who after

completing the antimicrobial treatment of GAS pharyngitis tests positive for GAS[7]. The pharyngeal carrier rate in adults is found to be less than 5% but it is more in children which range from 15-25%. Carrier may be a transient carrier or persistent carrier and a new type of GAS may replace the carrier strain.

Transmission

The transmission of organisms from carrier, mainly pharyngeal carriers, to other persons occurs via droplets of saliva or direct contact with nasal (respiratory) secretions. Studies have shown that unpasteurized milk and contaminated food have also been sources of several well documented *S.pyogenes* outbreaks[8].

Pathogenesis

GAS is a human pathogen which causes significant morbidity and mortality globally. The infections may range from mild self-limiting pharyngitis and impetigo to life threatening conditions including STSS (Streptococcal Toxic Shock Syndrome), necrotizing fasciitis and bacteremia. Repeated infections may result in non-suppurative sequalae like acute rheumatic fever and post streptococcal glomerulonephritis[9]. GAS causes serious infections in human by three mechanisms:

1. Suppuration, which is formed in pyoderma and pharyngitis
2. Elaboration of toxins which is seen in STSS
3. Immune mediated inflammation which happens in acute rheumatic fever and post streptococcal glomerulonephritis

Adherence and Colonization

The initial step in the colonization of host is the attachment of GAS to pharyngeal or dermal epithelium. There are strong adhesion mechanisms in GAS by which it can attach to host tissues and can be easily removed by mucous and salivary fluid flow mechanisms and exfoliation of epithelium. These adhesion mechanisms provide competition between normal flora and GAS for tissue sites where normal flora resides.

It is reported that multiple adhesins of GAS are involved in the adhesion of bacteria and could give them advantage of more avid adherence and more virulence. These adhesins facilitate the movement of streptococci from mucosa or skin into deeper tissues[8]. The adherences of organism to particular host cell induce inflammatory response and production of cytokines. At present, a total of 11 adhesins have been found in GAS including M protein, LTA, protein F/Sfb, Glyceraldehyde-3-phospate dehydrogenate etc. M-positive strains has greater adherence to Hep-2 cells while M negative strains has greatly reduced adherence. It plays an important role in the attachment of organism to keratinocytes and this upregulates the production of inflammatory mediators IL-1 and PGE2[10].

Intracellular Invasion

Recent studies suggested that GAS invade the epithelial cells after adhesion. Effective invasion requires expression of M protein and fibronectin binding protein Sfb1[11]. The reason for invasion is not clearly known, though the intracellular environment provides a

good place for streptococci to avoid host defense mechanisms. This internalization plays an important role in carriage and persistent infection. Two theories have been proposed for the role of internalization of GAS in disease pathogenesis. The first theory suggests that internalization is necessary for the carriage and persistence of streptococci. The second theory suggests that internalization leads to invasion of deeper tissues[11]. However, both the theories are correct depending on the properties of invading organisms and the regions it invades (throat or skin epithelium). Certain other studies have found that low virulence is associated with internalisation[12].

Host Response to Infection

Opsonisation and Phagocytosis:

M protein and hyaluronic acid are main factors which makes GAS anti-phagocytic. Two mechanisms have been proposed which describes the antiphagocytic behaviour of M protein.

1. The binding of factor H to C repeat region of M proteins inhibits deposition of soluble C3b and the activation of compliment pathway is inhibited[13].
2. The binding of fibrinogen to the surface of GAS also blocks the activation of complement in alternate pathway and greatly reduces the amount of C3b bound to streptococci, thereby reducing phagocytosis[14].

Recent studies suggested that mutations in Mrp gene affect resistance to phagocytosis. Insertion mutation in any one of the genes (emm – 49, enn – 49 &mrp – 49) resulted in

reduced resistance to phagocytosis in PMN's and blood[15]. It was also found that C5-a activated PMN's can kill M positive streptococcus by altering clearance and trafficking of GAS during infections.

Virulence Factors

Two substances - M protein and hyaluronic acid are the principle determinants by which GAS resist ingestion by phagocytes, with the former playing an important role.

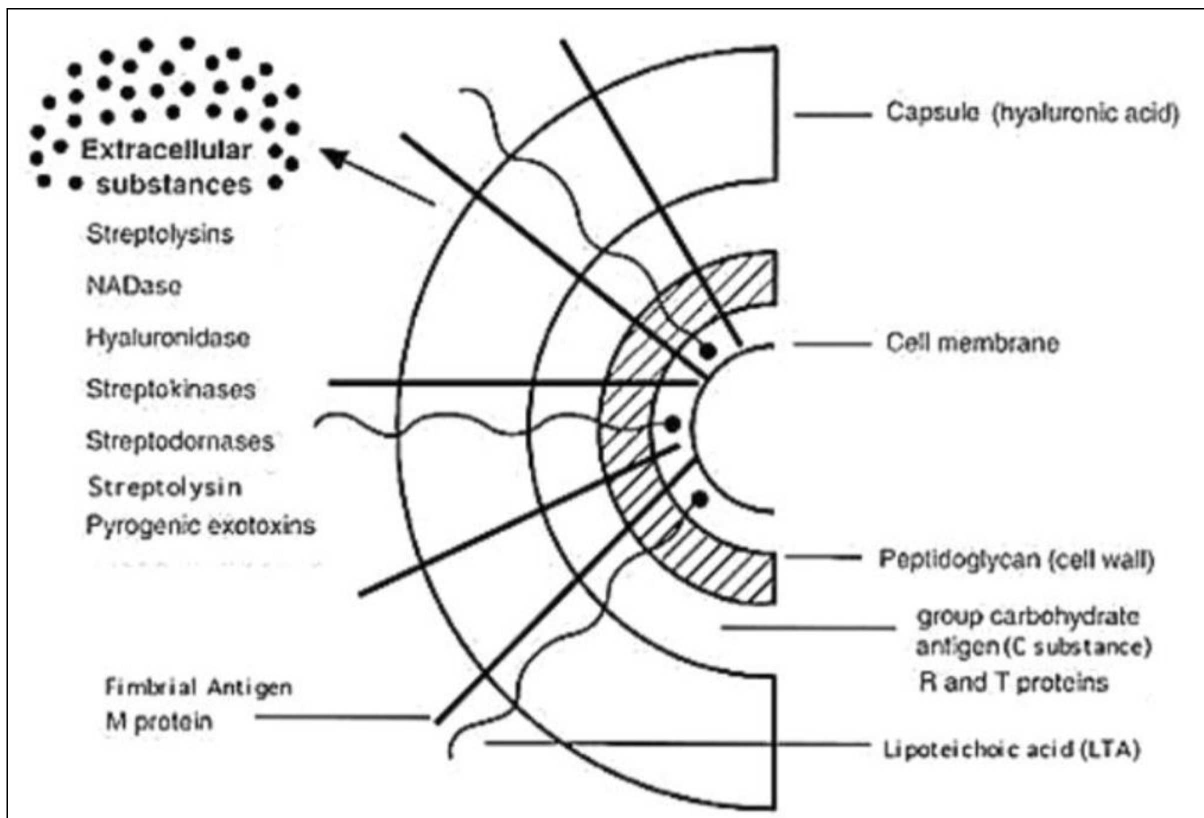
Hyaluronic Acid

GAS has an outer hyaluronic acid capsule. Group A carbohydrate antigen and type specific M protein are attached to the bacterial cell wall and membrane. M protein and capsule are considered as the most important virulence factors responsible for anti-phagocytic properties from the streptococci cell. Hyaluronic acid capsule contains repeating units of glucuronic acid and N acetyl glucosamine[16]. In addition to anti-phagocytic activity, the capsule acts as an important adherence factors in the pharynx, since it binds CD 44 in epithelial cells. Epidemiologic evidence suggests that capsule plays an important role in invasive infection in humans.

M Protein

Streptococci containing M protein tend to resist ingestion by human, mouse, guinea pig leukocytes. It is a major surface protein and virulence factor of GAS containing more than 80 serotypes[10]. M protein is anchored in cell membranes by LPSTGE motif[17]. It extends from cell surface as alpha helical coiled -coil dimer which mimics as alpha

helical coiled structure in host tissue proteins such as tropomyosin and keratin desmin vimentin. M protein has been divided into two classes based on the reaction with antibodies against the C repeat region of M protein[18]. Class 1 – M proteins contains an epitope (surface exposed) on GAS that reacts with these antibodies and do not contain epitope. Recent studies reported that rheumatic fever patients are infected with class 1 GAS strains. Class 2 – M proteins do not react with these antibodies and do not contain epitope. Class 1 proteins were positive for opacity factor while Class 2 were negative.



Cell Wall of Group A Streptococci

Group B Streptococcus (GBS)

Discovery:

It was first discovered in 1887 by Nocard and Mollereu in cattle as a causative agent of mastitis. In Latin, Streptococcus agalactiae means "want of milk"[19]. It has been described as a causative agent of puerperal sepsis by Pasteur and others in early 19th century. Lancefield studies have shown that most puerperal infections were caused by GAS which was then replaced by GBS. It is sometimes called as "BABY STREP" as it most often causes disease in neonates and infants. In period of 1950 to early 1970 it became the most common organism causing neonatal sepsis in United States.

Colonisation

It is a gram-positive beta-haemolytic, catalase negative facultative anaerobe. It colonises mainly in the gastro intestinal and genital tracts of up to 30% of healthy adults but causes life threatening infection in neonates[20]. The main risk factor for neonatal sepsis is the colonisation in the maternal vagina. Colonisation will not usually lead to disease except in susceptible people such as newborns and elderly people. Colonisation rates were lower in children aged 3 to 10 years (4%) compared to neonates. In adults, sexually active adults have more colonisation rates (38% women, 24% men) compared to those who have never engaged in sexual activity (17% women, 13% men)[21]. It can also cause infection of uterus and urinary tract in pregnant women who have been colonised. If pregnant women are colonised with GBS in late pregnancy, during labour the neonates get infected leading to early onset or late onset disease.

Transmission

1. Vertical transmission from mother to foetus during pregnancy is responsible for highest morbidity and mortality in neonates
2. Sexual transmission
3. Foodborne transmission also occurs as it is a known fish and bovine pathogen

Risk Factors

Most pregnant women are at increased risk of having baby that develops sepsis by GBS. Risk factors include: pregnant women tested positive for GBS in 35-37 weeks of pregnancy, detection of GBS in urine during current pregnancy, pre-term delivery and very low birth weight babies, fever during labor, premature rupture of membranes (PROM), long time gap (18 hours of more) between membrane rupture and delivery and history of early onset sepsis.

Clinical Manifestations in Pregnancy

One in three women carry GBS vaginally which infect amniotic fluid even in the intact membranes or infect baby during labor causing sepsis, meningitis, pneumonia[22]. It is the leading cause of neonatal infection and causes invasive diseases in children and non-pregnant women. GBS infection in pregnant women is life threatening. It causes urinary tract infections, chorioamnionitis, post-partum wound infections, pyelonephritis and sepsis. Spontaneous abortions or still birth occurs due to amniotic infection which may lead to premature rupture of membranes. GBS is known to cause intrauterine foetal

deaths and the risk of delivering infants with GBS disease is higher in women with previous history of GBS disease. Prospective study of GBS colonisation in prenatal clinics in Australia supports the evidence that GBS cause premature labour or premature rupture of membranes[23].

Clinical Manifestations in Elderly Adults

In adults, most common infections include skin and soft tissue infections. Bacteremia without identified focus is frequent in both younger and older adults. But urosepsis and pneumonia are more common in older adults than younger adults[24].

Skin and Soft Tissue Infections

Cellulitis is the most common clinical manifestation of GBS associated skin and soft tissue infections. Pre-disposing factors include lymphedema, chronic dermatitis, and radiation induced cutaneous injury. Patients who have undergone mastectomy develop cellulitis of the arm or chest wall has a consequence of impaired lymphatic drainage. GBS bacteremia can occur after many years of surgery[25].Infection such as paronychia and lives threatening such as necrotising fasciitis have been attributed to GBS.

Urinary Tract Infections

Infections such as pyelonephritis and prostatitis are common in older men, particularly hospital residents[25]. People more than 70 years of age are most commonly infected. The pre-disposing factors include indwelling urinary catheters, neurogenic bladder, prostatic hypertrophy, kidney stones, CRF and diabetes mellitus.

Pneumonia

Pneumonia is a manifestation of GBS disease which occurs exclusively in older, debilitated patients. It is associated with CNS dysfunction like dementia, encephalopathy which suggests that aspiration is an important antecedent factor for the development of pneumonia. Skin and soft tissue infections, UTI, pneumonia, bacteremia without identified focus are common in older individuals[26]. Arthritis and osteomyelitis are more common in younger patients. Meningitis, endocarditis, peritonitis, post-operative infections, and vascular catheter associated infections are rarely observed in both the younger and older individuals.

Meningitis in Adults

It does not usually penetrate the blood brain barrier except in infants. In comprehensive review of adults with GBS meningitis, one fourth of the 64 patients were more than 65 years of age. Comorbid conditions existed in 86% of patients which include colon cancer, chronic UTI, end-stage renal disease. GBS meningitis occur spontaneously in 94% patients where distant focus infection was found in half of the patients. In elderly patients, GBS meningitis is often fatal. While the overall case fatality rate is 34%, it is 56% for the adults who are more than 65 years old[27].

Clinical Manifestation in Infants

Early onset disease

GBS has been the leading cause of neonatal illness and death in many parts of the world especially in developed and industrialized countries for several decades[41]. Early onset disease occurs after the spread of pathogen ascending from vagina and cervix. It occurs during the first week or first five days of birth[28] but symptoms usually occur during the first 24 hours of life. Clinical illness present as pneumonia, sepsis and less commonly meningitis. Early onset illness occurs in case of prematurity or complicated delivery.

Late onset disease

It occurs from 1 week to several months after birth. Meningitis occurs most commonly with late onset cases compared with early onset cases. Late onset GBS disease occasionally occurs after the treatment of early onset disease[29]. Numerous risk factors for neonatal disease have been identified. They are PROM, chorioamnionitis, prematurity, infants born to young mothers who are heavily colonized or deficient in anti-capsular antibody.

Pathogenesis

Adherence to Epithelial Surface

The first step in pathogenesis of GBS disease is asymptomatic colonisation of female genital tract. It binds very effectively to the human vaginal cells; maximum adherence takes place at acidic pH. The potential non-protein ligand involved in GBS adherence is

LTA (Lipoteichoic Acid) which is an important cell wall polymer. Like other pathogens, it attaches to the host extra cellular matrix proteins such as fibronectin, fibrinogen and laminin[30].

Invasion of Epithelial and Endothelial Cells

It has ability to traverse several host cellular obstacles including placental membranes, alveoli of infant lung, neonatal Blood Brain Barrier (BBB). The virulence attribute of GBS is reminiscent of several enteric bacterial pathogens for which invasion of gut epithelial cells is primary step involved in establishing systemic infections[30].It has also been suspected that GBS can penetrate amniotic cavity through intact placental membranes, because early onset disease occurs in some infants delivered by Caesarean section with no identifiable risk factors. They are highly invasive for chorion cells and transcytoses through intact chorion cells mono layers without the disruption of intra cellular tight junctions. Following the aspiration of infected amniotic fluid, the initial focus of GBS infection in new born in lung and pneumonia is the hallmark of early onset infections in neonates. It must traverse three host barriers in lung: alveolar pulmonary epithelium, and pulmonary endothelium. Recent studies demonstrated that penetration into human Brain Micro-vascular Endothelial Cells (BMEC) is an important factor in producing meningitis. Time course studies in epithelial and endothelial cells indicate that GBS survive intra-cellularly for up to 24 hours after invasion without bacterial replication[31]. GBS polysaccharide capsule decreases the adherence of the organism to

alveolar epithelium but also provides an important survival advantage to GBS by inhibiting phagocytosis by macrophage and neutrophils.

Injury to Host Cell Barriers

Bacterial proliferation with associated injury to host tissues is apparent in pathological specimens from patients with GBS disease in particular placenta, lung and brain. Disruption of epithelial and endothelial cell barriers by direct cell injury could facilitate penetration into placenta and systemic spread of organism in the host.

Virulence Factors

Beta hemolysis by GBS is directly cytotoxic to human cells and considered as potential virulent factor for the organism. GBS causing human disease are invariably encapsulated, belonging to one of the nine recognized serotypes 1a, 1b, and 2 through 8. With minor exceptions, various GBS capsular polysaccharide antigens are composed of four monosaccharides mainly glucose, galactose, N acetyl glucosamine and sialic acid. Hyper immune rabbit antisera against given type specific polysaccharide antigen provide passive protection to mice. A low level of human maternal anti-capsular IgG is a major risk factor for the development of neonatal invasive GBS infections. There is a 2-4 fold increase in the incidence of invasive GBS disease in adults that have occurred in the past 2 decades and more than two thirds of cases of invasive GBS disease in United States occurred in adults[24].

Group C, G Streptococcus (GCS, GGS)

Non group A streptococci (GCS and GGS) have been shown to cause acute pharyngitis in children and adults. GCS and GGS are beta-haemolytic, gram positive, catalase negative, facultative anaerobes. They form large colonies on blood agar. They are mainly commensals of the nose, throat, intestine or skin. The main origin of virulent species of GCS which cause disease in humans are animals, unpasteurized milk and dairy products[32]. Streptococci C and G which causes human infections have now been considered to belong to the species *Streptococcus dysgalactiae subspequisimilis* (SDSE). These strains react with Lancefield's group C and G antigens. As they share the virulence factors, hemolysins, M proteins with group A streptococci, they are often termed "pyogenes-like"[33]. Small colony forming phenotypes regardless of Lancefield's group are placed in *Streptococcus anginosus* group and they are not considered to cause acute pharyngitis but can cause abscess and bacteremia infrequently. Patients from whom large colony forming GCS organisms were isolated had clinical features more of GAS infections than did patients from whom small colony forming organisms were isolated[34].Post streptococcal sequalae has also been caused by GCS and GGS. They also cause invasive infections like GAS.

GCS consists of 4 species: *S.equisimilis* (commonest), *S.dysgalactiae, S.equii* (agent of equine strangles rarely present in the throat of normal horses but seldom isolated from humans), and *S.zooepidemicus* (cause of respiratory infection and suppurative disease in

many animals but uncommon in humans). These four species can be identified by their biochemical properties and their reactions on blood agar plate.

GGS strains which cause infection in animals, particularly in dogs, have been considered to belong to *S.canis*. They are epidemiologically distinct from the human strains.

Virulence Factors

As they have the virulence factors like GAS, M protein is one of the virulent factors. Others include hyaluronic acid capsule, streptokinase, nephritis strain associated protein and various protein binding structures.

Human Infections Caused by GCS

Biotype *S.equisimilis* can cause serious infections including bacteremia, pneumonia, septic arthritis, and endocarditis. The outbreak of the infection by GCS is rare and unusual. Studies have shown that the outbreaks of pharyngitis have occurred in different countries. Overall, GCS are important opportunistic nosocomial pathogens and causes diseases associated with chronic pulmonary, cardiac disease, diabetes, malignancy and alcoholism. It was found that bacteremia occurred in 74% of cases and nosocomial acquisition was observed in 26%[35].

Human Infections Caused by GGS

It has been emerged as an important opportunistic nosocomial pathogen in the past 10-15 years. They have been isolated from variety of infections including endocarditis,

endophthalmitis, septic arthritis, pharyngitis, neonatal sepsis and cellulitis. More recently, GGS and GCS has also caused streptococcal toxic shock syndromes in Japan[36]. They have also been reported in AIDS patients. GGS are carried asymptomatically in female genital tract and causes puerperal and neonatal sepsis in presence of predisposing factors like PROM, prematurity, prolonged or difficult labor.

Maternal post-partum septicemia has also been reported. Most common outbreaks of GGS are those of pharyngitis. Single serotype of GGS namely T305 was isolated for a period of one year from infected leg ulcers and environment of outpatient ulcer clinic in London hospital and possible vehicle of transmission was appeared to be skin flakes from infected leg ulcers[37].Overall, GCS and GGS are important human pathogens because of their similarities to GAS and their abilities in causing severe invasive diseases and outbreaks of infections.

Group F Streptococci

Taxonomy

Lancefield and Hare[38] in 1928 described group F streptococci and identification in labs today depends on the specific reactivity with Lancefield group F antibody. In 1934, Long and Bliss described group F streptococci amongst the “minute haemolytic streptococci”. Serological grouping done by them from throat isolates showed that 91% were group F streptococci and remaining were group G[39]. Smith and Sherman[40] have isolated the similar organism from human faeces and retained the name “minute haemolytic

streptococci" to describe only their group F strains. According to European views of taxonomy of streptococci, all group F streptococci belong to *S.milleri* group.

S.milleri has been continued to be used by the European taxonomists to describe the cluster of organisms including *S.anginosus, Streptococcus MG, S.constellatus, S.intermedius, S.milleri,* minute beta-haemolytic colonies of groups F and G. These cluster of organisms were listed in 1986 edition of Bergey's Manual. Taxonomy of GFS is still confusing and universally accepted taxonomy does not exist.

GFS are catalase negative gram positive, facultative anaerobe or capnophilic cocci that occur in pairs or chains. They form minute haemolytic colonies in blood agar that may show considerable variation among the colonies and all types of haemolysis may be seen in them[39].

Classification

The classification of GFS isolates across haemolytic boundaries was done by Ottens and Wrinkler[41]. They observed only minor differences in the biochemical properties and identification of group F antigen in haemolytic and non-haemolytic strains.

Infections

GFS are the normal commensals of the oropharyngeal, respiratory, genitourinary and gastro intestinal tracts. These organisms mostly cause deep abscess and systemic infections. The areas involved in abscess in descending order are cutaneous system,

cervicofacial, dental, intra-abdominal areas. They also cause infections like bacteremia, endocarditis, myocardial abscess, osteomyelitis, wound infection, abdominal and pelvic abscess, meningitis and other CNS infections. They are unique among streptococci in their tendency to cause abscess. Despite the normal commensal in vagina, they are usually not associated with infection in this site. Only one study has recorded the isolation of GFS from Bartholin's abscess (in two cases)[42]. They have also been associated with dental and hepatic abscess, occasionally wound infections.

Pathogenesis

Pathogenesis is characterized by polysaccharide capsule which resist phagocytosis. GFS also produce pyrogenic exotoxins and hydrolytic enzymes (hyaluronidase) which enhances the spreading of organisms through the fascial tissue planes, and cause necrosis of tissues.

Group F Streptococci showed 2% (7 patients out of 350) of all beta-haemolytic streptococcal isolates from patients with bacteremia hospitalized at Mayo clinic-affiliated hospital during the period 1970-1980[43]. The underlying pathology associated with gastro intestinal tract was seen in 5 patients. All isolates of group F streptococci was susceptible to penicillin.

Streptococcus Anginosus Group (SAG)

Guthof (1956) proposed the name *S.milleri* for a group of non-haemolytic streptococci obtained from dental abscess and other inflammations in or around mouth He named these organisms as *S.milleri* in honor of the microbiologist W.D. Miller[44]. Colman and Williams observed that their *S.milleri* strains were heterogenous serologically and evidenced Lancefield group A, C, F, G antigens, other various type antigens or undetectable antigens[44]. These non-haemolytic strains differed from their beta-haemolytic "Pyogenes" like counterparts, *S.pyogenes, S.equisimilus* and GGS. They found that non-haemolytic *S.milleri* were physiologically identical to minute beta-haemolytic streptococci of group F and G described by Long and Bliss[45]. They suggested that *S.milleri* designation could also be applied these beta-haemolytic strains. The term *S.milleri* was not included in the approved list of bacterial names. The comprehensive study of genetic relatedness among these organisms has recognized that they could be included in single species which are officially now called *S.anginosus*[46]. Anginosus is the oldest approved name for the members in this group, and hence it took priority over the name, milleri. *S.anginosus* group (SAG) consists of three distinct species – *S.anginosus, S.intermedius,* and*S.constellatus*.

Morphology

Members of the SAG are gram positive, catalase negative, facultative anaerobes that possess variable haemolytic patterns (α, β and γ) on sheep blood agar. They give

distinctive butterscotch or caramel like smell when cultured in solid medium. They formed pin-point or minute colonies. Although members of genus Streptococcus are classified by haemolytic patterns and Lancefield antigen which are highly variable for members of SAG. These organisms are normal commensal in humans and isolated commonly from oral cavity, oropharynx, gastrointestinal tract and female genital tract (vagina). They have the capacity to cause a variety of infections in both humans and animal host. They are often associated with bacteremia and their unique feature is the ability to cause abscess. So, they should be considered as true pathogens when isolated from humans.

Virulence Factors

SAG is also known to release extracellular products which have immune suppressive effect and flourish through abscess. The presence of polysaccharide capsule makes pathogens to escape from the phagocytosis prior to infection. They also produce gelatinase and collagenase which contributes to their overall pathogenicity.

Infections by SAG

SAG cause infections in mouth and teeth, most commonly dental abscess, and also cause invasive pyogenic infections including head and neck infection, brain abscess, intra thoracic and intra-abdominal infections. Predisposing factors include previous surgery, trauma, diabetes, immune deficiency and malignancy but it is also related to patient’s oral hygiene and over all general health. Infective endocarditis has also been reported in some

patients with SAG bacteremia. *S.anginosus* is the most common species associated with endocarditis because it is usually found in high cell densities in infected tissues. Majority of the isolates were obtained from exudates, aspirates, or fluid samples, and some isolates were recovered from blood. The prophylactic use of antibiotic combinations like gentamicin and metronidazole in surgical patients may enhance the infection caused by *S.anginosus.*

A review of 153 clinical isolates of SAG bacteria found that the predominant strain isolated from the intra-abdominal infection was *S.anginosus*, and *S.intermedius* was mainly associated with CNS infections (brain abscess). *S.constellatus* has been associated with thoracic infections. A review of 30 cases of SAG bacteremia found that 3 patients who died were infected with *S.constellatus*. Only 2 of the 30 patients were infected with *S.anginosus* and none were dead. Two later studies showed the association of *S.anginosus* with GIT, *S.intermedius* was frequently isolated from purulent head and neck specimens[47]. Recent research showed that alcoholic patients have elevated levels of *S.anginosus* suggesting they have higher risk of *S.anginosus* infection. Members of SAG may cause abscess formation, but different species have different tendency for pyogenic infections. Since three species were associated with different clinical manifestations and symptoms, identification of these organisms provides pathway for the investigations of the etiology of disease and optimize patient outcomes.

Laboratory Diagnosis of Beta-haemolytic Streptococci

The gold standard for diagnosing beta-haemolytic streptococcus species is cultivation of the organisms in a media containing blood which can be hemolysed[48]. In many developing countries, human blood is used to prepare blood agar. But the use of human blood is no longer recommended due to poor isolation and hardly visible hemolysis.[49] Blood agar containing 5% defibrinated sheep blood is the most commonly used media for cultivation as it enables easy preliminary screen for beta-haemolytic colonies. Further confirmation can be done by several easy rapidly performed biochemical tests applied in microbiology labs despite the use of automated identification system. Brown described the appearance of different colonies of streptococci in blood agar[50].

Pyrrolidonyl Amino peptidase (PYR) Test

It is a rapid test which is used for the presumptive identification of Group A Streptococcus. It can be differentiated from other beta-haemolytic streptococci by the production of pyrrolidonyl aryl amidase, Bacitracin sensitivity and Lancefield grouping. A study reported that the sensitivity of this test is about 99.08%[51]. This test was described in 1981 by Godsey, Schulman and Eriquez described this test to differentiate GAS and enterococci from other streptococci based on their ability to split L-pyrrolidonyl -β-naphthylamide[52]. It is a substrate hydrolysed by 100% GAS. The study has been conducted to describe the evaluation of modification of PYR test[53]. This test was shown

to be more specific than Bacitracin sensitivity test for identification of GAS and appears to be equally sensitive to bacitracin test[54].

Bacitracin is a mixture of related cyclic peptides produced by organisms of Bacillus subtilis group. This act against gram positive bacteria by interfering with cell wall and peptidoglycan synthesis. Maxted introduced this test as presumptive test for identification of GAS[55]. He described this test by using bacitracin paper strips (filter paper strips coated in bacitracin diluted 1 in 5,000 in glycine phosphate buffer). He sub-cultured the colonies on blood agar plate and bacitracin paper was placed over the inoculated area. Plates were incubated at 37° Celsius for 24 hrs. Some strains showed ZOI of 2cm, some showed only slight inhibition and others none. Those showing any inhibition from the edge of paper were considered as negative or bacitracin resistant. He used 2,345 GAS strains out of which only 22 were resistant to Bacitracin[56]. Subsequent studies have made improvements in this test by making variations in the concentration of drug and diameter of ZOI. A study in India which used 54 GAS strains have shown that 94.44% of GAS were sensitive to bacitracin and this can be used as simple method for distinguishing GAS from other beta-haemolytic streptococcus[57]. When this test was performed in correct manner, it is about 95% accurate in most situations. But 2-7% of bacitracin testing may lead to false negative reports[54].

Christie Atkins Munch-Peterson (CAMP) test

It was first identified by Christie, Atkins, Munch Peterson in 1944 and this test has been named after three researchers. They used this test for the presumptive identification of GBS. They produce a diffusible extracellular protein (CAMP factor) which synergistically acts with staphylococcal beta lysin to lyse erythrocytes and shows the arrow shaped zone of enhanced hemolysis at the junction of staphylococcal and streptococcal streak. This is the most commonly used method as it is inexpensive and rarely gives false positive reactions with other streptococcal groups. A double blind study of 135 randomly selected streptococcal isolates showed there was complete agreement between CAMP test and standard Lancefield test. All group B streptococci tested had positive reactions[58].A series of experiments were made to standardise the CAMP test for the identification of GBS. Darling found that this test worked best when streptococci were inoculated in Trypticase Soy Agar containing erythrocytes and inoculation was done in candle jar. This was later modified by study which was done by incorporating the beta lysin of *S.aureus* into filter paper disks which eliminates the need for actively growing culture for CAMP reaction. These studies showed 100% accurate identification of GBS by the CAMP test after 18 hours of inoculation[59].

Hippurate Hydrolysis test was discovered by Ayers and Rupp where they described that human streptococcus can be differentiated from bovine streptococci by their ability to hydrolyse sodium Hippurate[60]. This procedure was later modified for the identification of group A, B and D Streptococci. This method takes a longer duration of 48 hours to obtain

the results. Rapid method was then developed by using heavy inoculum of organisms in an aqueous sodium Hippurate substrate and it detects the hydrolytic product glycine rather than benzoic acid and gives the results immediately within 2 hours[61].

Lancefield grouping (serogrouping)

This test was introduced by American Microbiologist Rebecca Lancefield. She arranged beta-haemolytic streptococci into twenty groups (A-U without I and J). This is mainly based on the antigenic differences in C carbohydrate, a group specific antigen located in the cell wall of streptococci. The original Lancefield precipitin test is rarely performed nowadays, which has been replaced either by Latex Agglutination test or Co agglutination. The study was performed to compare three methods for grouping streptococci. They are: Nitrous acid extraction method, Slide agglutination test and Lancefield grouping.

They compared the nitrous acid extraction method and Slide agglutination test with Lancefield grouping. They found that both new techniques can be recommended for diagnostic purposes, but the amount of carbohydrate extracted was significantly lower than the Lancefield method. They also confirmed that slide agglutination test is useful especially for routine work as it needs only the little amount of sensitizing serum[62].

Carbohydrate fermentation test

Carbohydrates are important in bacterial nutrition mainly as a source of energy. Fermentation reaction also plays a major role in identification of bacteria. Different members of beta-haemolytic streptococci can ferment sugars and the end product is mainly Lactic acid. The first extensive work on fermentation was done by Gordon. He selected 10 strains of streptococci (each from different sources) and tested the action on 14 carbohydrates, 13 glucosides and 6 poly atomic alcohols. All the strains showed identical reaction in many of these tests except 7 substances which showed greater differential value in strains. Those substances were lactose, saccharose, raffinose, inulin, salicin, coniferin and mannite. Another study was done which applied all the tests done by Gordon except that of coniferin to 300 strains of streptococci isolated from human stools. Both the studies have not suggested any satisfactory grouping for streptococci and this was done by Andrews and Horder. Buerger studied fermentation of 34 pathogenic strains but he was not able to give confirmatory results[63].

The attempts to classify streptococci based on the fermentative tests was made by Salomon where he used 78 organisms and found most of the reactions were of no differential value, but sugars like glycerine, mannite, raffinose, arabinose was fermented by some strains and non-fermented by others. He used litmus milk as indicator for fermentation reactions. Later studies had assumed that color change in litmus milk is not an accurate indication for fermentative properties of bacteria and they used phenolphthalein as an indicator. They used sugars like lactose, raffinose, mannose,

dextrose and tested 116 strains of streptococci, 100 strains from horses, 100 from cows and grouped all these strains in 9 classes[64]. The products of fermentation produced by a great majority of bacteria are very similar, irrespective of the carbohydrate fermented. Streptococci, Staphylococci produce lactic acid from variety of sugars. Recent studies have demonstrated that haemolytic streptococci of human origin can be differentiated from bovine origin by the final H^+ ion concentration produced when grown in glucose broth[65].

D – Test for inducible Clindamycin resistance

Inducible Clindamycin resistance in Streptococci can be detected by Disk diffusion test using Clindamycin and Erythromycin. Resistance to Erythromycin among GAS and GBS was first noted in 1959. Macrolides are used as alternatives in penicillin allergic individuals and clindamycin are recommended for invasive streptococcal infections. The mechanism for macrolide resistance may be due to active efflux (mef A gene) or target site modification (erm B gene), the latter conferring cross resistance to clindamycin and Streptogramin B (MLSB phenotype). mef A confers low level resistance to macrolide only[66].

Studies have shown that prevalence of macrolide resistance and distribution of resistance mechanism differ among beta-haemolytic streptococci with GBS and GGS have shown the highest resistance rate. So, macrolides or lincosamide cannot be empirically used for severe streptococcal infection before the strains are proved to be susceptible.

CLSI has recommended a disk diffusion test in which Erythromycin and Clindamycin disks are placed in proximity on the surface of inoculated agar. If Erythromycin has induced expression of ribosomal methylase enzyme among the bacterial cells on area adjacent to Clindamycin disk, there will be a flattening of ZOI around one side of the Clindamycin disk which resembles the letter "D"[67]. So, this test is commonly called as D – zone test. CLSI has recommended this test for Staphylococci and beta-haemolytic Streptococci.

Molecular diagnosis of Streptococci

GAS is one of the common pathogens causing pharyngitis in children and can lead to suppurative and non-suppurative complications. Accurate diagnosis and optimal antimicrobial therapy are important for the prevention of complication and reduces both the duration of the illness and transmission of the agent. But diagnosis based on the clinical features alone is difficult because of the overlapping of the symptoms with viral pharyngitis.

The gold standard for the detection of GAS is culturing throat swab in blood agar. But the long time-gap (48 hours) between collection of specimen and microbiological diagnosis has led to the development of Rapid Antigen Detection Test (RADT) and molecular assays. In early 1980s, RADT for group A streptococcus offered faster and simpler alternative for diagnosis of GAS pharyngitis in outpatient settings. Over time, several generations of RADT have emerged starting with latex agglutination followed by lateral

flow assays, color immuno-chromato graphic assays. But this method has certain limitations as given below.

1. They do not differentiate between viable and non-viable bacteria in specimen
2. Negative results are to be confirmed by culture

The persistent need for highly sensitive and rapid assays against culture method paved way for the development of molecular assays. The first assay designed was GAS direct probe test from Gen-probe. This test identified specific rRNA sequences of *S.pyogenes* in pharyngeal samples by single stranded chemiluminescent nucleic acid probe. A comparative study of detection of GAS by molecular method with standard culture method showed that the molecular assays have sensitivity and specificity of 94.8% and 100% respectively. This result indicated that molecular test can be used as a single primary test as it offered the result on the same day of specimen collection. Another molecular assay which is commercial isothermal DNA amplification assay called Illumigene assay has also demonstrated excellent sensitivity (93%) and specificity (91.4%). This test relies on Loop mediated isothermal Amplification (LAMP) technology with *S.pyogenes* primers to evaluate results within 60 minutes. The target region of this assay is GAS pyrogenic exotoxin B (sep B gene). A study was conducted to compare this assay with standard culture and PCR methods where specificity was 100% and sensitivity was around 95.9%.

In India, a four-year study has been done to assess the anti-microbial resistance of beta-haemolytic streptococcus and to ascertain molecular mechanisms of resistance. Beta-haemolytic streptococcus was recovered from various clinical samples and from various settings. AST was performed by disc diffusion method according to CLSI guidelines. MIC was determined by E-test for all antibiotics used. D-test was done for isolates which were clindamycin susceptible but erythromycin resistant. Resistant genes detected by PCR include erm (A), erm (B), tet (O), tet (M) and mef (A) genes. Total of 220 isolates of GAS, resistant to erythromycin, tetracycline and ciprofloxacin was seen in 102 (46%), 174 (79%), 21 (9.5%) respectively. 62 isolates (30.6%) were resistant to both erythromycin and tetracyclines. 9 isolates (4%) were resistant to erythromycin, tetracycline and clindamycin. Inducible clindamycin resistance was noted in 61 GAS isolates. The presence of erm (B) was observed in 10% and tet (M) in 14% with absence of erm (A) and tet (O) genes. Then, GAS was predominant organism followed by GGS in this study. This study showed that beta-haemolytic streptococcus was found to be sensitive to all anti-microbials erythromycin, tetracycline and ciprofloxacin[68].

Molecular Mechanism of Resistance to Group A and G Streptococcus

Group A Streptococcus

Penicillin is the mainstay of treatment of streptococcal infections. *S.pyogenes* remains universally susceptible to penicillin. In penicillin allergic individuals, Macrolides, Lincosamides and fluoroquinolones are commonly available treatment options.

Macrolides are ideal for the treatment of streptococcal pharyngitis and respiratory infections. Clindamycin is the drug of choice in treating soft tissue infections as it has additional advantage of suppressing toxin synthesis[69].Although they are universally susceptible to penicillin, a significant number of treatment failures have been reported from different parts of the world. In the past 15 years, the rate of penicillin failure has increased to almost 40% in the world. Recent studies demonstrated that oral cephalosporins are found to be effective[69].

The widespread use of antibiotics has led to the emergence of resistant organisms. Although resistance to penicillin has not been reported to date from Streptococcal isolates, 7-40% of treatment failure has been reported in patients with recurrent pharyngo tonsillitis. The first macrolide resistant streptococci were reported in 1968 in USA. The evidence of macrolide resistance has dramatically increased in several countries with resistant rates more than 20%. Erythromycin resistance is associated with increased cell invasiveness of *S.pyogenes* and it could be due to the presence of prtF1 gene, which is found more frequently in macrolide resistant strains. prtf1 gene encodes fibronectin binding protein F1 which is an adhesin that allows *S.pyogenes* to easily invade within human respiratory cells. Biofilm formation could also be important factor responsible for therapeutic failures and recurrence of GAS infection[70]. The resistance rates variation between 5% and 40% with the highest prevalence in Asia and lowest in Europe and USA suggestive of geographic variation in resistant rates and prevalence of resistant

mechanisms. Earlier studies from India reported that there is increase of 2-38% of macrolide resistance among GAS isolates.

Major mechanisms of macrolide resistance in GAS are target site modification and macrolide efflux pumps. Target site modification is mediated by rRNA methylase encoded by erm (B) gene or erm TR gene of erm A class. It is related to MLS phenotype. The second mechanism is by efflux pump which is associated with presence of mef (A) gene related to M phenotype.

Although tetracyclines are not used for GAS infections, high tetracycline resistance has been reported in many countries. Concurrence of tetracycline and macrolide resistance has also been reported as tetracycline resistant genes can reside on macrolide genetic elements which carry macrolide resistant genes. Resistance to tetracycline is conferred by ribosomal protection genes such as tet (O) and tet (M) and by efflux pumps imported by tet (K) or tet (L) genes. The other mechanisms of drug resistance include inactivation of drug and active efflux. The genes, tet (Q), tet (S), tet (T), tet (W) genes are rarely involved in tetracycline resistance. In *S.pyogenes*, tet (M) is a major determinant for resistance while tet (O), tet (S) have also been reported. The predominance of tet (M) is since this gene is carried by conjugative transposons or by composite structures (such as Tn3701) which gets easily translocated from chromosome to chromosome. Recently, studies were conducted to determine the prevalence of antibiotic resistance among GAS isolates and underlying genetic mechanisms. They have used two multiplex PCRs to

detect the presence of erythromycin resistant genes (erm B, erm A, mef A) and tetracycline resistance genes (tet O, tet M, tet K) using specific primers. The 16s rRNA genes specific for genus Streptococcus was included as internal control. Majority of erythromycin resistant isolates belong to inducible phenotype (iMLS) followed by M phenotype. Among macrolide resistant isolates, 61% harbored erm (B) and 37% mef (A) as the sole macrolide resistant genes and co-occurrence of erm (B) and mef (A) was seen in 2%. Among tetracycline resistant isolates, 97% harbored tet (M) and only 2% had tet (O) but 1% strain carried both tet (O) and tet (M) genes. None of the isolates were positive for tet (K) and tet (L) genes. This demonstrated that efflux pump mediator resistance was absent in the study population. This also explains tet (M) is the major genetic determinant for tetracycline resistance[69].

Group G Streptococcus

Lancefield group C and G can cause pharyngitis and a variety of severe infections in humans. They are the most common cause of streptococcal bacteremia in many humans. Resistance to erythromycin in GAS has been reported from many countries but there are also few reports of erythromycin resistance in GGS and GCS. In treatment of SDSE (*S.dysgalactiae subspecies equisimilis*) pharyngitis infections, the most commonly used antimicrobial agents are penicillin, cephalosporins, vancomycin, carbapenems. GGS and GCS resistance to erythromycin, tetracycline and clindamycin are now emerging[71]. A study conducted in North America among different members of beta-haemolytic streptococci have shown that compared to group A streptococcus, GGS was sensitive to

all antibiotics, only one GGS throat isolate was resistant to amikacin and intermediate sensitive to ampicillin, clarithromycin, amoxicillin, tetracycline and oxacillin[68].

Previous studies have demonstrated that there are major geographical differences associated with the mechanism of macrolide resistance. In one study, it was noted that 97% of erythromycin resistant GGS possessed erm genes of which 94% had erm TR genes (subclass of erm A) and only one possessed erm B gene[71]. Recent studies have shown that 50% of erythromycin resistant group C and group G streptococcus possess mef genes but only 5% possessed erm genes. The type of erm gene in GGS can also be related to the source of streptococcal infections. This study has reported that macrolide resistance in GGS has also resulted from the horizontal transfer of mef genes among different species of streptococci, staphylococci, enterococci. Efflux of macrolides mediated by mef genes has been described in different gram positive cocci[71].

Treatment of Streptococcal Infections

Beta-haemolytic streptococci cause wide range of diseases including mild infections to severe life-threatening infections. Among them, GAS cause serious infections like rheumatic fever, scarlet fever and rheumatic heart disease. In India, RHD is a leading cause of acquired cardiac disease. To date, effective vaccines are not available and all efforts for preventing streptococcal diseases (RHD, RF, suppurative complications) rely on the accurate diagnosis and appropriate treatment measures. GAS, GBS, GCS and GGS

causes infections of different severity and frequency. Penicillin is the universal active drug against all these organisms although therapeutic failures have also been reported.

Treatment of different streptococcal infections mainly depends on oral penicillin V, intramuscular benzathine penicillin, parenteral penicillin G, amoxicillin or cephalosporins (cephalexin, cefotaxime, ceftriaxone). Macrolides, mainly erythromycin and azithromycin are the first options in patients allergic to penicillin.

Lincosamides like clindamycin, lincomycin are also used for streptococcal infections. Clindamycin is the recommended antibiotic to treat severe skin and soft tissue infections (necrotizing fasciitis & toxic shock syndrome). The patients may also be treated with tetracyclines (tetracycline, minocycline, and doxycycline), vancomycin and fluoroquinolones depending on the susceptibility patterns. Aminoglycosides are also given along with beta lactam even along with the beta lactam antibiotics to treat rare cases of endocarditis due to GGS and GCS[72].

A study conducted in North India to determine the antimicrobial susceptibility pattern of various beta-haemolytic streptococci showed that GAS was generally susceptible to all antibiotics except macrolides and tetracyclines. Worldwide data suggests an increased resistance of GAS to macrolides. In the treatment of SDSE pharyngitis, the most active agents used are penicillin, cephalosporins, carbapenems, vancomycin. A recent study conducted to test the AST by using MIC method suggested that MIC for penicillin for GAS has not changed over time but increased MIC have been noted for GGS and GCS.

GBS should be treated promptly and aggressively with intravenous antibiotics as it is the most common cause of neonatal sepsis, meningitis and puerperal sepsis. The treatment is mainly intrapartum antibiotic prophylaxis of penicillin G 5 million units given IV as loading dose followed by 2.5 to 3 million units every 4 hours during labor until delivery. Ampicillin is considered as an alternative to penicillin G. They are not recommended for penicillin allergic individuals. Clindamycin is the recommended drug for penicillin allergic patients. The antibiotic prophylaxis should be started 4 hours before delivery as it prevents transmission of infection to foetus[73].

Materials and Methods

Type of Study

- Prospective study

Study Population

- Clinical samples of patients attending PSG Hospitals
- Study period: January 2018 to July 2019

Sample Size

- 175

Sample Size Calculation

$N = 4pq/d^2$ (where N = required sample size, p = expected prevalence, q = 100 – p, d = degree of prevalence)

Inclusion Criteria

Clinical samples of patients which includes wound swabs, pus, throat swabs, vaginal swabs. All beta-haemolytic streptococci obtained from clinical samples.

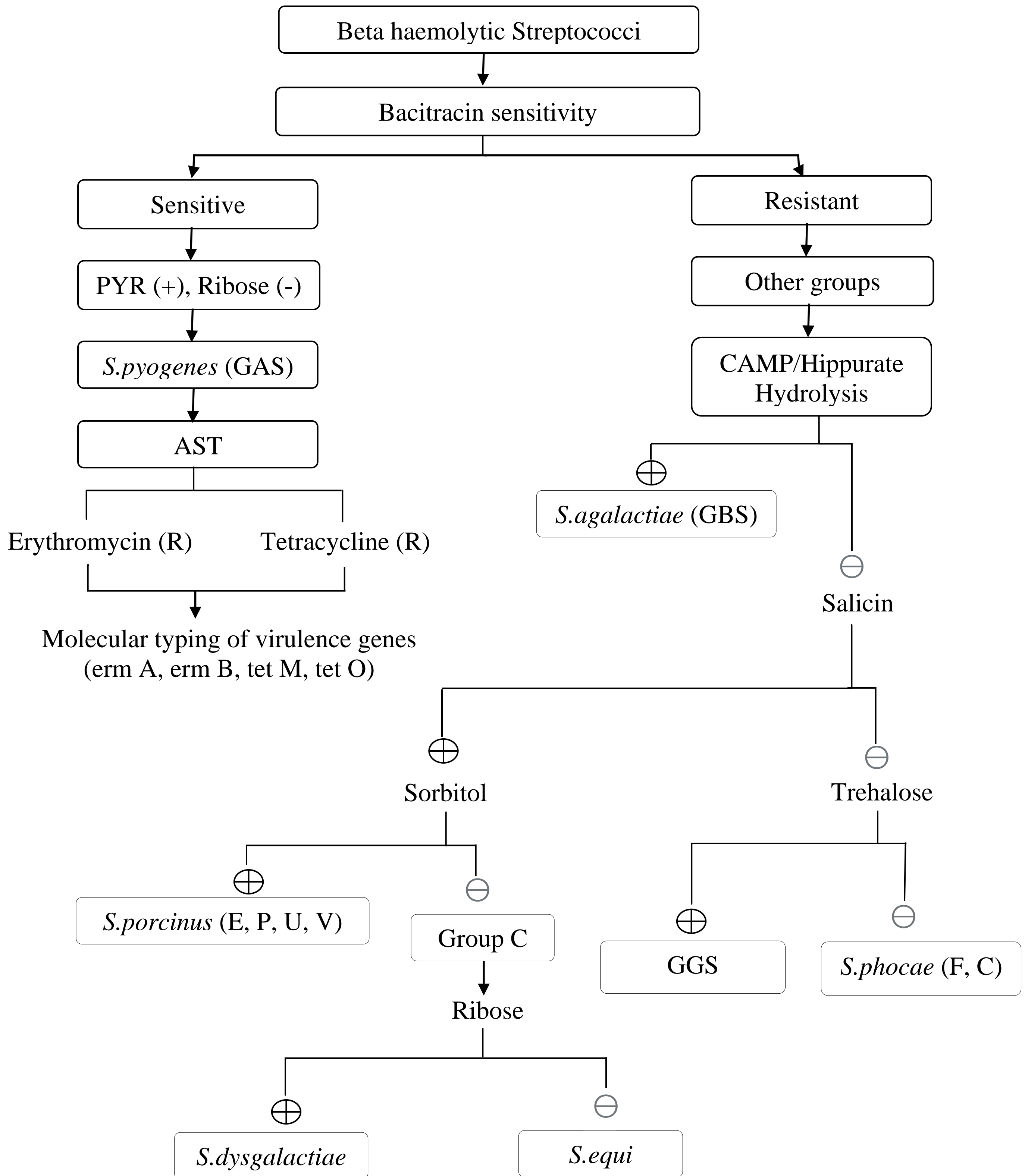
Exclusion Criteria

- Nil

Ethical Clearance

The institutional human ethical clearance was obtained before the study.

Algorithm for Identification of Streptococci



Quoted from Koneman's textbook of Microbiology 7th ed.

Sample Collection

Specimens like pus, wound swabs, throat swab, high vaginal swabs collected were inoculated on to blood and Mac Conkey agar and incubated at 37°C overnight.

Growth on Sheep blood agar

Following overnight incubation of sample, colonies of streptococci were seen as grey colonies of about 0.5 – 1mm in diameter with beta hemolysis.

Catalase test

A drop of 3% H_2O_2 was added to the slide. The growth was further applied to it with an applicator stick. Appearance of bubbles was taken as positive. Streptococcus colonies showed no bubbles which was taken as negative.

Gram stain

Smears made from colony and stained by conventional gram stain showed gram positive cocci in chains.

Storage

The isolates after subculture were stocked in Robertson's cooked meat medium in tubes. The tubes were sealed and stored at 4 – 8°C and when further tests were to be done, they were further sub-cultured.

Identification Tests

Group A Streptococcus

Bacitracin sensitivity

A well isolated colony of Streptococcus from the culture was taken and lawn culture was made on blood agar plate. Then 0.04 international units of Bacitracin disc was kept over the lawn culture. Following overnight incubation at 37°C, the zone of inhibition greater than 14mm was taken as sensitive for *S.pyogenes* (group A). All isolates resistant to Bacitracin were categorized as groups B – V (exceptions of I and J).

PYR test

The colonies were inoculated in PYR broth and incubated for a period of 4 hours. Later 3 drops of PYR reagent was added to it and change of color to red was confirmed positive indicating *S.pyogenes* whereas absence of color change indicates negative test. Except group A all the other beta-haemolytic Streptococci were negative for PYR test.

Ribose fermentation

Species identification was further done by Sugar fermentation test by inoculating the organism in peptone with 1% ribose sugar with Andrade indicator. Any color change from colorless to pink indicated the positive sugar fermentation test. Absence of color change was taken as negative. Ribose was not fermented in *S.pyogenes*. Ribose test was positive in groups B, C, G streptococci. Ribose test was negative in group A streptococci.

Group B Streptococci

CAMP test

The beta-lysin producing strain of Staphylococcus aureus was streaked down the centre of sheep blood agar plate. The colonies are Streptococcus was streaked perpendicular to S. aureus streak within 2 mm. The plate was incubated at 37°C for 24 hours in ambient air. The appearance of arrowhead zone of beta-hemolysis at the junction of two organisms (indicates enhanced hemolysis) was considered as positive test. Absence of arrowhead zones of hemolysis indicates negative test. CAMP positive strains were interpreted as Group B (*Streptococcus agalactiae*).

Hippurate Hydrolysis

The colonies of streptococcus were added in 0.1 ml of sterile water in plastic tube and make a heavy suspension. The rapid Hippurate disk was placed in to the mixture by using forceps. The tube was incubated at 37°C for 2 hours. 0.2 ml of Ninhydrin reagent was added, and tube was re-incubated for an additional 15-30 minutes. Group B Streptococcus hydrolyses hippurate and gives deep purple colour. The appearance of pink color or slightly yellow pink indicates negative test. All the other groups were negative for Hippurate hydrolysis.

Group C, G Streptococci (*S. dysgalactiae* subsp. equisimilis)

Esculin Hydrolysis Test

Colonies of streptococci were inoculated on to the surface of the slant of bile esculin medium with an S shaped motion and incubated at 37°C for 24 hours. Blackening of more than half of the agar slant indicates a positive reaction. Absence of blackening of medium indicates a negative test. Group C and G Streptococci give a positive reaction in this test.

Trehalose Fermentation Test

Species identification was further done by sugar fermentation test by inoculating the organism in 1% sugar with Andrade indicator. Any colour change from colourless to pink indicated the positive sugar fermentation test. Absence of colour change was taken as negative. Trehalose fermentation was seen in groups A, C, G streptococcus. The test was negative in group B streptococcus.

Susceptibility to Sulfamethoxazole-Trimethoprim

This test is used to distinguish group A and B streptococci from other beta-haemolytic streptococci. This test is performed in the same way as bacitracin test. The well isolated colony of streptococcus was taken, and lawn culture was made on blood agar plate. Then, commercial disk containing 1.25 microgram trimethoprim and 23.75 microgram of sulfamethoxazole is used. Any zone of inhibition is considered as susceptibility to SXT. Groups C, F, G were all susceptible to SXT. Groups A and B were resistant to SXT.

Grouping of Streptococci

The isolates from culture were grouped by manual nitrous acid extraction method and commercial kit – strep test kit PLASMATEC. These two methods were compared for its efficacy.

Manual nitrous acid extraction method

Nitrous acid extract preparation

1. 20 micro litres of 2% sodium nitrite solution was taken in a test tube.
2. 3 to 4 colonies of isolates of beta-haemolytic streptococci were added to the test tube.
3. 3 microlitres of Glacial acetic acid was added to the suspension and kept it at room temperature for 15 minutes.
4. 16 to 24 micrograms of sodium bicarbonate was added for neutralization.
5. Finally, 60 microlitres of distilled water was added to the tube.

Agglutination method

1. One drop of micro nitrous acid extract was added to the clean glass slide using Pasteur pipette.
2. To this one drop of group specific antisera was added to each circle.
3. The slide was mixed by rotation for about 1 minute.
4. Evidence of agglutination against diffuse light was considered as positive.

5. The group reagent which gave the strongest agglutination denoted the groups of the streptococcal isolate.

Commercial Latex kits method (*Name of the kit: STREP TEST KIT – PLASMATEC)*

Kit reagents:

1. Latex determination for the grouping of streptococci A, B, C, D, F, G.
2. Polyvalent positive control 2ml.
3. Freeze dried extraction Enzyme. Two vials, reconstituted each with 10ml of distilled H2O.
4. Disposable test cards 50 in number.
5. Mixing sticks 300 and kit insert.

Method

1. By using sterile bacteriological loop 2-6 colonies of streptococci was taken and emulsified in 0.4ml of extraction enzyme.
2. The tube which contains the bacterial colonies with extraction enzyme was kept for 10 minutes at water bath 56°C, the tube was shaken vigorously after 5 minutes of incubation.
3. The latex reagent was re-suspended by gentle agitation; one drop of latex reagent was added to each circle on the test slide.
4. One drop of the extract was added to each drop of latex reagent by using Pasteur pipette and the content was mixed well by using separate mixing stick.

5. The slide was rocked for not more than one minute, and the agglutination was observed.
6. Visible agglutination of the latex particle was noted in the circle which marked as A which indicates positive result.

Molecular detection of virulence genes

Group A is the most prevalent among beta-haemolytic streptococci. The resistance to erythromycin and tetracyclines were noted among most of the strains of group A streptococcus compared to the other groups. The resistant pattern for these two drugs is mainly due to the presence of erm and tet genes. The genes which are mainly responsible for erythromycin and tetracycline resistance are erm (A), erm (B) and tet (O), tet (M) respectively. Based on the AST resistant pattern, 24 isolates of group A streptococcus were taken up for molecular study for the detection of erm gene and 17 isolates were taken for detection of tet genes.

The isolates of group A streptococcus were streaked on blood agar plate and incubated for 24 hours at 37° Celsius. The pure colonies were taken and inoculated into 2ml of brain heart infusion broth in sterile tarson tubes and then incubated for 24 hours at 37° Celsius. After 24 hours, 1ml of bacterial culture from BHI broth was pipetted out and added into 1.5ml microcentrifuge tube and centrifuged for 5 minutes at 7500 rpm. The supernatant was discarded. The pellets were transferred into 1.5ml microcentrifuge tube. The volume of pellets was about 180µl.

DNA extraction

DNA from group A streptococcus was extracted by commercial kit method (QIAmp DNA mini-kit).

1. The pellets of 180µl volume obtained after centrifugation was taken in 1.5ml microcentrifuge tube.
2. 20µl of proteinase K was added to the sample.
3. 200µl of buffer AL (lysis buffer) was added to the sample and vortexed for 15 seconds.
4. The sample was incubated 55° Celsius for 10 minutes.
5. 200µl of ethanol was added to the sample and centrifuged for 15 seconds.
6. The sample was then transferred to QIAmp mini-spin column and centrifuged at 8000 rpm for one minute. The flow through liquid was discarded and spin column was placed in 2ml collection tube provided with the kit.
7. 500µl of AW1 buffer (wash buffer 1) was added to the spin column tube and centrifuged at the rate of 8000 rpm for one minute. The mini-spin column was placed in a clean 2ml collection tube and the tube containing filtrate was discarded.
8. 500µl of AW2 buffer (wash buffer 2) was added to the spin column tube. The tap was closed and centrifuged at the rate of 14000 rpm for 3 minutes.

9. The spin column tube containing sample was now placed in a new 2ml collection tube and old collection tube with filtrate was discarded. The new tube was then centrifuged at full speed for one minute.
10. The spin column tube was opened and 200µl of buffer AE (elution buffer) was added. It was then incubated at room temperature (i.e. 15 - 25° Celsius) for one minute and centrifuged at 8000 rpm for one minute to elute the DNA. The elute was then transferred into fresh capped 2ml collection tube for longer storage at -20° Celsius.

DNA Amplification

The extracted DNA from the isolates were amplified by using PCR. Sterile 1.5ml tubes were taken for preparation of amplicons.

Requirements	**Quantity**
Master mix	12.5µl
PCR water	5.5µl
Primer (reverse)	1µl
Primer (forward)	1µl
Extracted DNA	5µl
Total	**25µl**

Primers

Primers used for erm and tet genes.

Genes	Primers sequences	Amplicon size (bp)
erm (A)	F: GCA TGA CAT AAA CCT TCA R: AGG TTA TAA TGA AAC AGA	208
erm (B)	F: GAAAAGGTACTCAACCAAATA R: AGTAACGGTACTTAAATTGTTTAC	639
tet (O)	F: AACTTAGGCATTCTGGCTCAC R: TCCCACTGTTCCATATCGTCA	519
tet (M)	F: GAACTCGAACAAGAGGAAAGC R: ATGGAAGCCCAGAAAGGAT	740

The temperature setup was done as follows:

Initial denaturation: 94°C for 8 minutes

30 cycles of denaturation (94°C for 30 seconds)

Annealing at appropriate temperature

erm A: 56°C

erm B: 58°C

tet O and tet M: 62°C

Extension: 72°C for 60 seconds

Final extension: 72°C for 7 minutes

At the end of 30 cycles, the amplified products were obtained.

GEL Electrophoresis

Buffer preparation:

10x buffer – 1000 ml milli Q water + 10.78g Tris base + 5.5g boric acid + 3.5g EDTA

1x buffer – 900 ml milli Q water was added with 10x buffer to make 1x buffer

1. Sterile beaker was taken, 1x buffer was prepared, and 100ml of 1x buffer was added with 1.5 grams of agarose powder and then kept in microwave oven for melting for 3 minutes.
2. After 3 minutes, the agarose was cooled to 40°C. To this, 2µl of ethidium bromide was added.

3. In 100ml trough, the agarose gel was poured with 15 numbered comb to make wells. After the agar gets solidified, the comb was taken out slowly.
4. 1x buffer was added till the gel soaks.
5. Ladder was added to the fruit wells, and to the rest of the wells, the amplified DNAs were added.
6. The gel ran at 95 volts for 1 hour and the appearance of hand was noted under UV light.

Interpretation

Bands at 208 base pairs → erm (A) gene and Bands at 639 base pairs → erm (B) gene

Bands at 518 base pairs →tet (O) gene and Bands at 740 base pairs →tet (M) gene

Results

A total of 175 isolates of beta-haemolytic streptococci were isolated from various specimens like throat swab, pus, wound swab, urine, blood, vaginal swab, sputum, placental swab, tissue, synovial fluid and ear swab. During the study period between Jan 2018 and Jun 2019, among the total isolates obtained (12894), the incidence of beta-haemolytic streptococcal infections was found to be 1.35% **(Figure 1).**

Distribution of beta-haemolytic streptococci among the various clinical samples is depicted in **Figure 2**.

The frequency of occurrence of different Lancefield groups of beta-haemolytic streptococci is shown in **Figure 3**.

Distribution of isolates among inpatients and outpatients is depicted in **Figure 4**.

Table 1 depicts the characterisation of beta-haemolytic streptococci by standard conventional methods. **Illustrations 1 to 14** show the conventional tests and media used for the identification of beta-haemolytic streptococci.

Table 2 shows the antimicrobial susceptibility profile of the isolates obtained.

Table 3 shows the Comparative evaluation between manual and commercial kit methods for serogrouping of Streptococci.

Antibiogram of *Streptococcus pyogenes* is depicted in **Table 4.**

Illustration 1 shows the gram stain of beta-haemolytic streptococcus showing gram positive cocci in chains.

Illustration 2 shows the appearance of beta-haemolytic streptococci on blood agar.

Various conventional biochemical tests and media used for the identification of beta-haemolytic streptococci are shown in **Illustrations 3 to 12.**

Antimicrobial susceptibility testing of *Streptococcus pyogenes* and Clindamycin resistance as shown by positive D test is depicted in **Illustrations 13 and 14.**

Serogrouping of beta-haemolytic streptococci by manual extraction and commercial kit methods is depicted in **Illustrations 15 & 16.**

Detection of virulence genes was done by PCR for group A Streptococcus. erm (A) and erm (B) genes for detecting erythromycin resistance, tet (O) and tet (M) genes for tetracycline resistance. The distribution of resistant genes is shown in **Figure 5** and **Figure 6.** Molecular detection of virulence showing erm B and tet M genes is depicted in **Illustrations 17 and 18.**

Figure 1: Incidence of beta-haemolytic streptococci

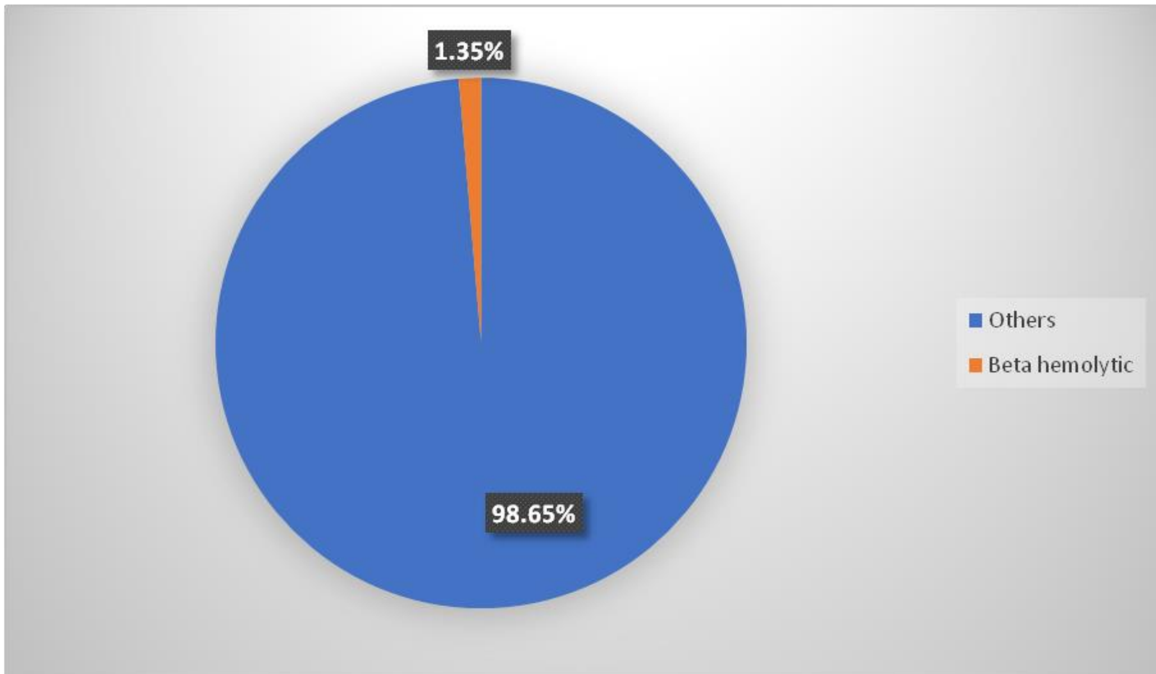


Figure 2: Distribution of beta-haemolytic streptococci among clinical samples

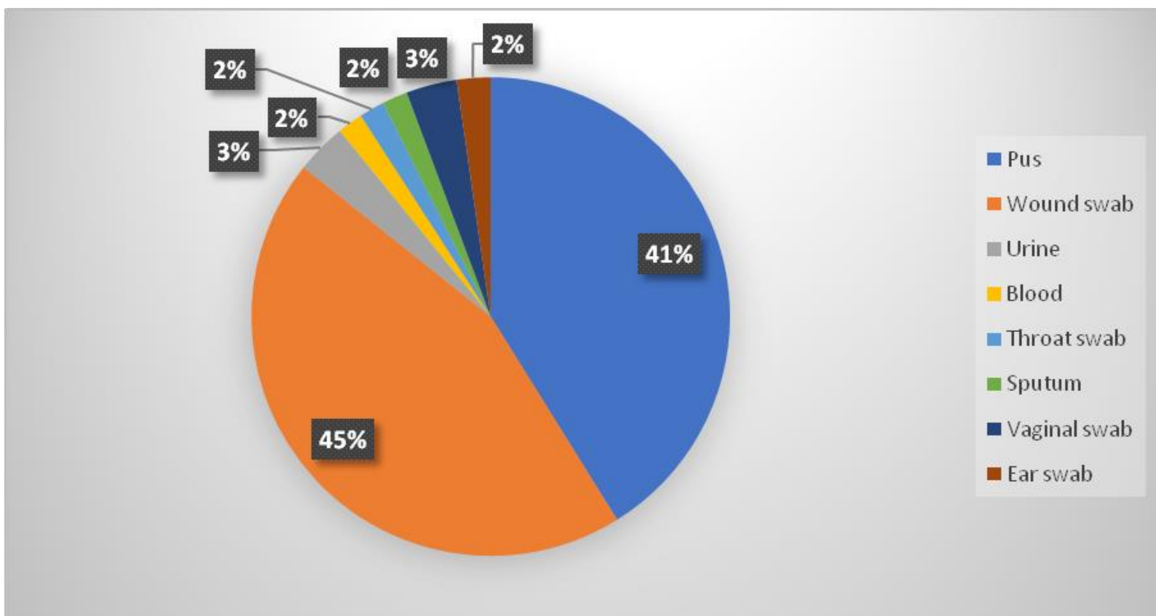


Figure 3: Frequency of Lancefield groups

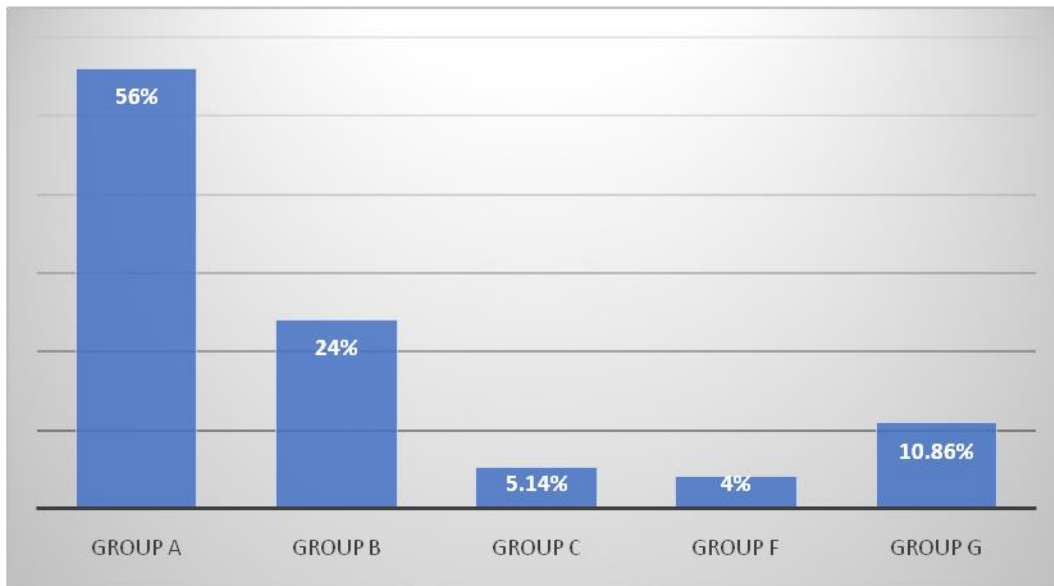


Figure 4: Distribution of streptococcal isolates among outpatients and inpatients

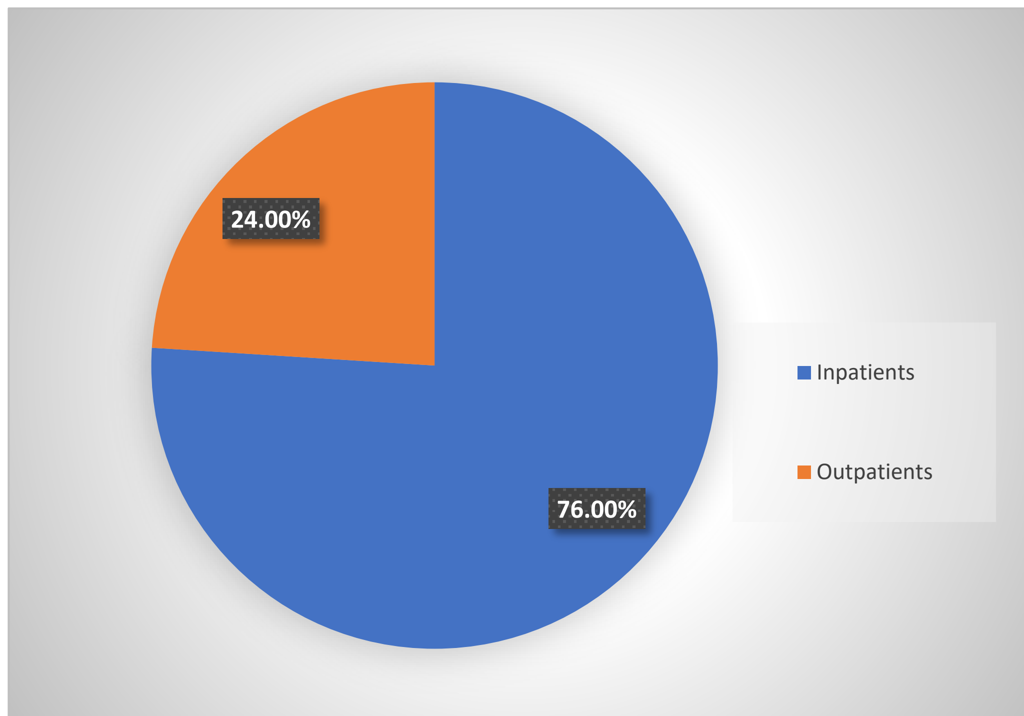


Table 1: Reaction patterns of isolates of beta-haemolytic streptococci

n=175

Groups	Nos. Tested	Test Reactions									
		Bacitracin	CAMP	PYR	Esculin	Hippurate	ADH	Ribose	Lactose	Salicin	Mannitol
A	98	S	-	+	+	-	+	-	+	+	-
B	42	R	+	-	-	-	+	+	-	-	-
C	9	R	-	-	-	-	+	+	+	+	-
G	7	R	-	-	-	-	-	+	-	-	-
F	19	R	-	-	-	-	-	-	-	-	-

Table 2: Antimicrobial susceptibility profile of streptococcal isolates (n=175)

Drugs		A (98)	B (42)	C (9)	F (7)	G (19)
Penicillin	S	100%	97.62%	100%	85.71%	100%
	R	0%	2.38%	0%	14.29%	0%
Ampicillin	S	100%	100%	100%	100%	100%
	R	0%	0%	0%	0%	0%
Vancomycin	S	100%	100%	100%	100%	100%
	R	0%	0%	0%	0%	0%
Linezolid	S	100%	100%	100%	100%	100%
	R	0%	0%	0%	0%	0%
Erythromycin	S	76.53%	71.43%	100%	85.71%	68.42%
	R	23.47%	28.57%	0%	14.29%	31.58%
Clindamycin	S	84.69%	83.33%	77.78%	100%	84.21%
	R	15.31%	16.67%	22.22%	0%	15.79%
Tetracycline	S	11.22%	78.57%	88.89%	85.71%	78.95%
	R	88.78%	21.43%	11.11%	14.29%	21.05%
Cotrimoxazole	S	26.53%	80.95%	100%	100%	84.21%
	R	73.47%	19.05%	0%	0%	15.79%
Ciprofloxacin	S	93.88%	97.62%	100%	100%	89.47%
	R	6.12%	2.38%	0%	0%	10.53%
Cefotaxime	S	100%	100%	100%	100%	100%
	R	0%	0%	0%	0%	0%
Chloramphenicol	S	73.47%	90.48%	100%	100%	100%
	R	26.53%	9.52%	0%	0%	0%

Table 3: Comparative evaluation of the manual nitrous acid extraction and commercial kit for serogrouping of Streptococci

Manual Method	PLASMATEC (Commercial Kit)	
	Positive	Negative
Positive	164	0
Negative	11	8

Sensitivity = 164/175*100 = 93.71%

Specificity = 8/8*100 = 100%

Positive Predictive Value = 164/164*100 = 100%

Table 4: Antibiogram of clinical isolates of *S.pyogenes*

n = 98

Name of the antibiotic	**% Sensitive**	**% Resistant**
Penicillin (10U)	100%	-
Ampicillin (10µg)	100%	-
Vancomycin (30µg)	100%	-
Linezolid (30µg)	100%	-
Erythromycin (15µg)	76.53%	23.47%
Clindamycin (2µg)	84.69%	15.31%
Tetracycline (30µg)	88.78%	11.22%
Cotrimoxazole (25µg)	73.47%	26.53%
Ciprofloxacin (5µg)	95.91%	4.09%
Cefotaxime (30µg)	100%	-
Chloramphenicol (30µg)	73.47%	26.53%

Illustration 1: Beta hemolysis

Illustration 2: Gram stain

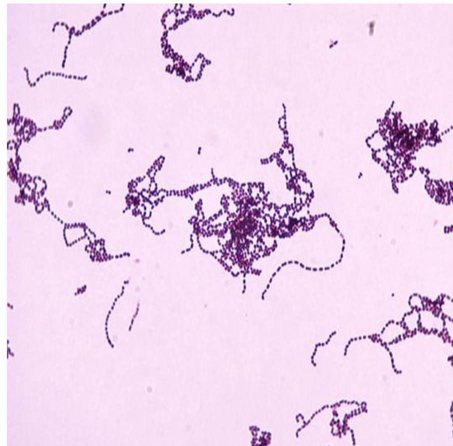


Illustration 3: Bacitracin sensitivity

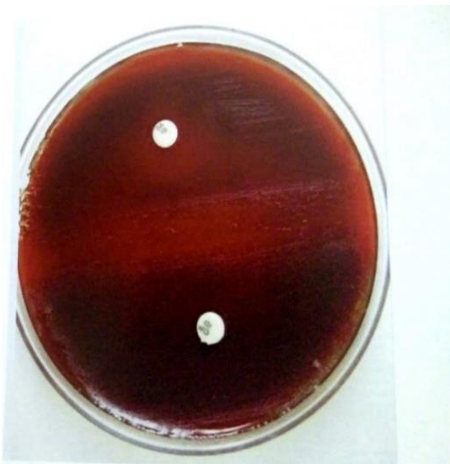


Illustration 4: CAMP test

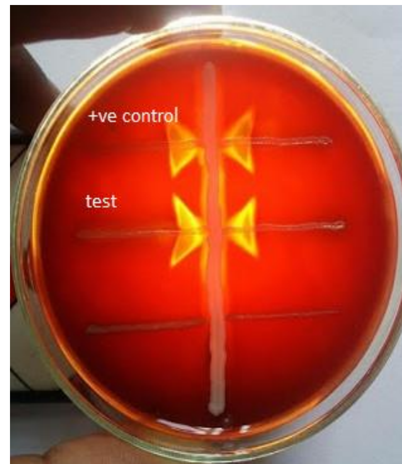


Illustration 5: Ribose fermentation

Illustration 6: Lactose fermentation

Illustration 7: Salicin fermentation

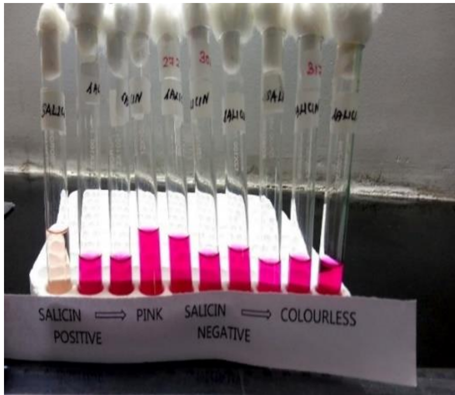


Illustration 8: Mannitol fermentation

Illustration 9: PYR test

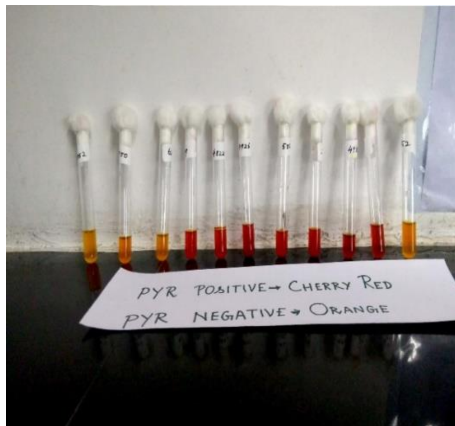


Illustration 10: Arginine dihydrolase

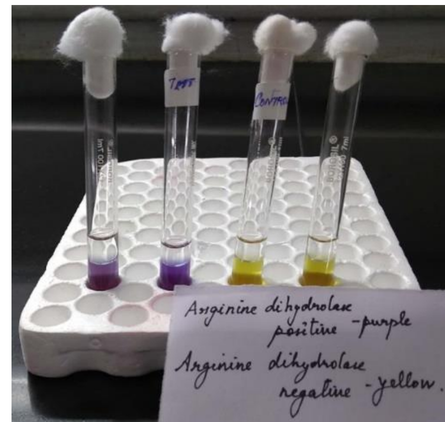


Illustration 11: Hippurate hydrolysis

Illustration 12: Esculin hydrolysis

Illustration 13: AST of *S.pyogenes*

Illustration 14: Positive D test

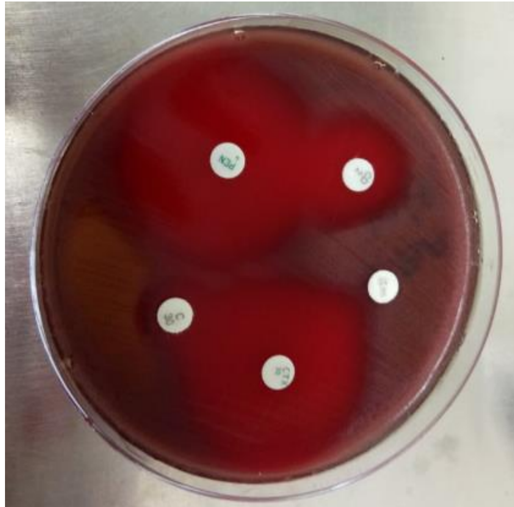


Illustration 15: Serogrouping by manual nitrous acid extraction method

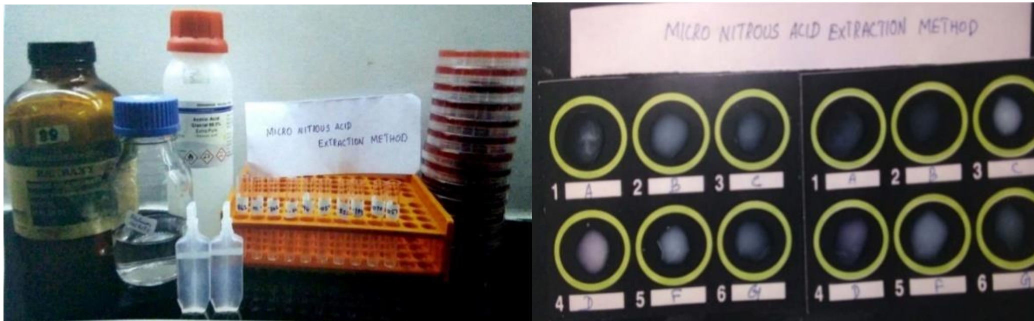


Illustration 16: Serogrouping by commercial kit method (PLASMATEC)

Figure 5: Incidence of erythromycin resistant genes (erm A, erm B) in *S.pyogenes*

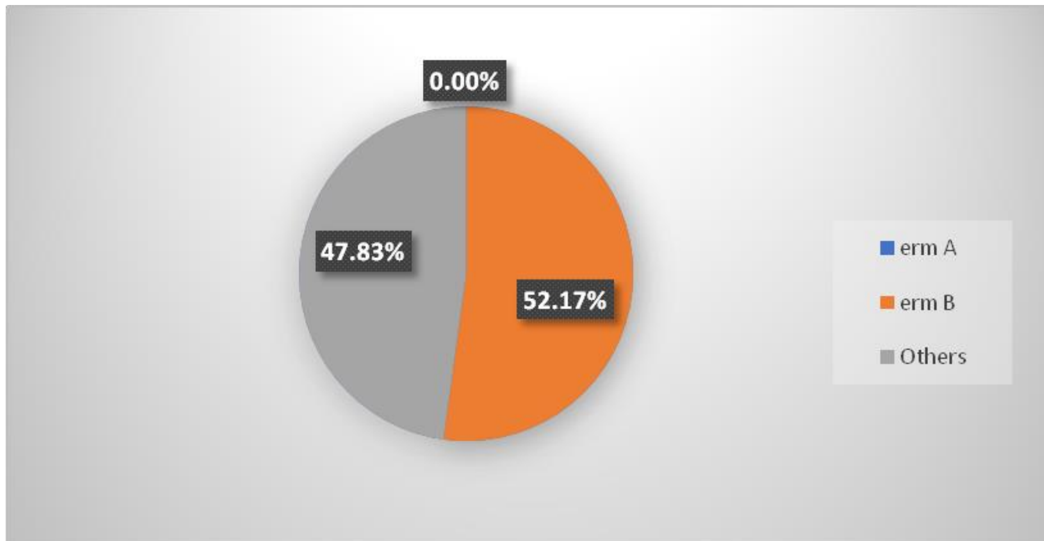


Figure 6: Incidence of tetracycline resistant genes (tet O, tet M) in *S.pyogenes*

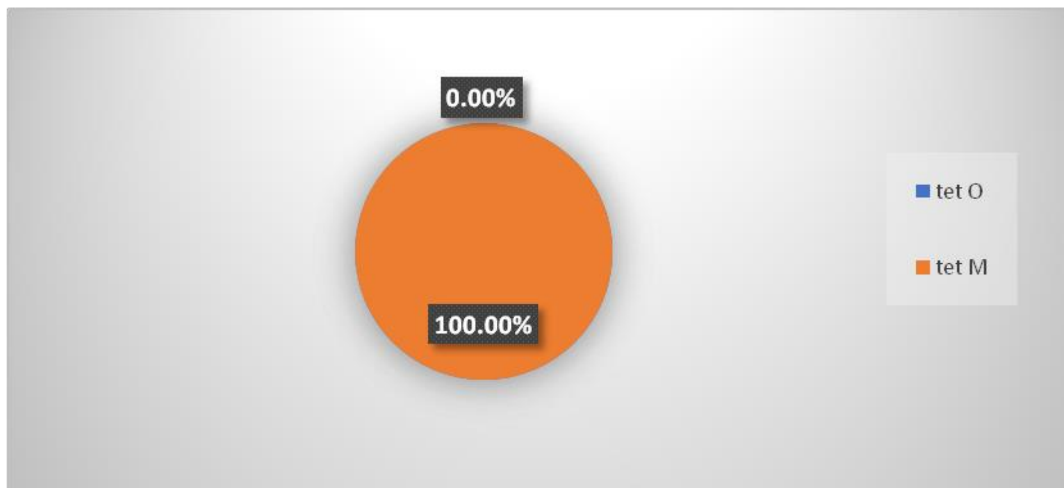


Illustration 17: PCR showing erm B gene of band 639 base pairs

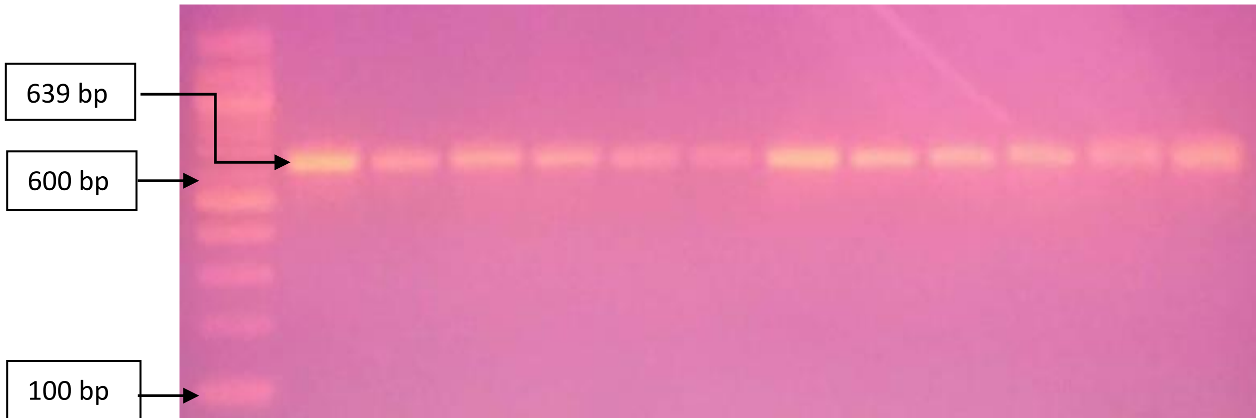


Illustration 18: PCR showing tet M genes at band 740 base pairs

Discussion

Majority of streptococcal infections are caused by beta hemolytic streptococci[74]. Although Lancefield group A streptococci are considered as the major pathogenic species, other non-group A or B (NABS) are also capable of causing significant disease. Among these groups, C and G are commonest organisms. However, the true burden of these infections remains unknown as only few laboratories identify up to species level.

The incidence of beta haemolytic streptococci among the clinical isolates obtained in our study was found to be 1.35%. Among these community acquired were around 24% and 76% were obtained from inpatients. Studies report around 15.5% of GAS isolated from patients with pharyngitis in the community[75]. Few studies from South India also report that the incidence of beta haemolytic streptococci to be around 28.2%[76]. Global incidence of streptococcal infections has also been reported to be around 15.59%[75].

GAS was the most common isolate in our study (56%) followed by Group B (24%), Group G (10.85%), Group C (5.1%), Group F (4%). This correlates well with similar studies done in India where the incidence of GAS was found to be 65%[83].

GBS (*S.agalactiae*) have gained increasing significance as a human pathogen which causes sepsis, meningitis, pneumonia and severe genitourinary infections, especially in debilitated or diabetic adults and in new born infants it causes severe meningitis or sepsis after the transmission of the organism from the mother's genital tract.

In recent years, infection with GBS has become one of the commonest causes of bacteraemia and meningitis in neonates, which usually acquire infection from the mother's vagina. Due to these findings, surveys have been conducted for evaluation of group B colonization in the vaginal flora. Among antenatal patients, the rate of colonisation of group B Streptococcus in our study was around 14.29%. Carrier rates of around 22.5% have been reported in patients during the third trimester of pregnancy[10]. Association of group B Streptococcus and clinical pharyngitis have been reported in few studies[10]. However, there was no such correlation in our study.

A high proportion of GCGS have been isolated from Indian studies (59.7%)[74] while the present study shows the prevalence of GCGS to be (Group G – 10.85% and Group C – 5.1%). Group G and Group C Streptococcus mimics GAS in clinical presentation of symptoms. A study in Salem has reported the predominance of groups A and G in school children presenting with pharyngitis[74]. Throat swabs taken from children with acute pharyngitis in Tamilnadu showed beta haemolytic streptococci where group C was predominant group (59.7%), followed by group G (25%) and group A (15.3%).

Accurate identification and typing forms a valuable tool for the epidemiological studies and to study pathogenesis. Bacitracin sensitivity remains the main test for identification of GAS. But this alone cannot be used as a predictor for the confirmation of *S.pyogenes* as resistant strains also exist. Studies conducted in Europe show that bacitracin resistant GAS has become common in number of European countries[77]. Studies have reported

9.8% strains of GAS resistant to Bacitracin, whereas 85.7% strains of GAS were sensitive to Bacitracin[78]. In our study, 100% of Group A Streptococcus were sensitive to Bacitracin.

PYR test is also more specific for GAS but it was reported that *Streptococcus porcinus* and *Streptococcus iniae* are also PYR positive. Studies shows that PYR test has sensitivity of 95.42% and specificity of 77.41%[77]. Recent studies have shown that L pyrrolidonyl-beta-naphthylamide test is also more specific than bacitracin test and appears to be equally sensitive to bacitracin test[53]. In our study, 100% of *S.pyogenes* were sensitive to both bacitracin and PYR test.

Serogrouping of the isolates of Streptococci were compared by the manual nitrous acid extraction method and commercial kit methods. The sensitivity and specificity of the manual method was found to be 93.71% and 100% respectively. Studies have reported that sensitivity of manual extraction method when compared with commercial kit method was 90% and specificity was 100%. Similar reports have also been published where the sensitivity was 97.71% and specificity was 80.64% for latex agglutination test. Commercial kits provide an easy and reliable method for serogrouping of *S.pyogenes*[79]. Studies conducted in Pittsburgh have shown that Nitrous acid extraction method yields more group antigen than either the Fullers formamide or hot hydrochloric acid extraction techniques[80]. Though serogrouping of Streptococci is useful for the identification, it is not considered as accurate for specific beta haemolytic species.

Infections caused by GAS are treated primarily based on the isolation of the likely pathogens and their antimicrobial susceptibility. Antibiotic susceptibility testing revealed that 100% of the isolates were sensitive to penicillin, cefotaxime and vancomycin. This reinforces the fact that penicillin resistance has not developed despite the frequent usage which also agrees with studies in other parts of India[78].

Although most of GAS isolates remains sensitive to penicillin, the world wide spread of erythromycin and clindamycin resistance is emerging as a major problem since erythromycin is the drug of choice for penicillin allergic individuals. Studies from San Antonio (USA) have reported that among 20 isolates collected during period of 2000 to 2009, inducible clindamycin resistance was observed in 10 isolates which was shown by flattening of zone of inhibition around the clindamycin disk which resembles letter D[83]. In our study inducible clindamycin resistance was observed in 34.78% of the erythromycin resistant group A isolates. Macrolide resistance in beta haemolytic streptococci is mediated by two different mechanisms: target site modification and active drug efflux. Target site modification is mediated by methylase enzyme (erm A and erm B) which reduces binding of macrolides, lincosamides and streptogramin B antibiotics (MLS_b resistance) to their target site in the bacterial ribosome, giving rise to the constitutive (CR) and inducible phenotypes (IR) MLS_b resistant phenotypes. The relative proportion of inducible MLS_b phenotype differs between various places. Studies conducted in North India have reported that out of 61 erythromycin resistant GAS isolates, inducible clindamycin resistance was observed in all 100% isolates[68].

Currently macrolides have been widely used because of better intracellular penetration, better eradication rate and choice of treatment in penicillin allergic individuals. Recent studies have shown that the changes in susceptibility of beta haemolytic streptococci to erythromycin and clindamycin have been observed with difference in rates of resistance depending on the geographical locations (10 – 45%).

A study conducted in North India with randomly collected 155 samples of beta haemolytic streptococci have reported that no isolate was resistant to penicillin, although resistance was observed against erythromycin (28%) and tetracycline (57%)[68]. Worldwide data suggests an increased rate of resistance of GAS to macrolides, ranging from 0.5% to 70% while 2.9% of isolates showed intermediate susceptibility to macrolides[81]. This may be the reason for infrequent use of erythromycin by most practitioners who prescribe penicillin to treat streptococcal infections. In our study, increased rates of erythromycin resistance was observed in group A (23.47%), group B (28.57%) and group G (31.58%) isolates.

Tetracycline is the drug of choice for empirical treatment for invasive beta haemolytic streptococcal infections. Studies from various parts of India have shown the high rate of resistance to tetracycline (19.4%) in GAS isolates[68]. Tetracycline is no longer considered for empirical treatment since higher resistance rates of up to 60% have been reported[75]. Our study showed the tetracycline resistance in 21.42% of GAS isolates.

A study from Japan found macrolide resistance in 16.2% isolates, but the resistant genes erm (A) and erm (B) were found in 6.2 and 7.5% respectively. A study from North India showed that out of two genes erm (A) and erm (B), only erm (B) was found in resistant isolates. None of the isolates showed erm (A) genes[68]. In our study, erm (B) was found to be the predominant gene observed in macrolide resistant clinical isolates which was about 47.83%. No isolates were positive for erm (A) and this shows the resistance might be mediated by other modes of resistance mechanisms like Prt F1 gene which encodes the fibronectin binding protein F1, mef A (macrolide efflux) gene, erm TR genes and biofilm formation[70].

A study from New York reported that out of 92 tetracycline resistant GAS isolates, 88% harbourtet (M) genes and tet (O) genes was present only in 3.82% isolates[82]. In a study from North India where both tet (O) and tet (M) genes studied, only tet (M) was present in 19.09% of isolates[68] and none of the isolates were positive for tet (O). Among the isolates of *S.pyogenes* resistant to tetracycline, in our study, tet (M) was isolated in all the 21 isolates, which also correlates well with studies from other parts of the world.

Summary

- The incidence of beta haemolytic streptococci from various clinical samples was found to be 1.35%.
- Among the beta hemolytic streptococci isolates, the commonest was GAS (56%) followed by GBS (24%), GGS (10.86%), GCS (5.14%) and GFS (4%).
- Antenatal colonisation of Group B Streptococcus was found in 14.29%.
- 100% of Group A Streptococcus isolates were sensitive to Bacitracin and PYR positive.
- Sensitivity and specificity of Manual nitrous acid Extraction method over the commercial kit method was found to be 93.71% and 100% respectively.
- 100% sensitivity was noted to Penicillin, Vancomycin, Linezolid and Cefataxim.
- Inducible clindamycin resistance was observed in 34.78% of erythromycin resistant GAS.
- Detection of virulence genes for macrolide resistance by PCR showed erm B to be predominant gene (47.83%).
- Detection of virulence genes for tetracycline resistance showed tet M to be predominant gene (100%).

Conclusion

The burden of streptococcal disease is quite high. Infections caused by beta hemolytic streptococci have the potential to cause systemic disease especially among the immunosuppressed patients with certain species of GCS and GGS. Before the start of prompt and accurate therapy microbiological identification is of prime importance. Variations in antibiotic susceptibility patterns do prevail, however continuous monitoring is warranted. High levels of resistance among erythromycin and tetracycline may be explained by the fact of injudicious use of these antibiotics. All laboratories should aim to identify beta hemolytic streptococci should identify accurately using phenotypic and molecular methods. Stringent measures should be adopted to curb the use of antibiotics by implementing antibiotic policy in health care organizations, preventing over the counter dispensing of drugs and stop the rampant misuse of antibiotics in livestock. Hospitals should limit the inappropriate and excessive antibiotic usage through antimicrobial stewardship programs.

So, it should be advisable to employ macrolides for pharyngitis problems to allergic patients after performing microbiological studies and avoid the empiric antibiotics therapeutic measures for these patients.

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Appendix

1. **GRAM STAIN**

Crystal violet:

Crystal violet	1.0 gm
5% sodium bicarbonate	1.0 ml
Distilled water	99 ml

Grams iodine:

Iodine crystal	2.0 gm
Sodium hydroxide	10 ml
Distilled water	90 ml

Acetone – 100%

DILUTE CARBOL FUSCHIN – 1 in 10 dilution

2. **BLOOD AGAR**

Sterile defibrinated sheep blood	7 ml
Nutrient agar	100ml

About 7ml of melted Nutrient agar, as a base into sterile petri dishes and aloe setting. Add sterile defibrinated blood (5-7%) to Nutrient agar, the latter cooled to 45 to 50°C before blood is added. Mix and pour 20 ml of blood agar in the petridish.

3. **ROBERTSON COOKED MEAT MEDIA:**

Beef infusion broth

Minced and dried meat

One part of meat is mixed with two parts of water. Cool, refrigerate and skim off any remaining fat. Boil for 30 minutes. Filter through two layers of gauze and pH is adjusted to 7.5. Dried meat particles are distributed in 15*150 mm tube to a height of 1.5 to 2.5 cm. The filtrate is then added to get 3 to 4 parts. The tubes plugged and sterilised by autoclaving

4. **PYR media:**

Casein peptone	2gm
Beef heart infusion	0.3gm
Sodium carbonate	0.25gm
Dextrose	0.2gm
Disodium phosphate	0.04gm
L- pyroglutamic acid-beta naphthylamide	0.01gm
Distilled water	100ml
pH	7.8

The above ingredients are mixed and sterilised by autoclaving at 121°C for 15 mts. These are dispersed in tubes and stored.

5. **ARGININE DECARBOXYLASE BROTH:**

Peptone	0.5 gm
Beef extract	0.5 gm
Bromocresol purple	0.001 gm
Cresol red	0.0005 gm
Glucose	0.05 gm

Pyridoxal	0.0005 gm
Distilled water	100 ml
pH	6.0

To the above 10gm of the L (levo) form of the amino acid arginine is added and dispensed in tubes. The above contents are autoclaved at 121°C for 15 minutes.

6. CARBOHYDRATE FERMENTATION MEDIA:

Peptone water	100 ml
Sugar	1 ml
Andrade indicator	1 ml

The above is distributed in tubes along with durhams tube and autoclaved at 115°C for 10 mts.

The sugars used are mannitol, lactose, ribose, trehalose, salicin, sorbitol and maltose.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
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To
Dr B Leelavathi
Postgraduate
Department of Microbiology
Guide: Dr S Parvathi
PSG IMS & R
Coimbatore

Ref: Project No. **17/354**

Date: December 13, 2017

Dear Dr Leelavathi,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 11.11.2017 to conduct the research study entitled "*Clinical & molecular epidemiology of beta-hemolytic streptococcal infections in a tertiary care hospital*" during the IHEC meeting held on 08.12.2017.

The following documents were reviewed and approved:

1. Project submission form
2. Study protocol (Version 1 dated 11.11.2017)
3. Application for waiver of consent
4. Confidentiality statement
5. Data collection tool (Version 1 dated 11.11.2017)
6. Current CVs of Principal investigator, Co-investigator
7. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the meeting held on 08.12.2017 at IHEC Secretariat, PSG IMS & R between 10.00 am and 11.00 am:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Mr R Nandakumar (Chairperson, IHEC)	BA., BL	Legal Expert	Male	No	Yes
2	Dr D Vijaya (Member – Secretary, IHEC)	M Sc., Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes
3	Dr S Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
4	Dr Sudha Ramalingam	MD	Epidemiologist, Ethicist Alt. member-Secretary	Female	Yes	Yes
5	Dr G Subhashini	MD	Epidemiologist	Female	Yes	Yes

The study is approved in its presented form. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the ICH-GCP/ICMR/Schedule Y guidelines. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of

13/12/17

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

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status report as decided by the IHEC.

Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 7 days of the occurrence of the SAE. If the SAE is 'Death', the IHEC Secretariat will receive the SAE reporting form within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
   c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
   d. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
   e. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented
   f. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review
7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Thanking You,

Yours Sincerely,

Dr D Vijaya
Member - Secretary
Institutional Human Ethics Committee

Urkund Analysis Result

Analysed Document: Thesis - Clinical and Molecular Epidemiology - LB.doc (D56923272)
Submitted: 10/13/2019 10:19:00 AM
Submitted By: leelavathi.b@gmail.com
Significance: 3 %

Sources included in the report:

Al urfy-2.docx (D4188205)
https://www.longdom.org/open-access/antimicrobial-susceptibility-of-beta-haemolytic-streptococci-isolated-from-paediatric-patients-with-pharyngoamigdalitis-2327-4972.1000151.pdf
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https://www.researchgate.net/publication/294276014_Prevalence_of_Beta-Hemolytic_Streptococci_Groups_A_C_and_G_in_Patients_with_Acute_Pharyngitis
42d958b5-cddd-4897-8393-cd0f2ec58b87
079e5f42-3824-4d70-bc67-8862d746c422

Instances where selected sources appear:

20