II. LITERATURE REVIEWS

A. General characteristic of group A streptococci

Streptococcus pyogenes belongs to Lancefield group A (GAS) and causes pharyngitis, scarlet fever, streptococcal toxic shock-like syndrome, erysipelas, and pyoderma. It is also responsible for the non-suppurative sequelae, acute rheumatic fever and glomerulonephritis (Fischetti 1989; 1991). In 1874, Billroth first described GAS in exudate from erysipelas and wound infections. In 1879, Pasteur found similar organisms in the blood of a patient with puerperal sepsis (McCarty, 1990; Willett, 1992; Murray *et al.*, 1994). GAS are gram-positive bacteria, spherical to ovoid shape, 0.5 to 1.0 μm in diameter (Willett, 1992; Murray *et al.*, 1994). They form short chains in clinical specimens and longer chains when grown in liquid media (Murray *et al.*, 1994). The length of the chain tends to be inversely related to the adequacy of the culture medium. In actively spreading lesions within the tissue, diplococcal and individual coccal forms are common, whereas in purulent exudates from walled-off lesions and in artificial culture media, chain formation is more common (McCarty, 1990). GAS are facultatively anaerobic, catalase-negative, non-motile, non-spore forming, and produce capsules which are chiefly composed of hyaluronic acid (Rotta and Facklam, 1980).

The minimal nutritional requirements of the streptococcus are complex because of the organism's inability to synthesize many of its required amino acids, purines, pyrimidines, and vitamins. The optimal pH for growth is 7.4 to 7.6 at 37°C. The growth enhancement of many strains can be achieved by culturing at a reduced oxygen tension or increased level of CO_2 (Willett, 1992).

On blood agar plates, GAS are β-hemolytic and may form one of three colony types, designated mucoid, matt and glossy. Mucoid colonies are formed

by strains that produce large capsules which give the colony a glistening, watery appearance. The matt colonies are formed by strains which produce a surface protein called M protein. Glossy colonies are small and formed by cells that do not generate hyaluronic acid capsules and produce less M protein (McCarty, 1990).

The ultrastructure of the GAS is typical of gram-positive bacteria in that there is a rigid cell wall, and inner plasma membrane with mesosomal vesicles, cytoplasmic ribosomes, and nucleoid. Most GAS strains produce capsules composed of hyaluronic acid that impedes phagocytosis. The streptococcal cell wall contains proteins (M, T, R), carbohydrates (group-specific), peptidoglycan, and lipoteichoic acid (Willett,1992).

B. Group A Streptococcal antigens

1. cell wall antigens

1.1 T protein

This protein has no relationship with the virulence of streptococci. T protein is acid-labile, heat-labile, but trypsin resistant (Willett, 1992; Jawetz *et al.*, 1995). It has also been useful for epidemiological studies of GAS by agglutination with specific antisera. T-agglutination is recommended for initial screening as it is known that certain M antigens are associated with certain T antigens (Maxted and Widdowson, 1972; Cleary *et al.*, 1979) as shown in Table 1.

Some T antigens are restricted to a single M type, whereas others are common to several M types.

Table 1 Relationship of T-pattern to M types

T complex	M types bearing T complex
1	1
2	2
3/13/B3264	3, 13, 33, 39, 41, 43, 52, 53, 56
8/25/Imp.29	2, 8, 25, 55, 57, 58
5/11/12/27/44	5, 11, 12, 27, 44, 59, 61
14/49	14, 49
15/17/19/23/47	15, 17, 19, 23, 30, 47, 54

(From Willett, 1992)

1.2 R protein

R protein is an antigen with no relationship with the virulence of GAS and does not induce the formation of protective antibodies. The function of R protein is unknown (McCarty, 1990). Two different R proteins have thus far been identified: one (designated 3R) is destroyed by either pepsin or trypsin and another (28R) is destroyed only by pepsin (McCarty, 1990).

1.3 Group specific carbohydrate

This carbohydrate, C-polysaccharide, is contained in the cell wall of many streptococci and forms the basis of serologic grouping (Lancefield group A-V) (Jawetz *et al.*, 1995). The C-polysaccharide is composed of a branched polymer of L-rhamnose and N-acetyl-D-glucosamine in a 2:1 ratio, the latter being the antigenic determinant. The polysaccharide is linked by phosphate-containing bridges to the peptidoglycan, which consists of N-acetyl-D-muramic acid, D-glutamic acid, L- lysine, and D- and L- alanine (Willett, 1992). This

antigen is extracted by various physical (e.g. autoclaving), chemical (e.g. HCl, formamide), or enzymatic (e.g. pronase) methods and subsequently detected by commercially available antisera using the technologies of latex particle agglutination or co-agglutination (Kaufhold, and Ferrieri, 1993). Immune response to group A carbohydrate is not protective and is not useful for diagnosis of streptococcal infections primarily because the antibody response is relatively slow (Salvadori *et al.*, 1995). Antibodies reach a maximum titer during teen-age rather than elementary school years, and they persist for long periods with little decline in titer for individuals with rheumatic heart valve disease when compared with patients with glomerulonephritis or rheumatic fever without valvular-involvement (Quinn, 1982). But recently, antibodies to N-acetyl-glucosamine epitopes were found to promote phagocytosis of several type-specific M positive strains (Salvadori *et al.*, 1995).

1.4 Fc-receptor for immunoglobulins

Many strains of GAS can express a surface receptor with affinity for immunoglobulinG (IgG), IgA, called IgG or IgA binding protein (Kronvall, 1973; Christensen and Oxelius, 1975). IgA and IgG binding proteins are structurally similar to M proteins in that they exhibit the significant sequence homology. However, they are not themselves considered to be M protein because an antiphagocytic property has not yet been formally demonstrated (Gomi *et al.*, 1990). The structure and binding properties of various Ig-binding proteins reveal heterogeneity (Gomi *et al.*, 1990). The Ig binding protein gene, which is an *emm*-like gene, is named *fcrA/mrp*. This gene usually encodes IgG1-IgG2 and IgG4-binding proteins (Heath and Cleary, 1989; O'Toole *et al.*, 1992; Podbielski *et al.*, 1993). The expression of Ig-binding protein genes is under the positive control of the *vir*-regulon.

The structure of the *fcrA/mrp* and *enn* gene has features similar to that of the *emm* gene. These genes demonstrate equivalent domains with increasing

sequence homology toward the C-terminal portions. However, even in the conserved central domains and the C-terminal proline-glycine-threonine-serine (PGTS)-rich domains, their characteristic sequences allow for the clear cut differentiation between the two classes of the *emm*-like genes (Podbielski *et al.*, 1994).

The IgG binding protein of GAS may impair fixation of complement and therefore is a virulence mechanism of the bacteria (Schalen *et al.*, 1985). Bessen and Fischetti (1990) showed that GAS isolated from skin displayed more IgG-binding activity than strains isolated from the nasopharynx (Bessen and Fischetti, 1990). Ig-binding protein bind Igs by a non-immune mechanism at the Fc region of Igs. It has been demonstrated in the mouse model that Ig-binding protein expression by GAS strains correlated with their ability to establish invasive skin infection (Reader and Boyle, 1993a) and may be a common response to group A organisms to pressure exerted by host defense mechanisms (Reader and Boyle, 1993b).

1.5 M-associated protein (MAP)

The M-associated protein is a non-type specific substance, but closely associated with the M-protein. Its roles in the infection or prevention of infection have not been defined (Stokes, 1975).

1.6 M protein

The GAS M protein was described by Lancefield in 1928 (Lancefield, 1928). The biochemical features of the M protein molecule become more apparent with the development of an extraction procedure, including pepsin digestion at the suboptimal pH of 5.8, and non-ionic detergent and lysin extraction, which specifically cleaves the M protein from the streptococcal surface (Beachy *et al.*, 1974; Cunningham and Beachy, 1974; Fischetti *et al.*, 1976; Van de Rijn and Fischetti, 1981). Depending on the extraction procedure

and the serotype, the size of M proteins varies from 20-40 kDa for pepsin digestion, 15-35 kDa for non-ionic detergent extraction, and 58 kDa for type 12 GAS by phage lysin extraction (Fischetti, 1989). However, the fragment is found to remain the biologically important determinant of which native M molecule is presented on the streptococcal surface (Fischetti *et al.*, 1976; Fischetti, 1977; Manjula and Fischetti 1980; Van de Rijn and Fischetti, 1981; Beachey *et al.*, 1984).

1.6.1 Structure of M protein

M protein is a thermal-stable molecule (Lancefield, 1928), elongated shape (Fox and Wittner, 1969; Fischetti *et al.*, 1976; Parry and Fraser, 1985) and appears like a network of fiber on the cell surface (Swanson *et al.*, 1969). M protein exists as a single, stable, dimeric molecule of about 50-60 nm in length. Seventy percent of its length is α-helical protein, containing a repeat of seven residues, with the periodicity of a non-polar amino acid, which is the basic structure of α-helical coiled-coil protein like tropomyosin (Brinton, 1965; Manjula and Fischetti, 1980; Phillips *et al.*, 1981). There is an inclination of the helix because there is a regular pattern of seven residue periodicity, the presence of a hydrophobic amino acid at position a and d, and a 3.6 residue necessary to achieve one turn of the helix (Huxley and Perutz, 1953; McLachlan and Stewart, 1975). For thermodynamic stability, a dimeric, rod-like superstructure would be formed as a result of the internalization of the hydrophobic residues from two helical chains coiling around each other (McLachalan and Stewart, 1975; Manjula and Fischetti, 1980; Fischetti and Manjula, 1982) as shown in Figure 1.

The basic model of the M protein consists of 3 major regions: the non-helical region; the long helical central rod region; and the anchor region (Figures 2 and 3).

The first region, the short non-helical N-terminal segment, is distinct from one M-type to another (Manjula *et al.*, 1984; Quinn *et al.*, 1985; Hollingshead *et al.*, 1986; Miller *et al.*, 1988; Mouw *et al.*, 1988), but is nearly 100% identical within an M type (Hollingshead *et al.*, 1987). It is not certain whether the tertiary conformation of N-terminal region is similar among the different M protein types because of the variation of this region. The N-terminal segment plays an important role in biological activity of the molecule since antibody generated to this region permits opsonization of the streptococcus of the M type from which it was derived (Fischetti, 1989).

The second region, the long α-helical central rod region, contains 2,3 or 4 tandem repeats also called A-, B-, C- or D- repeat regions, based on irregularities of the pattern. Each repeat consists of repeat block, which varies in size and number within serotypes. The percentage of the homology between the block within each repeat is high in A-and B-repeat regions but diverges slightly in the C- or D- region (Fischetti, 1989). Discontinuities in the seven-residue amino acid pattern of M proteins, especially in the B-repeat, probably account for the flexibility of the M molecules observed in electron micrographs (Manjula *et al.*, 1985).

The anchor region, which follows the helical central rod region, consists of the proline-glycine rich region. This region stabilizes the M protein's position in the cell wall. The membrane anchor region consists of about 20 hydrophobic amino acids, and the charged tail domain acts as a knot at the end of M protein to prevent the molecule from being pulled through the membrane.

The N-terminal end of all M molecule has an excess of negatively charged amino acids, which results in a net negative charge for the region. Mammalian cells also exhibit a net negative charge on their surface. It seems likely that one function of the central rod in the M protein is to act as a shaft for holding the negatively charged N-terminal end, and to create electrostatic repulsion which keeps phagocytes away from the bacterial surface (Fischetti., 1989; 1991).

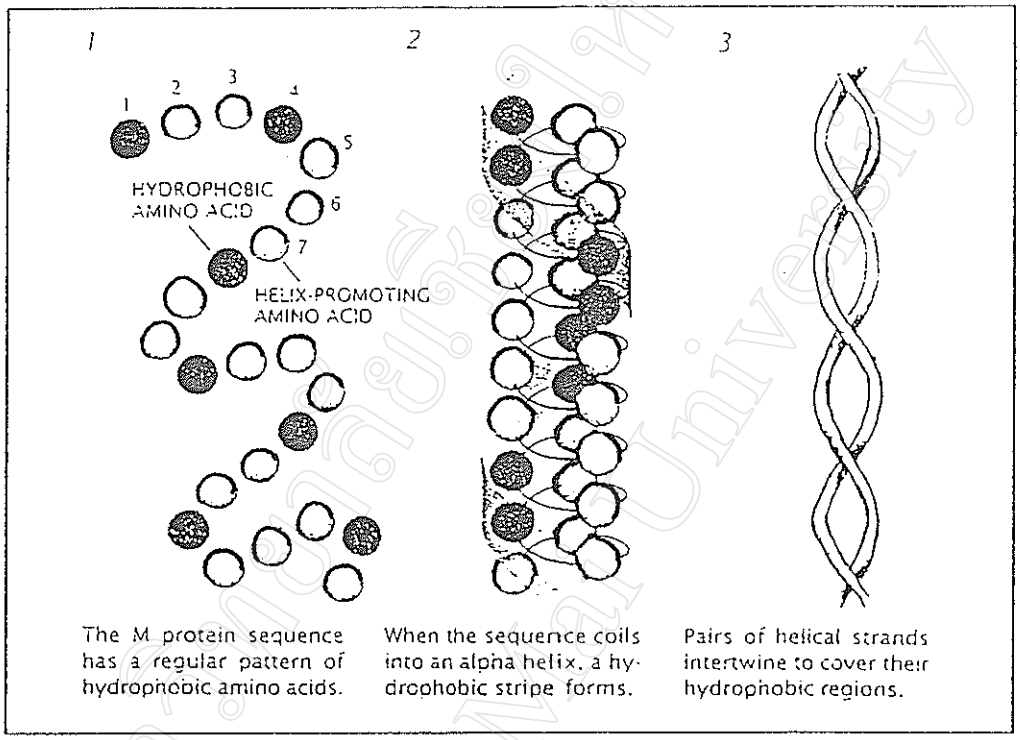

Figure 1 α-helical coiled-coil structure of group A streptococcal M protein (from Fischetti, 1991).

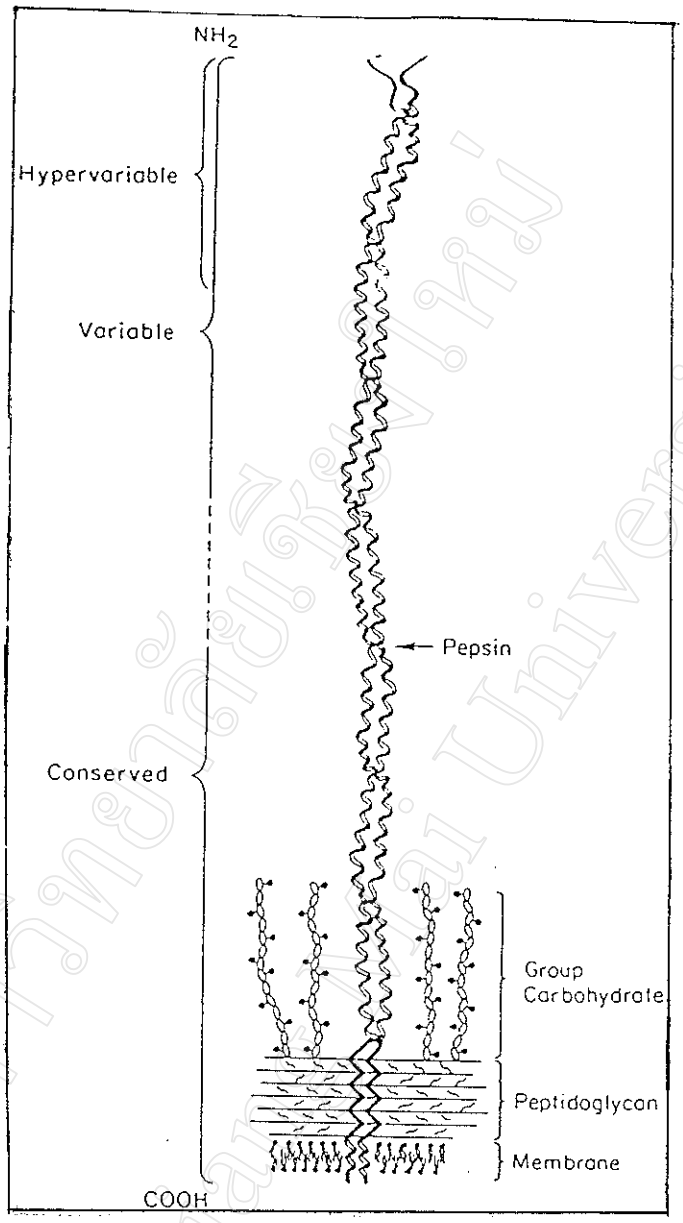

Figure 2 Basic model of the M protein (from Fischetti, 1989).

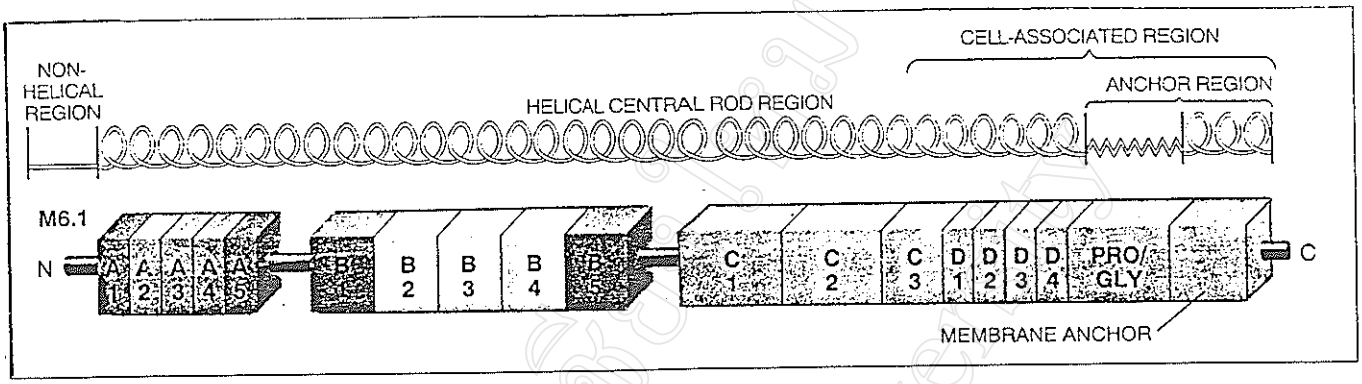

Figure 3 Tandem repeats of group A streptococcal M protein (from Fischetti, 1991).

1.6.2 M protein genes

The M protein genes, *emm* genes, from various M serotypes have been cloned, sequenced and shown to produce immunological M proteins. The length of the open reading frame (ORF) varies according to serotype. The *emm* gene consists of the promoter, signal peptide, and structural protein coding regions (Hollingshead *et al.*, 1986; Miller *et al.*, 1988; Mouw *et al.*, 1988; Haanes and Cleary, 1989; Manjula *et al.*, 1991). Codon usage is similar to that of other gram positive bacteria, which is low in GC content (Manjula *et al.*, 1991).

GAS are traditionally divided into two major classes based on their production of opacity factor (OF), a lipoproteinase whose activity is detected by horse serum opalescence(Bessen *et al.*, 1989; Bisno, 1991). The OF producing GAS (OF-positive GAS), and OF-non-producing GAS (OF-negative GAS) express two groups of M proteins. It has been suggested that there are

fundamental differences between M proteins of OF-positive and OF-negative GAS at the DNA level (Bessen *et al.*, 1989).

The *emm* genes possess a similar overall structure, consisting of a highly variable 5′ sequence coding the N-terminal region of M proteins, flanked by highly conserved sequences corresponding to N-terminal signal peptide and C-terminal wall-associating regions of the protein. By comparing different *emm* gene sequences of OF-positive and OF-negative GAS, it was found that a highly conserved domain extended only to the end of the hydrophobic amino-terminal regions of the signal peptide between these groups. The following sequence diverges between *emm* genes, but highly conserved within *emm* genes of OF-positive or OF-negative GAS groups, especially within the C-repeat region (Haanes-Fritz *et al.*, 1988; Haanes and Cleary, 1989; Podbielski *et al.*, 1991).

It was demonstrated that antibodies directed against defined epitopes in the conserved domain of M6 C-repeat region bound to surface epitopes of OF-negative but not OF-positive strains (Bessen *et al.*, 1989). In addition, a distinction can be made between OF-positive and OF-negative GAS by using DNA probes corresponding in position to the C-repeat region and to the cell wall and membrane anchor region of M6 which is an OF-negative strain (Bessen *et al.*, 1989).

The proline-glycine rich and membrane anchor regions of *emm* genes of OF-positive GAS were different from those of OF-negative GAS (Haanes and Cleary, 1989; Relf and Sriprakash, 1990; Podbielski, *et al.*, 1991).

The *emm* genes are located within a virulence regulon, named *mry* or *vir*-regulon, which is controlled by mry or virR protein. The regulatory gene lies upstream from a cluster of one to three genes so call the *emm* gene family. Downstream from the *emm* gene cluster is the *scpA* gene, which encodes a C5a peptidase. It is widely distributed among group A streptococci but is not part of the *emm* gene family (Hollingshead *et al.*, 1993). Okada *et al.*, (1993)

demonstrated that *vir* is autoregulated and environmentally regulated in response to the level of CO_2. These data suggested a model for the regulation of virulence in *S. pyogenes*, where positive transcriptional control of *vir* in response to environmental stimuli regulates the expression of the M protein. OF-positive GAS contain a triplet of *emm*-like genes between *vir-R* and *scp-A*, but the structure of the *vir*-regulon varies among OF-negative GAS (Haanes *et al.*, 1992; Whatmore *et al.*, 1995)(Figure 4).

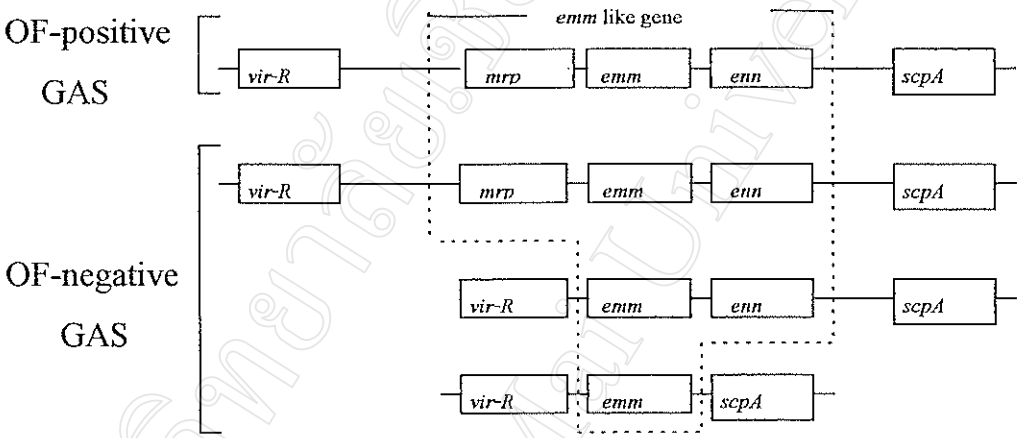

Figure 4 *vir*-regulon structure of OF-positive and OF-negative GAS (Whatmore *et al.*, 1995).

1.6.3 Virulence factor of M protein and pathogenesis

M protein has been identified as the streptococcal colonization ligand that forms a complex network with the other components and binds to fibronectin on epithelial cells causing pharyngeal colonization and disease (Ellen and Gibbons, 1972; Willett,1992; Reed *et al.*, 1994). M protein is a key virulence factor due to its involvement in mediating resistance to phagocytosis (Lancefield, 1962). This may be the repulsion between negative charge of leukocytes and the N-terminal end of the M protein (Fischetti, 1989; 1991). Furthermore, M protein exerts this effect by interfering with both the alternative and classical complement pathways (Bisno, 1979; Peterson *et al.*,

1979; Hong *et al.*, 1990). M protein binds to factor H, which is a regulatory protein of the complement system. This binding interferes with the deposition of C3 protein on the bacterial surface (Bisno, 1979; Peterson *et al.*, 1979; Jacks-Weis *et al.*, 1982; Horstmann *et al.*, 1988). It also leads to inhibition of the alternative C3 convertase and the classical C5 convertase of complement (Hong *et al.*, 1990).

It has been suggested that M protein has some properties of the superantigens (Tomai *et al.*, 1990; 1991). Superantigens are proteins that polyclonally activate T-cells by an MHC class II-dependent but haplotype-unrestricted mechanism. Proliferative responses to superantigens are limited to T-cell expression, particularly T-cell receptor (TCR) V_β gene families, and are independent of antigen specificity (Herman *et al.*, 1991). In addition, pepsin-extracted M protein has been shown to induce T-cell proliferation and the release of some monokines by peripheral blood mononuclear cells (Kotb *et al.*, 1993). This superantigen property of M protein has been proposed to be an important role of it in the development of post streptococcal diseases (Kotb, 1992).

2. Extracellular products

2.1 Erythrogenic toxins (Streptococcal pyrogenic exotoxins)

Streptococcal pyrogenic exotoxins (SPEs) are extracellular proteins produced by a number of GAS strains. There are at least four serological types of SPEs, designated SPEA, SPEB, SPEC and SPEF (Hauser *et al.*, 1991; Norrby-Teglund *et al.*, 1994). They have molecular weights of 8 kDa, 17.5 kDa, 13.2 kDa, and 23.36 kDa, respectively (Johnson, *et al.*, 1992). They are heat labile but are stable to acid, alkali and pepsin (Willett, 1992). SPEA and SPEC have been shown to be encoded by bacteriophage, while SPEB is

chromosomally encoded (Johnson and Schievert, 1984; Goshorn and Schlievert, 1989).

There are a number of exotoxins collectively called "superantigens"such as SPEs. SPEC stimulates TCR V_β 2-, 8-, 12-, 14- and 15- bearing cells (Fleischer, *et al.*, 1991; Tomai *et al.*, 1992). SPEB stimulates only cells expressing TCR V_β 8 element. SPEC stimulates cells expressing V_β 1-, 2-, 5.1- and 10 (Tomai *et al.*, 1992) while SPEF stimulates cells expressing V_β 2-, 4-, 8-, 15- and 19- (Norrby-Teglund, *et al.*, 1994).

SPEs are capable of inducing toxic shock-like syndrome or scarlet fever (Tomai *et al.*, 1992). One common feature among these molecules is that they all are powerful inducers of T cell proliferation (Imanishi *et al.*, 1990; Abe *et al.*, 1991) and activate a variety of cells to produce cytokines such as interleukin-1 (IL-1), gamma-interferon (γ-IFN), and tumor necrosis factor (TNF) (Parsonnet *et al.*, 1985; Jupin *et al.*, 1988; Fast *et al.*, 1989). SPEs are also found to modulate inflammatory mediators released from human neutrophils. For example, SPEA enhances the generation of chemoattractant leukotriene B4 (LtB4) (Hensler *et al.*, 1991). In addition, these toxins induce a nonspecific T cell dependent immunosuppression of antibody production as well as enhance susceptibility to lethal endotoxin shock (Tomai, *et al.*, 1992). This information suggests that they are important pathogenic factors of group A streptococci.

2.2 Hemolysin

Two hemolytic and cytolytic toxins, streptolysin-O (SLO) and streptolysin-S (SLS), are produced by most strain of GAS (Willett, 1992). They are responsible for the clear zones of β-hemolysis around the colonies in blood agar media (Willett, 1992). Both of them can injure the membrane of red blood cells and other cells (McCarty, 1990).

Streptolysin-O (SLO) is an immunogenic single-chain protein (Ca 60 kDa) released into the culture medium during growth and inactivated reversibly by atmospheric oxygen, and irreversibly by cholesterol and structurally related sterol (McCarty, 1990; Willett, 1992). For this reason, the SLO is responsible for the subsurface hemolysis on blood agar plate (Quinn, 1982). Their cytolytic activity, which is attributed to their ability to damage the cholesterol-containing membranes, extends to a wide range of eukaryotic cells, including red blood cells, polymorphonuclear leukocytes and platelets (Murray, *et al.*, 1994). By binding to cholesterol-containing membranes, the soluble molecules of the SLO assemble to oligomeric curved rod structures. These 25-100 toxin monomers form rings and arcs which penetrate the apolar domain at the lipid bilayer. These generate slits and pores in the membrane that allow leakage of cellular component. In the macrophage and the granulocytic leukocyte, the membranes of the cells and cytoplasmic granules are breached presumably by the same mechanism, with severe damage to the cell and release of lysosomal enzymes, resulting in cell death (McCarty, 1990; Willett, 1992).

SLO is exceedingly toxic to mammalian and amphibian myocardium, causing cardiac standstill (Bernheimer, 1972). It has also been postulated that SLO may reach the blood circulation and be involved in immune complexes in patients with rheumatic fever (Willett, 1992).

Following pharyngeal or systemic infection, the SLO induces a brisk antibody response which is useful for documenting a recent infection. This is referred to as anti-streptolysin O (ASO titer), and more anamnestic response occurs after repeated infection (Willett, 1992; Murray, *et al.*, 1994). In pediatric populations where these organisms are frequently encountered, antibody titers to 300-500 may be seen normally. The titers are lower in adults (Willett, 1992; Jawetz *et al.*, 1995).

Streptolysin-S (SLS) is an oxygen-stable, non-immunogenic cell-bound hemolysin capable of lysing erythrocytes, as well as leukocytes and platelets

following direct cell contact. SLS can also stimulate the release of lysosomal contents after engulfment, with subsequent death of the phagocytic cells (Murray *et al.*, 1994). It is responsible for the surface hemolysis seen on blood agar plate (Willett, 1992). The extraction of hemolysin (18 kDa) from intact streptococcal cells with serum depends on the association with serum, albumin, and a RNase-resistant RNA. It may be inhibited by non-specific inhibitors, such as phospholipids, frequently present in the sera of human and animal suggestive of their involvement in the cytolytic activity of the toxin (Willett, 1992; Jawetz *et al.*, 1995).

2.3 Deoxyribonucleases

Four immunologically distinct deoxyribonucleases (A through D) have been identified. These enzymes are capable of depolymerizing free DNA and RNA present in pus (Willett, 1992; Murray *et al.*, 1994). The DNases facilitate the liquefaction of pus, and the spread of the organisms and presumably generate growth substrates (Willett, 1992; Murray *et al.*, 1994).

Most GAS produce two or more enzymes, B being the most common (Quinn, 1982). All have molecular weights of 20-30 kDa. Antibody titer to DNase B is an important marker of pharyngeal or skin infection (Quinn, 1982; Willett, 1992; Murray *et al.*, 1994).

2.4 Streptokinases (Fibrinolysins)

At least two forms streptokinases (A and B) have been described (Murray *et al.*, 1994). They transform the plasminogen of human plasma into plasmin, an active proteolytic enzyme that digests fibrin and other proteins. Plasmin may be responsible for the rapid spread of GAS in infected tissue (Murray *et al.*, 1994; Jawetz *et al.*, 1995). This process of digestion may be interrupted by non-specific serum inhibitors and a specific antibody, antistreptokinase (Jawetz

et al., 1995). Different serotypes of GAS produce different amounts of these toxins (Huang *et al.*, 1989).

2.5 Lipoteichoic acid (LTA)

LTA is present in the cell wall of GAS. Adherance to buccal epithelial cells is mediated by this molecule. It is an amphipathic and amphoteric molecule which is highly cytotoxic for a variety of host cells. It has a wide array of biological activities (Matsuno and Slade, 1970; Ofek *et al.*, 1975; Willett, 1992). It was found to bind the lipoteichoic acid receptor, fibronectin, on epithelial cells (Ofek *et al.*, 1975; Beachey and Ofek, 1976; Beachey, 1981; Murray *et al.*, 1976).

The mechanism of GAS colonization via LTA has been proposed. The glycolipid end of the LTA molecule can form ionic complexes with the M protein permitting the reorientation and interaction of its lipid ends with cell membrane receptors (Beachey *et al.*, 1983).

2.6 Opacity factor (OF)

The OF is an aspartic proteinase. It produces opacity when combined with various mammalian sera (Johnson and Kaplan, 1988). This OF production is consistently and exclusively associated with specific M-serotype streptococcal strains (Johnson and Kaplan, 1988). Because OF is antigenically specific and closely associated with M protein (Widdowson, *et al.*, 1971; Hallas and Widdowson, 1979), the demonstration of OF production allows the classification of that strain into OF-positive and OF-negative categories.

2.7 C5a peptidase

The C5a peptidase of GAS (SCPA) is a surface bound serine peptidase of 128 kDa which is highly specific for C5a. It cleaves C5a on the carboxy side at His-67 and Lys-68, a position known to be within the PMN binding site (Chen

and Cleary, 1990; Cleary *et al.*,1992). This cleavage abrogates C5a's ability to serve as chemoattractant (Wexler and Cleary, 1985).

SCPA is produced by most but not all M-positive strains of GAS (Cleary *et al.*, 1991). Its structural gene is located adjacent to the M-protein gene in *vir*-regulon (Haanes and Cleary, 1989; Chen and Cleary,1990). Transcriptional control is in ordination with *vir-R* gene (Simpson *et al.*, 1990).

C. Diseases and Pathogenicity

1. Pharyngitis

Group A streptococcal pharyngitis is one of the most common bacterial infections of childhood, which account for 20-40 percent of cases of exudative pharyngitis (Wessels, 1994). This is primarily a disease of children between the age of 5 to 15 years, but infants and adults are also susceptible. The pathogen is spread from person-to-person via respiratory droplets (Wessels, 1994). The incubation period is 1 to 4 days. In infants and small children, the sore-throat occurs as a subacute nasopharyngitis with a thin serous discharge and little fever and a tendency to extend to the middle ear, mastoid, and meninges. The cervical lymph nodes are usually enlarged. The illness may persist for a week. In older children and adults, the disease is more acute and is characterized by intense nasopharyngitis, tonsillitis, and intense redness and edema of the mucous membranes, with purulent exudate accompanied by enlarged tender cervical lymph nodes, and high fever. Twenty percent of infections are asymptomatic (Jawetz *et al.*, 1995).

The specific diagnosis can be made only by bacteriological or serological test.

2. Scarlet fever

Scarlet fever had been thought to reflect infection of an individual lacking toxin-specific immunity with a toxin-producing strain of GAS (Murray *et al.*, 1994; Wessells, 1994; Jawetz *et al.*, 1995). Antitoxin prevents the rash but does not interfere with the streptococcal infection (Murray *et al.*, 1994; Jawetz *et al.*, 1995). Subsequent studies have suggested that development of the scarlet fever rash may reflect a hypersensitivity reaction requiring prior exposure to the toxin (Wessells, 1994). One to two days after initial clinical symptoms of pharyngitis, a typical diffuse erythematous rash will appear on the upper chest and spread to extremities. Associated findings include circumoral pallor, strawberry tongue and accentration of the rash in the skin folds (Pestia's lines) (Murray *et al.*, 1994; Wessells, 1994). The rash subsides after 6-9 days (Wessells, 1994).

3. Erysipelas

This is an acute superficial cellulitis of the skin with prominent lymphatic involvement. Erysipelas occurs most commonly in young children or older adults, involves the face and less frequently the trunk or extremities, and is usually preceded by either respiratory or skin infections with GAS. The cutaneous manifestations are accompanied by chills, fever and systemic toxicity (Murray *et al.*, 1994).

4. Pyoderma

Pyoderma, also called impetigo, is a superficial infection of the skin caused primarily by GAS, occasionally by other streptococci or by *Staphylococcus aureus*. Cultures of impetiginous lesion often show *S. aureus* as well as GAS, but longitudinal studies have shown that in almost all cases, streptococci can be isolated initially, with staphylococci appearing later, presumably as secondary colonizing flora (Wessells, 1994). The strains of

streptococci that cause skin infections, M49, M57, M59, M60 and M61, are different from those that cause pharyngitis. However, a pyoderma serotype can colonize the pharynx and establish a persistent carriage stage (Murray *et al.*, 1994; Jawetz *et al.*, 1995), and may precede glomerulonephritis but does not often lead to rheumatic fever (Jawetz *et al.*, 1995).

Clinical disease is preceded by initial colonization of the skin with GAS via direct contact with another infected child or fomite, or transfer by an arthropod vector. Introduction of GAS into the subcutaneous tissue occurs by minor break in the skin integrity.

5. Streptococcal toxic shock-like syndrome.

In the late 1980s, severe streptococcal soft tissue infections (e.g. cellulitis, necrotinizing fasciitis) associated with multisystem toxicity were reported (Murray *et al.*, 1994). The general features of the illness includ fever, hypotension, renal impairment, and respiratory distress syndrome. Streptococcal toxic shock-like syndrome is associated with a 30 percent mortality, most deaths are secondary to shock and respiratory failure. The GAS responsible for this syndrome differ from the strains causing pharyngitis in that most of them are serotypes M1, M3 or M18 and some have prominent hyaluronic acid capsules. The pyrogenic toxins are considered to be the causative agents of this syndrome (Belani *et al.*, 1991; Murray *et al.*, 1994) because of their ability to induce erythema, pyrogenicity, tissue damage, systemic toxicity, multiorgan system failure, myocardial depression, vascular permeability change and shock (Podbielski, *et al.*, 1991). Streptococcal pyrogenic exotoxin type A appears to have the most potent inflammatory and cytotoxic properties and is capable of causing substantial tissue injury. The production of this exotoxin correlates most strongly with the severity of GAS infection (Podbielski *et al.*, 1991).

6. Post streptococcal infection diseases

6.1 Rheumatic fever (RF) /Rheumatic heart disease (RHD)

RF is a non-suppurative complication of GAS disease. It is characterized by inflammatory changes of the heart, joints, blood vessels, and subcutaneous tissue (Murray *et al.*, 1994). This is the most serious sequelae of GAS infection because it results in damage to heart muscle and valves (Jawetz *et al.*, 1995).

A number of hypotheses relating to the mechanism of RF/RHD have been proposed.

1. The organism or streptococcal enzymes cause the destruction of the tissue (Willett, 1992; Murray *et al.*, 1994).

2. Serum sickness-like reaction is mediated by complexes of antibodies and antigens leading to RF/RHD (Willett, 1992; Murray et al., 1994). Circulating immune complexes have been found in the sera of patients with acute rheumatic fever (ARF)(Friedman *et al.*, 1984).

3. An autoimmune reaction is currently favoured because antibodies directed against heart tissue have been identified in patients with complicated streptococcal disease and rheumatic heart disease. These antibodies can bind to cardiac and skeletal muscles, as well as the smooth muscle in blood vessels (Murray *et al.*, 1994).

Rheumatic fever is associated with upper respiratory tract infections (Murray *et al.*, 1994). During endemics of pharyngitis, rheumatic fever may occur in as high as 3% of affected persons (Willett, 1992). It most commonly occurs in young school-age children with no male or female predilection and it occurs during the fall or winter months (Murray *et al.*, 1994).

The revised Jones Criteria for diagnosis of RF is currently used as shown in Table 2 (Murray *et al.*, 1994).

Table 2 Jones's criteria (revises).

Major manifestations	Minor manifestations
Carditis Polyarthritis Chorea Erythema marginatum Subcutaneous nodules	Clinical finding: Arthralgia fever Laboratory findings: -Elevated acute phase reactants (erythrocyte sedimentation rate, C-reactive protein) -Prolonged PR interval on electrocardiography
PLUS Supporting evidence of antecedent GAS infection - Positive throat culture - Positive streptococcal antigen test - Elevated or rising streptococcal antibody titer	

The diagnosis of ARF is suspected when the patient on the first examination has two major criteria or one major and two minor criteria.

It has been shown that M proteins share epitopes with human heart tissue, notably sarcolemmal membrane protein, cardiac myosin and brain (Dale and Beachey 1982; 1985; 1986; Bronze *et al.*, 1987; Mische *et al.*, 1987; Cunningham *et al.*, 1989; Bronze and Dale, 1993; Vashishtha and Fischetti, 1993).

6.2 Acute glomerulonephritis (AGN)

AGN is another non-suppurative complication of streptococcal disease which may be seen after either pharyngeal or cutaneous infection. It is

characterized by acute inflammation of the renal glomeruli with edema, hypertension, hematuria and proteinuria (Willett, 1992; Isselbacher *et al.*, 1994; Murray, *et al.*, 1994; Jawetz, 1995). It is primarily associated with serotypes 1, 2, 3, 4, 12, 15, 49, 55, 57, 60 and 61. The incidence of AGN in epidemic or in sporadic streptococcal infections may vary from less than 1% to as high as 10% to 15% (Willett, 1992). AGN occurs more commonly in children (Willett, 1992; Isselbacher *et al.*, 1994).

There are several mechanisms that may be evoked in the pathogenesis of the AGN.

1.) The development of streptococcal Ag-Ab complexes may induce immunological damage of renal tissue (Lange *et al.*, 1976; Jawetz, 1995).

2.) The streptococci may induce the endogenous immune complex system (Friedman *et al.*, 1984; Willett, 1992).

3.) Streptococcal antigens may cross react with components of the human glomerulus (Villarreal *et al.*, 1979; Goroncy-Bermes *et al.*, 1987).

D. Presumptive identification of GAS

The presumptive differentiation of GAS from the other group of streptococci is based on the susceptibility of GAS to bacitracin (Facklam, 1976; Murray *et al.*, 1976; Rotta and Facklam, 1980) and resistance to sulfamethoxazole-trimethoprim (Facklam *et al.*, 1979; Billi *et al.*, 1984). Serological confirmation depends on the antigen-antibody interaction in the agglutination or precipitation method (Rotta and Facklam, 1980).

E. Group A streptococcal typing

The antigenic classification of GAS is based on the occurrence of multiple M and T antigens (Lancefield, 1940; 1962). Epidemiological studies on the

transmission of streptococci in the population are largely dependent on the recognition of these antigens in the isolated strain.

1. M typing

Immunity to infection of GAS is determined by immune response to the individual M antigens (Lancefield, 1962). Therefore, the specificity of the M-antigen-antibody reaction has special significance for epidemiological studies.

A positive result by M typing depends on the presence of the M protein in the strain. Strains that do not possess the M protein at the time of typing cannot be type-identified. It is best to type freshly isolated strains. Passage and subculture in artificial media reduce the production of the M protein. There have been several tests designated to detect the type-specific M protein.

1.1 M typing with antibody

This method uses antibody and M protein extracts. It has been employed a capillary precipitin test for several decades (Swift *et al.*, 1943). This procedure is carried out as a precipitin test in capillary tube. It is time consuming and requires potent absorbed typing sera in order to produce reliable results. The absorption of sera by carbohydrate eliminates mainly an antibody to the group polysaccharide.

1.1.1 Preparation of M antigen extract

Hydrochloric acid has been used for the extraction of M protein since 1962 (Lancefield, 1962). The bacterial-acid mixture is placed in a boiling water bath for 10 minutes. The pH has to be adjusted to 7.0 before the centrifugation. The supernatant fluid contains M protein and can be used for M typing.

It is critical that the extraction of M protein should be done at pH 2. If it is done in a more acidic medium, hydrolysis and destruction of M protein occur. In cases of poor M-typability, it may prove advantageous to increase the

content of neopeptone in the Todd-Hewitt broth by adding neopeptone in the broth.

1.1.2 The precipitation reaction in capillary tubes.

The precipitation reaction is carried out by the method of Swift *et al* (Swift *et al.*, 1943) in capillary tubes about 8 cm long with an external diameter of about 1 mm. The capillaries must be thoroughly washed. The capillary is applied in a slanting position to the surface of the serum and a column of about 2 cm is drawn up then 2 cm of the M protein extract are drawn up in a similar manner. The column of the two fluids is drawn up so as to occupy the middle of the capillary. The capillary is inserted in a vertical position in a rack with a groove filled with plasticine and incubated in a thermostat at 37°C. The result is read after two hours. After reading, the capillary is placed in an icebox at 14°C for 18 hours and read again. The evaluation is made according to the following scales:

\+ evident precipitation, the height of the precipitation column measures 2 mm maximum

++ the precipitation column measures 3-10 mm

+++ more than 10 mm of the capillary is filled with precipitate.

Only unequivocal precipitin reactions should be evaluated. A strain can only be regarded as identified if the reaction has been specific, i.e, if the strain has reacted with only one M antiserum.

The extract of the strain to be identified by the capillary precipitation method, has to be tested against individual M antisera of all types; no polyvalent sera for preliminary orientation can be prepared. It is therefore an advantage to use the result of typing by the agglutination test to decide which precipitation sera should be employed in the test. In principle, the sera are employed with types whose agglutination sera have given a positive reaction, or which are antigenically related to the T antigen; one or two other sera may be

added. If the result of the agglutination test is negative, the extract must be examined against all M antisera.

1.2 M typing with unabsorbed sera or an Ouchterlony immunodiffusion assay

This method is carried out as a double-diffusion test in agarose gel (Rotta and Facklam, 1980). The procedure is comparable to the capillary test in terms of results, but is simpler, faster and easier to perform. Both methods show very good agreement in routine practice.

The identification of precipitation bands corresponding to the group and type reaction is possible because the bands develop in different positions due to different diffusion patterns of the M protein and the group A polysaccharide. However, some unabsorbed sera may give cross-reactive bands mimicking the M-anti-M reaction. In this instance, the M antisera must be absorbed.

1.2.1 Preparation of M antigen extract.

The HCl extracted procedure is used as indicated in 1.1.1

1.2.2 The preparation of reaction in agar gel.

One percent solution of Noble agar with 0.01% sodium azide in 0.01 M phosphate buffer saline is used and poured into four plastic frames holding six glass microscope slides each. After solidification, two sets of wells in each slide are cut out, each set consisting of eight peripheral wells and one central well.

The central well is filled with serum and the peripheral wells are filled with extracts of the strains to be typed. Thus the number of sets of wells corresponds to the number of sera available for typing. Eight strains can be typed at a time against one serum. It is not possible to place the extract in the central well and the sera in the peripheral wells because of frequent cross-reactions due to the large volume of serum in the plate. The slides are incubated in a moist chamber at room temperature (approximately 22°C) overnight.

The band corresponding to the M-anti-M reaction develops nearest to the well containing the extract, while the band representing the group reaction forms closest to the central well.

The M type is identified if the extract of the strain gives a clear precipitation reaction with one serum only. If cross reactions are seen, the typing in agar gel should be repeated with a new hydrochloric-acid extract.

2. T-typing

GAS can also be characterized by the T protein, another surface antigen (Lancefield, 1940). These T proteins are identified by the use of one of several established serological assay utilizing approximately 20 antisera (Johnson and Kaplan, 1993). The T-typing system, based on the agglutination reaction, is used to identify strains according to the T protein. T types correlate well with M types. However, since various streptococcal types have additional T referred to as a certain "T pattern" (Rotta and Facklam, 1980). It results in a pattern of T-proteins rather than a single type. This occurrence of T-patterns may be the source of confusion since "incomplete" patterns are commonly found. T typing sera are commercially available and are prepared by certain reference laboratories (Johnson and Kaplan, 1993).

The set of the polyvalent and diluted monovalent sera for characterization of T-typing pattern consists of the following sera.

Polyvalent	Monovalent
T	1, 3, 13, B3264
U	2, 4, 6, 28
W	5, 11, 12, 27, 44
X	8, 14, 25, Imp19
Y	9, 18, 22, 23

The variation in T type or pattern is most strongly influenced by variation in the quality of typing antisera and in the method used to prepare the streptococcal T protein (Efstratiou, 1980).

3. Serum opacity reaction (SOR)

The SOR test detects opacity factors of GAS based on the capacity of the factor to produce opalescence in various mammalian serum. The test serves as a useful epidemiological tool for the group A strains as it has an association with specific M types (Widdowson *et al.*, 1971), although currently there are less than 30 identified and characterized OF-producing serotypes.

4. Genomic-typing methods

Genotyping methods are independent of growth conditions and are less susceptible to phenotypic variation than phage typing, antigens, enzymes, or membrane protein typing method (Cleary *et al.*, 1988).

4.1 Restriction fragment length polymorphism (RFLP) or DNA fingerprinting.

This method has been shown to be a highly specific and useful indicator of strain relatedness (Cleary *et al.*, 1988; 1992; Bingen *et al.*, 1992a; 1992b; Martin and Single, 1992; Mylvaganam *et al.*, 1994; Seppälä, 1994b). Restriction enzyme cleavage sites are determined by a 4-8 base code uniquely located in the DNA of all known species. The number of sites for a given enzyme depends on the G+C ratio and the size of genome in question.

Some of these studies showed that DNA fingerprints can distinguish between strains from different geographic locations and between epidemiologically unrelated strains that are not typable with the standard battery of sera, and can be subclassed within serotype (Cleary *et al.*, 1988; Bingen *et al.*, 1992a; 1992b; Martin and Singer, 1992; Mylvaganam *et al.*,

1994). This analysis is a promising method for distinguishing recurrence from relapse in failures of treatment (Bingen *et al.*, 1992a; Cleary *et al.*, 1992). Although this method appears to be a more efficient tool for strain differentiation, it is limited by difficulties in comparing fragment mobility from gel to gel.

4.2 Ribotyping

Ribotyping refers to a Southern blot analysis in which strains are characterized for the RFLPs associated with the ribosomal operon(s) (Stull, *et al.*, 1988). Operons are clusters of genes that share related functions and are often coordinately regulated; the ribosomal operons comprise nucleotide sequences coding for 16S rRNA, 23S rRNA and one or more tRNAs. Ribosomal sequences are highly conserved, and probes prepared from isolated *E. coli* rRNA (Stull *et al.*, 1988) hybridize to the chromosomal ribosomal operons of a wide range of bacterial species. All bacteria carry these operons and are therefore typable. In general, ribotypes are stable and reproducible, with isolates from an outbreak typically having the same ribotype (Rabkin *et al.*, 1989; Tenover *et al.*, 1994). The number and locations of these random sites vary among different strains, and thus the number and size of the fragments detected by electrophoresis of the amplicon also vary. The approach is attractive because it is conceptually simple and theoretically suitable for use with any organism. Ribotyping has been used for epidemiological study of GAS (Seppälä *et al.*, 1994b; Bruneau *et al.*, 1994). However, it is less discriminatory than DNA fingerprinting (Seppälä *et al.*, 1994b).

4.3 Oligonucleotide probe.

The probes derived from N-terminal region of M protein genes have been used for typing of GAS. However, a single *emm*-specific oligonucleotide probe

cannot identify all members of M types, as defined by conventional serotyping using polyclonal antisera (Penney *et al*., 1995).

4.4 Random amplified polymorphic DNA anlysis (RAPD)

The RAPD assay is based on the observation that short primers (typically 10 bp) whose sequences are not directed to any known genetic locus will nevertheless hybridize with sufficient affinity at random chromosomal sites to permit initiation of polymerization. If two such sites are located within a few kilobases of each other on opposite DNA strand and in the proper orientation, then amplification of the intervening fragment will occur (Welsh and McClelland, 1990; Williams *et al*., 1990).

In practice, however, achieving reproducible discriminative results is difficult with RAPD. Compare with the polymerase chain reaction (PCR) that uses conventional site-specific primers, the reaction conditions used in RAPD are typically less stringent in order to facilitate initiation of the polymerization reaction at sites having one or more sequence mismatches. However, polymerization is initiated with various efficiencies at such sites, and the final quantities of DNA produced may vary widely among the different fragments amplified from a given isolate. Consequently, when the products of the reaction are visualized by ethidium bromide staining, the individual bands produced for that isolate may vary widely in intensity. Such variation is inherent in RAPD and introduces two specific problems. First, it can be quite difficult to compare and interpret patterns whose bands demonstrate such differences in intensity (Saurnier *et al*., 1993; Gardiner *et al*., 1995). Second, because some of the products may represent relatively inefficient reactions, the actual fragment obtained from a single isolate may vary in different amplification reactions (Welsh and McClelland, 1990; Maslow *et al*., 1993; Arbeit, 1995).

The RAPD has been used for GAS typing. It was some what better than DNA fingerprint for differentiation of isolates of the same or different serotypes. However, not all of the serotypes were differentiated by this method (Seppälä *et al.*, 1994a).

4.5 *vir*-Typing

This method is based on long PCR followed by restriction endonuclease digestion and agarose gel electrophoresis. It takes the advantage of sequence and architectural divergence of constituents of the *vir*-regulon of GAS. Thus, the *vir*-regulon is the target of the amplification. This method provides a highly reproducible typability rate, and the interpretation of the patterns is unambiguous (Gardiner *et al.*, 1995). However, this method requires a high-quality DNA and a curious PCR condition for a successful long PCR.

F. Epidemiology of GAS infections

GAS is an important cause of bacterial infections in both adults and children throughout the world. It commonly colonizes the oropharynx of healthy children and young adults (age of 5-14 years)(Quinn, 1982; Pruksakorn *et al.*, 1990). The GAS carriage rates in asymtomatic children and patients with pharyngitis in different regions of the world are shown in Table 3 and Table 4. The variation of the carriage rates depended on the school and time of the isolation (Pruksakorn *et al.*, 1990).

It is difficult to compare the findings between the countries because of methodological differences. There does not appear to be an unusually high frequency of GAS isolation in developing countries among patients with sore throats (Markowitz, 1991). The pharyngeal carriage of β-hemolytic streptococci appears to be much more common in tropical countries than in those with temperate climates. There are also striking differences in the

prevalence of RF/RHD among different ethnic groups (Table 5). The differences are inversely related to the socioeconomic status (Markowitz, 1991), factors such as nutrition, hygiene, access of medical care and the degree of crowding in households (Bisno, 1991). The importance of rheumatic fever as a major health problem is also evident from hospital admission statistics for cardiac patients in Third World countries as shown in Table 6.

It has been shown that M types of GAS vary from country to country and the site of infection as shown in Table 7.

Less than 20% of the Asian GAS strains could be identified by available M typing sera, compared with more than 80% of the United States isolates although T proteins have been detected (Figure 5)(Kaplan *et al.*, 1992).

Table 3 Group A streptococci carriage stage in asymtomatic school children in different regions of the world.

Country	Carriage Rate(%)
Liberia	49
South India	49
Kuwait	47
Philippines	25
Japan	21
United States	13-29
Thailand	0.99-15.73

(From Pruksakorn *et al.*, 1990; Markowitz, 1991)

Table 4 Percentage of patients with pharyngitis positive for group A streptococci.

Country	% of GAS isolated
India	14
Egypt	19
Kuwait	22
United States	23
Thailand	6.39

(From Pruksakorn *et al.*, 1990; Markowitz, 1991)

Table 5 Prevalence of RF/RHD in different regions of the world

Region/Country	**Year**	**Prevalence per 100,000**
Africa	1984	470
	1970-1975	. 30-150
America	1984	15
	1980	0.23-4.88
	1965	6
	1935-1960	40-65
Eastern Mediterranean	1984	440
Southeast Asia	1984	12
Western-Pacific	1984	70
Latin America	1968-1980	100-1700
Pacific (except Japan)	1978-1985	100-1860
Japan	1979	70
Kuwait	1985	31
Iran	1972	58-100
Sri-Lanka	1973	140
New-Zealand Maori non-Maori	1972-1983	 88 9
Hawaii Chinese Samoans	1976-1980	 4 96
Baltimore	1978-1980	0.5

(Modified from WHO study group, 1988; Markowitze, 1991; WHO: cardiovascular diseases unit and principal investigators, 1992.)

Table 6 Rheumatic heart disease as a percentage of all cardiac admission in Third World countries.

Country	Period	% of RHD
China	1948-1958	50
Thailand	1968	38
Burma	1968-1970	30
Egypt	1950	53
Turkey	1960-1970	50
Tunisia	1970-1972	45
Libya	1972	36

(Modified from Agarwal, 1988)

Table 7 Distribution of M types in diseases associated with GAS infection among different countries

Country	M types frequency associated with disease			
	Pharyngitis	RF/RHD	Skin infection	AGN
Thailand	1, 4, 11, 12, 44, 49, 55, 60, 63, NT	1, 44, 55, NT	11, 44, 49, 55, 60, 63	n
USA	1, 2, 4, 5, 11, 12, 28, NT	1, 2, 3, 4, 5, 6, 18, 24, 41, 53, 75, 77, 78, NT	n	n
New-Zealand	1, 49, 57, 58, NT	53, 58, NT	49, NT, 55, 57, 60	49, 57, NT
Kuwait	1, 2, 4, 5, 6, 12, 28R, 33, NT	1, 12, NT	n	1, 4, 12, 25, NT

n=no data reported; NT = non typable

(Modified from Lennon *et al.*, 1988; Pruksakorn *et al.*, 1990; Majeed *et al.*, 1988; Johnson *et al.*, 1992; Kaplan *et al.*, 1989; 1992)

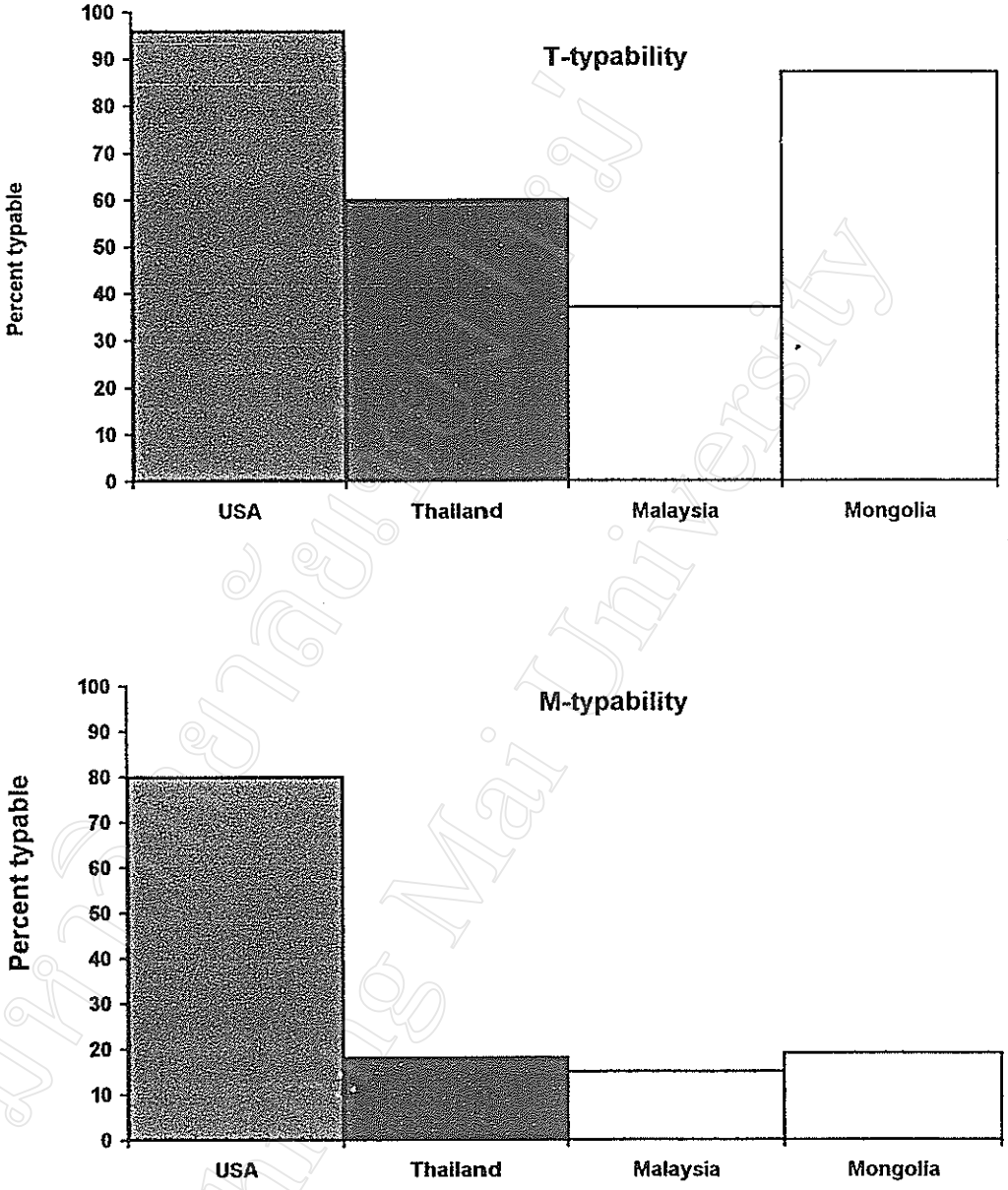

Figure 5 The percentage of typability by T-typing (top) and M-typing (bottom) of GAS isolated from the United States and from three countries in Southeast Asia (Kaplan *et al.*, 1992).