VIII. DISCUSSION

Group A steptococci (GAS) are responsible for a wide array of infections including pharyngitis, impetigo, scarlet fever, erysipelas, pneumonia, septic arthritis, toxic shock-like syndrome and septicemia (Bisno, 1991). GAS is also associated with non-suppurative sequelae: rheumatic fever and glomerulonephritis. Although several virulence factors have been identified, the pathogenesis research for more than seven decades has primarily focused on the M-protein, an α-helical coiled-coil surface fimbriae protein (Lancefield, 1962; Fischetti, 1989; Kehoe, 1994).

Serotyping with antisera against the surface proteins M and T and the production of opacity factor have been the standard approach in epidemiological studies of GAS infections (Kaufhold, *et al.*, 1992; Mylvaganam, *et al.*, 1994; Seppala, *et al.*, 1994b). Being dependent on the phenotypic expression of bacterial surface antigen or protein, this method is affected by environmental growth conditions. The development and maintenance of a suitable bank of M antisera is cumbersome, expensive, and limited to only a few laboratories in the world. T typing is easier to perform, but it offers a lower discriminatory power than M typing. A major limitation of serotyping is that a fairly large proportion of isolates are nontypable (Pruksakorn *et al.*, 1990; Prakash and Dutta, 1991; Kaplan, *et al.*, 1992; Seppala *et al.*, 1994a; Jamal, *et al.*, 1995), although the M protein genes and M protein were demonstrated (Pruksakorn, unpublished data; Tran *et al.*, 1993). Serotyping may be insufficient for type differentiation especially in epidemiologically unrelated isolates. In light of the changing epidemiology of GAS infections (Schwartz *et al.*, 1990; Markowitz, 1994) and continual evolution of M proteins (Fischetti, 1989; Relf *et al.*, 1994). There is an urgent

need to develop new methods of strain characterization that are generally applicable.

Several alternative methods have been investigated for GAS typing such as biotyping (Bouvet *et al.*, 1994), multilocus enzyme electrophoresis (Musser *et al.*, 1991; 1992; Haase *et al.*, 1994), ribotyping (Bingen *et al.*, 1992a, Bouvet *et al.*, 1994; Seppala *et al.*, 1994b), oligonucleotide probes specific to *emm* sequences method (Kaufhold, *et al.*, 1994), random amplified polymorphic DNA method (Seppala *et al.*, 1994a), and *vir*-typing (Gardiner, *et al.*,1995).

Restriction fragment length polymorphism (RFLP) of chromosomal DNA has been used in the epidemiological study of a number of other pathogenic bacteria including *Streptococcus zooepidermicus* (Skjold *et al.*, 1987), *Corynobacterium diptheriae* (Pappenheimer and Murphy., 1983), *Neisseria meningitidis* (Bjorvatn *et al.*, 1984; Kristiansen *et al.*, 1984), *Neisseria gonorrhoeae* (Falk, *et al.*,1985) *Leptospira interrogans* (Le Febvre *et al.*, 1985), *Mycoplasma pneumoniae* (Chandler *et al.*, 1982), *Vibrio cholerae* (Kaper *et al.*, 1982), *Candida albicans* (Scherer and Stevens, 1987), group B streptococci (Denning *et al.*, 1989; Blumberg *et al.*, 1992; Gordillo *et al.*, 1993) and group A streptococci (Cleary *et al.*, 1988; Bingen *et al.*, 1992a; 1992b; Martin, and Single., 1992; Mylvaganam *et al.*, 1994). RFLP analysis is a useful supplementary tool for the classification of GAS and can add to the discriminatory power of serotyping (Clearry *et al.*, 1988; Martin and Single., 1992; Mylvaganam *et al.*, 1994). Different M and T types and OF-production of GAS were associated with specific restriction enzyme profiles (Cleary *et al.*, 1988; Mylvaganam *et al.*, 1994). However, this approach is limited by labor intensive nature of the technique and the patterns produced are complex and difficult to interpret. The method we described here can be used for all isolates of GAS tested. It is simpler than the previous methods, such as RFLP, MLEE, ribotyping, RAPD and *vir-t*yping. It requires only small quantities of crude

DNA and individual strains can be compared easily by both the PCR product sizes and the *Mbo*I digestion patterns.

Relf and Sriprakash (1990;1992; 1994) have amplified the *emm* genes from many strains of GAS using PCR. The forward primer corresponding to the conserved N-terminal sequence of both OF-positive and OF-negative GAS and the reverse primers corresponding to the C-terminal sequences of OF-negative (P6) and OF-positive (P49) were used. PCR products of M1, M4, M12, M53, M57 were cloned and sequenced and found to be 95-99% similar to the published DNA sequences. The PCR products using *emm*6 (P6) and *emm*49 (P49) oligonucleotide primers were classified into two mutually exclusive groups which corresponded to the presence and absence of serum opacity factor using hybridization study. These data suggested that the major PCR products represented *emm* genes.

In this study, the method based on the polymorphism of *emm* genes both in length and sequence was employed to analyze the *emm* genes of M-nontypable GAS isolated from Thailand. Twenty-one M-nontypable GAS strains and 22 M-typable strains were studied. The *emm* genes were amplified using the primers which have been proven to amplify the *emm* genes of GAS by Relf and Sripakash. The amplified *emm* genes were digested with different restriction enzymes. The chosen digestion patterns were compared with each other. The compared digestion patterns were correlated with the percent homology of N-terminal sequence of *emm* gene. The correlated pairs of GAS were again compared with each other for homology of chromosomal DNA by RFLP method.

All OF-negative and OF-positive *emm* genes of M-typable GAS were amplified by P6 and P49, respectively. The *emm* genes of M-nontypable GAS showed more than 90% homology of N-terminal sequences to those of OF-negative and OF-positive M-typable GAS (Table 11). Thus, these results correlated with that of Relf and Sripakash.

H140, 104, CSF3, K3, K16 and J82 showed no significant homology of N-terminal sequences of *emm* gene to any of M-typable GAS strains. The *emm* genes were amplified by only P6 primer for H140 and 104 and by P49 primer for CSF3, K3, K16 and J82. These data may suggest the OF-phenotype of these M-nontypable GAS strains.

The sizes of PCR products of all isolates varied from 900 to 1600 bp depending on the M-types and the strains. The sizes of PCR products were comparable to those of the published *emm* genes as predicted (Table 9). The sizes of *emm* genes of OF-negative GAS tend to be larger than those of OF-positive GAS, except those of O20, AK14 and M55 GAS, which correlated with the study of Podbielski (1993). It showed that the median size of the *emm* genes exhibited a correlation with the OF-phenotype. The isolated *emm* genes from OF-negative strains of M3, M5, M6, M14, M17, M19, M24, M29, M46 and M57 having small *vir* regulon were, on everage, 0.25 kb larger than those of OF-positive GAS strains (Podbielski, 1993). In this study, N-terminal sequences of *emm* genes of GAS O20 and AK14 were 99% homology to that of M12. Because M12 and M55 were grouped in unusual *vir*-regulon not in small *vir*-regulon as in the study of Podbielski (1993), therefore, this might be the reason why the sizes of the PCR products of O20, AK14 and M550 were different from other OF-negative GAS.

The PCR products of all *emm* genes tested were digested with different restriction enzymes. *Sal*I, *Hind*III, *Kpn*I, *Bgl*II, *Pst*I, *Eco*RI, *Bam*HI, the six-base recognition enzymes, showed low frequency or inability to digest the tested *emm* genes of M-typable GAS strains, whereas *Mbo*I, a four-base recognition enzyme, could digest almost all tested *emm* genes in this study. These showed that the four-base recognition enzymes were appropriate for the analysis of short length genes. However, the six-base recognition enzymes were appropriate for the analysis of the entire chromosomal DNA by RFLP method because they produced fewer distinct fragments. Under the condition

used here, *Pst*I gave the best resolution bands for comparison of related GAS strains.

The *Mbo*I digestion patterns showed correlation with the percent homology of chromosomal DNA by RFLP method and with the percent homology of the N-terminal sequences of *emm* genes. The results showed 33 different *Mbo*I digestion patterns out of 43 tested GAS strains. None of the GAS pairs which showed lower than 90% homology of the N-terminal sequences of *emm* genes had identical *Mbo*I digestion patterns. The results agree with RFLP analysis which also gave low percentage (57-74%) of DNA fragment homology (Table 12, Figures 28, 29). Of 18 pairs of GAS with more than 90% homology of N-terminal sequences of *emm* genes, 16 pairs showed identical *Mbo*I digestion patterns. Fifteen out of 16 pairs of GAS also showed high percent homology (93.02-100%) of RFLP. Two pairs, ARF3-ARF19 and M22-ARF19, gave distinct *Mbo*I digestion patterns and also showed relatively low percentage of homology (85.71 and 90%) of RFLP. *Mbo*I digestion patterns could discriminate the GAS strains which possess different M type and genetic background. Alternatively, this results may explain the close evolution of *emm* genes and GAS.

ARF3-ARF15 and ARF19-M22 showed different *Mbo*I digestion patterns, despite having more than 90% homology of N-terminal sequences of *emm* genes. The size differences of the PCR products and restricted fragments within each pair may result from recombination events in A-, B-, and/or C-repeats leading to deletion or addition of repeat segments. These were observed in both laboratory and natural selection (Fischetti *et al.*, 1985; 1986; Hollingshead *et al.*,1987; Fischetti,1989; 1991) or from short nucleotide insertion (Harbaugh *et al.*,1993) or combination with point mutations (Scott,1990; Harbaugh *et al.*,1993). These two pairs of GAS showed 85.71% and 90% homology of RFLP, respectively. The results indicated that *Mbo*I digestion patterns could discriminate the closely related *emm* genes.

M25 and strain 42 showed 100% homology of N-terminal sequences of *emm* genes, and also gave identical *Mbo*I digestion patterns. However, this pair showed relatively low percentage of homology of chromosomal DNA by RFLP method (87.18%)(Figure 27). The discrepancy may result from horizontal transfer of *emm* genes between divergent GAS strains. Whatmore *et al.* (1994a; 1994b) showed that identical or very closely related 5′ *emm* sequences were frequently found in very divergent genetic background. A possible explanation is that genetic exchange between strains may occur during mix infection (Haanse *et al.*, 1994). Alternatively, Seppala *et al.* (1994b) claimed that it was not possible to predict the serotype on the basis of the RFLP patterns. The predictability of serotypes by RFLP was also impaired by the fact that the differences between RFLP patterns of isolates of different serotypes were not always greater than between those of the same serotype.

The sizes of PCR products of *emm* genes of M5, M58, M79, H92, S665 and J63 were not similar. However, the discrimination may improve by digestion with the other four-base recognition enzymes.

*Mbo*I digestion method could discriminate all GAS that are typable or non typable with standard sera. Although, some of these *emm* genes showed close relation by nucleotide sequencing, they can be differentiated by this method. Almost all of the digestion patterns by *Mbo*I method correlated with percent homology of N-terminal sequences of *emm* genes and percent homology of chromosomal DNA. These could be a key to understanding the genetic basis of antigenic diversity of GAS.

Although *emm* genes in M-nontypable GAS were demonstrated by PCR, they could not be typed by standard anti-M antisera, that it might be due to insufficient or defective expression of M proteins. Therefore, they are not detectable by the immunodiffusion technique. Nevertheless, using a more sensitive method such as Fluorescence Activated Cell Sorter (FACS scanning) or immunofluorescence technique for the detection of M protein may provide

the satisfactory answer. There are some strains of M-nontypable GAS that shows no homology of *emm* genes to any known *emm* genes of standard M-typable GAS. It is possible that this group of GAS possesses new M-types and their M-proteins are not recognized by a set of anti-M protein available.

The GAS infections and their sequelae remain a very significant health problem in developing countries worldwide (WHO Special study group, 1988). The precise identification of M-nontypable GAS isolates is important to vaccine design to induce the protection against serotypes of GAS which are epidemiologically associated with severe infections or their sequelae (Tran *et al.*, 1993).

In addition, if more GAS strains were included for the amplification of *emm* genes and digestion with *Mbo*I or the other four-base recognition enzymes, the results might give significant association of digestion patterns between strains. However, using this method only may be insufficient for GAS typing. The combination of this method with the serological method or other DNA typing methods may improve the capability of typing, especially the Southeast Asian isolates of GAS.