VI. MATERIALS AND METHODS

1. GAS strains and types

Twenty-two standard M-typable GAS and 21 M-nontypable GAS were used in this study and summerized in Table 8.

Table 8 Standard M-typable and nontypable GAS

Standard M-type GAS	Nontypable GAS	
	strains	Site of isolation
M1, M5, M6, M13,M18, M22 M24, M25, M39, M49, M52, M55, M58, M59, M66, M73, M75, M76, M77, M79, M80, M3R	38, 42, 104, J63, J82	skin
	CSF3	CSF
	ARF3, ARF15, ARF19	Throat (ARF patients)
	H92, H140	Throat (RHD patients)
	S14, S122, S142, S219, S330, S665, O20, AK14, K3, K16	Throat (sore throat patients)

2. DNA preparation

GAS were grown in 50 ml Todd-Hewitt broth supplemented with 1% neopeptone at 37°C for 24-48 hours(hr). Cells were harvested by centrifugation at 3000 rpm for 20 minutes(min) and washed 3 times with phosphate buffer saline (PBS) pH 7.4. The pellet was resuspended in 500 µl of 100 µg/ml lysozyme and incubated at 37°C for 1 hr. Ten microliters of 10

mg/ml proteinase K and 20 µl of 20% SDS were added to the mixture and incubated overnight at 58°C. Saturated NaCl was added at the 1/3 volume and incubated at 4°C for 20 min. The mixture was centrifuged at 13000 rpm for 20 min and the supernatant was drawn and put into a sterile tube. Three volumes of cold absolute ethanol were added and rocked gently until DNA flocculation appeared. The ethanol was discarded. The DNA was then washed twice with 70% ethanol. The DNA flocculation was fished out, dried for 3 min, resuspended in 200 µl TE buffer pH 8, and kept at 4°C until used.

3. Polymerase Chain Reaction (PCR)

3.1. Primers

The forward primer (spf1) for amplification of the *emm* genes was derived from the conserved region of the leader signal sequence of both OF-positive and OF-negative GAS as shown in Figure 6.

Its sequence is AAT CTG CAG TAT TCG CTT AGA AAA TTA AAA.

The reverse primer for OF-positive *emm* genes, P49, was derived from the conserved region of the membrane anchor as shown in Figure 7.

Its sequence is TTG GGA TCC TGC TGA TCT TGA ACG GTT AGC.

The reverse primer for OF-negative *emm* genes, P6, was derived from the conserved region of the proline/glycine rich region as shown in Figure 8.

It sequence is TGC GGA TCC AGC TGT TGC CAT AAC AGT AAG.

The primers were diluted to 40 *p*mol/µl in sterile distilled water and stored at -20°C until used.

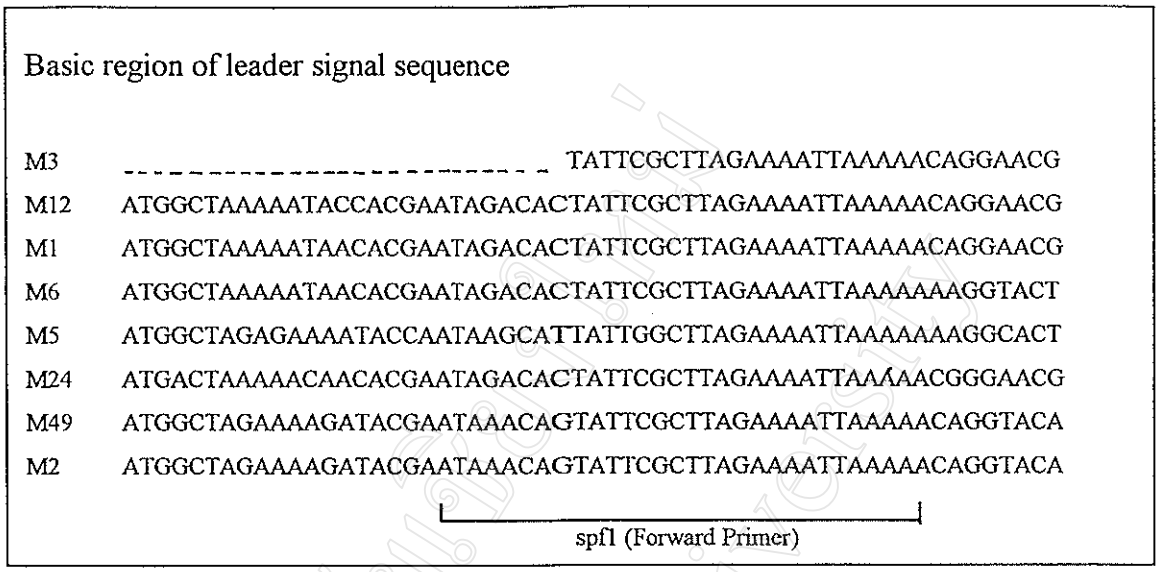

Figure 6 Conserved region within the leader signal sequence of *emm* genes of both OF-positive and OF-negative GAS (from Katsukawa, 1994).

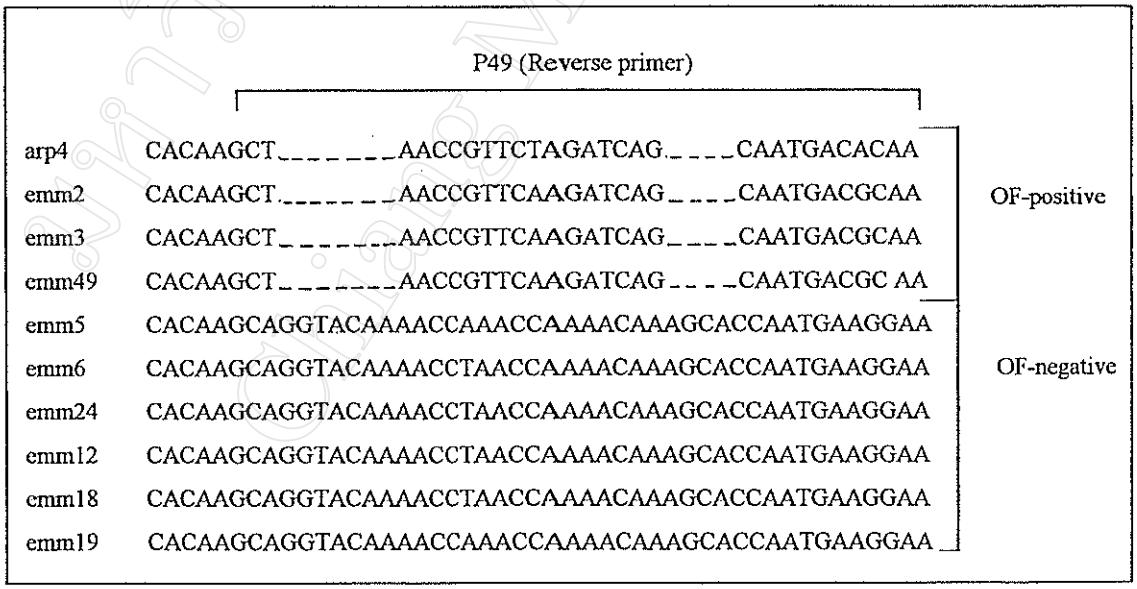

Figure 7 Conserved region of OF-positive GAS within the proline/glycine rich region (from Podbielski *et al.*, 1991).

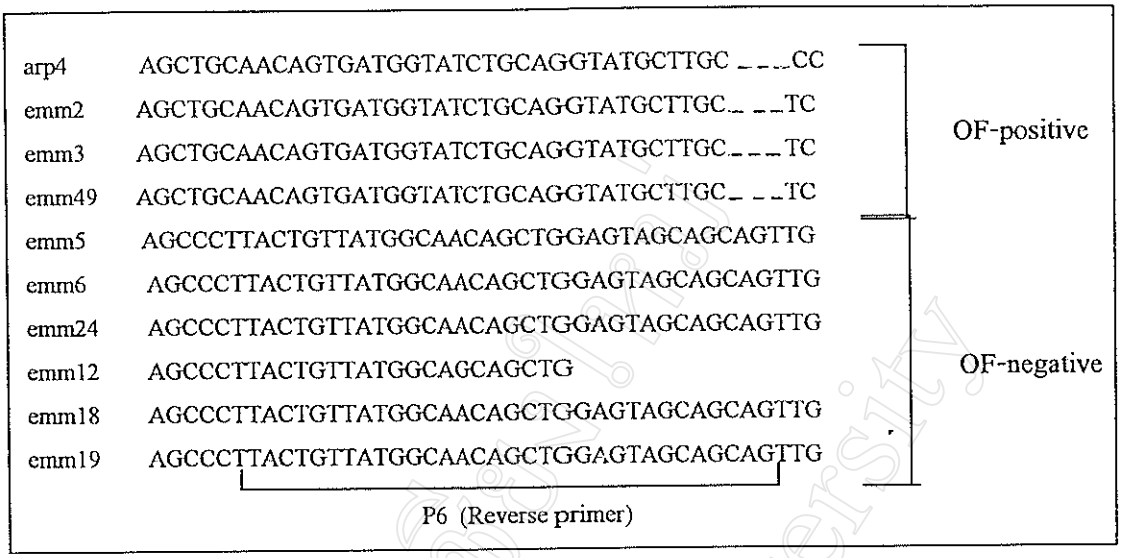

Figure 8 Conserved region within the membrane anchor region of OF-negative GAS (from Podbieski *et al.*, 1991).

3.2. Deoxynucleoside triphosphate (dNTPs).

The equal volume of 100 mM of each dNTP were mixed and stored at -20°C until used.

3.3. Template DNA

The chromosomal DNA concentration was compared with a known concentration of the *Hind*III-digested λ DNA markers on 1% agarose gel electrophoresis. The chromosomal DNA was diluted to 0.01 μg/μl in sterile distilled water.

The following reagents were mixed in a 450 μl of PCR tube.

- 50 ng of chromosomal DNA
- 20 pmol of forward and reverse primers
- 200 μM of dNTPs
- 1.5 U of *Taq* DNA polymerase (Promega)
- 2 mM $MgCl_2$
- 10 μl of 10x Buffer

- H_2O was added up to 100 µl
- 2 drops of mineral oil

The amplification for *emm* gene of each GAS strain was started by P49 and spf1 primers. If the PCR product was not seen, the amplificatipon was done again by P6 and spf1 primers.

3.4. PCR condition

The following conditions were set in a thermocycler (Perkin Elmer):

Pre-heating at 95°C for 10 min followed by 35-cycles of the following conditions :

Denaturing: 95°C 1.0 min

Annealing: 55°C 1.5 min

Extension: 72°C 2.0 min.

After completing with these cycles, another 10 min at 72°C was performed for the final extension. The reaction tubes were stored at 4°C until used. Three microlitres of the PCR product were analyzed on 1% agarose gel electrophoresis at 100 V for 1 hr and visualized by ethidium bromide staining. The amount of the PCR product was calculated by comparing with the *Hind*III-digested λ DNA markers.

4. Restriction enzyme digestion of PCR products

The following reagents were added in a 450 µl sterile microcentrifuge tube:

- 5µg (2-7 µl) of PCR product, without further preparation
- 2 µl of 10x buffer
- 10U of restriction enzyme
- distilled water was added up to 20 µl.

The mixture was incubated at 37°C for 2 hr. The reaction was stopped with 5 µl of gel loading buffer and heat inactivated at 75°C for 15 min.

The PCR product and digested DNA fragments were resolved in a 1% agarose gel electrophoresis at 100 V for 1.5 hr. and then stained with ethidium bromide and visualized under the UV light illuminator.

5. Restriction fragment length polymorphism (RFLP)

The following mixture was added in a 450 µl sterile microcentrifuge tube:

- 5µg (2.5 µl) of chromosomal DNA
- 2 µl of 10x buffer
- 10 U of a restriction enzyme
- distilled water was addded up to 20 µl.

The mixture was incubated at 37°C for 2 hr and the reaction stopped with 5 µl of gel loading buffer and then heat inactivated at 75°C for 15 min. The fragments were run on 0.8% agarose gel electrophoresis at 80 volts for 6 hr, stained with ethidium bromide, and visualized under the UV light illuminator. Fragment sizes between 4-23 kb were analyzed.

The percentage of homology by RFLP method was calculated by the following formula (Myvaganam *et al.*, 1994).

$$\% \text{ Homology} = \left(\frac{2NS}{N1+N2} \right) \times 100$$

NS = Number of bands shared

N1, N2 = Number of bands from DNA1 and DNA 2, respectively.

6. Agarose Gel Electrophoresis

The percentage of agarose varied from 0.8% to 1%, depending on the resolution required: 0.8% for restricting fragments of chromosomal DNA; 1%

for PCR products and restriction PCR product fragments. The agarose gel was prepared by dissolving the agarose powder (SEAKEM, FMC BioProducts) in 1x TBE buffer to the desired concentration and volume. After melting, the gel was poured into a tray. A comb was put in the melted gel to form the slots. After the gel was completely set (approximately 1 hr at room temperature), the comb was carefully removed and the gel was set in the chamber. TBE (1x) was poured into the electrophoresis tank to just cover the gel.

7. Calculation of the percent homology of N-terminal sequence of *emm* genes.

The N-terminal sequences of *emm* genes of M-nontypable GAS were sequenced (Pruksakorn, unpublished data) and compared with those of standard strains (Whatmore *et al.*, 1994a).

8. Data analysis

The *emm* restriction fragments were compared with each other, with the percent homology of N-terminal sequence of *emm* genes and with the percent homology of RFLP method.