III. PRINCIPLE AND RATIONALE OF STUDY

1. PCR Technology

The polymerase chain reaction (PCR) is a method that can amplify the selected fragment of DNA in vitro. Since it was introduced by Mullis at the Cetus corporation in 1983, the technique has been modified for many uses and has essentially revolutionized molecular biology (Guyer and Koshland, 1989). The general principles and optimization of PCR are summarized below (Saiki; 1989; Innis and Gelfand; 1990; Taylor, 1991).

The reaction requires deoxynucleotides, DNA polymerase, primers, DNA template and buffer containing magnesium. The deoxynucleotides and primers are present in large excess, so the synthesis step can be repeated by heating the newly synthesize DNA to separate the strands and cooling to allow the primers to anneal to their complementary sequences. With each cycle of heating and cooling, the amount of DNA in the region flanked by each primer will increase almost exponentially. Thus, repetition of cycles leads to the selective enrichment of a specific DNA sequence that can be readily manipulated or detected.

The standard reaction

The standard PCR is typically done in a 50- or 100-µl volume. In addition to the sample DNA, the reaction mixture contains 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 100 mg/ml gelatin, 0.25 mM of each primer, 200 mM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP), and 2.5 units of *Taq* polymerase. A few drops of mineral oil are often added to seal the reaction and prevent condensation. The amplification can be conveniently performed in a DNA thermal cycler using the "step-cycle" program set to

denature at 94°C for 20 sec, anneal at 55°C for 20 sec and extend at 72 °C for 30 sec for a total of 30 cycles. These conditions can be used to amplify a wide range of target sequences with excellent specificity.

Primers

There is no set of rules that will ensure the synthesis of an effective primer pair. Fortunately, the following guidelines will help in the design of primers:

1) Where possible, select primers with a random base distribution and with a GC content similar to that of the fragment being amplified.

2) Avoid sequence with significant secondary structure, particularly at the 3′ end of primer.

3) Check the primers against each other for complementarity.

Most primers should be between 20 to 30 bases in length and the optimal amount to use in an amplification will vary, 0.1 to 1.0 µM is generally optimal.

Reaction buffer and magnesium concentration

The free magnesium ion concentration must be adjusted for specific PCR experiments. An optimal concentration is usually between 1.5 and 4.5 mM. Too little free magnesium will result in no PCR product and too much free magnesium may produce a variety of non-specific products.

Deoxynucleoside triphosphate (dNTPs)

The 4 dNTPs are usually present at 50-200 mM each. Higher concentrations may tend to promote misincorporations by the polymerase. The four dNTPs should be used at equivalent concentration to avoid the same problem. Low dNTP concentrations minimize mispairing at nontarget sites and reduce the likelihood of extending misincorporated nucleotides.

DNA polymerase

The most commonly used DNA polymerase is *Taq* DNA polymerase. Its advantages of heat stability and high temperature optimum make it an ideal choice. The recommended concentration range is between 1 and 2.5 units per 100 µl reaction after other parameters are optimized. A high concentration of the enzyme can result in greater production of non-specific PCR products and reduce the yield of the desired target fragment.

Target DNA

For genomic DNA, amounts ranging from 0.05-1.0 µg are typically used for amplifications of a single locus. The DNA should be intact over length which is to be amplified. It is also important that inhibitors of the reactions (e.g. detergent, EDTA, trace of phenol) are not present.

Cycling parameters

PCR is performed by incubating the sample at three temperatures corresponding to the three steps in a cycle of amplification-denaturation, annealing , and extension. The denaturation conditions are 90°-95°C for 30-60 sec. Insufficient heating during the denaturation step is a common cause of failure in PCR reaction. The temperature at which annealing is done depends on the length and GC content of the primer. The estimated annealing temperature for each primer can be calculated by the following formula:

[2(no. of base AT) + 4(no. of base GC)] - 5°C

or [5°C below the true Tm of the primers]

Polymerization usually occurs at 72°C and the temperature usually depends on the length of desired PCR products.

Detection of the reaction product

The product of a PCR should be a fragment or fragments of DNA of defined length. The simplest way to check the product is electrophoresis on 0.8-1% agarose gel containing ethidium bromide. The product can then be visualized under an ultraviolet transilluminator.

2. Restriction fragment length polymorphism (RFLP)

A restriction endonuclease enzymatically digests DNA at a specific ("restricted") nucleotide recognition sequence. The number and sizes of the restriction fragments generated by digesting a given piece of DNA are influenced by both the recognition sequence of the enzyme and the composition of the DNA. The fragments can be separated by size using agarose gel electrophoresis and the pattern can be detected by staining the gel with ethidium bromide and examined it under UV light.

Different strains of the same bacterial species have different RFLP profiles because of the variation in their DNA sequences that alters the number and the distribution of restriction sizes (Arbeit, 1995).

RFLP can be performed by digestion the PCR product with the selected restriction enzyme to create a profile for differentiation of bacterial strains.

3. Rationale of study

GAS are still important pathogenic organisms in developing countries. However, the most appropriate method of GAS typing has not been concluded and the epidemiological study of GAS remains important for understanding the pathogenesis and vaccine development. M protein which is encoded by an *emm* gene shows the polymorphism between the GAS strains and types. Thus, amplification of the gene by PCR and then digestion with restriction enzyme

should give different digested fragment patterns among GAS. These differences may be useful for GAS typing in the future.

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