(alleles) of selected human genes. To study the allelic frequency among the Pakistani population, it is a prerequisite to optimize PCR condition for the given STR primer pairs. Once the PCR conditions will be optimized, then it will grant enormous help to researchers to conduct any inquiry in advance without wasting time on PCR condition optimization. Thus the technology holds promise to decide disputed paternity and to resolve forensic cases that will act as guideline to police and court in capturing culprits.

MATERIALS AND METHODS

A total of two blood samples were collected from Centre of Excellence in Molecular Biology (CEMB), Lahore. We collected 5 mL blood sample in EDTA (7 mM final concentration) in sterile collection tubes. The particulars of individuals were recorded in the prescribed consent form dully signed by the participating volunteer.

One aliquot of 0.7 mL whole blood sample in microcentrifuge tube was preserved at -70 °C for each sample as backup source and remaining whole blood was preserved at -20°C in the sterile tubes and in microcentrifuge tubes.

DNA extraction. *Total genomic DNA was extracted from frozen blood by a modified method of Singer* et al. *(1989) and Grimberg* et al. *(1989). Firstly, 700 uL EDTA blood samples were thawed by keeping at 37 °C for 10 minutes. Then we added 600 µL of 1xSSC and mixed gently followed by spinning at 10,000 rpm for 2 minutes. The WBC's pellet was collected followed by addition of 1 mL of 1xSSC and re-suspended the pellet gently. The above mentioned step was repeated. A total of 375 µL of 0.2 M Sodium Acetate (pH 5.2) 50 µL of 10% SDS and 10-15 µL of 40 mg/mL, of self digested proteinase K (100 mM Tris pH 8.0, 40 mM EDTA, 0.05% SDS) was added. The solution was mixed gently and incubated at 56 °C in a rotating wheel for 2 hours to dissolve the pellet thoroughly. The samples were de-proteinized by adding 120 µL of buffered phenol (pH 8.0) and supernatant was recovered after centrifugation at 14,000 rpm for 5 min. The upper aqueous layer was transferred to a new microfuge tube (used wide mouth tips).*

A total volume of 120 µL phenol/chloroform/isoamyl alcohol (25:24:1) was added to the microfuge tube. The above step was repeated. The DNA was precipitated by chilled 95% ethanol and kept at room temperature for 10 min. The samples were centrifuged for 10 min at 14,000 rpm and decanted supernatant very carefully (did not disturb the pellet). The DNA was re-suspended by adding 100 µL of TE (10 mM Tris, pH 8.0, 1.0 mM EDTA), mixed gently and incubated for 10 min at 56°C (better on a rotating wheel). To it 20 µL of 2.0 M sodium acetate was added and mixed gently and then 500 µL chilled 95% ethanol mixed gently by inverting microfuge. The samples were kept at room temperature for 15 min and centrifuged for 10 min at 14,000 rpm. The supernatant was discarded. The pellet was washed with 70% ethanol and centrifuged for 1 minute. The supernatant was removed and the pellet was dried. The pellet was re-suspended in 50 µL TE (when the pellet was invisible) or 100 µL TE buffer (when the pellet was visible). The tubes were incubated for 2-3h at 56 °C and mixed gently at the end of the incubation period. This protocol can yield 35 µg of genomic DNA per 700 µL whole blood. The concentration of the DNA was measured by preparing 1:100 dilutions of stock DNAs' and measured the absorbance at 230, 260 and 280 nm wavelengths using Hitachi Spectronic 2000 spectrophotometer. Used distilled water for blank and for making dilutions. Absorbance values were obtained by scanning the wavelength-Absorbance curve.

Prepared 100 ng/ µL dilution from stock DNA based on spectrophotometric calculation. Loading 60 and 120 ng of total genomic DNA on 0.8% agarose gel with a known standard confirmed the quality of the DNA.

PCR condition optimisation. *DNA concentration in the working solution of approximately 10 ng/µL in ddH $_2$O was confirmed by spectrophotometric analysis at 260 nm. For the optimisation, concentration of the genomic DNA, 5X buffer without $MgCl_2$, $MgCl_2$, dNTPs (dATP, dCTP, dGTP, and dTTP), STR primers, and Taq DNA polymerase were optimised for the three STRs, respectively. The primers were synthesised from Research Genetics. Taq polymerase, together with 5 X PCR buffer, $MgCl_2$, and dNTPs were synthesised locally. PCR was performed in volumes of 25 µL containing Tris-Cl (pH 8.3), $(NH_4)_2SO_4$, $MgCl_2$, 200-250 uM of dNTP's mix, 10 pmole each primer (reverse and forward), 25 ng of genomic DNA, and 0.5-1 unit of Taq polymerase. Amplification was performed in thermalcycler PTC-100 TM (MJ Research, Inc.) for 30 cycles. For three different loci different concentrations of the reagents were optimized but amount of genomic DNA (25 ng/µL) and concentration of dNTP's were kept constant.*

Estimation of PCR product. *The amplifications of the genomic DNA were confirmed on 2% agarose gel stained with ethidium bromide. Due to small base pair size the alleles were not resolved on the agarose. So loading concentrations for polyacrylamide gel electrophoresis (PAGE) were made according to the brightness of the bands. For exact determination of alleles 5-8% PAGE was used. Gel was run using 0.5x TBE running buffer in Hoefer apparatus followed by silver staining method (Anonymous, 1999).*

RESULTS AND DISCUSSION

During the first experiments with a new PCR system, an optimization is necessary in most cases. In polymerase chain reaction (PCR), annealing temperature and $MgCl_2$ are important parameters, which needs optimization. The concentration of other reagents necessary for PCR were

added as reported by Rahman et al. (2001). Primers were provided by Research Genetics with recommended concentration of 10 pmole/reaction final concentrations.

The optimal amplification depends on several factors including temperature profile, and the concentration of reagents in the buffer. The most straightforward way of optimizing a PCR with a given primer pair is to change the concentration of $MgCl_2$ or the annealing temperature.

Optimization of $MgCl_2$ concentration. *In most reports, the concentrations of the single compounds in the PCR buffer mix are basically the same (Saiki et al., 1988). Briefly: 50 mM KCl, 10 mM Tris (pH 8.3), 2, 5 mM $MgCl_2$, 1 µM of each primer, 200 µM of each mononucleotide, 200 µg/mL gelatin and 2 units/100 µL of Taq Polymerase. However, in our case we use buffer with $(NH_4)_2SO_4$, Tris (pH 8) and Tween 20. Tween 20 removes inhibition from SDS, which is used in cell lysis. It is used between 0.5 - 2% or under. Similarly increased concentration of Tris in the buffer is reported to decrease the specificity (Blanchard et al., 1993), and therefore, the Tris concentration can also be used to optimize the PCR (Rasmussen et al., 1993).*

Modest concentrations of salts stimulate the synthesis rate of Taq Polymerase but higher salt concentrations are increasingly inhibitory (Gelfand, 1989). The Mg^{2+} binds tightly to the phosphate sugar backbone of nucleotides and nucleic acids, and variation in the $MgCl_2$ concentration has strong and complex effects on experiments involving nucleic acid interactions. Variations of the Mg^{2+} concentration below 4 mM can improve the performance of PCR by affecting the specificity (lower concentrations raise specificity, higher concentrations lower the specificity) (Blanchard et al., 1993). The effect of variations in the dNTPs concentration is closely related to the Mg^{2+} concentration, due to the interaction between mononucleotides and the Mg^{2+}. A higher concentration of Mg^{2+} allows amplification with a higher concentration of dNTPs, that is not seen at lower Mg^{2+} concentrations (Blanchard et al., 1993). In the present study dNTPs concentration was kept constant at 200 µm and $MgCl_2$ concentrations were varied between 1.5 to 3.0 mM. For the two loci D3S1358 and vWA 3 mM and for D16S539 1.5 mM of the $MgCl_2$ was found optimum to amplify the expected size of PCR product. 2-2.5 units of Taq Polymerase in 100 µL of reaction are normally used. Concentrations higher than 4 units/100 µL can generate non-specific products and may reduce the yield of the desired product (Saiki, 1989). However in the present study, 1 unit/25 µL reaction was used to amplify the loci without non-specific products.

Annealing temperature optimization. *Annealing temperature is one of the most important parameters that need adjustment in the PCR reaction. Moreover, the flexibility of this parameter allows optimization of the reaction in the presence of variable amounts of other ingredients (especially template DNA). The normal range of annealing temperature is 37-75 °C. It appears that stringent initial conditions mean less non-specific product, especially when amplifying from eukaryotic genomic DNA. The initial denaturation temperature 94 °C for 5 min and extension temperature 72 °C for 45 sec was considered best as polymerases add 2000 nt/min (Henegariu et al., 1997). In the present studies, denaturation temperature of 94 °C for 30 sec, annealing time was 30 sec (annealing temperature was kept variable to optimize it) and extension temperature 72 °C for 45 sec. A total of 30 cycles were exercised of the profile followed by extension of 5 min at 72 °C.*

In the present studies, two loci vWA and D3S1358 were successfully amplified at 57 °C. However, non-specific products were observed at 8% PAGE. Raising the temperature up to 59 °C controlled their non-specificity. STR locus D16S539 was amplified at 57 °C but due to non-specific products, the annealing temperature was raised up to 61 °C. In this case duplex formation was observed at 6% PAGE which was controlled by increasing concentration up to 8% PAGE.

Band stuttering is the common problem with STRs loci. Stutter bands (sometime shadow bands) were amplified with the three STRs primer pairs. The amplification of stutter bands was also observed at vWA STR locus (Weber & May, 1989; Sprecher et al., 1996). Band stuttering is common in dinucleotide repeats and produced due to slippage mechanism of the polymerase during amplification (Luty et al., 1991).

Optimization of the PCR conditions and reagents was performed on two random samples and these can be used to generate population data for 100 randomly selected individuals. Such kind of population data is necessary for the establishment of Forensic science in Pakistani courts. This study could help to resolve disputed paternity by using the optimized conditions for the three STR loci. Furthermore, additional STRs primer pairs are needed to explore the polymorphism at other loci to increase the scope of the DNA typing technology.

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