International Journal of Modern Engineering Research (IJMER)

www.ijmer.com Vol.2, Issue.3, May-June 2012 pp-996-1000 ISSN: 2249-6645

Optimization of In-House PCR-SSP Technique for HLA B27 detection in saurashtra patients

Dharashree Vanvi[1], Pooja Raja[2], Soham Trivedi[3]

[1]*Biotechnology Engineering Department, V.V.P. Engineering college, Rajkot, India*
[2]*Biotechnology Engineering Department, V.V.P. Engineering college, Rajkot, India*
[3]*Biotechnology Engineering Department, V.V.P. Engineering college, Rajkot, India*

ABSTRACT

The HLA system is studied from various viewpoints including, organ transplantation, population genetics, disputed parentage, disease association studies and to answer basic questions of immunobiology. HLA-B27 is a MHC class 1 molecule that is strongly associated with the Seronegative Spondyloarthropathies (SSA). Survey suggested frequent cases of SSA in Saurashtra and many blood samples of suspected Sero-negative patients needed to be analysed on daily basis for the detection of HLA-B27. The techniques that were generally employed in routine clinical practice were based on Microlymphocytotoxicity assays. Some laboratories generally send such samples to other states that adds to the overall cost for the exact diagnosis. Moreover, there were various limitations of these serological approaches and false-negative results are a frequent problem for HLA-B27 typing. Thus there was a need to develop and encourage PCR based techniques for the detection of HLA-B27 that relies on specific DNA sequences, can be a direct determination of allelic DNA and thus serve as a ideal test for HLA-B27. PCR-SSP technique utilizes oligonucleotide primers to start the PCR that have sequences complimentary to known sequences, which are characteristic to certain HLA specificities. For e.g. the primers those are specific to HLA-B27, will not able to initiate the PCR for HLA-DR17. Using a set of different PCR's each with primers specific for different HLA antigens does typing. Thus the present studies will revolutionize the Saurashtra immuno-diagnostics and the optimized In-House PCR-SSP technique can be used in parallel with serological typing in various diagnostics laboratories across the state.

Keywords **- HLA B27, PCR SSP, Serology, SSA,**

I. INTRODUCTION

The HLA-B27 is a major histocompatibility complex class 1 molecule that is strongly associated with the disease ankylosing spondylitis (AS) and related spondyloarthritis (SpA). There is almost 90% association of HLA-B27 with ankylosing spondylitis [1]. The last two decades have seen a massive growth in the application of DNA technology in Histocompatibility and Immunogenetics [2]. DNA based HLA typing is fast replacing conventional Microlymphocytotoxicity based method, which has been regarded as the gold standard. Many laboratories in India have already switched over to molecular methods, as the results are far superior [3]. This is so as the serological methods for HLA class I detection is hindered in many cases by serological cross reactivity and decrease in expression of HLA antigens, particularly in patients with different hematological tumors [4]. Serological typing is also difficult in those cases of poor cell viability and also in refuting phenotypic homozygosity. Sometimes due to lack of availability of reliable commercial antisera laboratories need to screen to find their own reagents and exchange these with other such-minded laboratories. This can mean difference in the quality of reagents between laboratories and thus can lead to some laboratories producing more accurate results than others. In addition these reagents could not be replenished. Whilst serology performed adequately in HLA–B27 determination can prove unsatisfactory [2]. The principle of PCR-SSP is that each individual allele (making up a serological specificity) is amplified by a primer pair exactly matched to that region. Amplification of HLA loci with PCR-SSP has thus proved to be a rapid and accurate method for genotyping HLA-A, -B and -C alleles [5,6,7,8] and indicates that HLA typing by serology may not be sufficiently reliable [9,10,11].

Currently, most HLA-B27 testing is performed with surface antigen tests that require viable cells for analysis. Two commercially available antibodies are commonly used but they cross-react with other HLA-B surface antigens, especially B7 and B40 [12]. The cross-reactivity of these antibodies can compromise the accuracy of the results generated in the antigen assays. With DNA sequence information available for alleles of the HLA system and the development of molecular biological techniques, it is possible to tissue type for allelic differences in HLA genes. The SSP based HLA typing is an economical, rapid, precise, technically simple and reproducible method. In 1994 M. Bunce described a comprehensive HLA-B PCR-SSP typing system based on available HLA nucleotide sequences which can detect all serologically defined antigens in most heterozygous combination in 48 one-step PCR reactions. Serological approach recognizes immunological relevant antigenic differences, in contrast DNA based testing may also identify differences of little biologic relevance [13]. Joannis Mytilineos in 1998 demonstrated an impressive advantage of the PCR-SSP method for HLA-A and HLA-B locus typing over serological typing in black individuals. Mehrnaz Narouei-Nejad et al in 2003 reported that there was a 31% difference in the SSP typing and serological methods. Reports suggest that HLA-DR SSP typing is far superior and almost 90% of alleles are identified as compared to 40% by serology [14]. In 2005 Michael T. Seipp reported that using DNA sequencing as standard, the sensitivity and specificity of PCR were 99.6 and 100.0, and those of the single antigen assay were 98.2 and 97.6. In the same year Derek Middleton reported that implementation of a DNA technique for HLA-

www.ijmer.com Vol.2, Issue.3, May-June 2012 pp-996-1000 ISSN: 2249-6645

C alleles reduced the homozygosity role in stem cell donor from 50% by serology to 21%. Moreover typing of non-Caucasians by serological methods has always proved difficult when the majority of HLA sera available have been derived from Caucasian donors. Thus as the PCR-SSP technique is more strategic for HLA typing as compared to the serological approach, the present study was carried out to optimize an In-House PCR-SSP Technique for routine HLA-B27 Typing and to compare it with the traditional serological technique.

II. MATERIALS AND METHODS

DNA Extraction: Salting-out methodology was carried out for extracting the DNA. This method avoids using phenol and chloroform by using high salt concentrations to remove proteins. It is rapid, safe and inexpensive. Average yields are similar to that obtained with the phenol-chloroform extraction procedure (50- 200 ug), and the quality of DNA is excellent.

Principle: In eukaryotic cells the bulk of DNA is localized in the nucleus, which is separated from the rest of the cell sap by a complicated membrane structure. DNA Extraction by this method involves the following steps: 1). Cell Breakage: Chemical (detergents) and /or enzymatic procedures are best suited for opening cells and obtaining intact DNA. Detergent can solublize lipids in cell membranes resulting in gentle cell lysis. In addition, detergents have an inhibitory effect on all cellular DNAses and can denature proteins, thereby aiding in the removal of proteins from the solution. Therefore lysis of cells was performed using anionic detergents such as Sodium dodecyl sulfate (SDS). Moreover buffers like Red Cell Lysis Buffer (RCLB) was employed to facilitate the extraction process. Haemoglobin (and other pigments) inhibits restriction enzymes and TAQ polymerase. Hence washing with RCLB was carried out. 2). Removal of Protein: Proteins can be removed from DNA preparations using a protease that can digest all proteins. Two such enzymes in use are proteinase k and pronase. Both enzymes are very stable, and devoid of DNAse contamination, making them safe to use in the purification of nucleic acids. They can digest intact and denatured proteins and do not require any co-factors for their activities. An Special buffer Nucleas Lysis Buffer (NLB) was used to salt out proteins. NLB comprises of NaCl used to lyse all cells. High salt concentration aids the salting out of the proteins. DNA is stabilised and remains as a double helix with the utilisation of such buffers. 3). Concentration of the DNA: Ammonium acetate was utilised for the removal of heavy metals, detergents. Finally, Isopropanol and 70% ethanol (final concentration) in presence of the appropriate concentration of salts were used for DNA precipitation. Such precipitation is based on the phenomenon of decreasing the solubility of nucleic acids in water. It is also reasoned that low temperature and the presence of salts further lower the activity of water molecules thereby facilitating more efficient DNA precipitation.

Requirements: • Red Cell Lysis Buffer: Add 14.4 ml of 1 M NH4Cl and 100µl NaHCO3 and make up the final volume upto 100 ml with distilled water. Adjust pH upto7.5 to 8. • Nucleas Lysis Buffer: Add 4 ml of 5 M NaCl, 200 µl 0.5 Na2EDTA and 250 µl of 2 M Tris-HCL and make up the final volume upto 50 ml with distilled water. • 10% SDS – 10gm SDS in 100ml DW. • 20 mg/ml proteinase k. • 4 M Ammonium Acetate. • Chloroform. • Isopropanol. • Chilled absolute alcohol . • Chilled 70% ethanol

Procedure: Only fresh EDTA Samples were used for DNA Extraction.

Day 1: • 45 ml of the Red Cell Lysis Buffer (RCLB) was mixed with 5 ml of blood (EDTA) in falcon tubes that were mixed gently 2 to 3 times and left at room temperature for 10-20 minutes. • Then the mixture was centrifuged at 1800 rpm for 20 minutes. • After discarding the supernatant, the pellet was then suspended in 25 ml of RCLB and was left for 10-20 minutes at room temperature. • Again the mixture was centrifuged at 1800 rpm for 20 mins. • Now after discarding the supernatant, the pellet was suspended in 3 ml Nucleas Lysis Buffer. 200 µl of 10% SDS and 20 µl of Proteinase K Enzyme (10 mg/ml) were added respectively. • The mixture was then incubated at 37ºC - 42ºC for 4 hours to overnight.

Day 2: • After the incubation, 4 ml of Ammonium Acetate and 3 ml of Chloroform were added and were mixed nicely by slight vortexing. • The mixture was then centrifuged at 4000 rpm for 30 minutes. •The supernatant was then taken out carefully and mixed with Isopropanol (5-10 ml). • Now the DNA was taken out and was washed with 70% Ethanol twice. • It was then dried and dissolved in distilled water.

Determination of the purity of DNA : Ideally for samples of limited cell mass such as mononucleated bone marrow specimens The micro-sample capability of the NanoDrop provides accurate DNA quantitation with minimal consumption of sample, which is critical for HLA typing, The DNA quantification and its purity can be assessed using an NanoDrop device that employs a sample retention system requiring only 1ul of sample for DNA absorbance spectral analysis, providing a calculated DNA concentration and purity ratios. A ratio of 1.8 is generally accepted pure for DNA. If the ratio is appreciably lower it may indicate the presence of protein or other contaminants that absorb strongly at or near 280 nm. The DNA template can then be amplified using sequence specific primers for sequence specific PCR-SSP. This device can be integrated into the HLA-B27 detection workflow in the following manner: DNA extracted from the EDTA samples by the salting-out procedure, then quantified on the NanoDrop to ensure adequate template prior to amplification. However due to unavailability of the instrument in the present studies only the quality of DNA was assessed.

Checking the quality of DNA: 500ng of DNA was run on a 1 % agarose gel (low EEO) for 1hour in 0.5x TBE buffer in submerged electrophoresis chamber. A single band near the well after ethidium bromide staining and viewing on an Ultraviolet (UV) transilluminator (Biorad, Italy) indicates the presence of high molecular weight DNA. If a smear is seen, it indicates that the samples have been degraded. (2 µl DNA + 5 µl D/W + 2 µl Bromophenol dye).

www.ijmer.com Vol.2, Issue.3, May-June 2012 pp-996-1000 ISSN: 2249-6645

Polymerase Chain Reaction with Sequence Specific Primers (PCR-SSP): Allelic variability has traditionally being determined phenotypically, but often may be more accurately investigated via molecular biology techniques either indirectly by Restriction Fragment Length Polymorphism analysis (RFLP) or directly by PCR based techniques. In the determination of allelic polymorphism by PCR amplification with sequence specific primers (PCR-SSP), Oligo-nucleotide primers are designed to obtain amplification of specific alleles. The typing method is based on the principle that a completely matched primer will be more effeciently used in the PCR reaction than a primer with one or several mismatches especially in the first critical cycles (Figure 1). Thus the specificity of the typing system is part of the PCR reaction, which reduces post-amplification processing of samples to a minimum, making the technique more attractive for tissue typing in clinical practice that includes donor-recipient matching for transplantations and HLA-B27 determination. Assignment of alleles is merely based on the presence or absence of amplified product, which can be detected by agarose gel electrophoresis. (Figure 1)

Amplification primers: Forward Primer: 5'-GCTACGTGGACGACACGCT 3', Reverse Primer 1:5'-CTCGGTCAGTCTGTGCCTT-3', Reverse Primer 2: 5'-TCTCGGTAAGTCTGTGCCTT-3', Control primer sequences: HGH- Forward Primer - 5'-TGCCTTCCCAACCATTCCCTTA-3', HGH- Reverse Primer - 5'-CCACTCACGGATTTCTGTTGTGTTTC-3'

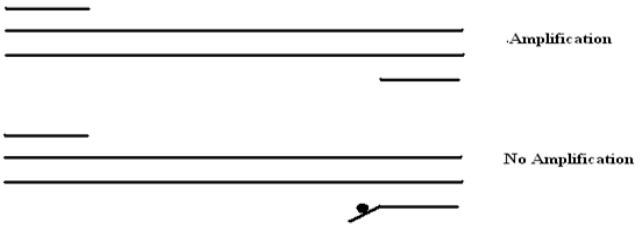

Figure 1: Illustration of the principle for allele specific PCR Amplification - A completely matched primer will be more efficiently used in PCR Reaction than a primer with one or several mismatches allowing the discrimination of alleles differing by a single base pair mismatches, denoted by ●, should be placed in the extreme 3' end of primer as the Taq Polymerase lacks 3' – 5' proof reading exonuclease activity.

Reagent Mixes: A total of 100 µl of mixes was divided into 10 PCR tubes each containing 10 µl of reagent mix. Two out of 10 tubes were of positive control and negative control.

Composition of reagent mixes for HLA-B27 typing: DDW – 62 µl, 10X PCR complete buffer – 10 µl, MgCl2 (25mM) – 2 µl, dNTP (10mM) – 0.8 µl, Primer 1 (10 p mol/ml) – 5 µl, Primer 2 (10 p mol/ml) – 5 µl, Primer 3 (10 p mol/ml) – 5 µl, Control 1 – 5 µl, Control 2 – 5 µl, Taq – 1 µl, DNA (50-100 ng) – 1µl , Total – 100 µl.

Cycle parameters: Denaturation 94°C – 5mins 30 cycles of • 94°C for 1 min, • 65°C for 2min, •72°C for 1min, • Final extension of 72°C for 10 min.

Visualization of amplifications by agarose gel electrophoresis: The absence or presence of PCR Products was visualized by agarose gel electrophoresis: 1) A 2 % (w/v) agarose gel in 0.5x TBE Buffer (89 mM Tris Base/89 mM Boric Acid/2 mM EDTA, pH 8) was prepared. • Agarose was dissolved by boiling, then cooled to 60ºC. Ethidium Bromide (0.5 µg/ml gel solution) was then added. • A 4 mm thick gel with 3 mm wide slots was casted and was allowed to set for 10 – 20 mins. 2) The PCR Reactions were then loaded on the gel after addition of a 2.5µl loading buffer (30% v/v glycerol stained with bromophenol blue and xylene cycnol) to PCR tube. 3) The gels were run in 0.5x TBE buffer (without buffer recirculation). Minigels (8.5 × 10 cm) were allowed to run for 10 to 15 minutes and large gels (20 × 20 cm) for 20 to 30 minutes at 7 to 8 V/cm. 4) Finally the gel was examined under Ultra-Violet Illumination and the results were documented by photography.

III. RESULTS

The results of 10 samples typed by serology and PCR-SSP technique is shown in the following table. (Table 1)

Table 1: Results of Serology and PCR-SSP

	HLA B27 positive	HLA B27 negative	Total
Serology	2	8	10
PCR-SSP	3	7	10

The following table shows the comparision of the two techniques for 10 samples typed by PCR-SSP and Serology. (Table 2)

Table 2: Comparison of Serology and PCR-SSP

Serology	PCR-SSP	Results	Percentage
Positive	Positive	2/10	20% TP
Positive	Negative	1/10	10% FN
Negative	Positive	1/10	10% FP
Negative	Negative	7/10	70% TN

(TP – true positives, FN – False negatives, FP – false positives, TN –true negatives)

Thus it was found that 2 out of 10 (20 %) samples were HLA B27 positive by microlymphocytotoxicity and 3 out of 10 (30 %) were HLA B27 positive by PCR-SSP. The sensitivity of test was obtained as 66.66% and its specificity was 87.5%. Furthermore, the positive predictive value was found to be 66.66% and its Negative predictive value was 87.5 %. The Accuracy or efficiency of the test was calculated to be 81.81%

The false-positive rate was found to be 12.5 % and the false-negative rate was found to be 33.33 %. Based on the sensitivity and specificity of test the likelihood ratio of a positive test result (LR+) was calculated as 0.77 and the likelihood ratio of a negative test result (LR-) was 0.75. Moreover, The cost analysis of the various methods for HLA-B27 typing was carried out. The following table shows the comparision of the cost of methods (Table 3).

www.ijmer.com Vol.2, Issue.3, May-June 2012 pp-996-1000 ISSN: 2249-6645

Table 3: Cost analysis of various methods for typing

Method for HLA B27 typing	Cost/test
Serology: In House tray	Rs.500/-.
Serology: Commercial kit	Rs.600/-
PCR-SSP: In House (Including DNA Extraction)	**Rs.150/-**
PCR-SSP: Commercial kit (Excluding DNA Extraction)	Rs.600/-
Flow cytometry	Rs.1200/-
PCR-SSOP	Rs.2000/-

The sensitivity of the PCR-SSP technique was tested using B27 positive DNA samples with concentrations ranging from 0.1 to 100 ng/µl. It was found that the lowest DNA concentration at 1.0 ng/µl showed the faint positive bands of the control and B-27 specific primers. However, the bands were most clearly detected at the concentration ranging from 10 to 100 ng/µl. The following figure depicts the gel photograph of genomic DNA.

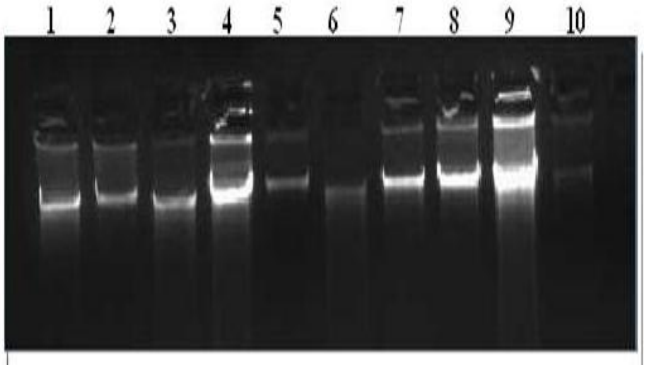

Figure 2: Gel photograph showing genomic DNA

As depicted in the Figure 2 sample 3 and sample 4 can be clearly visualized as HLA-B27 positive samples. Here the Sample 1 is a positive control for HLA-B27 and sample 2 is the negative control.

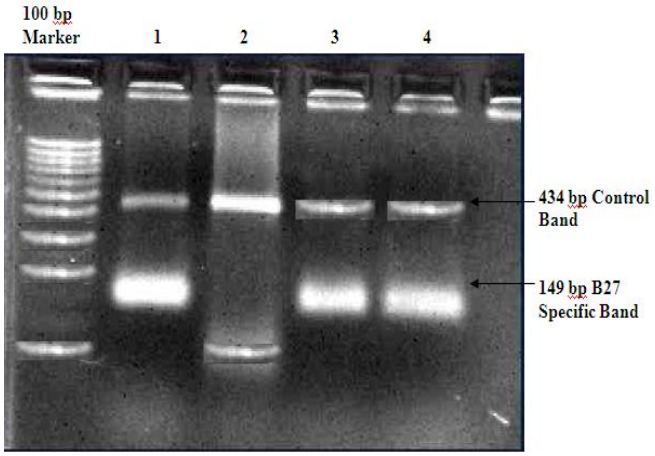

Figure 3: Gel photograph showing B27 specific band with the control bands

IV. DISCUSSION

Unlike recently, Serological typing had been the primary technique used for HLA Class I analysis. But due to the advent of molecular biology, the last twenty years has seen an exponential growth in the application of DNA technology to the field of Histocompatibility and Immunogenetics (H&I). DNA based typing focuses on defining differences in genes and may identify differences of little biologic relevance whereas immune based testing by serology or cellular reactions is related to known immunological differences [14]. Some limitations for serological tests include lack of availability of specific antisera for all alleles of HLA-B27 because the number of known HLA-B27 alleles has increased. Moreover, the test must be performed within 6 hours after drawing the blood, and the amount of blood used must not be less than 5 ml [15]. Serological typing can also be difficult in those cases of poor cell viability or poor expression. Moreover lots of expertise is inevitable in confirming phenotypic homozygosity. In addition when the HLA antisera available have been derived from Caucasian donors typing of non-Caucasians by serological methods can prove to be .difficult [2]. Serology is also hindered in those patients owing to immunosuppression due to serological cross reactivity. In different situations, such as aplastic anemia and leukemia, when the expression of HLA antigens on the cell surface is down-regulated, it is impossible to type by serological methods [4]. Most commercial typing sera are obtained from Caucasian population and some alleles peculiar to the India population may not be identified [3]. Moreover the weak reactions may be due to Platelet contamination that can deplete antibody and complement. Also Erythrocyte contamination can make microscopic evaluation difficult because of visual confusion with negative lymphocytes. False-negative serological HLA-B27 typing results may be due to altered antigenic epitopes but it can be detected by polymerase chain reaction [16]. Thus, molecular typing techniques, which give more accurate results, have replaced or supplemented by the Microlymphocytotoxicity.

Most genotyping methods are based on the group-specific amplification of HLA-B alleles by the PCR. The PCR-SSP is more widely used to detect HLA-B27 alleles. Additionally, a real-time PCR method for the detection of HLA-B27 alleles was described [17]. Availability of SSP kits for tissue typing with advantages at a cost similar to serology based dry kits and changing donor pattern have made it imperative for most commercial labs to switch over to DNA based methods. In the present studies an In-House PCR-SSP technique was developed for confirming the results obtained by serology at our laboratory for the HLA-B27 detection. A recent study in the detection of all HLA-B27 alleles, and their methods involved an initial screening with two sequence-specific PCRs, followed by two additional PCR amplifications in order to identify a few rare subtypes of B*27, B*4202 or B*7301, which is not convenient for routine testing (Zino E, 2004).

V. CONCLUSION

In the present studies the PCR-SSP technique developed provides accurate results and can be employed for routine analysis. It is more reliable technique than the serological

approach as effective detection of HLA-B27 antigen can be visualized by the B27 bands. The HLA-B27 testing using Microlymphocytotoxicity gave a false-positive rate of 12.5% and the false-negative rate of 33.33%. The cause of false-negative results may be due to improper proportion of cells and antiserum, and possibly a weak reaction or insufficient mixing (Milken SL, 1987). Furthermore the cost of this In- house developed PCR-SSP technique as compared to other approaches is merely Rs.150 per test. This technique is about 50 times less costly then that of the imported commercial PCR-SSP test kit by few labs.

The technique is superior to serology as fresh and aged samples can be tested and only a small amount of blood is used. The results can be obtained even with a low concentration of DNA (0.1 ng/ml). Moreover the PCR-SSP technique developed in the present study is reliable, simple, convenient, and more cost effective for routine laboratories. The technique is fast and easy to perform and to handle specimens, because the viable cells necessary for serological typing are not needed. Thus such PCR-SSP techniques can be employed by the Rajkot Diagnostics laboratories as a part of routine clinical practice for exact diagnosis of Seronegative Spondyloarthritis and it can be envisaged that in the near future HLA-typing by serology will be replaced by PCR-SSP in routine clinical practice.

ACKNOWLEDGEMENTS

We would like to acknowledge the management of V.V.P. Engineering college, Principal Dr. Sachin Parikh, Head of Biotechnology Department Mr. Dharmesh Sur that gave us an opportunity to carry out the work. Our sincere thanks to entire staff members of the biotechnology department for their consistent support and encouragement throughout the present work.

REFERENCES

[1] Michael T. Seipp, Maria PCR Melting Assay and Two Flow Cytometric Antigen Assays, Cytometry, Part B (Clinical Cytometry), 63B, 10–15.

[2] Derek Middleton (2005), "HLA Typing from Serology to Sequencing Era", Iranian Journal of Allergy, Asthama and Immunology, 4(2), 53-66.

[3] M.N. Mishra, H. Mani, A.S. Narula, V.K. Saxena (2004), "HLA Typing – A Comparison of Serology and DNA Techniques", Int J Hum Genet, 4(2),151-153.

[4] Mehrnaz Narouei-Nejad, Farideh Khosravi, Abdolali Danesh, Behrooz Nikbin (2003), "PCR-SSP Versus Serology in Typing of HLA-A, -B and -C in Iranian patients", Arch Iranian Med , 6 (1), 23 – 28.

[5] Cadavid LF, Watkins DF (1997). "The duplicative nature of the MHC class I genes: an evolutionary perspective". Eur J Immunogenet.; 24: 313 – 22.

[6] Cereb N, Maye P, Lee S 1995. Locus-specific amplification of HLA class I genes from genomic DNA: locusspecific-sequences in the first and third introns of HLA-A, -B and -C alleles. Tissue Antigens.; 45: 1 – 11.

[7] Guttridge MG, Burr C, Klouda PT 1994. Identification of HLA-B35, B53, B18, B5, B78, and B17 alleles by polymerase chain reaction using sequence specific primers (PCR-SSP). Tissue Antigens. 44: 43 – 6.

[8] Bidwell J.1994. Advances in DNA-based HLA typing methods. Immunol Today. 15: 303 – 7.

[9] Adorno D, Canossi A, Papola F, et al 1997. Comparison between HLA class I PCR-ARMS and serologic typing in cadaveric kidney transplantation. Transplant Proc. 29: 1923 –5.

[10] Bunce M, Welsh KI 1994. Rapid DNA typing for HLA-C using sequence-specific primers (PCR-SSP): identification of serological and non-serologically defined HLA-C alleles including several new alleles. Tissue Antigens. 43: 7 – 17.

[11] Lo YM, Patel P, Newton CR, et al 1991. Direct haplotype determination by double ARMS: specificity, sensitivity and genetic applications. Nucleic Acid Res. 19: 3561 – 7.

[12] Levering WH, Wind H, Sintnicolaas K, Hooijkaas H, Gratama JW 2003. Flow cytometric HLA-B27 screening: cross-reactivity patterns of commercially available anti-HLA-B27 monoclonal antibodies with other HLA-B antigens. Cytometry ,54B, 28–38.

[13] Hurley KC, Tang T, Jennifer NG, Hartzman RJ 1997. HLA typing by molecular methods. Manual of Laboratory and Clinical Immunology. 5, 1098-1111.

[14] M.N. Mishra, H. Mani, A.S. Narula, V.K. Saxena 2004, HLA Typing – A Comparison of Serology and DNA Techniques, Int J Hum Genet, 4(2),151-153.

[15] O. Nathalang, S. Tantimavanich, K. Nillakupt, P. Arnutti, C. Jaruchaimontree 2006, HLA-B27 testing in thai patients using the PCR-SSP technique , Tissue Antigens, 67, 233-236.

[16] Kirveskari J., Kellner H., Wourela M et al 1997. False-Negative serological HLA-B27 Typing results may be due to altered antigenic epitopes and can be detected by polymerase chain reaction. Br. J. Rheumatol, 36, 185-189.

[17] Sylvain K, Aurelie H, Marc M, Christophe R , 2004. Rapid screening for HLA-B27 by a Taqman-PCR assay using sequence specific primers and a minor grove binding probe, a novel type of Taqman probe. J Immunol Methods, 287, 179-186.

[18] O. Bishop, Neurophysiology of binocular vision, in J.Houseman (Ed.), *Handbook of physiology*, 4 (New York: Springer-Verlag, 1970) 342-366.