Volume : 2 | Issue : 8 | Aug 2013 • ISSN No 2277 - 8160

Research Paper | Medical Science

Rapid HLA-B27 Test with Real-Time PCR in Suspected Ankylosing Spondylitis Iraqi Patients

Prof Dr Sadiq Al-Mukhtar	Professor, (MB Ch B, FICMS)Dean Of Al-Kindi college of medicine
Prof Dr. Batool Mutar Mahdi	Professor, (M.B.Ch.B., M.Sc.FICMS.Clinical Immunology) Al-Kindi College of Medicine
Dr Hyam Raouf	Asst Lecturer, (MB CH B MSc) HLA typing research Unit
Dr Haider Hashim Zalzala	Lecturer, (MB CH B, FICMS)HLA typing research Unit
Laheeb Ali Abid	Pharmacist, (BS, Diploma) HLA typing research Unit
Chemist Zena Nehad	Chemist, (BS) HLA typing research Unit

ABSTRACT

*HLA-B27 is a MHC class 1 molecule that is strongly associated with the Ankylosing Spondylitis and Seronegative Spondyloarthropathies. Various methods have been developed for the identification of the HLA-B27 allele. The PCR-based HLA-typing methods, including the standard PCR with sequence-specific primers (SSP), sequence specific oligoneuclotide (SSO) have become widely used alternatives to serology. This study is to assess a real-time TaqMan PCR assay for genotyping HLA-B*27 in the Iraqi patients with suspected Ankylosing Spondylitis. The study consisted of 50 Iraqi Arab Muslims patients suspected to have ankylosing spondylitis consulted Orthopedic and Rheumatology department in Al-Kindi Teaching Hospital in Baghdad from September 2012 to May 2013. The second control group consisted of twenty apparently healthy volunteers' age matched with first group from staff employees served as controls. Blood samples have been obtained from them and genomic DNA was extracted using using reliaprep spin column kit (Promega,USA). Real-time PCR reactions were performed using an Exicycler TM 96 Real-time Quantitative Thermal Block (Bioneer, Daejeon, Korea) and the AccuPower, HLA-B27 real-time PCR kit (Bioneer, Daejeon, Korea). The age of patients in this study were range from 16-35 years with a mean age of 28.2 ± 3.5 years. Control ages range from 17-37 years with a mean of 30.28 ± 2.87. All positive samples had Ct FAM values <28. All samples had Ct TAMRA values <26. A Total of 50 clinical samples who were suspected to have AS and twenty controls were used to compare the real time Taq Man PCR with traditional PCR-SSO method. Ten out of fifty (20%) of patients showed positive HLA-B27 results and did not detected in control group. This results gave concordant with PCR-SSO results.Real-time PCR methods appear to be reliable and very fast in the detection of HLA-B27 and could be potential alternatives to conventional PCR and serology methods.*

KEYWORDS: Real time, PCR, Ankylosing Spondylitis

Introduction:

HLA-B27 is strongly associated with ankylosing spondylitis (AS) with a relative risk of 95%, which is the highest value of relative risk among all HLA-disease associations (1). A strong association has been found between the subtypes B*2705, B*2704, B*2702, and B*2707 and AS, whereas other subtypes are not associated with AS or may provide protection against the disease (2). As typing for HLA-B27 is routinely performed by serological methods, such as the microlymphocytotoxicity test, flow cytometry, and enzyme immunoassays used for typing of HLA-B27, these tests lack specificity for the antigens that cross-react with HLA-B27, such as HLA-B7, false-positive results can be generated (3,4). Therefore, several more accurate molecular methods have been developed for HLA-B27 genotyping. The PCR-based HLA-typing methods, PCR with sequence-specific primers (SSP), have become widely used alternatives to serologic methods in clinical practice. Molecular detection of HLA-B27 is traditionally based on allele specific amplification of exon 2 or exon 3 by PCR, followed by gel analysis.; but these techniques are long and arduous. The disadvantages of this method are long processing time and the requirement for post-PCR manual procedures(5). This have been overcome by the introduction of real-time PCR. Therefore, real-time PCR, which allows the simultaneous amplification and detection of a specific DNA target, it is a striking alternative method to conventional PCR-SSP. This single tube PCR method for the detection of HLA-B27 should be particularly suitable for the routine analysis of large numbers of samples in the laboratory(6). The duplex real-time TaqMan PCR approach appears to be a reliable, sensitive, rapid and high-throughput method to genotype HLA-B*27 in the population(7).

In this study we try to assess "B27" associated with ankylosing spondylitis using real time PCR in Iraqi patients.

Patients and methods:

The study consisted of 50 Iraqi Arab Muslims patients suspected to have ankylosing spondylitis consulted Orthopedic and Rheumatology department in Al-Kindi Teaching Hospital in Baghdad from September 2012 to May 2013. The study took place at the HLA typing research Unit, Al-Kindi College of Medicine, Baghdad University. The age of patients in this study were range from 16-35 years with a mean age of 28.2 ± 3.5 years. Control ages range from 17-37 years with a mean of 30.28 ± 2.87.

The exclusion criteria were patients previously had prior or recent history of rheumatoid arthritis, autoimmune diseases and other metabolic diseases. Those patients had been previously typed with the conventional PCR-SSO method using SSO HLA type B update pluse Kit and Master mix for HLA-B multiplex plus Kit -Innogenetics-Belgium using automated method by Autolipa-48 –Innogenetics-Belgium..

The second control group consisted of twenty apparently healthy volunteers' age matched with first group from staff employees served as controls.

The Ethical Committee of Al-Kindi College of Medicine, Baghdad University, approved the study and all samples were obtained with informed consent in accordance with the Al-Kindi Teaching Hospital Declaration.

Volume : 2 | Issue : 8 | Aug 2013 • ISSN No 2277 - 8160

Blood samples have been obtained from them and genomic DNA was extracted using using reliaprep spin column kit (Promega,USA). Real-time PCR reactions were performed using an Exicycler TM 96 Real-time Quantitative Thermal Block (Bioneer, Daejeon, Korea) and the AccuPower, HLA-B27real-time PCR kit (Bioneer, Daejeon, Korea). The following 2 detection probes were used: an HLA-B27-specific probe and a glyceraldehyde- 3-phosphate dehydrogenase (GAPDH)-specific probe, which were labeled with a fluorescent reporter dye FAM (6-carboxyfluorescein) and TAMRA (6-carboxytetramethyl- rhodamine) at the 5'end, respectively and a fluorescent quencher dye BHQ (black hole quencher) at the 3'end, for both. The real-time PCR reaction was performed in a total volume of 50 mL with the HLA-B27 Pre- Mix (Bioneer, Daejeon, Korea), which contains HLA-B27- specific primers targeting exon 2 of HLA-B gene, GAPDH specific primers, dual-labeled fluorogenic probes, DNA polymerase, deoxynucleotide triphosphates (dNTPs), and stabilizer, with 5 mL of template DNA and 45 mL of 0.1% diethyl pyrocarbonate-treated distilled water. The amplification protocol for this reaction consisted of an initial denaturation step at 95 Co for 5 min, followed by 35 amplification cycles of denaturation for 5 sec at 95 Co , annealing and extension for 15 sec at 65 Co . During the PCR, the HLA-B27 target region and an internal control region for GAPDH were amplified simultaneously. Fluorescence signals were monitored in real-time to determine the threshold cycle number (Ct). A cut-off value was assigned on the basis of Ct values for FAM and TAMRA reporter dyes to interpret the results. Specimens yielding Ct FAM value of <30 and a Ct TAMRA value of <27 were interpreted as being HLAB27-positive. Samples yielding a Ct FAM value of >30 were considered HLA-B27-negative if the internal control signal was above the assigned cut-off (Ct TAMRA, <27). A sample was considered to contain inhibitory substances or degraded DNA, if the Ct TAMRA value was >27. HLA-B27 positive and negative controls were coamplified.

Statistical analysis:

Was done using MiniTab version 3. P-value less than 0.05 was considered positive.

Results :

All positive samples had Ct FAM values <28. All samples had Ct TAMRA values <26 (Fig. 1).

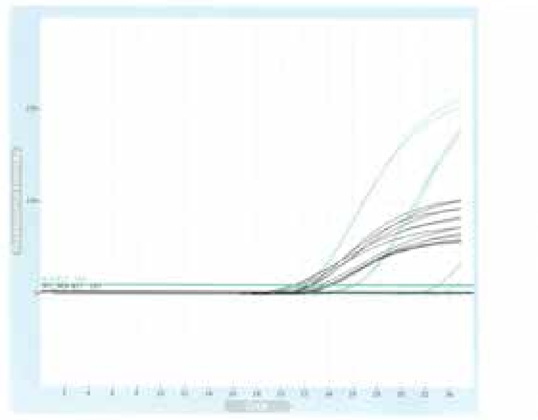

Fig. 1. Fluorescence signal versus cycle number plot during the amplification of HLA-B27 positive cases of human DNA samples of patients suspected to have AS with primers specific for HLA-B27 (green signal) and GAPDH (black signal)(NTC).

Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase. NTC, Non Template Control.

A Total of 50 clinical samples who were suspected to have AS and twenty controls were used to compare the real time Taq Man PCR with traditional PCR-SSO method. Ten out of fifty (20%) of patients showed positive HLA-B27 results and did not detected in control group with ''B27'' FAM Ct value = 26 and thirty samples had FAM Ct value=33 and ten samples their ''B27'' FAM Ct did not determined and give negative results Figure-2- . These results gave concordant with PCR-SSO results. The conventional PCR-SSO method takes approximately two days. In comparison, the real-time PCR systems can analyze 96 samples at same time with in one and halve hour.

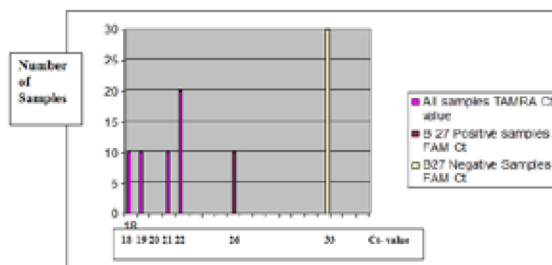

Fig. 2. Histogram representation showing the distribution of Ct FAM (HLA B-27) and Ct TAMRA (GAPDH) (NTC) from 50 samples on genotyping by Taqman allele-specific amplification. All samples had Ct TAMRA values <26, and 10 positive samples had Ct FAM values <28. Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase. NTC :Non Template Control.

Discussion:

Serological typing had been the main technique used for HLA Class I analysis. However, due to the beginning 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. DNA based typing focuses on defining differences in genes and may identify differences of little biologic relevance (8). There are many limitation 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, the test must be performed within 6 hours after drawing the blood, the amount of blood used must not be less than 5 ml, difficult in confirming homozygosity and it is difficult to be done in immune suppressed patients (9). False-negative serological HLA-B27 typing results may be due to altered antigenic epitopes but it can be detected by polymerase chain reaction (10). Thus, molecular typing techniques, which give more accurate results, have replaced the Microlymphocytotoxicity. Most genotyping methods are based on the group-specific amplification of HLA-B alleles by the PCR. The PCR-SSP and PCR-SSO is more widely used to detect HLA-B27 alleles. However, conventional PCR-based genotyping requires post-PCR manipulations that increase the risk of cross-contamination between samples; further, these post-PCR steps are laborious, especially when genotyping large numbers of samples. Therefore, real-time PCR, which allows the simultaneous amplification and detection of a specific DNA target, is an attractive alternative to conventional PCR-SSP (11, 12). In our study we use real time PCR method for detection HLA-B27 in suspected AS Iraqi patients and detected 20% of them were ''B27'' positive and this results was in accordance with results done by SSO method. There is no other studies in Iraq about ''B27'' subtypes. The 20 percent is so low because other studies found 90% is association between "B27" and AS because the study patients group are suspected to have AS and not proved by their physions by other tests like x-ray and other investigations. Thus, Real time-PCR accurately determined HLA-B27 genotypes and substantially reduced the labor-intensive steps and the total processing time (2 hr, including interpretation) when compared to the usual time required for PCR-SSP (5 hr). Our results in agreement with other study done in Dutch and other countries (6, 13). Thus real time PCR technique is superior to serology, SSP and SSO 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, this technique 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 this techniques can be employed 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 real time PCR in routine clinical practice.In addition to that HLA-B27 gene can be detected by using a spectral plasmon resonsnce imaging system(14).

Conclusions:

Real-time PCR methods appear to be reliable and very fast in the detection of HLA-B27 and could be potential alternatives to conventional PCR and serology methods.

Conflict of interest:
There is no conflict of interest .

REFERENCES

1- Gonzalez S, Martinez-Borra J, Lopez-Larrea C. Immunogenetics, HLA-B27 and spondyloarthropathies. Curr Opin Rheumatol 1999; 11:257-64. | 2- Khan MA, Mathieu A, Sorrentino R, Akkoc N. The pathogenetic role of HLA-B27 and its subtypes. Autoimmun Rev 2007;6:183-9. | 3- Dunky A, Neumuller J, Hubner C, Fischer GF, Bayer PM, Wagner E, et al. HLA-B27 determination using serological methods. A comparison of enzyme immunoassay and a microlymphocytotoxic test with flow cytometry and a molecular biological assay. Rheumatol Int 1996;16:95-100. | 4- Levering WH, Wind H, Sintnicolaas K, Hooijkaas H, Gratama JW. Flow cytometric HLA-B27 screening: cross-reactivity patterns of commercially available anti-HLA-B27 monoclonal antibodies with other HLA-B antigens. Cytometry B Clin Cytom 2003;54:28-38. | 5- Kuzio S, Hanguehard A, Morelle M, Ronsin C. Rapid screening for HLA-B27 by a TaqMan-PCR assay using sequence-specific primers and a minor groove binder probe, a novel type of TaqMan trade mark probe. J Immunol Methods. 2004 ;287:179-86. | 6- Roelandse-Koop EA, Buisman B, van Hannen EJ, van der Zee A, Kortlandt W, Hermans MH, van Houte AJ, van Rhee-Luderer R. Rapid HLA-B27 screening with real-time TaqMan PCR: a clinical validation in the Dutch population.Clin Chem Lab Med. 2011 ;49:1979-85. | 7- Fan W, Huang L, Zhou Z, Zeng X, Li G, Deo P, Hu L, Li Y. Rapid and reliable genotyping of HLA-B*27 in the Chinese Han population using a duplex real-time TaqMan PCR assay. Clin Biochem. 2012;45:106-11. | | 8- MN Mishra, H Mani, AS Narula, VK Saxena. HLA Typing – A Comparison of Serology and DNA Techniques. Int J Hum Genet.2004; 4:151-153. | | 9- O Nathalang, S Tantimavanich, K Nillakupt, P Arnutti, C Jaruchaimontree . HLA-B27 testing in thai patients using the PCR-SSP technique , Tissue Antigens, 2006;67, 233-236. | | 10- Kirveskari J, Kellner H, Wourela M et al . 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,1997; 36: 185-189. | | 11- Sylvain K, Aurelie H, Marc M, Christophe R . 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.2004; 287: 179-186. | | 12- Bon MA, van Oeveren-Dybicz A, van den Bergh FA. Genotyping of HLA-B27 by real-time PCR without hybridization probes. Clin Chem 2000;46:1000-2. | | 13- Funer R, Casamitjana N, Colobran R, Ribera A , Pujol-Borrel R, Paluo E and Juan M. Hla-B27 genotyping by fluorescent resonance emission transfer probes in real time PCR. Hum Immun. 2004;65:826-838. | | 14- Yang y, yuan l, Fang x, Liang X and Yang F. Detection of HLA –B27 gene using a spectral plasmon resonance imaging system .Biosenser Bioelectronics .2013;46:80-83. |