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RESEARCH ARTICLE

RAPID DETECTION OF HLA-B27 SEQUENCE SPECIFIC ALLELES BY IN-HOUSE OPTIMIZED MOLECULAR ASSAY FOR THE DETECTION OF ANKYLOSING SPONDYLITIS

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ABSTRACT

Introduction: Ankylosing Spondylitis (AS) is a long term disease, involved in inflammation of the joints between the spinal bones and the joints between the spine and pelvis. Chronic inflammation in these areas causes pain and stiffness. Over the time, chronic inflammation of the spine can lead to a complete cementing together of the vertebrae, a process referred to as ankylosis.

Materials and Method: 40 blood specimens were collected from suspected patients with symptoms related to AS attending orthopedics and neurology departments of Shri Mahant Indiresh Hospital, Dehradun. Samples were processed using In house protocol by Conventional PCR using silica columns for high yield nucleic acid extraction.

Results: Out of 40 specimens, 21came positive and remaining 19 were negative by both Olerup SSP approach and in house PCR protocol for HLA B-27 SSA. Age groups wise, positivity rate was maximum i.e. 66.6 % in the patients above 60 years of age. However, in the age group of 0–20 years, 21-40 years and 41–60 years, the positivity rate was 50, 52 and 62.5 %, respectively, which were comparatively less from the age group above 60 years.

Discussion: In developing nations, the patients can't afford for expensive molecular diagnostic tests. The In-House protocol standardized for the amplification of HLA-B27 is a novel, rapid, accurate and highly sensitive & specific technique for low cost as compared to commercially available assays. By using optimized protocol the cost can be reduced to 2-3 folds and hence will help in early and accurate diagnosis and management of the disease.

Keywords: Sequence Specific Primers, Ankylosing Spondylitis, Seronegative Spondyloarthropathy, Internal Control, Amplicons.

INTRODUCTION:

Ankylosing Spondylitis (AS) is a long term disease that involves inflammation of the joints between the spinal bones and the joints between the spine and pelvis (1). These joints become swollen and inflamed or AS is a form of chronic inflammation of the spine and the sacroiliac joints. The sacroiliac joints are located in the low back where the sacrum (the bone directly above the tailbone) meets the iliac bones (bones on either side of the upper buttocks).Chronic inflammation in these areas causes pain and stiffness in and around the spine. Over time, chronic inflammation of the spine (spondylitis) can lead to a complete cementing together (fusion) of the vertebrae, a process referred to as Ankylosis, leads to loss of mobility of the spine. The tendency to develop ankylosing spondylitis is believed to be genetically inherited. Majority (nearly 90%) of people with ankylosing spondylitis are born with a gene known as the HLA-B27 gene. Blood tests have been developed to detect the HLA-B27 gene marker and have furthered our understanding of the relationship between HLA-B27 and ankylosing spondylitis. The HLA-B27 gene appears only to increase the tendency of developing ankylosing spondylitis, while some additional factor(s), perhaps environmental, are necessary for the disease to appear or become expressed. For example, while 7% of the United States population has the HLA-B27 gene, only 1% of the population actually has the disease ankylosing spondylitis. In northern Scandinavia (Lapland), 1.8% of the population has ankylosing spondylitis while 24% of the general population has the HLA-B27 gene (2). Even among HLA-B27-positive individuals, the risk of developing ankylosing spondylitis appears to be further related to heredity. In HLA-B27-positive individuals who have relatives with the disease, the risk of developing ankylosing

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spondylitis is 12% (six times greater than for those whose relatives do not have ankylosing spondylitis). Recently, two more genes have been identified that are associated with ankylosing spondylitis. These genes are called ARTS1 and IL23R. These genes seem to play a role in influencing immune function. It is anticipated that by understanding the effects of each of these known genes researchers will make significant progress in discovering a cure for ankylosing spondylitis. Ankylosing spondylitis and each of the spondyloarthropathies are areas of active research (3). The relationship between infectious agents and the triggering of chronic inflammation is vigorously being pursued. Factors that perpetuate ''autoimmunity'' are being identified. The characteristics of the gene marker HLA-B27 are being further defined. These genes seem to play a role in influencing immune function (4). It is anticipated that by understanding the effects of each of these known genes, researchers will make significant progress in discovering a cure for ankylosing spondylitis. Testing for HLA-B27 is of clinical importance for the early diagnosis of ankylosing spondylitis. Excluding HLA-B27 virtually excludes ankylosing spondylitis. Serological techniques such as microcytotoxicity and flow cytometry for testing HLA-B27 require viable cells that adequately express HLA-B27 and may give false negative results if HLA-B27 is down regulated or ''masked''. Flow cytometry is rapid and relatively inexpensive, but has been reported to lack specificity, especially in the presence of antigens that cross-react with HLA-B27, such as HLA-B7 (5). Moreover, results of ongoing research will lead to a better understanding and treatment of the entire group of diseases collectively known as spondyloarthropathies. Present observations confirmed the significance of HLA-B27 allele as a novel and rapid molecular marker for diagnosis of ankylosing spondylitis

MATERIALS AND METHODS:

40 specimens were collected from the patients attending orthopedics and neurology departments of Shri Mahant Indiresh Hospital, and were transported at 4°C to Molecular Research Laboratory, SGRRIM&HS for further processing. The study was approved by Institution ethical clearance committee & the written consent from all the patients was taken. Blood samples were collected from the patients in anti-coagulant tubes and double oxalate or EDTA.

Reagents required for amplification and detection of Amplicons:

Olerup SSP kit:

PCR Buffer (10X) (Larova Cat # Taq 500), SSP HLA-B27 PCR Kit, DNA 1: a B-27 positive DNA as a positive control, Nuclease free water as a negative control DNA. Taq DNA polymerase(5 unit/µl) ,100bp DNA Ladder (Bangalore GENEI Cat # MBD13J) ,Tris Base (Life Technology Cat # 1000LT) , EDTA (Amresco Cat # 9572E) , Agarose (Amresco Cat # GR 100-LELT) , Bromophenol Blue ((Amresco Cat # 0532) ,Gel loading dye,1X TAE Buffer, Milli Q Water were used (6).

PCR master mix completes with Taq contains:

DNA Taq polymerase: 0.4 units per 10 µl SSP reaction, Nucleotides: Final concentration of each dNTPs is 200µM , PCR buffer : Final conc. 50mM KCl, 1.5Mm MgCl2, 10Mm Tris-HCL pH 8.3, 0.001%w/v gelatin , Glycerol : Final conc. of glycerol is 5%, Cresol red: Final concentration of cresol red is 100µg/ml.

In-House method for sequence specific allele detection.

Chemicals and reagents required for DNA amplification:

PCR Buffer (10X) (Larova Cat # Taq 500), DNA: B-27 positive DNA as a positive control, Nuclease free water as a negative control DNA. Taq DNA polymerase(2 unit/µl) ,100bp DNA Ladder (Bangalore GENEI Cat # MBD13J) ,Tris Base (Life Technology Cat # 1000LT) , EDTA (Amresco Cat # 9572E) , Agarose (Amresco Cat # GR 100-LELT) , Bromophenol Blue((Amresco Cat # 0532) ,Gel loading dye,1X TAE Buffer, Milli Q Water were used.

DNA Extraction for the amplification of SSP by olerup and in-house method

Silica column based Nucleopore DNA extraction kit was utilized for high yield nucleic acid extraction. Eluted DNA was used for the amplification of Sequence Specific Allele of HLA-B27 by Olerup method as well as by In House PCR method (7).

In-House Method for HLA B27 Sequence specific alleles:

PCR master mix preparation:

DNA Taq polymerase (2U/µl), Nucleotides: Final concentration of each dNTPs is 100µM, PCR buffer: Final conc. 50mM KCl, 1.5Mm MgCl2, 10Mm Tris-HCL pH 8.3. Primers: forward and reverse primer (5′ primer,-B27ex294F: 5′TACGTGGACGACACGCT-3′;primer, B27ex2199RC: 5′-AGTCTGTGCCTTGGCCTTGC-3′, $MgCl_2$ (50 mM), nuclease free water and internal control primers.

PCR was performed on Bench top model 9600 thermocycler with an initial denaturation of 94°C for 5 min, 35repetitive cycles of amplification (30s at 94°C, 30 s at 61°C and 72ºC for 30 s) and a final elongation at 72°C for 5 min and storage at 4ºC(8-10). The PCR products were analyzed by agarose gel (1.5%) electrophoresis for results interpretation.

RESULTS:

Total of 40 specimens were considered for the proposed study; out of them 21samples showed HLA-B27 specific allele positive results while the remaining 19 were negative. Appearance of 149–150 bp specific band indicates the presence of HLAB27 gene HLA-B*2701 to HLA-B*2728 along with 439 bp band of internal control targeting Human Growth Hormone gene, which was used for the validation of the result as it is present in normal population as well as in diseased individuals (Fig.1). In the present study, the positivity rate of HLA-B27 specific allele in different age groups and gender wise distribution of the HLA-B27 specific alleles in these patients were determined using optimized in house SSP-PCR approach. When samples were analyzed in terms of age groups, it was found that out of 21 positive samples, the positivity rate was maximum i.e. 66.6 % in the patients above 60 years of age. However, in the age group of 0–20 years, 21-40 years and 41–60 years, the positivity rate was 50, 52 and 62.5 %, respectively, which were comparatively less from the age group above 60 years (table 1). In the comparative study between the optimized In house PCR protocol and Olerup SSP PCR method, we noticed that out of 40 samples, 21 samples were positive while remaining 19 were negative and by in house PCR protocol for HLA-B27 SSP Alleles 21 samples were positive while remaining 19 were negative(table 2).

Analytical specificity of an In-House method for sequence specific alleles detection:

The specificity of an In-House method for sequence specific alleles detection is ensured by the selection of the primers. The specificity of In house developed protocol was validated with the template of different microorganisms as well as with presently positive HLA-B27 DNA. To determine the specificity of an In-House method for sequence specific alleles detection, it has been tested for cross-reactivity. As a result, there was no case of PCR amplification when using template from different sources, which exclude the target organism (Table 3).

DISCUSSION:

It has been well established that association of the HLAB27 antigen in 90–95 % of patients with ankylosing spondylitis (AS) are HLA-B27 specific allele positive (9). Identification of HLA-B27 by PCR supports the diagnosis of ankylosing spondylitis in symptomatic individuals and negative results exclude the diagnosis (10). Various studies showed that 90–94 % of AS sufferers have HLA-B27 allele positive, while 5–9 % of the general population with ankylosing spondylitis may have other contributory factors for positivity of HLA-B27. The disease is most likely triggered in genetically predisposed individuals by an environmental factor, since only 1 % of the people with the HLA-B27 allele develop ankylosing spondylitis. The exact mechanism of triggering the disease is unidentified, but many theories have been proposed to explain the contribution of HLA-B27 in the disease. SSP–PCR is a novel, rapid, cost effective, and standard method for the detection of HLA-B27 alleles (11). It is a rapid and selective method for the detection of HLA-B*2701 to HLA-B*2728 subtypes. Sequence based typing (SBT) or SSP technique is capable of detecting a single base difference in DNA sequence between two alleles but they are not likely to detect a new undefined allele, unless the variation happens to be at the specific site detected by the probe or the primer .Many factors influence the specificity of the PCR like sequence of the primer (i.e. GC content), free $Mg2^{+}$ ion concentration, ratio of primer to target, buffer, and polymerase concentration. Conditions must be optimized for each primer pair. It is extremely important to take precautions to prevent contamination of samples with previously amplified DNA. Contamination of reagents can be minimized by preparing solutions in facilities, which have not been exposed to amplified products, aliquoting reagents for single use, and using dedicated equipment and consumables. Ankylosing spondylitis and each of the spondyloarthropathies are areas of active research. The relationship between infectious agents and the triggering of chronic inflammation is vigorously being pursued. Factors that perpetuate ‘‘autoimmunity’’ are being identified. The characteristics of the gene marker HLA-B27 are being further defined. These genes seem to play a role in influencing immune function. It is anticipated that by understanding the effects of each of these known genes, researchers will make significant progress in discovering a cure for ankylosing spondylitis. Testing for HLA-B27 is of clinical importance for the early diagnosis of ankylosing spondylitis. Excluding HLA-B27 virtually excludes ankylosing spondylitis. Serological techniques such as microcytotoxicity and flow cytometry for testing HLA-B27 require viable cells that adequately express HLA-B27 and may give false negative results if HLA-B27 is down regulated or ‘‘masked’’. Flow cytometry is rapid and relatively inexpensive, but has been reported to lack specificity, especially in the presence of antigens that cross-react with HLA-B27, such as HLA-B7 (12). Moreover, results of ongoing research will lead to a better understanding and treatment of the entire group of diseases collectively known as spondyloarthropathies. Present observations confirmed the significance of HLA-B27 allele as a novel and rapid molecular marker for diagnosis of ankylosing spondylitis.

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 as effective detection of HLA B27 antigen can be visualized by the B27 bands. HLA-B27 is an inherited gene marker associated with a number of related rheumatic diseases. They share in common features such as spinal and peripheral arthiritis, skin and GI disorders, anterior chamber eye disease and psoriasis like skin lesions. This gene is found with highest prevalence in patients with AS (>90%), reactive arithiritis (80%) and patients with the combination peripheral arthiritis and either psoriasis or inflammatory bowel disease (50%) statistics are however sometimes misleading. Although is true that patients with classic disease will show the presence of the HLA- B27 gene between 50 and 90+% of the time if we randomly take 100 persons with the gene marker we may find evidence of disease in only 25% of the study group. This suggests that most patients with the gene never develop any clinical rheumatic symptoms of significance.

HLA-B27 gene, one of the HLA class I Molecules, is strongly associated with Ankylosing Spondylitis (AS). It is one of the most frequent gene investigated by clinicians for diagnosis and prognosis of AS. It is one of the inflammatory rheumatoid disorders that cause arthritis of the spine and sacroiliac joints. Laboratory test may reveal an elevated erythrocyte sedimentation rate, anemia, and a positive HLA-B27 assay. X-ray and bone scan may show characteristic changes.

Conventional HLA-B27 typing has been performed by cell cytotoxicity test or fluorescence serology with specific antibodies. Amplification and detection of HLA–B27 PCR is a simple, rapid and accurate method for the diagnosis of AS, Reiter's Syndrome, certain eye disorder such as acute anterior Uveitis, Iritis, Behcets Syndrome, Psoriatic arthritis and inflammatory bowel disease at molecular level. PCR technique which is based on the detection of allelic differences at the nucleotide level and circumvents the common problems associated with MLCT (microlympho-cytotoxicity test) and Flowcytometry (13-18). Furthermore the cost of this In House developed PCR SSP technique as compared to other approaches is merely Rs. 700 per test this technique is about 2 times less costly than that of the imported commercial PCR-SSP test kit by few laboratories. 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. Thus such PCR-SSP techniques can be employed by the Molecular Research Laboratory, Department of Biochemistry, Shri Guru Ram Rai Institute of Medical and Health Sciences (SGRRIM&HS), Patel Nagar, Dehradun (U.K) as a part of routine clinical practice for exact diagnosis of Seronegative Spondyloarthritis or Ankylosing Spondylitis (AS).

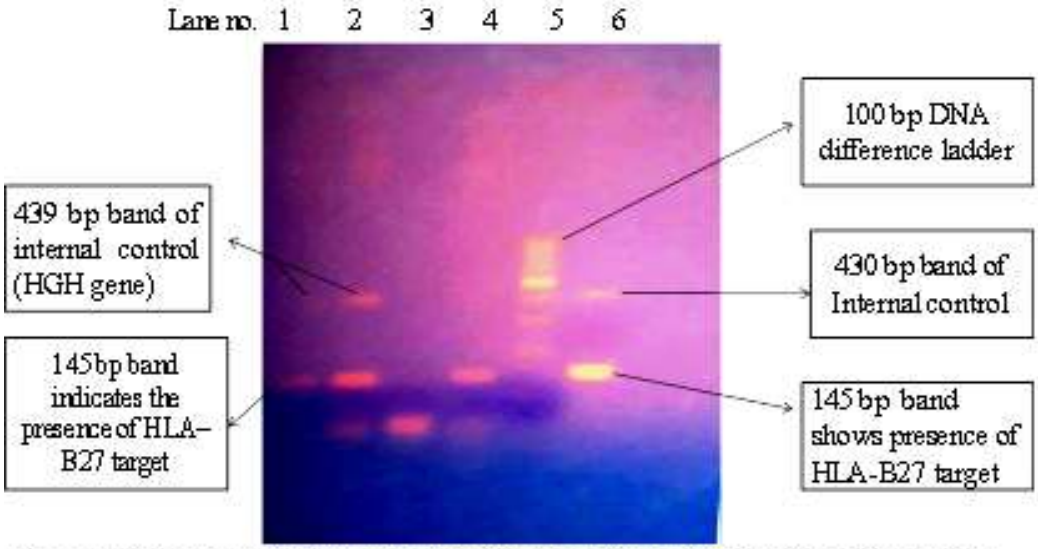

Figure 1 Agarose gel picture showing PCR amplicons of 5 samples at Molecular Research Lab SGRRIMHS, Dehradun

Lane no. 1,2 shows positive result with IC
Lane no. 3 shows negative control
Lane no. 4 shows positive control
Lane no. 5 shows universal DNA Ladder
Lane no. 6 shows positive result by Olerup SSP Approach

Table 1: Age wise distribution of HLA-B 27 alleles (2705-2725)

Sr. No.	Age Group (in years)	No. of HLA-B27 positive/no. of patients in that particular age group	Positivity rate %
1	0-20	1/2 = 0.5	50%
2	21-40	13/25 = 0.52	52%
3	41-60	5/8 = 0.625	62.5%
4	Above 60	2/3 = 0.66	66.6%

Table 2: Comparative study between optimized in house PCR and Olerup SSP PCR method

Sr. No.	Methods employed for the amplification of HLA B27 SSP alleles	Total No. of Specimens	No. of HLA B27 positive patients	No. of HLA B27 positive patients	No. of HLA B27 negative patients
1	PCR SSP In house approach	40	21	21	19
2	PCR SSP :Commercial kit based approach	40	21	21	19

Table no. 3 Analytical Specificity of In House PCR

Sr. No.	Microorganism (DNA) used for the analytical specificity	Results Interpretation		
		nternal Control	Olerup SSP Kit	Optimized In house PCR for HLA B27 sequence specific alleles
1	E.coli	Positive	Negative	Negative
2	Mycobacterium tuberculosis	Positive	Negative	Negative
3	HSV I	Positive	Negative	Negative
4	HSV II	Positive	Negative	Negative
5	HPV	Positive	Negative	Negative
6	HLA-B27	Positive	Positive	Positive

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CONFLICT OF INTEREST: None

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