Usage of Conventional PCR Technology for the Detection of HLA-B27 Allele: A Significant Molecular Marker of Ankylosing Spondylitis

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Abstract Ankylosing spondylitis is a chronic inflammatory disease that has been linked to the human leukocyte antigen class I allele HLA-B27. More than 90 % of patients with ankylosing spondylitis possess the HLA-B27 allele, but only 1 % of people with HLA-B27 develop the disease. Ankylosing spondylitis predominately affects young males. The present study was planned to find out the involvement of HLA-B27 specific allele in relation to age and sex in symptomatic suspected patients of ankylosing spondylitis using conventional PCR technology. Forty symptomatic patients of ankylosing spondylitis were included in the present study. SSP-PCR technique was used to detect the HLA-B27 specific allele. This study showed (out of 40 symptomatic suspected cases of ankylosing spondylitis only 12 patients were detected positive with HLA-B27 allele, while remaining 28 were negative) that the positivity rate for ankylosing spondylitis with HLA-B27 allele is low. Furthermore, it was observed that the males above 50 years are more prone to develop AS with HLA-B27 specific allele. It could be concluded that the conventional PCR technology is a rapid, sensitive, and confirmatory method for the diagnosis of ankylosing spondylitis.

Keywords Human leukocyte antigen · Ankylosing spondylitis · Polymerase chain reaction

Introduction

Human leukocyte antigens-B27 is a class I surface antigen encoded by B locus in the major histocompatibility complex (MHC) on the short (p) arm of chromosome no. 6 at position 21.3 from base pair 31,429,845 to base pair 31,432,923 and presents microbial antigen to T cells [1]. The disease ankylosing spondylitis (AS) has been associated to the human leukocyte antigen (HLA) class I allele HLA-B27, and the relationship of HLA-B27 with AS is the strongest for any HLA locus. Almost all nucleated cells in the body, except neurons and striated muscle cells contains class I HLA molecules on it. The strongest evidence of the involvement of HLA-B27 in AS came from studies done with mice and rats that had been given HLA-B27 as a transgene. They developed diseases such as ankylosing enthesopathy, and rats with a high copy number of HLA-B*2705 developed axial and peripheral arthritis, gut inflammation, and lesions on the skin. Rats and mice that were kept in sterile environments did not develop joint disease or gut inflammation [2, 3]. Development of the disease was dependent on the presence of gut bacteria and a high copy number of HLA-B27 in cells of bone-marrow lineage. Major histocompatibility complex (MHC) class I molecules presents endogenous peptides derived from intracellular proteins to the β T cell receptor on CD8+ cytotoxic T lymphocytes in infected cells. Ankylosing spondylitis is a disease that predominately affects males and usually appears in young adulthood. It usually begins with unilateral pain at the sacroiliac joint, progressing to bilateral pain that radiates down the legs, and up the spine,

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eventually affecting the rib cage [4]. It is also a systemic disease, meaning it can affect other tissues throughout the body. Accordingly, it can cause inflammation in or injury to other joints away from the spine, as well as to other organs, such as the eyes, heart, lungs, and kidneys [5]. Ankylosing spondylitis shares many features with several other arthritis conditions, such as psoriatic arthritis, reactive arthritis, and arthritis associated with Crohn's disease and ulcerative colitis. Each of these arthritic conditions can cause disease and inflammation in the spine, other joints, eyes, skin, mouth, and various organs [6].

Materials and Methods

Forty suspected cases of Ankylosing Spondylitis attending various wards of Shri Mahant Indresh Hospital, Shri Guru Ram Rai Institute of Medical and Health Sciences, Patel Nagar, Dehradun were included in the present study. Five milliliters of blood was collected in a vacutainer from each patient and transported at 4 °C to the Molecular Research Laboratory of the Department of Biochemistry for further processing. This study protocol was approved by Institutional Ethical Committee and written consent was obtained from each patient for the present study.

DNA Extraction Materials

Silica column based AuPreP GEN DNA extraction kit, extraction buffer, ethanol (Mol. bio. grade), autoclaved ultra pure water (MQ water with 18 MΩ resistance), proteinase K (25 mg/ml) [7] were used for DNA extraction. Wash buffer (60 ml of 98 % ethanol is added to the wash buffer to prepare working solution). Biosafety cabinet (class II), refrigerated centrifuge (10,000×g), rotors for 50, 15, 1.5, and 0.2 ml tubes) were used for sample preparation. Aerosol barrier tips to prevent carry over contamination or cross contamination (20, 200, and 1,000 µl), pipettes (20, 200, and 1,000 µl), latex gloves and micro centrifuge tubes (1.5 ml) of molecular grade level were also used [8].

DNA Extraction/Isolation Technique

For DNA extraction from 40 blood samples, 200 µl of the specimen was taken as starting volume. Twenty microliters of proteinase K and 200 µl of extraction buffer (lysis buffer 1 and 2 of the kit) were added. The preparation was vortexed (for 15–20 s) and incubated at 60 °C for 20 min and thereafter was transferred to 90 °C for 10 min. Two hundred microliters of absolute ethanol to each sample was added for proper precipitation of nucleic acids. Contents were properly mixed and transferred into prearranged silica

columns and further centrifuged at 10,000 rpm for 3 min at 4 °C. The columns were transferred to fresh collection tubes. Five hundred microliters of wash buffer was added and centrifuged at 13,000 rpm for 3 min at 4 °C after which the contents were transferred to fresh collection tubes; the washing step with wash buffer was repeated again followed by dry wash at 13,000 rpm for 2 min. The columns were transferred to autoclaved microfuge tubes and 60 µl of pre heated milli Q water was added at 70 °C to each column and centrifuged at 13,000 rpm at 4 °C for 5 min. The column was discarded and DNA was eluted in micro centrifuge tubes, which will be used as template for PCR reaction.

Amplification of HLA-B27 Allele

Chemicals and Reagents Required for DNA Amplification

Polymerase chain reaction buffer (10×) (Larova Cat # Taq 500), SSP HLA-B27 PCR kit, DNA 1: HLA B-27 positive DNA as a positive control, nuclease free water as a negative control. Taq DNA polymerase (5 U/µl), 100 bp 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, 1× TAE Buffer and milli Q water were used. A sequence specific primer typing approach (Olerup PCR-SSP kit) was used because this technique avoids the probability of cross reactivity with other HLA-B27 alleles, which are responsible for 3–5 % equivocal results. Allele specific PCR for HLA-B27 is a direct genotyping method based on specific primer recognition of a unique HLA-B27 gene sequence. It uses HLA-B27 unique primer specific for HLA-B alleles. Combination of specific primer sequence and melting temperature analysis was used in PCR for accurate determination of HLA-B27 genotype. The internal β -globulin control, which was detected in all HLA-B27 negative samples, demonstrated the correct PCR condition and the presence of sufficient sample DNA, thus preventing false negative results.

PCR Master Mix

Taq DNA polymerase: 0.4 units per 10 µl SSP reaction, nucleotides: final concentration of each dNTPs is 200 µM, PCR buffer: final concentration 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl pH 8.3, 0.001 % (w/v) gelatin, glycerol: final concentration of glycerol is 5 %, cresol red: final concentration of cresol red is 100 µg/ml components were included for the preparation of master mix. For a single reaction, ready primer mix 5.0 µl and PCR master mix 3.0 µl with 2 µl DNA as a template were added in a

0.2 ml PCR tube. All the components were mixed carefully. The PCR tube was vortexed gently in order to settle down all the ingredients in bottom of the tube.

Results and Discussion

Out of 40 samples collected for the proposed study, 12 samples showed HLA-B27 specific allele positive results while the remaining 28 are negative. Appearance of 149–150 bp specific band indicates the presence of HLA-B27 gene HLA-B*2701 to HLA-B*2728 long with 236 bp band of internal control ($\beta 2$ micro globulin gene), which was used for the validation of the result as it is present in normal population as well as in patients (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 allele in patients using SSP-PCR approach were determined. Twelve (30 %) AS patients were found to be HLA-B27 specific allele positive, while remaining 28 (70 %) were negative. When samples were analyzed in terms of age groups, it was found that out of 12 positive samples, the positivity rate was maximum i.e. 44.44 % in the patients above 50 years of age. However, in the age group of 15–30 years and 31–50 years, the positivity rate was 25 and 26.6 %, respectively, which were comparatively less (Table 1). Furthermore, we noticed that in terms of gender wise variation, eight were men and remaining four were women (Table 2).

It has been well established that association of the HLA-B27 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

Table 1 Age wise distribution of positivity rate of the presence of HLA-B27 allele

Sr. no.	Age group (years)	No. of HLA-B27 positive patients/no. of patients of that particular age group	Positivity rate (%)
1	15–30	4/(16)	25
2	31–50	4/(15)	26.6
3	Above 51	4/(9)	44.44
	Total	12/(40)	30

Table 2 Sex wise distribution of HLA-B27 specific allele

Sr. no.	Sex	No. of positive patients (12)	Positivity rate (%)
1	Male	08/(12)	66.66
2	Female	04/(12)	33.33

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 [12].

Fig. 1 Agarose gel picture result interpretation. Lane 3 positive cases, Lane 1, 2 negative case. Lane 4 universal ladder

Many factors influence the specificity of the PCR like sequence of the primer (i.e. GC content), free Mg^{2+} 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 [13]. 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 [14]. 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 [15]. 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.

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Conflict of interest None

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