HLA-B27 real-time PCR using TaqMan-MGB sequence specific probes

Stewart M Smith, Andrew D Laurie, Howard C Potter and Ben D McGettigan

Abstract

Objectives: The HLA-B*27 genotype is strongly associated with ankylosing spondylitis (AS) and also other spondyloarthropathies (SpA) such as psoriatic SpA. Our objective was to develop and validate a real time PCR for HLA-B*27 with greater capacity than our current allele specific approach without loss of fidelity.

Methods: We describe a real-time PCR method for the detection of HLA-B*27 using allele specific primers combined with TaqMan fluorescent minor groove binder (MGB) probes. One probe is specific for human leucocyte antigen (HLA) B*27 conjugated with the fluorochrome FAM and the other specific for human growth hormone (HGH) conjugated with the fluorochrome VIC which acts as endogenous control.

Results: 441 consecutive samples were tested of which 139 tested HLA-B*27 positive and 302 tested negative. We tested a further 12 external quality control samples of which four were positive and eight negative for HLA-B*27.

Conclusions: RT-PCR is suitable for HLA-B*27 genotyping and has the advantages of lesser hands-on time, does not require any post amplification processing, and does not use the toxic DNA intercalating dye ethidium bromide.

Keywords: HLA-B*27, TaqMan, PCR, MGB, minor groove binder, NFQ, non-fluorescent quencher, real time PCR, ankylosing spondylitis.

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Introduction

The human leucocyte antigen (HLA) B*27 is strongly associated with the spondyloarthropathies (SpA’s). HLA-B*27 is found in 90-95% of ankylosing spondylitis (AS) patients and also lesser frequency in forms of psoriatic arthritis and reactive arthritis (1,2). HLA-B*27 may have a direct role in the pathogenesis of SpA which is supported by epidemiological studies and transgenic rat studies (3). Different hypotheses exist, such as the arthritogenic peptide hypothesis, the misfolding hypothesis or molecular mimicry (3-5). Less than 5% of those members of the general population that are HLA-B*27+ develop AS, and HLA-B*27 has been estimated to account for only 20-50% of the overall genetic susceptibility to AS (6). In addition to HLA-B*27, a recent genome wide association study has confirmed 25 loci that confer increased genetic risk for AS, including genes involved in peptide processing prior to MHC class I presentation and alterations in the IL-23 pathway (7).

There are more than 50 subtypes of HLA-B27 identified which mostly differ by only a small number of nucleotides. (IMGT/HLA database: http://www.ebi.ac.uk/imgt/hla). The subtypes HLA-B*27:02, B*27:04, B*27:05, B*27:07, B*2:708 are associated with AS although B*27:07 is suggested to be associated but not in Greek Cypriots (8,9). Important conformational differences exist in subtypes not associated with AS (4,10-14).

Many laboratories use an allele specific PCR based on that of Sayer et al (17), which offers excellent specificity for HLA-B*27 although they report a lack of sensitivity for subtype HLA-B*27:12. This lack of sensitivity is also likely for the subtypes, HLA-B*27:16, HLA-B*27:18, HLA-B*27:23, HLA-B*27:29.

The aim of our study was to adapt our conventional allele specific HLA-B27 protocol to a fluorogenic real time (RT-PCR) method. The allele specific PCR detects the presence of the B*27 genotype by amplifying a region between primer sets that recognise only B*27 specific sequences. The PCR reaction amplifies this region to sufficient quantity so that it may be visualised after electrophoresis in agarose gel. Visualisation is achieved by staining with ethidium bromide (a fluorescent DNA intercalating dye) and viewing the gel under UV light (Figure 2). Both allele specific and RT-PCR methods detect the presence or absence of B*27 and do not discriminate between heterozygous and homozygous individuals. The RT-PCR method involves the addition of specific dual labelled hydrolysis probes to the PCR. These TaqMan probes have a fluorescent reporter (ie. FAM or VIC) attached to the 5’ end of the probe sequence and a non-fluorescent quencher (NFQ) attached to the 3’ end. Addition of a minor groove binder (MGB) to the probe sequence at the 3’ end of the probe permits it to anneal at a higher melting temperature (Tm), allowing the use of shorter more specific probes (2,18). The probe in free form is normally quenched as random coiling brings the probe and fluorophore close together (19). The MGB works together with the quencher to improve quenching. During the extension step of the PCR, the probe binds to its complementary sequence at a position that sits between the forward and reverse primers. (Fig 1) Extension Taq polymerase cleaves the probe, because of intrinsic 5’ to 3’ nuclelease activity, releasing the fluorescent reporter (FAM or VIC) into the mix thus causing an irreversible increase in fluorescence(19). As the cycles continue fluorescence increases rapidly until the probe is exhausted. The advantages of this method are that primers and probes provide specificity to the reaction products and allow immediate visualisation. It is possible to use several different probes in each PCR to simultaneously detect specific products. In this case both the HLA-B*27 and HGH (internal control) products are detected independently. The analytical capacity of this technique is much greater than the allele specific PCR and allows for near complete automation.

![Figure 1A. Target DNA, primers and probes are mixed together.](image)

![Figure 1B. After melting, primers and probes are allowed to bind to their complementary sequences.](image)
Allele specific PCR

Allele specific PCR was carried out according to the method of Sayer et al (17). Briefly, sequence-specific primers amplify a 141bp band from exon 2 of the HLA-B*27 family. Internal PCR amplification control primers specific for the human growth hormone (HGH) are used for each sample to ensure there is sufficient sample DNA for amplification to proceed or to show amplification failures for any other reason. This internal control produces a 437bp band which must be present to validate the PCR and assure of primer specificity.

Real time PCR

Allele specific PCR primers were used as above, according to the method of Sayer et al (17). The Alt HGH internal control primers / HGHVIC1 probe used were different from the ones above and they were designed using ABI Primer Express v3.0 software (Table 1). The same B27 primers above were used combined with a 22 nt. fluorescent TaqMan probe B27FAM1, which was designed manually in a configuration allowing efficient binding between the B27EX294F / B27EX2199RC primers (Table 1). Both TaqMan probes were purchased from Applied Biosystems while primers were purchased from Sigma. Probe concentrations of 0.25μM and Primer concentrations of 0.5μM were used in PCR reactions. Samples were then amplified using the Roche Lightcycler 480 in 15μl reaction volumes using 10μl of PCR buffer (Roche Lightcycler 480 Probes master mix), primer/probe mix and 5μl of undiluted DNA. One cycle of denaturation at 95°C for 5 minutes was followed by 40 cycles of 95°C for 15 seconds, 66°C for 15 seconds, and 72°C for 30 seconds. The intensity of fluorescence of the two different fluorophores was measured each cycle as the PCR proceeded in real time. Samples are considered positive when fluorescence intensity rose above the threshold value, which usually occurs from cycle 26 to 30.

Table 1. Primers and TaqMan probes used in the RT-PCR HLA - B*27 genotyping procedure.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>ALT HGH For</td>
<td>5'-CTGCACACAGGTCGCCCTT-3'</td>
</tr>
<tr>
<td>ALT HGH Rev</td>
<td>5'-CACCATTCCCAAAGGCTT-3'</td>
</tr>
<tr>
<td>HGHVIC1</td>
<td>5'-VIC-CACCTACCAGAGTTTG-MGB-Q 3'</td>
</tr>
<tr>
<td>B27EX294F</td>
<td>5'-CTGCATAAGCCAGGCGACT-3'</td>
</tr>
<tr>
<td></td>
<td>5'-GATGTATACCTTTAAGCTAAC-3'</td>
</tr>
<tr>
<td>B27FAM1</td>
<td>5'-FAM-TTCTTGAGGTTCGACAGCGAG-MGB-Q 3'</td>
</tr>
</tbody>
</table>

RT-PCR reactions were performed in 96 well plates and results output according to the plate layout. Fluorescence measurements from each well were recorded once per cycle of PCR and the signal from each probe was separated by its distinct emission spectra collected using appropriate filter sets. Curves were then constructed with Cycle number (x-axis) vs log fluorescence intensity (y-axis) for each well. Fluorescence intensity for the B*27 positive samples was usually about 15 times that of negative controls and blanks by cycles 35 to 40. When the fluorescence level rose above the discriminator (horizontal dashed line) the sample was scored as positive for that probe.
A curve for each well is constructed for HGH (Figure 3A) and HLA-B*27 (Figure 3B) specific probes with cycle number (x-axis) vs. log fluorescence intensity (y-axis). The discriminator (horizontal dashed line) is set manually and results calculated for each well. Positivity is expressed as a red square for those wells where fluorescence levels are above the threshold.

Results

Of the 453 comparative samples tested, 100% concordance with the conventional allele specific PCR was recorded.

Table 2. Comparison of results between allele specific and RT-PCR.

<table>
<thead>
<tr>
<th>Allele Specific</th>
<th>PCR</th>
<th>RT-PCR</th>
</tr>
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<tbody>
<tr>
<td>HLA-B*27 positive</td>
<td>139</td>
<td>139</td>
</tr>
<tr>
<td>HLA-B*27 negative</td>
<td>302</td>
<td>302</td>
</tr>
<tr>
<td>RCPA QAP HLA-B*27 positive</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>RCPA QAP HLA-B*27 negative</td>
<td>8*</td>
<td>8*</td>
</tr>
<tr>
<td>Total</td>
<td>453</td>
<td>453</td>
</tr>
</tbody>
</table>

*Four of the HLA-B*27 external quality assurance samples provided by the RCPA Immunology QAP program were reported as heterozygote for HLA-B*7.

Effects of DNA Dilution

Five HLA-B*27 positive and two HLA-B*27 negative samples were chosen to perform dilution experiments. These samples were all extracted as described previously and had DNA concentrations ranging from 15 – 65ng/μl. Each sample was serially diluted eight times, with dH₂O so as to obtain a halving of DNA in each successive dilution and these samples were then analysed by the RT-PCR assay. This gave a range of DNA concentrations over which the sensitivity of this test could be measured, from 0.12 – 65ng/μl. Ideally for each doubling dilution of DNA one would expect to see a one cycle increase in the cycle number at which fluorescence levels increase above an arbitrary threshold. This would hold true so long as the threshold is set in the exponential phase of amplification.

The question of sensitivity of detection can be answered by observing the lowest concentration at which fluorescence is clearly distinguishable from negative samples and blank reactions containing no DNA. Figures 4A and 4B below show how reactions proceeded for both the HGH and B*27 specific fluorescent probes. Fluorescence increased exponentially until buffer constituents were exhausted whereupon the fluorescence plateaus. The figures show that positive signals were obtained over the full range of concentrations used. Of note, Figure 4B shows that when DNA concentration increased from 123ng to 246ng there was no difference in the cycle number threshold suggesting saturation of amplification at higher concentrations. The sensitivity of detection was therefore at least 0.12ng/μl. This is well below the lowest concentration of DNA isolated by our DNA isolation system by a factor of at least 10.
Other investigators have shown applications using a Lightcycler technique, which can be performed that gives 100% concordance with our current allele specific PCR. Furthermore it also showed 100% agreement with external control samples. The method showed no cross reactivity with the four HLA-B*7 positive samples available. DNA concentration did not affect interpretation of this test when DNA is prepared by our automated DNA extraction method. The method sensitivity to DNA concentration was well within the range isolated routinely in our laboratory. There were signs of departure from linearity for some samples at higher DNA concentrations and for one sample at low concentrations which did not affect the ability to discriminate whether the samples were positive or negative.

Discussion

We have shown that a RT-PCR for the detection of HLA-B*27 can be performed that gives 100% concordance with our current allele specific PCR. Furthermore it also showed 100% agreement with external control samples. The method showed no cross reactivity with the four HLA-B*7 positive samples available. DNA concentration did not affect interpretation of this test when DNA is prepared by our automated DNA extraction method. The method sensitivity to DNA concentration was well within the range isolated routinely in our laboratory. There were signs of departure from linearity for some samples at higher DNA concentrations and for one sample at low concentrations which did not affect the ability to discriminate whether the samples were positive or negative.

Other investigators have shown applications using a Lightcycler PCR thermocycler such as Sylvain et al (2) and Bon et al (20). Bon et al (20) used SYBR green I and melting curve analysis for genotyping of HLA-B27 and Sylvain et al (2) used a TaqMan -PCR assay using sequence-specific primers and a MGB fluorescent probe with the use of an artificial exogenous internal positive control (IPC) DNA added to each PCR reaction. This was used to distinguish true negatives from false negatives. Our assay is advantageous in that it does not use artificial exogenous control material, but instead the HGH gene as the internal control. This is an important distinction as both the quantity and quality of DNA isolated, including the presence of any PCR inhibitors, is reflected by the HGH internal control. This is something that exogenous control material cannot do.

Of note Sayer DC et al (17) reported a lack of sensitivity for HLA-B*27:12 because of a different B pocket configuration in its peptide binding groove and differs from HLA-B*27:05 AS associated F pocket configuration. For these reasons it is unlikely to be associated with AS(17,21), although it may be associated with reactive arthritis (22).

In conclusion, this real time PCR reliably and accurately types whole blood samples for HLA-B27. It is very easy to automate using the AGOWA® mag Maxi DNA isolation kit for extraction of DNA through to end result as there is no post PCR gel electrophoresis and photographic interpretation. This minimises contamination risk of post amplification PCR products and overcomes the use of the DNA intercalating dye and known carcinogen ethidium bromide. This technique is suitable for HLA-B*27 genotyping in the routine laboratory.

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References


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