Technical reports

HLA-B*27 typing by sequence specific amplification without DNA extraction

D C Sayer, H S Cassell, F T Christiansen

Abstract
HLA-B27 appears to play a direct role in the pathogenesis of ankylosing spondylitis and almost all patients with this disease have HLA-B27. Therefore, a diagnosis of ankylosing spondylitis can virtually be excluded in the absence of HLA-B27. Many techniques have been used for HLA-B*27 typing. Of these, molecular methods are the most sensitive and specific but require extracted DNA as the testing material. A technique where HLA-B*27 is amplified directly from whole blood using sequence specific primers has been developed. This technique uses small sample volumes, is not restricted by choice of anticoagulant or sample age up to at least six weeks, and can be applied to other clinical polymerase chain reaction based procedures.

Keywords: ankylosing spondylitis; HLA-B*27; whole blood; sequence specific primers

Testing for HLA-B27 is of clinical importance for the diagnosis of ankylosing spondylitis. Excluding HLA-B27 virtually excludes ankylosing spondylitis. Serological techniques such as microcytotoxicity and flow cytometry for testing for HLA-B27 require viable cells that adequately express HLA-B27 and may give false negative results if HLA-B27 is downregulated or “masked”. Flow cytometry is rapid and relatively inexpensive, but has been reported to lack specificity, especially in the presence of antigens that crossreact with HLA-B27.

Molecular techniques are sensitive and specific. However, DNA extraction makes the procedure cumbersome, time consuming, and therefore unattractive to routine clinical laboratories.

We have developed a procedure for the detection of HLA-B*27 after amplification with sequence specific primers directly on whole blood (WBSSP).

Previous attempts to amplify directly from whole blood have been inconsistent, probably because of the physical entrapment of DNA by blood proteins that are denatured as a result of the high temperatures encountered during the polymerase chain reaction (PCR). However, incorporating formamide into the PCR mix reduces the melting temperature of DNA and allows the PCR cycles to be performed at much lower temperatures, resulting in successful amplification.

Materials and methods
TEST SAMPLES
One hundred and forty two samples of blood collected into tubes containing acid citrate dextrose (Becton Dickinson, New Jersey, USA) were used in the initial WBSSP evaluation. All samples were selected randomly from routine HLA typing requests and were tested by microcytotoxicity and WBSSP. Ankylosing spondylitis was clinically indicated in 79 of these cases.

Microcytotoxicity was performed on cells after positive selection with magnetic beads (Dynabeads; Dynal, Oslo, Norway) using conventional techniques.

SAMPLE PREPARATION
Whole blood
Samples were mixed on a rotating mixer for one hour. An aliquot of 0.5 µl was removed and mixed with 6 µl of formamide (Sigma, St Louis, USA) and 3.5 µl of water and incubated at 95°C for five minutes. The final concentration of formamide in the PCR mix was 12%. This had been determined to be the optimum concentration by titration (data not shown) and may differ according to the application.

Buffy coat
The blood samples were centrifuged at 200 × g for 15 minutes. An aliquot of 1 ml of buffy coat was removed and placed into a sterile tube. From this, 0.5 µl was treated in the same way as whole blood.

HLA-B*27 SPECIFIC AMPLIFICATION
The test primers are similar to those described by Olerup (5’ primer, B27ex294F: 5’-CTACGTGGACGACACGCT -3’; 3’ primer, B27ex2199RC: 5’-AGTCTGTGCCTTGGCCTTGC-3’) and amplify a region of 141 bp in exon 2 of HLA-B. However, in contrast to the primers described by Olerup, these primers do not contain the deliberately introduced mismatch in the 5’ primer. The primers are specific for all subtypes of HLA-B*27 (HLA-B*2701–HLA-B*2713) except HLA-B*2712. The control primers HGH I (5’-CAG

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The presence or absence of HLA-B*27 was indicated by the presence or absence of the HLA-B*27 test product (fig 1). There was adequate amplification in 132 of the 142 samples. Sixteen samples were positive for HLA-B*27 by WBSSP and microcytotoxicity. The presence of HLA-B40. Importantly, however, Balas et al have shown the HLA-B2721 differs from all other HLA-B27 subtypes in the configuration of the B pocket of its peptide binding groove, and that it differs from the ankylosing spondylitis associated HLA-B2705 in its F pocket configuration. On this basis, they suggest that HLA-B2712 is unlikely to be associated with ankylosing spondylitis. If this is correct, then our assay will maintain its sensitivity for ankylosing spondylitis.

HLA-B27 typing by WBSSP has the sensitivity and specificity of a DNA based test without the additional reagent cost and “hands on” time required for DNA extraction. In addition, testing can be performed on aged samples, long after the sample has become unacceptable for flow cytometry and microcytotoxicity.

### Results and discussion
The presence or absence of HLA-B*27 was indicated by the presence or absence of the HLA-B*27 test product (fig 1). There was adequate amplification in 132 of the 142 samples. Sixteen samples were positive for HLA-B*27 by WBSSP and microcytotoxicity. All of the remaining samples (116) were negative by both microcytotoxicity and WBSSP. A result was not recorded for 10 samples because of PCR failure indicated by the lack of amplification with the control primers. Amplification failures may be explained by variations in cell numbers obtained in the small sample volume when samples are inadequately mixed. To improve the robustness of the assay,uffy coats were evaluated as the test material. Forty samples were tested in duplicate comparing

### Amplification with sequence specific primers using whole blood (WBSSP)

**Figure 1 Amplification with sequence specific primers using whole blood (WBSSP)**

TGCTTCCCAACATTCCCTTA-3′ and HGH2 (5′-ATCCACTCAGGATTCTGT TTGTGGTC-3′) have been described previously, and amplify a conserved region in the human growth hormone gene, yielding a product of 439 bp.

The PCR was performed in a final volume of 50 µl and contained 1 µM of each test primer, 0.4 µM of each control primer, 240 µM of each dNTP, 10 mM Tris HCl at pH8.3, 50 mM KCl, 1 mM MgCl₂, 0.01% gelatin, 0.1% Triton X-100, 2.5 U of AmpliTAQ polymerase (PE Biosystems, Foster City, USA), and 10 µl of the sample mix. Amplification was performed in a Perkin-Elmer 480 thermocycler as follows: seven cycles of 85°C for two minutes, 55°C for 45 seconds, and 60°C for one minute; 15 cycles of 85°C for one minute, 50°C for 45 seconds, and 60°C for 90 seconds; and 10 cycles of 85°C for one minute, 45°C for 45 seconds, and 60°C for two minutes. Products were resolved by electrophoresis in a 2% ME agarose gel at 150 V for 25 minutes.

The HLA-B*27 specific primers are not complimentary to HLA-B*2712. This allele was described by Balas et al as a Bw6 associated serological blank, which reacts with only some monoclonal antibodies with HLA-B40 reactivity. Therefore, it is unlikely that this allele would be detected by microcytotoxicity and flow cytometry, particularly in the presence of HLA-B40. Importantly, however, Balas et al have shown the HLA-B2721 differs from all other HLA-B27 subtypes in the configuration of the B pocket of its peptide binding groove, and that it differs from the ankylosing spondylitis associated HLA-B2705 in its F pocket configuration. On this basis, they suggest that HLA-B2712 is unlikely to be associated with ankylosing spondylitis. If this is correct, then our assay will maintain its sensitivity for ankylosing spondylitis.
Variability in the interpretation of microsatellite patterns with different electrophoretic conditions

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Abstract
Microsatellite markers permit the analysis of microsatellite instability and loss of heterozygosity. Frequently, the allelotypes of microsatellites are interpreted in the presence of numerous bands in gels. The importance of different gel electrophoresis conditions in the interpretation of microsatellite patterns was tested. Microsatellite markers were used to amplify DNA from gastric cancer samples and adjacent gastric mucosa. Polymerase chain reaction (PCR) products were separated by electrophoresis through 7% polyacrylamide gels containing either 5.6 M urea and 32% formamide or 7 M urea. PCR reactions separated on urea/formamide gels resulted consistently in clear allele definition (one or two bands), whereas 7 M urea gels resulted in allele patterns that comprised multiple bands. Analysis of microsatellite abnormalities using non-formamide gels gave false negative results in just under a third of cases (four of 13). In conclusion, the interpretation of microsatellite alterations in cancer DNA is improved by using electrophoresis conditions that result in complete DNA denaturation, such as urea/formamide/acrylamide gel electrophoresis.

Keywords: microsatellite instability; interpretation; electrophoresis

Microsatellite DNA sequence analysis has become an extremely valuable tool for the study of the human genome, and it has been used to characterise a number of human genetic disorders.1–7 The validity of data generated by PCR amplification of microsatellite markers rests upon the correct interpretation of the normal alleles of the proband. However, many studies have reported data collected using gel electrophoretic conditions that result in alleles constituted by numerous bands, when a maximum of two major bands should be visible from normal tissue DNA, because they result from normal diploid cells.

We compared different gel electrophoresis conditions to determine their effect on the analysis of MSI and LOH in tumour relative to non-tumour tissue from the same individual.

Materials and methods
Tissue samples and genomic DNA extraction
Thirteen patients with gastric cancer were selected. Tissue was obtained from gastric carcinomas and from non-tumour, non-metaplasia, non-dysplastic containing gastric mucosa from the same patient. Serial 5 µm thick tissue sections were obtained on glass slides and the areas of interest were microdissected after matching with an adjacent section stained with haematoxylin and eosin. DNA extraction was performed with the QiAmp tissue kit (QIAGEN, Chatsworth, California, USA), according to the manufacturer’s instructions. Cancer samples were included in the study when the tumour cells constituted greater than 70% of the tissue section.

PCR analyses of microsatellite sequences and gel electrophoresis
Both tumour and non-tumour DNA were amplified by PCR using specific oligonucleotides for the microsatellite loci D2S123,1 D13S170,2 and TP53,7 PCR reactions were performed using 2 mM MgCl2, 1.25 U of Taq Gold DNA polymerase (Perkin Elmer Corporation, Branchburg, New Jersey, USA), 20 pmol of each dideoxynucleotide, and 50 pmol of both γ-32P ATP labelled and unlabelled primer in a 50 µl reaction mixture. PCR was performed using a DNA engine (MJ Research, Watertown, Massachusetts, USA) with 45 cycles of one minute at 94°C, one minute at 50°C, and one minute at 72°C. PCR products were diluted in the same volume of formamide loading buffer (80% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, and 2 mM EDTA) and denatured at 95°C for two minutes. The PCR products were then separated in formamide containing urea gels, essentially as described by Litt and colleagues3 and in formamide free urea gels. “Formamide” gels contained 5.6 M urea, 32% formamide, and 7% polyacrylamide, whereas “formamide
Discordant results were found in over half of the cases when the D13S170 marker was used and in more than a third of the cases when the TP53 or D2S123 markers were used. Allelotyping using non-formamide containing gels produced false negative results in almost a third of the cases for LOH or MSI (four of 13 cases), and one of the 13 cases was a false positive that was read as LOH in non-formamide gels when the formamide containing gel electrophoresis but MSI when analysed using non-formamide gels.

All possible differences in the interpretation of microsatellite instability, LOH, and non-informativity status of alleles were found when the two conditions were compared for each microsatellite marker (table 1). For example, with the D13S170 marker, three cases of MSI were scored as LOH and in five instances a case was read as normal when in fact it displayed MSI or LOH (table 1). Overall, the most frequent outcome was that MSI was missed under the non-formamide containing gel electrophoresis conditions (table 1).

Different interpretation of microsatellite instability (MSI), loss of heterozygosity (LOH), and non-informativity (NI) for each microsatellite marker, when comparing the band pattern of PCR amplifications separated by the two different electrophoretic conditions is indicated.

Discordant results were found in over half of the cases when the D13S170 marker was used and in more than a third of the cases when the TP53 or D2S123 markers were used. Allelotyping using non-formamide containing gels produced false negative results in almost a third of the cases for LOH or MSI (four of 13 cases), and one of the 13 cases was a false positive that was read as LOH in non-formamide gels when the formamide containing gel electrophoresis but MSI when analysed using non-formamide gels.

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in gastric tumours, and compared the effect of different gel electrophoresis conditions on the interpretation of allele patterns relative to that of DNA extracted from tumour free gastric mucosa. In studies on genetic disease, including the molecular characterisation of tumorigenesis pathways, comparative microsatellite analysis between non-tumour and tumour samples from the same individual can be performed to identify genomic alterations in tumours. This analysis requires the correct identification of the alleles from both the tumour and non-tumour tissue. It has been shown previously that electrophoresis of PCR amplifications of microsatellite markers displaying more than two major bands in normal tissues can be the result of different migration rates of conformationally different forms of the same DNA molecule, and this phenomenon can result from incomplete DNA denaturation during gel electrophoresis. In our study we found that gels lacking formamide did not provide reliable DNA denaturing capacity, frequently resulting in the presence of several bands in gels, when a maximum of two major bands is expected from normal somatic cells. Because allelic analysis with the markers used in our study resulted in clearly defined alleles in all cases when formamide-containing gels were used, we selected these gels as the standard experimental conditions. We found that interpreting data with incompletely denatured DNA during electrophoresis (from urea gels lacking formamide) resulted in multiple types of data misinterpretation, with MSI frequently being missed in non-formamide containing gels. It is possible, however, that PCR amplification with other microsatellite markers could result in pronounced stutter bands, as a result of polymerase slippage, that would not be eliminated by separating the reactions in formamide-containing gels. Nonetheless, the analysis of microsatellite data should be performed using technical conditions that allow the demonstration of no more than two major DNA bands, corresponding to the two expected alleles from normal tissue. Therefore, if microsatellite amplification results in multiple bands with no obvious major band within the expected allele size range, the use of formamide containing gels might help achieve a better DNA separation, with easier scoring of allele status.

Many publications are currently being generated by the use of microsatellite marker analyses with PCR to investigate both MSI and LOH at many loci in the genome of tumours and pre-neoplastic lesions. It is therefore of great importance to assess such changes correctly, to avoid the accumulation of biased data in the literature. Furthermore, the future use of this technology in diagnostic applications might be hindered by lack of reproducibility among laboratories, indicating that strict conditions should be followed and guidelines should be available for laboratory reference. For example, MSI is a typical finding in colon tumours from individuals with hereditary non-polyposis colorectal cancer, and a lack of identification of MSI could result in an index case being missed, potentially affecting the clinical follow up of the individual as well as other family members. LOH studies using multiple microsatellite markers are performed frequently in the research setting to search for putative tumour suppressor genes, and it is therefore important that scoring of allele changes should be accurate. In our study, we show that accurate analyses of MSI and LOH with some microsatellite markers can be achieved by using formamide containing gels run under denaturing conditions, in contrast to the more variable results obtained with typical urea/acylamide gel electrophoresis.

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