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Short communication

Low-cost simultaneous detection of CCR5-delta32 and HLA-B*5701 alleles in human immunodeficiency virus type 1 infected patients by selective multiplex endpoint PCR

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Host genetic traits impact susceptibility to human immunodeficiency virus type 1 (HIV-1) infection, disease progression as well as antiretroviral drug pharmacokinetics and toxicity. Remarkable examples include a 32-bp deletion in the CCR5 coreceptor molecule (CCR5-delta32) impairing attachment of monocytotropic HIV-1 to the host cell membrane and the HLA-B*5701 allele, strongly associated with a potentially fatal hypersensitivity reaction triggered by abacavir, a nucleoside inhibitor of HIV reverse transcriptase. We developed a simple selective multiplex endpoint PCR method for simultaneous analysis of both genetic traits. Two primers were designed for amplification of a region surrounding the CCR5 32-bp deletion site. One common forward primer and two reverse primers with different 3′ termini targeting the HLA-B*570101 and HLA-B*570102 alleles were designed for HLA-B*5701 analysis. A panel of 110 reference DNA samples typed in the HLA-B locus was used for development and blind validation of the assay. All the 45 HLA-B*5701 positive and the 55 HLA-B*5701 negative samples were correctly identified. The CCR5-delta32 allele was readily detected in 7 samples and did not interfere with detection of HLA-B*5701 while providing an internal amplification control. Multiplex PCR products were easily identified in agarose gels with no background noise. This simple and low-cost end-point selective multiplex PCR can conveniently screen HIV patients for the protective CCR5-delta32 allele and the risk of developing abacavir hypersensitivity reaction.

Some host genetic traits have been documented to have an impact on susceptibility to human immunodeficiency virus type 1 (HIV-1) infection, HIV-1 disease progression or antiretroviral drug metabolism and adverse reactions (Limou and Zagury, 2013; Barreiro et al., 2014). For example, a 32-base pair deletion in the gene coding for the main HIV-1 coreceptor CCR5 (CCR5-delta32) results in a truncated protein failing to bind the outer HIV-1 envelope glycoprotein gp120, thus preventing infection by the widely occurring CCR5-tropic (R5) HIV-1 variants (Arenzana-Seisdedos and Parmentier, 2006). While individuals who are homozygous for the CCR5-delta32 allele are naturally resistant to infection by R5 HIV-1, heterozygous subjects infected with HIV-1 experience slower disease progression and appear to achieve better treatment response compared to homozygous wild type CCR5 patients (Mulherin et al., 2003; Wang et al., 2014). Indeed, delta32-CCR5 is one of the genetic traits partly explaining viral load variability in the natural history of HIV-1 infection (Fellay et al., 2009). Among factors influencing adverse reactions to antiretroviral drugs, the most important is the HLA-B*5701 allele, particularly the B*570101 and B*570102 variants, strongly associated with a potentially fatal hypersensitivity reaction (HSR) to the widely used reverse transcriptase nucleoside analog abacavir (Sousa-Pinto et al., 2015). Although only a fraction of HLA-B*5701 positive subjects will develop HSR upon exposure to abacavir, the absence of this allele has a negative predictive value for HSR equal or close to 100%. Thus, testing for the HLA-B*5701 allele is mandatory before administration of abacavir. While the HLA-B57 or HLA-B57/58 gene family can be detected by serological methods, this approach lacks resolution resulting in prudentially denying abacavir to HLA-B57 positive but HLA-B*5701 negative subjects who could benefit the drug (De Spiegelaere et al., 2015). Incidentally, HLA-B*5701 has also been associated with viral control and delayed disease progression and it is thus considered as a protective allele (UK Collaborative HIV Cohort Study Steering Committee; Vaidya et al., 2013).

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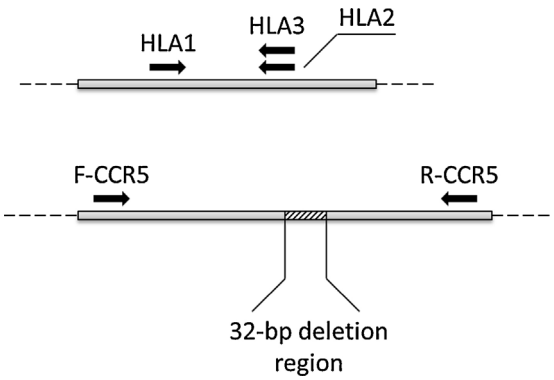

Fig. 1. Relative localization of the five primers used in the multiplex CCR5 delta32 and HLA-B*5701 selective PCR. Note that the CCR5 gene and the HLA-B locus are on different chromosomes (21 and 6, respectively).

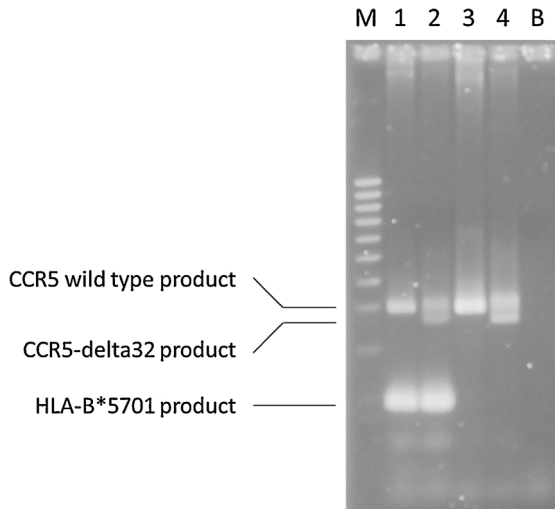

Fig. 2. Schematic representation of agarose gel results. Lane M, molecular weight marker (100 base pair DNA ladder, from 100 to 1000 base pairs). Lane 1, CCR5 wild type/wild type homozygous and HLA-B*5701 positive. Lane 2, CCR5 wild type/delta32 heterozygous and HLA-B*5701 positive. Lane 3, CCR5 wild type/wild type homozygous and HLA-B*5701 negative. Lane 4, CCR5 wild type/delta32 heterozygous and HLA-B*5701 positive. Lane B, blank PCR product.

Methods used to detect HLA-B*5701 and discriminate it from highly related alleles include costly high resolution sequencing, cumbersome systems based on PCR amplification of a large number of different HLA-B targets and more convenient sequence-specific PCR procedures (Stocchi et al., 2012; De Spiegelaere et al., 2015). These assays are mostly in a real-time PCR format and usually based on amplification refractory mutation systems (ARMS), use of discriminating probes or melting curve analysis. However, their setup is not trivial and inconsistencies have been documented (Darke and Corbin, 2014). We have developed a simple ARMS specifically targeting the HLA-B*5701 allele through endpoint PCR. As an internal amplification control, the assay includes simultaneous amplification of the CCR5 gene region across the site where the 32-base pair deletion may occur. Detection of the CCR5 and HLA-B*5701 PCR products is simply accomplished by agarose gel electrophoresis of the reaction mixture without the need for a real-time machine and any labeled probe.

Frozen whole blood samples were obtained from the previously reported EPI109367 prospective study specifically addressing the prevalence of HLA-B*5701 in HIV-1-infected patients from UK by using high resolution sequence-specific primer typing (Orkin et al., 2010). Use of the samples for setup and validation of the assay was approved by the institutional review board of the University of Siena and regulated with ViiV Healthcare as an amended research protocol for HLA-B genotyping with a new method. The sample panel included 700 coded samples of which 45 were HLA-B*5701 positive, 10 were HLA-B57 positive but HLA-B*5701 negative and 640 were from individuals carrying non-B*57 alleles. Three unblinded HLA-B*5701 positive and three HLA-B*5701 negative samples were used for the assay setup. To generate an HLA-B*5701 enriched validation panel, all the HLA-B*5701 positive, 5 HLA-B57 positive but HLA-B*5701 negative and 60 randomly selected B57 negative samples were recoded and blindly tested with the assay developed.

Total DNA was extracted from 200 μl of whole blood by using the EZ1 Advanced XL Qiagen instrument with the EZ1 DSP Virus Kit according to the manufacturer instruction. The DNA was measured spectrophotometrically and diluted to 10 ng/μl. Five primers were used for the multiplex PCR reaction (Fig. 1). Three primers target the HLA B*5701 allele: HLA1 (5′-GTCTCACATCATCCAGGT-3′), HLA2 (5′-ATCCTTGCCGTCGTAGGCGG-3′) and HLA3 (5′-ATCCTTGCCGTCGTAGGCAG-3′) (Martin et al., 2005). The reverse primers HLA2 and HLA3 target the HLA B*570101 and B*570102 alleles, respectively, both coupled with the invariant forward primer HLA1, yielding a PCR product of 96 base pairs in length. Two primers target the flanking region of the delta32 CCR5 deletion: F-CCR5 (5′-CCTGGCTGTCGTCCATGCTG-3′) and R-CCR5 (5′-TTCTCATTTCGACACCGAAGCAG-3′). The CCR5 primers generate two different PCR products, either 300 or 268 bases pairs in length depending on the presence or absence of the 32-base pair deletion. The PCR reaction was performed in 30 μl of total volume containing 1 U GoTaq Hot Start in its own 1× Green Buffer (Promega, Madison, WI, USA), 2.5 mM $MgCl_2$, 7.5 pmol of each HLA1, HLA2 and HLA3 primer, 5 pmol of each F-CCR5 and R-CCR5 primer, and 5 μl (50 ng) of DNA sample. A touchdown PCR strategy was chosen to increase specificity, consisting of (95 °C × 25 s, 65 °C × 50 s, 72 °C × 25 s) × 13 cycles, (95 °C × 25 s, 60 °C × 50 s, 72 °C × 15 s) × 10 cycles and (95 °C × 25 s, 55 °C × 50 s, 72 °C × 10 s) × 27 cycles. Reaction mixtures were directly loaded on a 3% agarose gel (2.4% Nusieve + 0.6% Seakem gel; Lonza, Basel, Switzerland) for 1 h and 30 min at 6 V/cm and stained with ethidium bromide.

Initial experiments were designed to evaluate the impact of relative primer concentrations and different thermal profiles on sensitivity and specificity. HLA and CCR5 primer sets were combined in the range 2.5–10.0 pmol each in 2.5 pmol increments. The best balance between the HLA and CCR5 products was obtained with 7.5 pmol of HLA primers and 5 pmol of CCR5 primers. However, to warrant consistent generation of a sharp HLA-B*5701 signal a three-temperature touchdown PCR procedure was necessary. Using the final protocol as described above, the system correctly identified all of the 45 HLA-B*5701 positive samples and scored as HLA-B*5701 negative both the 5 HLA-B57 positive but HLA-B*5701 negative and the 60 B57 negative samples, achieving a 100% specificity and 100% sensitivity on this control panel. The wild type homozygous CCR5 and the mixed heterozygous wild type/delta32 PCR products, detected in 7 cases, were always visible and clearly distinguishable from each other by the different gel mobility. The system was then used on a panel of 618 samples from HIV-infected patients as part of their initial characterization at HIV diagnosis as recommended by Italian guidelines. Of these, 20 (3.24%) scored HLA-B*5701 positive and 48 (7.77%) had CCR5 delta32/wild type heterozygosis. Co-occurrence of both HLA-B*5701 and CCR5 delta32/wild type heterozygosis was documented in only 1 (0.16%) patient and no patient was CCR5 delta32/delta32 homozygote. Of note, abacavir was later used in 121 of the HLA-B*5701 negative patients and no suspected HSR was reported. Schematic representation of end-point PCR result is shown in Fig. 2.

HLA-B*5701 screening is necessary prior to administration of abacavir according to current treatment guidelines. Because HLA testing is normally performed at immunogenetics units, detection of the B*5701 allele is often based on screening of the whole HLA locus by a variety of cumbersome, expensive and time-consuming methods. Alternatively, serological methods with lower cost and faster turnaround time can be applied but these systems have lower two-digit resolution and thus conservatively deny use of abacavir to all HLA-B57 positive patients rather than only to those who are HLA-B*5701 positive. Indeed, such low-resolution methods should be complemented by a higher-resolution second-level assay (De Spiegelaere et al., 2015). Following the paper by Martin et al. (2005), HLA-B*5701-specific, fast and cost-effective methods based on real time PCR have been developed and marketed allowing incorporation of this parameter into the test panel performed at the HIV monitoring laboratory (Stocchi et al., 2012). The protocol described here is derived from the original assay by Martin et al. (2005) and maintains simple agarose gel electrophoresis as the detection method, not requiring a real time PCR instrument. While the generic HLA-B57 primers have been removed, the system combines HLA-B*5701 with CCR5-delta32 detection in a single amplification, providing an internal amplification control as well as an extra marker of disease progression at no increased cost. This assay has undergone extensive validation thanks to the availability of a large sample panel deliberately enriched with HLA-B*5701 positive cases. The validation study is indeed larger than what done with certified real time PCR assays. Field application of the system confirmed its ease of use and fast turnaround time. The prevalence of the HLA-B*5701 allele in our case file appears to be lower than previously reported in Italy (Orkin et al., 2010) but one quarter of the patients tested were black Africans, known to be HLA-B*5701 positive at very low frequency (Lucotte, 1997). The system can be used both in high income countries as a replacement for inconvenient immunogenetics methods and in low-middle income countries were expenses for equipment and reagents are a top priority.

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