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Detection of *HLA-B*57:01* by real-time PCR: implementation into routine clinical practice and additional validation data

Aim: *HLA-B*57:01* status needs to be determined before initiating abacavir therapy. We developed a pharmacogenetic real-time (Q)-PCR screening test using two sets of sequence specific primers. This test has been implemented into routine clinical practice. **Materials & methods:** HIV-infected patients admitted at our University Hospital were thus genotyped using the above mentioned test. A panel of 80 DNA samples with a known genotype were used to characterize Q-PCR conditions using different master mixes. **Results:** A total of 353 patients were genotyped, detecting 15 (4.25%) *HLA-B*57:01* positive carriers. Among the negative patients, 17.2% were treated with abacavir without any hypersensitivity reaction. Using different Q-PCR master mixes, significantly lower cutoff Ct values were found, thus new analytical settings are provided. **Conclusion:** The pharmacogenetic test developed in our laboratory for the fast screening of *HLA-B*57:01* can be successfully implemented into routine clinical practice. All 16 sequences (including an additional six) currently known for the *HLA-B*57:01* allele are detected by sequence specific primers used in this test. The Brilliant II SYBR® Green QPCR MM (Stratagene) can safely replace the master mix originally used to develop the test.

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KEYWORDS: abacavir ■ HIV-1 ■ *HLA-B*5701* ■ hypersensitivity reaction ■ pharmacogenetic test ■ real-time PCR

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Combination antiretroviral (ARV) therapy (cART) has significantly increased the life expectancy of HIV-infected patients, reducing both morbidity and mortality [1]. Since the recognition of AIDS as a clinical syndrome, roughly 30 years ago, there have been 26 approved ARV drugs in six mechanistic classes, which are now used to design combination regimens [2]. At present, cART is recommended for all HIV-infected individuals to reduce the risk of disease progression and HIV transmission [101], thus increasing the extent of patient exposure to ARV drugs. Strategies that limit or prevent drug toxicity, have been successfully implemented into routine clinical practice, including therapeutic drug monitoring [3–5] and pharmacogenetic testing before initiating abacavir (ABC)-containing regimens [6].

ABC is a guanosine analog acting as a reverse transcriptase inhibitor, approved for clinical use since 1998 in combination with other ARV drugs for the treatment of HIV infection. It is generally a very well tolerated drug, except for the occurrence of an immune-mediated hypersensitivity reaction that in clinical trials has been observed in 5–8% of treated patients when only clinical criteria for the diagnosis were used [7]. The hypersensitivity reaction to ABC

is a multiorgan clinical syndrome, appearing within the first 6 weeks of therapy that requires an immediate and permanent discontinuation of the drug in order to avoid a more severe, and often fatal, reaction [8]. Four years after its commercialization, two independent research groups reported a significant association between the hypersensitivity reaction to ABC and the positive-carrier status of the *HLA-B*57:01* allele [9,10]. Since then, a powered, double-blind, prospective, randomized study (PREDICT-1), undertaken in Europe and Australia, demonstrated the clinical utility of *HLA-B*57:01* screening before initiating ABC-containing cART regimens to prevent immune-mediated hypersensitivity reactions [7]. However, the limited number of non-white individuals (<20%) enrolled in the trial restricted the possibility of directly translating these findings across different races. In this regard, a retrospective case–control study (SHAPE), undertaken in the US, confirmed the sensitivity and specificity of the *HLA-B*57:01* allele as a genomic marker for ABC hypersensitivity reaction in the African–American population [11], which suggests the clinical utility of screening in different populations. Finally, a randomized (1:1), open-label, multicenter, North American study

(ARIES) definitely confirmed the clinical utility of *HLA-B*57:01* screening before initiating cART containing regimens in order to prevent immunologically mediated ABC hypersensitivity reactions across races [12]. Therefore, current international HIV treatment guidelines recommend screening for *HLA-B*57:01* before initiating patients on an ABC-containing regimen [101] and such requirements have also been included in the licenses of ABC-containing products.

The HLA coding region, located on the short arm of chromosome 6, is a highly polymorphic region, particularly the class I locus for which 7553 *HLA-A*, *HLA-B* and *HLA-C* alleles have been so far reported in the immunogenetics (IMGT)/HLA database [13]. In this regard, *HLA-B* appears to be the most polymorphic gene (3086 alleles of the 7553 described) of the HLA region and of the entire genome. Moreover, different DNA sequences code for the same allele. At the present, 16 different sequences are reported in the IMGT/HLA database that code for the *HLA-B*57:01* allele [13], with the *HLA-B*57:01:01* DNA sequence differing from the reference sequence, that is, *HLA-B*07:02*, by 59 point mutations. Similarly to other HLA class I genes, the highest genetic variability is found in the exon 2 and exon 3 sequences, which are regions that encode the peptide binding domains of the HLA class I molecules [14]. Owing to the relatively high similarity of these sequences, the gold-standard technique to identify a particular class I allele remains as DNA-sequence-based typing. However, this technique is not always readily available; therefore, other molecular techniques with rapid turnaround times have recently been developed to quickly detect the *HLA-B*57:01* allele. These approaches are mostly based on sequence specific primers (SSPs) or oligonucleotide probes (as reviewed by Stocchi [15]).

In this regard, we characterized and validated a specific, sensitive and rapid pharmacogenetic test for the identification of *HLA-B*57:01* positive patients, using two sets of SSPs and the real-time (Q)-PCR technique for the detection of amplified DNA products. The detailed methodology and the results of its validation by comparison with a reference method have been published previously [16]. Since February 2010 the test has been implemented into routine clinical practice, and within the first 3 years of clinical activity (up to March 2013) has genotyped a total of 353 patients and detected 15 (4.25%) *HLA-B*57:01*-positive carriers. An analysis conducted on the remaining 338 negative patients showed that 19 patients were

previously treated with ABC, and two of them are still currently being administered the drug. Interestingly, among the remaining 319 *HLA-B*57:01*-negative patients, 55 (17.2%) initiated an ABC-containing treatment without any occurrence of hypersensitivity reactions, supporting the clinical use of this test. However, the main limitation of this and other SSP-based approaches is represented by the continuous need for sequence updates owing to the highly polymorphic nature of *HLA-B*. Moreover, owing to the relatively high homology of the sequences between all these different alleles, other technical problems can arise because of even small changes in the PCR conditions (i.e., thermocyclers and PCR reagents). In fact, the present study was carried out to overcome the technical problem raised by discontinuation of the Brilliant SYBR® Green Q-PCR Master Mix (Reference MM; Stratagene, CA, USA), whereas the suggested equivalent reagent (Brilliant III Ultra Fast SYBR Green Q-PCR MM, MM1) could not be used under the PCR conditions previously described [16]. Thus, other reagents with similar kinetics (MM2: Brilliant II SYBR Green QPCR MM, Stratagene; MM3: SYBR Green Jumpstart Taq Ready Mix, Sigma-Aldrich, MO, USA) were selected and the characterization of their performance within our test is provided. Briefly, both master mixes were used to reanalyze the panel of 80 DNA samples with known genotype, originally provided by GlaxoSmithKline (GSK) for the validation of the assay. Samples were analyzed using the cycling conditions previously described [16], but significant lower average values of the cutoff threshold cycle (Ct) of positive samples were measured. Thus different cutoff Ct values were calculated in order to detect positive amplifications, which are the following: MM2: <14 for the exon 3 and <12 for the exon-2-specific Q-PCR; MM3: <12.8 for the exon 3 and <12 for the exon-2-specific Q-PCR. The latter appeared to be less specific in the identification of the desired target, thus it was not further characterized. On the other hand, MM2 has been used in parallel with MM1 in nine different Q-PCR runs. Results obtained with the two MMs are 100% concordant, which suggests that MM2 can safely replace MM1. In conclusion, data presented in this manuscript suggest that the pharmacogenetic *HLA-B*57:01* screening test developed in our laboratory can be safely used in the clinical setting, even with introduction of a different Q-PCR master mix.

Materials & methods

■ DNA samples

Blood samples were withdrawn from HIV-infected patients routinely admitted at the outpatient clinic of the Institute of Clinical Infectious Diseases, Catholic University Medical School of Rome (Rome, Italy), when an ABC-containing regimen was planned (cART-experienced patients) or at the first visit after HIV infection was detected (cART-naïve patients). Whole-blood samples were collected in EDTA treated tubes and stored at -20°C until the day of DNA extraction.

We have used 80 DNA samples with known genotype to characterize PCR conditions with different Q-PCR master mixes (MM2 and MM3). The 80 DNA samples were selected from a representative sample (776) of a multiethnic population used to validate an alternative screening method during the EPI109367 clinical trial, funded by GSK [17]. This panel was enriched in *HLA-B*57:01* positive samples (46.3%), including six alleles belonging to the *B*57* family, and was used to validate our pharmacogenetic test using the reference MM [16]. In addition, 61 more DNA samples from HIV-infected patients who visited at the outpatient clinic of our University Hospital were genotyped using both Reference MM and MM2, bringing the total sample of interest to 837.

■ Total DNA extraction

Total DNA was prepared from whole-blood samples, using the QIAamp® DNA Blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, aliquots of 200 µl for each whole-blood sample were used, and total DNA was eluted in 200 µl elution buffer AE (provided by the kit). DNA concentration was measured using the Quant-iT™ PicoGreen® DNA Assay Kit (Invitrogen Corporation, Paisley, Scotland). A standard curve in the range of 0–10 ng was run in each assay using Lambda DNA standards, provided by the kit. Consistent with data reported in the kit handbook, final DNA yield was approximately 5 µg per sample.

■ *HLA-B*-specific amplification by PCR

HLA-B-specific amplification between the first and the third intron was carried out by PCR, using the following validated primers: 5Bin1–57 forward primer (F: 5'-GGG AGG AGC GAG GGG ACC G/CCA C-3', Intron 1: 36–57) and 3Bin 3–37 reverse primer, (R: 5'-GGA GGC CAT CCC CGG CGA CCT AT-3', Intron 3: 37–59), yielding a 922 bp product [18]. The

PCR reaction contained 50 ng of total DNA, 1X PCR Buffer, 300 nM of each primer, 1.25 U of AccuPrime Pfx DNA Polymerase (Invitrogen Corporation) in a 50 µl final volume. After initial denaturation (10 min at 95°C), a total of 35 PCR cycles were conducted, using the following two-step PCR conditions: denaturation at 95°C 20 s, and annealing/extension of 1 min at 68°C, in a MasterCycler ep thermocycler (Eppendorf, Hamburg, Germany). The amplicons were separated by electrophoresis through 1.5% agarose gels containing 0.1 µg/ml ethidium bromide. This step allowed us to verify the efficiency of DNA amplification, in order to avoid the processing of not correctly preamplified samples thus reducing the incidence of false-negative results.

■ *HLA-B*57:01* typing by SSPs-real time PCR

HLA-B preamplified DNA was used for *HLA-B*57:01* allele detection by Q-PCR, performed with two sets of primers specifically designed to encompass the highest variable regions of the *HLA-B* DNA sequence [16]. The first set consists of forward primer 345F (5'-GTC TCA CAT CAT CCA GGT GAT GT-3') and reverse primer 419R (5'-GAC TGG TCA TGC CCG CGG AG-3'), allowing for the amplification of a 75 bp amplicon, corresponding to nucleotides 1020–1094 on exon 3 of the AJ458991 sequence (NCBI GenBank database). The second set of primers consists of forward primer 193F (5'-GCG AGT CCG AGG ATG GCG CC-3') and reverse primer 319R (5'-GGA GCG CGA TCC GCA GGT TCT-3'), yielding a 127 bp product, corresponding to nucleotides 622–748 on exon 2 of the AJ458991 sequence (NCBI GenBank database). Q-PCR reactions were carried out in a 20 µl reaction volume, using *HLA-B* preamplified DNA diluted 1:1000, 163 nM of each primer and the Brilliant SYBR Green Q-PCR Master Mix 2X (Reference MM, Stratagene) in a MX3000P Q-PCR machine. Cycling conditions were the following: 20 cycles of denaturation at 95°C for 20 s; annealing at 64°C; and extension at 72°C for 30 s for the exon-3-specific Q-PCR, taking Ct values <15 to determine positive amplifications; 15 cycles of denaturation at 95°C for 20 s; annealing at 65°C; and extension at 72°C for 30 s for the exon-2-specific Q-PCR, taking Ct values <14 as positive amplifications [16].

In May 2011, Stratagene discontinued the production of the Reference MM. The suggested equivalent product, namely the Brilliant III

Ultra Fast SYBR Green Q-PCR Master Mix (MM1, Stratagene), which contains an enzyme specifically engineered for faster replication, allowing for the performance of one-step PCRs at the optimal annealing/extension temperature of 60°C, appeared not suitable for the present test. Thus, other reagents with similar kinetics to the Reference MM (i.e., MM2: Brilliant II SYBR Green QPCR MM, Stratagene; and MM3: SYBR Green Jumpstart Taq Ready Mix, Sigma-Aldrich) were selected. MM2 and MM3 were fully characterized using the panel of 80 DNA samples with known genotypes provided by GSK. The samples were reanalyzed using the same cycling conditions as described above. Cutoff Ct values for each new MM were calculated as published, considering the average Ct value of the positive samples and the cycle differences among the lowest and the highest positive sample. Using these new parameters, MM2 has been used in parallel to the Reference MM in nine different Q-PCR runs.

■ Data analysis

All 141 results obtained with MM2 were compared with the relative results obtained using the reference MM. To rate the compliancy between the two methods we used the Cohen's κ -statistic of inter-rater agreement [19]. Cohen's κ , a general measure of agreement for categorical data, is computed using the following formula: $\kappa = (P_0 - P_e) / (1 - P_e)$, where P_0 is the observed proportion of pair results from MM2 and Reference MM exhibiting agreement and P_e is the proportion expected to show agreement by chance alone. The statistic can vary between $\kappa = 1$ indicating complete agreement and $\kappa = 0$ indicating no agreement other than that expected by chance only, given the size of the sample [20]. Results were analyzed by Stata 10 (Stata Statistical software: Release 11, 2007; StataCorp LP, TX, USA).

Results

■ Analysis of routine samples

DNA samples from HIV-infected patients admitted at the outpatient clinic of our University Hospital were analyzed, using the Q-PCR method previously described [16]. As shown in TABLE 1, in 3 years of clinical activity a total of 353 patients were genotyped, among which 15 (4.25%) were found to be *HLA-B*57:01*-positive carriers and thus excluded from ABC-containing therapeutic regimens. Our technique also allows us to detect potential carriers of other *B*57* alleles by a positive amplification in the exon-2-specific

Q-PCR. In fact, 16 *HLA-B*57:01* negative patients were typed as potential carriers of different *B*57* alleles (TABLE 1). An analysis carried out on the total 338 *HLA-B*57:01*-negative patients showed that 19 patients were treated with ABC-containing cART between the years of 1999–2007, that is, before the recommendation of *HLA-B*57:01* screening. Only two of them are currently being administered ABC (FIGURE 1). Among the remaining negative patients (319), 55 (17.2%) were treated with ABC without any occurrence of hypersensitivity reactions. However, nine out of 55 patients discontinued the treatment (FIGURE 1).

■ Standardization of Q-PCR cycling conditions with new commercially available master mixes

Using the Q-PCR settings previously described [16], we have reanalyzed in three different PCR runs 80 DNA samples provided by GSK and previously genotyped in the EPI109367 trial using MM2. However, the threshold had to be manually adjusted in order to exclude false-positive samples. In particular, in the exon-3-specific Q-PCR, the threshold was set up to 0.150, in order to discriminate the other *HLA-B*57* alleles from *HLA-B*57:01*. With this setting, a total of 37 positive samples were identified, that is, amplification detected in both Q-PCRs. As summarized in TABLE 2, with MM2 we found in the exon-3-specific Q-PCR an average Ct value of 12.68 ± 0.08 (standard error of the mean [SEM]), with an average melting temperature (T_m) of the amplification product equal to 85.96 ± 0.07 (SEM); and in the exon-2-specific Q-PCR, an average Ct value of 10.52 ± 0.08 (SEM), and an average T_m equal to 87.25 ± 0.04 (SEM). The cutoff Ct value (<14) for the exon 3 Q-PCR was calculated considering the average Ct calculated for the positive samples: 12.68 ± 1.13 cycle, since 2.25 cycle differences were measured among the lowest and the highest positive sample; and cutoff Ct value (<12) for the exon-2-specific Q-PCR was calculated considering the average Ct calculated for the positive samples: 10.52 ± 1.22 cycle, since 2.44 cycle differences were measured among the lowest and the highest positive sample (TABLE 3).

Similarly, 79 DNA samples provided by GSK (one positive sample was excluded since there was not enough DNA remaining), were reanalyzed in three different PCR runs, using the MM3 and the Q-PCR settings previously characterized [16]. With this different reagent, the thresholds used to measure the Ct for each

run were manually increased up to 0.15 for the exon 3 Q-PCR and up to 0.20 for the exon 2 Q-PCR, in order to eliminate false-positive samples. With these settings, a total of 36 positive samples were identified, with an average Ct value of 11.88 ± 0.05 (SEM), and an average Tm of the amplification product equal to 88.41 ± 0.03 (SEM) for the exon-3-specific Q-PCR; and an average Ct value of 10.72 ± 0.06 (SEM), and an average Tm equal to 89.95 ± 0.02 (SEM) for the exon-2-specific Q-PCR (TABLE 2). The cutoff Ct value (<12.9) for the exon-3-Q-PCR was calculated considering the average Ct calculated for the positive samples: 11.88 ± 1.05 cycle, since 2.09 cycle differences were measured among the lowest and the highest positive sample; and cutoff Ct value (<12.2) for the exon-2-specific Q-PCR was calculated considering the average Ct calculated for the positive samples: 11.07 ± 1.12 cycle, since 2.23 cycle differences were measured among the lowest and the highest positive sample (TABLE 2). However, with this master mix, despite the adjusted parameters, we detected one false-positive sample; therefore, additional validation data were generated using only MM2.

With the newly found Q-PCR analytical conditions, MM2 has been used in parallel to the Reference MM in nine different Q-PCR runs. Results obtained with the two reagents are 100% concordant (59 *HLA-B*57:01* negative and two positive patients). In total using MM2, 141 DNA samples were analyzed, 39 samples were identified positive and 102 negative.

Table 1. Number of patients genotyped using real-time PCR between January 2010 and March 2013.

Patients genotyped	n (%)
Total	353 (100)
<i>HLA-B*57:01</i> positive	15 (4.25)
<i>HLA-B*57:01</i> negative	338 (95.75; 16 potential carriers of different <i>HLA-B*57</i> alleles)

Cohen's κ statistic was found to be equal to 1 (standard error = 0.0842, $p < 1\%$) indicating that the results obtained with MM2 are equivalent to those obtained with the Reference MM. Finally, in order to assess the error rate owing to possible changes in the PCR efficiency, one positive sample was measured in triplicate in 11 different Q-PCR runs. The average Ct value was 12.67 ± 0.07 (mean $\pm$ SEM, $n = 11$) for the exon-3-specific PCR, and 10.59 ± 0.05 (mean $\pm$ SEM, $n = 11$) for the exon 2 PCR. Ct values were found consistently below the cutoff value, indicating the high reproducibility of the results in different PCR runs.

Discussion

International HIV-treatment guidelines recommend *HLA-B*57:01* typing before ABC administration in order to reduce the incidence of ABC hypersensitivity reaction [101]. A number of different techniques with rapid time of response have been developed and are at the present available to detect the *HLA-B*57:01* allele. In a recent article, Stocchi and collaborators provided an update on what is currently available in the field, outlining

Figure 1. Analysis of patients genotyped using real-time PCR between January 2010 and March 2013.

†One re-challenge after a previous diagnosis of HSR was obtained using clinical criteria.
ABC: Abacavir; HSR: Hypersensitivity reaction.

Table 2. Positive results from the set of 80 samples provided by GlaxoSmithKline.

Master mix	Exon Q-PCR	Background (cycles)	Threshold	Average Ct $\pm$ SEM	Average Tm $\pm$ SEM
MM2	3	3–10	0.150	12.68 $\pm$ 0.08 (n = 37)	85.96 $\pm$ 0.07 (n = 37)
	2	3–7	0.150	10.52 $\pm$ 0.08 (n = 37)	87.25 $\pm$ 0.04 (n = 37)
MM3	3	3–10	0.150	11.88 $\pm$ 0.05 (n = 36)	88.41 $\pm$ 0.03 (n = 36)
	2	3–7	0.150	10.72 $\pm$ 0.06 (n = 36)	89.65 $\pm$ 0.02 (n = 36)

Samples were analyzed in three different PCR runs carried out per set of primers, using two different master mixes (MM2: Brilliant II SYBR® Green QPCR MM, Stratagene; MM3: SYBR Green Jumpstart Taq Ready Mix, Sigma-Aldrich). Data were analyzed using fixed parameters for background correction and fluorescence threshold. Data, average Ct (each sample measured in triplicate) and average Tm values, relative to 37 positive amplifications are reported.

Ct: Threshold cycle; Q-PCR: Real-time PCR; SEM: Standard error of the mean; Tm: Melting temperature.

the scientific and pharmacoeconomic pros and cons of such techniques [15]. In this regard, we have characterized a test in Q-PCR, which is a fast and direct method to amplify and analyze genomic DNA, and suggested that this test could be easily implemented into routine clinical practice, particularly in those laboratories already involved in detection of viral load and virus genotyping [16]. In the present paper, data from 3 years of clinical use of this test in our University Hospital are provided. A total of 353 HIV-infected patients were genotyped, with 15 patients typed as *HLA-B*57:01* positive carriers. As recommended, these patients were excluded from therapeutic regimens containing ABC. Among all the negative patients, 16 were typed as potential carriers of other *HLA-B*57* alleles, which appears to be clinically relevant information. The HLA class I molecules are directly involved in mediating susceptibility and clinical outcome of several inflammatory conditions, autoimmune diseases and infectious diseases, including HIV clinical course [21]. In this regard, HLA-B, has been significantly related to HIV clinical progression. Interestingly, *HLA-B*57:01* is frequently retrieved in persons that spontaneously control the infection by HIV, whereas the *HLA-B*57:02*

and *03* alleles are often associated with a slower rate of disease progression [21]. However, a perspective evaluation on the disease clinical course has not been performed in the present study. None of these *HLA-B*57* potential carriers have been treated with ABC.

An analysis carried out on the total 338 *HLA-B*57:01* negative patients showed that 19 patients were treated with ABC-containing cART between the years of 1999–2007, that is, before the recommendation of the *HLA-B*57:01* screening, and were genotyped afterwards. Two of them are currently being administered ABC, while all the others discontinued the drug after several months (or years) of use for different reasons, including gastrointestinal toxicity, virological failure, recruitment in clinical trials, simplification strategies and structured treatment interruption. In our cohort, we found two patients, treated with ABC in prescreening times that discontinued the therapy after 1 year due to patient's decision or after 3 years due to a virological failure, respectively. These patients were subsequently genotyped as *HLA-B*57:01*-negative carriers and successfully rechallenged with ABC. Conversely, one of these patients that interrupted the therapy after 8 weeks owing to a

Table 3. Standardized real-time PCR conditions for detection of *HLA-B*57:01* with different master mixes.

MM	Commercial reagents	Exon Q-PCR	Annealing temperature	Cutoff Ct value	Threshold	Background (cycles)
Reference MM	Brilliant SYBR® Green Q-PCR Master Mix (Stratagene)	3	64°C	<15	0.050	3–10
		2	65°C	<14	0.150	3–7
MM1	Brilliant III Ultra Fast SYBR Green Q-PCR Master Mix (Stratagene)	3	60°C	None	None	None
		2	60°C	None	None	None
MM2	Brilliant II SYBR Green Q-PCR Master Mix (Stratagene)	3	64°C	<14	0.150	3–10
		2	65°C	<12	0.150	3–7
MM3	SYBR Green Jumpstart Taq Ready Mix (Sigma-Aldrich)	3	64°C	<12.9	0.150	3–10
		2	65°C	<12.2	0.200	3–7

Ct: Threshold cycle; MM: Master mix; Q-PCR: Real-time PCR.

hypersensitivity reaction diagnosed using clinical criteria, was indeed subsequently discovered to be a *HLA-B*57:01*-positive carrier and was not rechallenged with ABC. Moreover, 17.2% (55 out of 319) of the remaining *HLA-B*57:01*-negative patients were treated with ABC. This percentage appears to be consistent with the current International HIV-treatment guidelines, that recommend the drug as an alternative NRTI in cART-naïve patients [101]. None of these patients developed a hypersensitivity reaction, including a total of six patients that were rechallenged with the drug, one of which was after a previous interruption due to a suspected hypersensitivity reaction. All together these observations are consistent with data gathered in the clinical studies that supported the use of the *HLA-B*57:01* screening before the initiation of an ABC-containing regimens [7,11–12].

From this analysis, we also observed that 16.36% (nine out of 55) of the treated patients discontinued ABC and are not currently being administered the drug. Reasons for these interruptions do not include the development of hypersensitivity reactions to the drug. However, the diagnosis of ABC hypersensitivity in HIV patients is mostly based on clinical criteria and it may be complicated by confounding factors, such as multiple drug administration and the occurrence of opportunistic infections [22]. As reported in the label of ABC licensed products, the clinical diagnostic criteria for ABC hypersensitivity require two or more symptoms in the following groups: fever, rash, gastrointestinal symptoms (including nausea, vomiting, diarrhea or abdominal pain), constitutional (including generalized malaise, fatigue or achiness) and respiratory (including dyspnea, cough or pharyngitis) symptoms. Moreover, it is important for a correct diagnosis to consider the temporal relationship between symptoms and drug administration, and the effect of dechallenge and rechallenge with the drug. The ABC hypersensitivity reaction appears within the first 6 weeks of therapy, resolving within 72 h of withdrawal of the drug and requires an immediate and permanent discontinuation of the drug [8]. In our cohort, five patients interrupted the treatment after several months, for the following reasons: one gastrointestinal toxicity after 6 months, one virological failure after 10 months, one structured treatment interruption after 4 month, and two deaths after 12–14 months of therapy. On the other hand, four patients discontinued ABC between the second and the fourth week of therapy (which is compatible with the timing of clinical appearance of drug hypersensitivity), for the following reasons:

one gastrointestinal toxicity, one a self-reported localized rash (not confirmed by medical evaluation), two for constitutional symptoms (headache and fatigue), and in one patient because of renal failure; however, these isolated symptoms do not meet the clinical diagnostic criteria for ABC hypersensitivity reaction. Taken together these results demonstrate that the pharmacogenetic test that we characterized for the fast screening of *HLA-B*57:01*-positive carriers using the Q-PCR technique can be introduced into routine clinical practice, and the genotyping data obtained can be safely used to guide therapeutic decisions.

However, the main limitation of this and other SSP-based approaches is represented by the continuous need for sequence updates owing to the highly polymorphic nature of HLA-B. At present (11 October 2013, the last released update of HLA sequences in the IMGT/HLA database) [13], all the 16 sequences related to *HLA-B*57:01* are detected by our test. Moreover, owing to the great homology of the DNA sequences of all the different alleles (e.g., 59 point mutations in the *HLA-B*57:01* sequence vs the reference sequence), other technical problems can arise by even small changes in the PCR conditions (i.e., the thermocycler and/or the PCR reagent). In this regard, we had to face this kind of problem owing to discontinuation of the master mix originally used to validate the test, the Brilliant SYBR Green Q-PCR Master Mix (Reference MM). The suggested equivalent reagent, namely the Brilliant III Ultra Fast SYBR Green Q-PCR Master Mix (MM1), appeared not suitable for our test. This master mix includes a mutated form of Taq DNA polymerase that has been specifically engineered for faster replication, allowing for the performance of one-step PCRs using a single temperature (60°C) for the annealing and extension steps. In fact, when tested in our assay, using both 64–65°C (optimal annealing temperature for the *HLA-B*57:01* specific primers) and 60°C (optimal annealing temperature for the new enzyme), we could not detect any DNA amplification (data not shown). Two other master mixes, selected for similar kinetics to the reference MM, required some adjustment in the analytical parameters (threshold values, as described in the 'Results' section) in order to eliminate false-positive samples. Moreover, with the manually adjusted threshold cycles, the Ct cutoff values to detect positive amplification were significantly lower (TABLE 3). However, despite the attempt to optimize these parameters, MM3 gave one false-positive sample, thus it was not further characterized. On the other hand, the MM2 was used in parallel to the Reference MM in nine

consecutive Q-PCR runs, analyzing in a single-blinded manner an additional 61 patients (two of which were found to be positive carriers) with a 100% concordant results.

Conclusion

Data presented in this manuscript suggests that the screening test developed in our laboratory for the fast screening of *HLA-B*57:01* [16] can be successfully implemented into routine clinical practice, that the two sets of SSPs employed are still able to detect all the reported sequences for the *HLA-B*57:01* allele and that the new reagent, MM2: Brilliant II SYBR Green QPCR MM (Stratagene) can safely replace the reference MM, originally used to develop the test.

Author disclosure

Data discussed in this article were in part presented at the Italian Conference on AIDS and Retroviruses (ICAR 2013), which was held in Turin, Italy, 12–14 May 2013. The abstract (P122) was published in *New Microbiologica* [23].

Financial & competing interests disclosure

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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Executive summary

- International HIV treatment guidelines recommend *HLA-B*57:01* typing before the initiation of abacavir (ABC)-containing therapeutic regimens, in order to reduce the incidence of ABC-related hypersensitivity reactions.
- The requirement of *HLA-B*57:01* screening has also been included in the licenses of ABC-containing pharmaceutical products.
- Molecular techniques based on sequence specific primers and with rapid turnaround times have recently been developed to quickly detect the *HLA-B*57:01* allele in the HIV-infected population, among which is a test using real-time (Q)-PCR that was developed in our laboratory.
- The main limitation of sequence specific primer-based approaches is represented by the continuous need for sequence updates owing to the highly polymorphic nature of *HLA-B*.
- At present, 16 different sequences are reported in the IMGT/HLA database as coding for the *HLA-B*57:01* allele.
- Another possible limitation is related to the relatively high homology of the sequences between different alleles, leading to the detection of false-positive or false-negative carriers if small changes in PCR conditions are introduced.

Results

- The *HLA-B*57:01* screening test characterized in our laboratory has been used in routine clinical practice, so far genotyping a total of 353 patients and detecting 15 *HLA-B*57:01*-positive carriers.
- Among the *HLA-B*57:01* negative patients, 17.2% were treated with ABC without any hypersensitivity reactions.
- The sequence specific primers employed in this test are able to detect all the 16 sequences coding for the *HLA-B*57:01* allele.
- Novel Q-PCR analytical settings were characterized in order to overcome a technical problem, which was caused by the discontinuation of the Q-PCR master mix originally used to validate the test.
- A new master mix was used in parallel to the reference master mix in nine consecutive Q-PCR runs, analyzing, with a single-blinded approach, an additional 61 patients (two of which were found to be positive carriers) with a 100% concordant results.

Conclusion

- The *HLA-B*57:01* screening test developed in our laboratory can be successfully implemented into routine clinical practice, and is currently able to detect all the reported sequences of the *HLA-B*57:01* allele.
- With newly discovered Q-PCR conditions, the Brilliant II SYBR® Green QPCR MM (Stratagene) can safely replace the reagent originally used to develop the test.

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■ Website

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