Comparative Analysis of Real-Time Polymerase Chain Reaction Methods to Typing *HLA-B*57:01* in HIV-1-Positive Patients

Francesca Falasca,[1] Cinzia Dello Russo,[2] Barbara Mora,[3] Antonella Pirazzoli,[4] Alessandra Fantauzzi,[5] Pierluigi Navarra,[2] Antonio Pizzuti,[3] Corrado De Vito,[6] Guido Antonelli,[1] and Ombretta Turriziani[1]

Abstract

The *HLA-B*57:01* allele is strongly associated with the hypersensitivity reaction to Abacavir (ABC). Therefore, treatment guidelines recommend that patients initiating ABC are preventively tested for the presence of this allele. To date, four different commercial assays based on the real-time quantitative polymerase chain reaction (Q-PCR) technique are available for the detection of *HLA-B*57:01*: Duplicα-RealTime Reagent Set *HLA-B*57:01* by Euroclone, *HLA-B*57:01* Real-TM by Sacace Biotechnologies, COBAS AmpliPrep/COBAS TaqMan *HLA-B*57:01* Screening Test by Roche Diagnostic, and *HLA-B*57:01* by Nuclear Laser Medicine. The study was carried out to compare the performance of the first three commercially available Q-PCR kits in a routine clinical setting. A total of 98 samples from Policlinico Umberto I Hospital were tested. Results obtained by the Duplicα-RealTime Genotyping kit and AmpliPrep/TaqMan system were 100% concordant. In contrast, genotyping by the *HLA-B*57:01* Real-TM kit showed poor agreement with the other systems, that is, 12 out of 33 positive samples were detected as *HLA-B*57:01* negative. To confirm the correct genotype of these discordant samples, two additional methods with rapid turnaround times and already implemented into routine clinical practice were used, that is, a PCR-based microsequence-specific primer DNA typing test and a laboratory-developed screening test in Q-PCR. All 12 discordant samples were genotyped as *HLA-B*57:01*-positive samples using these two additional methods in a single-blinded manner, thus confirming the low sensitivity of *HLA-B*57:01* Real-TM test. These findings underline the need to compare results obtained with commercial assays before choosing a test suitable for use in a routine clinical laboratory.

ABACAVIR (ABC) IS A NUCLEOSIDE reverse transcriptase inhibitor used as an antiretroviral drug in HIV-infected patients. ABC-hypersensitivity reaction syndrome (HRS) has been observed in 5%–8% of treated patients during the first 6 weeks of treatment.[1] Symptoms of HRS to ABC include combinations of fever, rash, constitutional symptoms, gastrointestinal tract symptoms, and respiratory symptoms that become more severe with continued dosing.[2] Symptoms related to the hypersensitivity reaction worsen with continued therapy and usually improve within 24 h of ABC discontinuation.

In 2002, two independent research groups reported a strong association between the hypersensitivity response to ABC and the status of positive carrier of the major histocompatibility complex class I allele *HLA-B*57:01*.[2,3] Since then, several clinical studies confirmed the strength of this association across different ethnicities and, more importantly, showed the clinical utility of the pre-emptive genotyping for the *HLA-B*57:01* allele to reduce the incidence of the immunologically mediated HRS to ABC.[4–6] Consistently, most treatment guidelines recommend that patients initiating

[1]Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy.
[2]Institute of Pharmacology, Catholic University Medical School, Rome, Italy.
[3]Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy.
[4]ViiV Healthcare, Verona, Italy.
Departments of [5]Clinical Medicine and [6]Public Health and Infectious Diseases, Sapienza University of Rome, Rome, Italy.

ABC are preventively tested for the presence of the _HLA-B*57:01_ allele,[7–9] and such a requirement has been included in the summary of product characteristics of all ABC-containing formulations.

The HLA-B gene is extremely polymorphic, exhibiting many closely related alleles, making the _HLA-B*57:01_ difficult to discriminate from other related alleles. Therefore, the gold standard technique for its identification remains the DNA sequence-based typing, which is not always readily available in routine clinical laboratories. Thus, a number of different techniques have been recently applied to the _HLA-B*57:01_ genotyping, allowing for a significant reduction of time and costs for the screening and favoring its implementation in routine clinical practice. These strategies involve both serological and molecular methods. Standard serological approaches used to detect HLA-B*57 lack specificity, as commercially available monoclonal antibodies cross-react with HLA-B*57 and HLA-B*58 subtypes.[10] Currently, serological tests have been replaced by flow cytometry for analysis and sorting of blood cells. In 2011,[11] a monoclonal antibody (mAb, 3E12) specific for HLA-B*57 and HLA-B*58 and strongly associated with clinically important immune phenotypes had been developed. As this methodology cannot type HLA-B locus at the allele level, it can be used only to exclude the negative subjects, with positive patients to be further analyzed with high-resolution typing to discriminate _HLA-B*57:01_ allele from the other related alleles.[11] Several molecular methods, such as sequence-specific oligonucleotide probe hybridization, sequence-specific primers polymerase chain reaction (SSP-PCR), allele-specific polymerase chain reaction, SSP-PCR with fluorescence detection through capillary electrophoresis, and real-time quantitative PCR (Q-PCR),[10,12,13] are available to identify this allele.

In this scenario, screening tests based on the Q-PCR technology can be easily implemented in the routine laboratory practice of virological laboratories already involved in other assessments required for the management of HIV infection, that is, the viral load and the viral genotyping. To date, four Q-PCR-based methods are commercially available for the detection of the _HLA-B*57:01_ allele: Duplicα-RealTime Reagent Set _HLA-B*57:01_ (Euroclone S.P.A., Milan, Italy), _HLA-B*57:01_ Real-TM (Sacace Biotechnologies Srl, Como, Italy), COBAS® AmpliPrep/COBAS TaqMan® System (Roche Diagnostic, S.P.A., Monza, Italy), and _HLA-B*57:01_ by Nuclear Laser Medicine (NLM S.R.L., Milan, Italy). In this study, we assessed the performance of _HLA-B*57:01_ typing among the first three commercial Q-PCR-based methods.

Ninety-eight whole blood specimens (33 _HLA-B*57:01_ positive and 65 negative) were selected, from samples collected and genotyped by Duplicα-RealTime during routine clinical testing. All samples were reanalyzed by the other two commercially available assays in Q-PCR. Blood samples were derived from 98 HIV-infected individuals (74% male and 26% female) with a median age of 54 years (interquartile range 49–62). All patients were routinely admitted at the Policlinico Umberto I Hospital of Rome when an ABC-containing regimen was planned (combination antiretroviral therapy-experienced patients) or at the first visit after HIV infection diagnosis.

Total DNA was prepared from whole blood samples, using the QIAamp® DNA Blood Mini Kit (Qiagen, Vienna, Aus-

tria) according to the manufacturer's instructions. The extracted DNA was used for the _HLA-B*57:01_ genotyping by the Duplicα-RealTime and Real-TM.

Briefly, the Duplicα-RealTime is based on an allele-specific Q-PCR followed by melting curve analysis of the amplified products. Amplification and detection of specific amplicons were carried out on the Stratagene MX3005 platform (Agilent Technologies, Milan, Italy). Each specific amplicon (_HLA-B*57:01_ allele and an internal control, human growth hormone) was detected by a different melting temperature. The presence of _HLA-B*57:01_ was characterized by a melting profile temperature between 91.5°C and 92.5°C, while the internal control showed a melting temperature between 88.8°C and 89.8°C.

The Real-TM screening test is based on a different allele-specific Q-PCR protocol followed by analysis of cutoff threshold cycles (Ct) for the detection of _HLA-B*57:01_. In addition, the Human Beta-globin gene is used as internal control. Q-PCRs were performed according to the manufacturer's instructions on the Stratagene MX3005 platform. Specifically, _DNA HLA-B*57:01_ was detected on the JOE (yellow)/HEX/Cy3 channel and _IC_ on the FAM (green) channel.

The sample was considered positive for _HLA-B*57:01_ if the Ct value detected in JOE (yellow)/HEX/Cy3 did not exceed the Ct value detected in FAM channel +5 cycles. The sample was considered negative for _HLA-B*57:01_ if the Ct value in the JOE (yellow)/HEX/Cy3 channel was absent or if the Ct value detected in this channel was greater than the Ct value detected in FAM channel +5 cycles.

In contrast, the AmpliPrep/TaqMan is a fully automated Q-PCR system based on two major processes: specimen preparation to isolate DNA from 250 μl of whole blood using a generic silica-based capture technique and simultaneous PCR amplification of target and detection of cleaved dual-labeled oligonucleotide probe specific to identify the _HLA-B*57:01_ allele.

Results obtained using Duplicα-RealTime and AmpliPrep/TaqMan were 100% concordant. In fact, the AmpliPrep/TaqMan confirmed the _HLA-B*57:01_-positive genotype of 33 samples and the absence of this allele in 65 specimens (Table 1). In contrast, the Real-TM assay showed poor

TABLE 1. PERFORMANCES OF THREE REAL-TIME PCR-BASED ASSAYS AVAILABLE TO IDENTIFY THE _HLA-B*57:01_ ALLELE

	HLA-B*57:01-_positive_ samples (n=33)		HLA-B*57:01-_negative_ samples (n=65)	
	Positive	_Negative_	_Positive_	_Negative_
Duplicα-RealTime	33	0	0	65
AmpliPrep/TaqMan	33	0	0	65
Real-TM	**21**	**12**	0	65

Duplicα-RealTime: Duplicα-Real Time Reagent Set HLA-B*57:01 (Euroclone). AmpliPrep/TaqMan: COBAS® AmpliPrep/COBAS TaqMan® System (Roche Diagnostics). Real-TM: HLA-B*57:01 Real-TM (Sacace Biotechnologies). Discordant samples were confirmed by the PCR-SSP typing method (One Lambda) and Q-PCR assay.[14]

PCR-SSP, polymerase chain reaction sequence-specific primers; Q-PCR, quantitative polymerase chain reaction.

Discordant samples are highlighted in _bold_.

agreement with the other two systems since it identified 12 out of 33 positive samples as *HLA-B*57:01*-negative patients.

To assess the correct genotype of these discordant samples, two additional methods with rapid turnaround times and already implemented into routine clinical practice were used, that is, a PCR-based micro SSP DNA typing test (One Lambda, Inc., Conoga Park, CA) and a laboratory-developed screening test in Q-PCR.[14] Specifically, the PCR-based micro SSP DNA typing test (One Lambda, Inc.)[15] provide dried primers in different wells of a 96-well tube tray. DNA samples were added immediately after isolation to each well for PCR in the presence of recombinant Taq polymerase and specially formulated dNTP-buffer mix (Micro SSP D-mix). Each typing tray included a negative control reaction tube that detects the presence of the internal control PCR product. PCR amplification was carried out according to standard procedures (One Lambda, Inc.). After the PCR program, 2.5% agarose gel in the Micro SSP Gel System was used to electrophorese the samples. The presence or absence of specific PCR products was documented using the UV transilluminator. Interpretations of the typing were done with the lot-specific interpretation and specificity tables.

This method positively amplified all HLA-B*57 alleles and was able to distinguish between *HLA-B*57:01* and closely related HLA-B*57 alleles, from HLA-B*57:02 to B*57:64.

All 12 samples, *HLA-B*57:01* positive, were appropriately amplified and complete concordance was observed between the typing results obtained by the Duplicα-RealTime Genotyping Kit and AmpliPrep/TaqMan methods.

These results were also confirmed by the Q-PCR screening assay reported by Dello Russo *et al.*[14] DNA samples were anonymously transferred to the Institute of Pharmacology, Catholic University Medical School, Rome, Italy, identified as the reference laboratory for the Italian *HLA-B*57:01* Network.[16] HLA-B-specific amplification between the first and the third intron was carried out by PCR, using previously validated primers.[17] The *HLA-B*57:01* typing was performed using the HLA-B preamplified DNA and a Q-PCR method based on two sets of allele-specific primers, which were specifically designed to encompass the highest variable regions of the HLA-B DNA sequence.[18] By this assay, all 12 samples were identified as positive. Particularly, in the Exon 3-specific Q-PCR, positive samples were identified with an average Ct value of 12.95 ± 0.045 (SEM) and an average Tm of the amplification product equal to 86.07 ± 0.017 (SEM). In the Exon 2, positive samples displayed an average Ct value of 11.41 ± 0.054 (SEM) and an average Tm equal to 86.83 ± 0.08 (SEM).

Therefore, these additional analyses further confirmed the accuracy of the Duplicα-RealTime and AmpliPrep/TaqMan assays to identify the presence of *HLA-B*57:01* allele and the lower sensitivity of Real-TM test. The latter provided a high percentage of false *HLA-B*57:01*-negative samples (36%), when used on the MX3005 Q-PCR platform. To better assess the discrimination ability, receiver operating curves were drawn to obtain the area under the curve (AUC). The AUC of the Real-TM test was found statistically significantly lower (at 0.05 level) when compared to the others using the Bonferroni test ($p < .001$).

Numerous genotypic tests exist for *HLA-B*57:01* screening. The laboratories, different in terms of equipments, expe-

rience, and automation, can choose the most fitting method on the basis of available technologies and/or preferred type of samples. The assortment of still new different typing methods and kits available might increase the possibility of errors occurring in the accurate definition of individual HLA-B*57 alleles.[12,19]

However, a recent study on a 6-year-long external quality assessment scheme of *HLA-B*57:01* typing in 47 laboratories from 12 different countries showed that routine clinical *HLA-B*57:01* typing using various genotypic tests resulted in a single false-negative *HLA-B*57:01* report from 1,283 reports, indicating that current HLA typing is excellent.[19] In addition, the methods used in our study were also able to provide a correct *HLA-B*57:01* genotyping. By analyzing a *HLA-B*57:01*-enriched panel of 98 samples, we found a complete agreement between the Duplicα-RealTime and AmpliPrep/TaqMan systems. Despite different methods of DNA extraction and chemistry of detection, these assays identify the same samples as *HLA-B*57:01* positive and/or negative. In addition, our results showed the noninferiority of SYBR Green assay compared to probe-based methods to identify *HLA-B*57:01* allele, since both approaches showed the same analytical performance.

The major limitation of this study is the lack of comparison with results from sequence analysis. A comparison with sequence-based typing would strengthen the data obtained. However, in a previously study, Allice *et al.* reported a 100% sensitivity and sensibility of Duplicα-RealTime and Roche system when compared to sequence-based typing, thus supporting the accuracy of these methods.[20] Furthermore, the Duplicα-RealTime test was additionally validated by an External Quality Assurance program[16] thus enabling its use as reference method to compare the performance of other tests.

An incorrect HLA-B*57 genotyping by Real-TM was found in this study. Indeed, a poor sensitivity of *HLA-B*57:01* Real-TM methods was also reported by Dello Russo *et al.*, at the Italian Conference on AIDS and Retrovirus (ICAR 2013).[21] Specifically, 28 samples previously genotyped with the laboratory-developed Q-PCR described earlier were reanalyzed by the *HLA-B*57:01* Real-TM kit (Sacace), according to the manufacturer's instructions, and 4 false-negative samples out of 11 positive samples (36%) were detected.[21] Therefore, the present study, performed on a largest number of samples, confirmed the low sensitivity of the Real-TM. Interestingly, in both cases, the Real-TM was used on Stratagene Q-PCR platforms.

In conclusion, also given the permanence that HLA typing results could have an undesirable effect on a patient's care, choosing a test suitable for use in a routine clinical laboratory is critical and important to compare the performance of the assays available to identify the *HLA-B*57:01* allele.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Ombretta Turriziani
Department of Molecular Medicine
Sapienza University of Rome
V.le dell'Università 31
00185 Rome
Italy

E-mail: ombretta.turriziani@uniroma1.it