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 Turriziani¹

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*: Duplic α -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 Duplic α -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

¹Department of Molecular Medicine, Sapienza University of Rome, Rome, Italy.

²Institute of Pharmacology, Catholic University Medical School, Rome, Italy.

³Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy.

⁴ViiV Healthcare, Verona, Italy.

Departments of ⁵Clinical Medicine and ⁶Public 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: Duplic α -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 Duplic α -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 Duplic α -RealTime and Real-TM.

Briefly, the Duplic α -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 μ l 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 Duplic α -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

	<i>HLA-B*57:01</i> -positive samples (n=33)		<i>HLA-B*57:01</i> -negative samples (n=65)	
	Positive	Negative	Positive	Negative
Duplic α -RealTime	33	0	0	65
AmpliPrep/TaqMan	33	0	0	65
Real-TM	21	12	0	65

Duplic α -RealTime: Duplic α -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 Duplic α -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 Duplic α -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, experience,

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 Duplic α -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, Alice *et al.* reported a 100% sensitivity and sensibility of Duplic α -RealTime and Roche system when compared to sequence-based typing, thus supporting the accuracy of these methods.²⁰ Furthermore, the Duplic α -RealTime test was additionally validated by an External Quality Assurance program¹⁶ 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.

Acknowledgment

The study was supported by a research fund granted by ViiV Healthcare, Verona, to Sapienza University of Rome, while operating with full scientific autonomy.

Author Disclosure Statement

No competing financial interests exist.

References

- Lucas A, Nolan D, Mallal S: HLA-B*5701 screening for susceptibility to abacavir hypersensitivity. *J Antimicrob Chemother* 2007;59:591–593.
- Hetherington S, Hughes AR, Mosteller M, *et al.*: Genetic variations in *HLA-B* region and hypersensitivity reactions to abacavir. *Lancet* 2002;359:1121–1122.
- Mallal S, Nolan D, Witt C, *et al.*: Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse transcriptase inhibitor abacavir. *Lancet* 2002;359:727–732.
- Mallal S, Phillips E, Carosi G, *et al.*: PREDICT-1 Study Team: HLA-B*5701 screening for hypersensitivity to abacavir. *N Engl J Med* 2008;358:568–579.
- Saag M, Balu R, Phillips E, *et al.*: Study of hypersensitivity to abacavir and pharmacogenetic evaluation study team.: High sensitivity of human leukocyte antigen-b*57:01 as a marker for immunologically confirmed abacavir hypersensitivity in white and black patients. *Clin Infect Dis* 2008;46:1111–1118.
- Young B, Squires K, Patel P, *et al.*: First large, multicenter, open-label study utilizing HLA-B*5701 screening for abacavir hypersensitivity in North America. *AIDS* 2008;22:1673–1675.
- Clumeck N, Pozniak A, Raffi F: EACS Executive Committee: European AIDS Clinical Society (EACS) guidelines for the clinical management and treatment of HIV-infected adults. *HIV Med* 2008;9:65–71.
- Panel on Antiretroviral Guidelines for Adults and Adolescents: Guidelines for the use of antiretroviral agents in HIV-1-infected adults and adolescents. Department of Health and Human Services (Sections C22, E and F). Available at <http://aidsinfo.nih.gov/ContentFiles/AdultandAdolescentGL.pdf>, accessed June 26, 2013.
- Hammer SM, Eron Jr JJ, Reiss P, *et al.*: International AIDS Society-USA.: Antiretroviral treatment of adult HIV infection: 2008 recommendations of the International AIDS Society-USA panel. *JAMA* 2008;300:555–570.
- Stocchi L, Cascella R, Zampatti S, *et al.*: The pharmacogenomic HLA biomarker associated to adverse abacavir reactions: Comparative analysis of different genotyping methods. *Curr Genomics* 2012;13:314–320.
- Kostenko L, Kjer-Nielsen L, Nicholson I, *et al.*: Rapid screening for the detection of HLA-B57 and HLA-B58 in prevention of drug hypersensitivity. *Tissue Antigens* 2011;78:11–20.
- Pozzi S, Longo A, Ferrara GB: HLA-B locus sequence-based typing. *Tissue Antigens* 1999;53:275–281.
- Martin AM, Nolan D, Mallal S: *HLA-B*5701* typing by sequence-specific amplification: Validation and comparison with sequence based typing. *Tissue Antigens* 2005;65:571–574.
- Dello Russo C, Lisi L, Fabbiani M, *et al.*: Detection of HLA-B*57:01 by real-time PCR: Implementation into routine clinical practice and additional validation data. *Pharmacogenomics* 2014;15:319–327.
- Tang J, Bansal A: Protocol for analyzing human leukocyte antigen variants and sexually transmitted infections: From genotyping to immunoassays. *Methods Mol Biol* 2012;903:359–380.
- Meini G, Bruzzone B, D'Arrigo R, *et al.*: First External Quality Assurance Program of the Italian HLA-B*5701 Network Assessing the Performance of Clinical Virology Laboratories in HLA-B*5701 Testing. Proceedings of the 7th Italian Conference on AIDS and Retroviruses, May 17–19, 2015, Riccione, Italy.
- Cereb N, Maye P, Lee S, Kong Y, Yang SY: Locus-specific amplification of HLA class I genes from genomic DNA: Locus-specific sequences in the first and third introns of HLA-A, B, and C alleles. *Tissue Antigens* 1995;45:1–11.
- Dello Russo C, Lisi L, Lofaro A, *et al.*: Novel sensitive, specific and rapid pharmacogenomic test for the prediction of abacavir hypersensitivity reaction: HLA-B*57:01 detection by real-time PCR. *Pharmacogenomics* 2011;12:567–576.
- Darke C, Corbin SA: External quality assessment of patient HLA-B*57:01 testing prior to abacavir prescription. *Int J Immunogenet* 2014;41:277–280.
- Allice T, Milia MG, Burdino E, *et al.*: HLA-B*5701 Typing with the New Commercial COBAS CAP/CTM HLA-B*5701 Test: Comparison with Real-Time PCR and Sequence Based Typing Assays. Proceedings of the 6th Italian Conference on AIDS and Retroviruses, May 25–27, 2014, Rome, Italy.
- Dello Russo C, Lisi L, Fabbiani M, Cauda R, Navarra P: Detection of HLA-B*57:01 in real-time PCR: Implementation into routine clinical practice, additional validation data and comparison with commercially available methods. *New Microbiol* 2013;36:193.

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