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Improvement in allele-specific PCR assay with the use of polymorphism-specific primers for the analysis of minor variant drug resistance in HIV-1 subtype C

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Abstract

In recent years, highly sensitive assays have been developed that detect HIV-1 drug resistance mutations when present at less than 1% of the viral population. These assays are powerful tools when attempting to determine the clinical implications of these low level resistant virions after the administration of single-dose nevirapine. This report demonstrates that non-drug resistant polymorphisms in the primer-binding site for the allele-specific PCR (ASPCR) assay impact primer binding resulting in significant discrepancies in the assay's performance. Specifically, the use of a "universal" set of ASPCR primers caused an overestimation of the K103N (ntAAC) mutation at position 103 of reverse transcriptase when primer binding site polymorphisms resided close to the 3′ end of the allele-specific primer. Drug resistance was predicted at values ranging from 0.69% to 7.69% for a sample containing only 1% resistance mutations and 3.35–31.84% for a sample containing 5% mutations. Conversely, the use of polymorphism-specific primers detected 1.15–1.36% and 5.20–5.71% resistance for the same 1% and 5% samples. The results demonstrate the need to account for sequence polymorphisms when designing and implementing this highly specific assay.
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1. Introduction

Commercial genotyping assays are limited to the detection of HIV-1 drug resistance when resistant viruses comprise at least 20% of the viruses in an infected individual (Brun-Vezinet et al., 2004; Church et al., 2006; Grant et al., 2003; Palmer et al., 2005). There is widespread interest in understanding the clinical relevance of minor variant drug resistance, that is, when drug resistant viruses are present at less than 20%. This has become increasingly important in light of the growing concern regarding the development of nevirapine resistance in mothers and babies following the use of single-dose nevirapine for the prevention of mother-to-child transmission (PMTCT) of HIV in developing countries (Eshleman et al., 2001; Jackson et al., 2000). The exact nature of the clinical importance of minor variants remains unclear, but data suggests these populations of drug resistant viruses contribute to virologic failure (Jourdain et al., 2004; Lecossier et al., 2005), and may allow for the prediction of virologic failure at an earlier stage. As a result, several new assays have been developed to detect and quantify minor variant resistance (Bergroth et al., 2005; Flys et al., 2005; Nissley et al., 2005; Palmer et al., 2005; Shi et al., 2004), and the application of such techniques to HIV-1 subtype C samples has already provided insight into the persistence of drug resistance following single-dose nevirapine (Loubser et al., 2006; Palmer et al., 2006a).

Allele-specific polymerase chain reaction (ASPCR), one of the most powerful of these new techniques, can detect and quantify drug resistant viruses when present at less than 1% (Halvas et al., 2006; Paredes et al., 2007). ASPCR consists

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of two quantitative PCR (qPCR) reactions differing only in the primer that overlaps the drug resistance mutation: one reaction contains an "allele-specific" primer matching the drug resistance mutation and the other contains an "allele-specific" primer matching the wild type sequence. It has previously been reported that the presence of nucleotide polymorphisms in the allele-specific primer binding site, causing primer-template mismatch, reduces the accuracy of ASPCR, specifically resulting in the underestimation of the percentage of drug resistant viruses (Palmer et al., 2006b). Given the high degree of background genetic variation that is characteristic of HIV, this has a significant impact when applying ASPCR on a large-scale basis to clinical samples for the purpose of assessing the clinical relevance of low levels of drug resistance.

In an effort to understand the underlying cause of this discrepancy, an in vitro analysis was performed to assess the impact of naturally occurring, non-resistance nucleotide polymorphisms in the ASPCR binding site on the accuracy of the assay. This reports shows that primer site mismatches caused by naturally occurring nucleotide polymorphisms can result in dramatic overestimates of the percentage of drug resistant viruses present in the sample at the 103 position of reverse transcriptase. However, the use of polymorphism-specific primers and corresponding standard curves eliminates the problem, allowing accurate quantitation of minor variants, and thus should be applied when ASPCR is used in highly polymorphic regions of HIV.

2. Materials and methods

2.1. Clinical samples

Plasma samples from women in Botswana who received single-dose nevirapine for PMTCT were obtained 1–19 months after drug exposure and prior to the initiation of highly active antiretroviral therapy (HAART). The samples were genotyped for nevirapine drug resistance by ViroSeq according to manufacturer protocol (Applied Biosystems, Foster City, CA). In 18 samples, no drug resistance was detected by this conventional sequencing method. Of these 18 sequences, 17 were HIV-1 subtype C and one was HIV-1 subtype A. The genotypes of these 18 sequences were reproduced in plasmids for in vitro testing of the ASPCR method.

2.2. Site-directed mutagenesis

The HIV-1 subtype C (HIV-1C) infectious molecular clone pMJ4 (Ndung'u et al., 2001) provided the reverse transcriptase (RT) that served as the backbone for subsequent mutagenesis; the Apa I site located in the pBlueScript vector of pMJ4 was deleted by partial Apa I digest followed by Klenow fill-in. This allowed the 1.6 kb fragment encompassing RT to be cut out by Apa I and Hpa I digest. It was cloned into a pCR2.1 (Invitrogen, Carlsbad, CA) vector modified to include a Hpa I restriction site. The resulting HIV-1C RT subclone, pCLB11, served as the template for QuikChange II (Stratagene, La Jolla, CA) site-directed PCR mutagenesis to recreate the HIV-1C nucleotide polymorphisms of interest. Briefly, the DNA template is denatured and forward/reverse mutagenic primers containing the desired mutation are annealed to the template followed by extension with *PfuUltra* DNA polymerase. The parental strand is digested with Dpn I endonuclease, specific for methylated DNA.

Using this technique, 32 plasmids were generated corresponding to the 8 unique sequences seen in the Botswana samples. These sequences corresponded to the changes in the ASPCR primer-binding region for position 103 of reverse transcriptase. A plasmid was generated for each of the 8 sequences with an A, C, T, and G at the third position of amino acid 103 of reverse transcriptase.

2.3. Allele-specific PCR (ASPCR)

ASPCR is a nested PCR assay combining a standard first-round PCR using universal primers and a quantitative, second round PCR with allele-specific primers. The first round primers were RT-18 (5′-GGA AAC CAA AAA TGA GGG GAA TTG GAG G-3′) and NE1 (5′-CCT ACT AAC TTC TGT ATG TCA TTG ACA GTC CAG CT-3′). In a 50 μL reaction, 38.5 μL of water, 5 μL of 10× reaction buffer, 2 uL of dNTPs, 1 μL of each primer NE1 and RT-18 (10 μM concentration each) and 0.5 μL of FastStart High Fidelity Enzyme Blend (Roche Applied Science, Indianapolis, IN) were mixed with 10 ng of each plasmid. The cycling parameters for the PCR were 94 °C for 5 min followed by 35 cycles of 94 °C for 20 s, 55 °C for 20 s, and 72 °C for 2 min, with a final extension at 72 °C for 7 min. The first round PCR generated a 957 bp amplicon comprising nucleotides 2359–3316 (HXB2 numbering) which was gel purified by electrophoresis on 1% agarose followed by QiaQuick gel extraction per manufacturer protocol (Qiagen, Valencia, CA).

The first-round PCR amplicon (10^7 copies as measured by spectrophotometry) served as the template for the second-round, quantitative PCR step. The forward primer was constant for all reactions (2090F-5′-AAG TGG AGA AAA TTA GTA GAT TTC AGG GA-3′). Different sets of reverse primers were created. The universal primer set is exactly the same set of primers used in the protocol devised for subtype C (Palmer et al., 2006a), with the addition of a specific primer for the ntAAG. Allele-specific reverse primers were also constructed to match the varying template sequence in the primer-binding site. The reverse primers always included a single nucleotide mismatch at the penultimate 3′ position to enhance reaction specificity. The final 3′ position of the allele-specific primer falls on the third position of the RT aa103 codon, the variation of which confers the lysine to asparagine (K103N) drug resistance mutation. Primers were constructed containing each of the four nucleotides at the final position. For example, the group I reverse primers, designed to match the "universal primers" described previously, consisted of the following:

AAA-103K: 2218R 5′-CCC ACA TCT AGT ACT GTC ACT GAT TCT-3′,
AAC-103N: 2218R 5′-CCC ACA TCT AGT ACT GTC ACT GAT TGG-3′,

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