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Comparison of LigAmp and an ASPCR Assay for Detection and Quantification of K103N-Containing HIV Variants

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ABSTRACT

We compared the ability of the LigAmp assay and an ASPCR assay to detect and quantify K103N-containing HIV variants in samples from 63 women who received single-dose nevirapine in a clinical trial. Samples were first analyzed with the ViroSeq HIV Genotyping system, and ViroSeq PCR products were used as templates for the LigAmp and ASPCR assays. A cutoff of 0.5% K103N for detection of K103N was used for both assays. Results for the percentage K103N were similar for the two assays ($R^2 = 0.92$). Forty-six samples (73.0%) were positive for K103N by both assays and 13 samples (20.6%) were negative by both assays. Four samples (6.3%) were positive by ASPCR only. No samples were positive by LigAmp only. Eight discordant samples were analyzed in more detail. Sequence polymorphisms near oligonucleotide binding sites provided a possible explanation for the discordance in four of eight samples. The percentage K103N was also determined by analyzing 40 HIV clones from each of these eight samples, using a combined amplification/sequencing method (AmpliSeq). The percentage K103N determined by clonal analysis was consistent with the LigAmp result for five of eight samples, and was consistent with the ASPCR result for three of eight samples. Among 320 clones analyzed, we identified eight different codons at position 103 (mean = 3.8 codons/sample), which encoded six different amino acids, illustrating the extensive genetic diversity in HIV. Further studies are needed to compare performance of assays for detection and quantification of HIV drug resistance mutations in clinical samples.

INTRODUCTION

Single-dose nevirapine (SD NVP) is an inexpensive, safe, and effective way to prevent mother-to-child transmission of HIV in resource-poor settings.[1,2] One disadvantage of this regimen is that NVP-resistant HIV variants can emerge and persist in some women and infants.[3–5] This may contribute to subsequent antiretroviral treatment failure.[6,7]

HIV genotyping methods designed to detect antiretroviral drug resistance mutations usually employ population sequencing-based methods. Those assays are designed to detect the major viral population, do not quantify mutant species, and are relatively insensitive for detection of minority variants.[8,9] More sensitive methods have been used to detect and quantify HIV variants with specific drug resistance mutations. These include the oligonucleotide ligation assay, single genome sequencing, parallel allele-specific sequencing, ultradeep pyrosequencing, mutation-specific polymerase chain reaction (PCR) assays (e.g., allele-specific PCR, ASPCR; amplification refractory mutation system, ARMS), and the LigAmp assay.[4,10–18]

Sensitive detection and quantification of point mutations in HIV are more complex than analysis of point mutations in eukaryotic DNA for several reasons, including (1) HIV has an RNA template. Typically, reverse transcription is required prior to analysis. (2) The number of HIV RNA copies/ml (HIV viral load) in clinical samples can vary over several orders of magnitude, and may be quite low. (3) There are significant genetic differences among different HIV-1 subtypes and strains. (4) HIV-infected individuals typically harbor complex mixtures of genetically related viruses (quasispecies). The diversity of HIV viruses complicates the design of DNA oligonucleotides used in HIV assays, and can interfere with their hybridization to some HIV templates.

The sensitivity of some methods for detection of the K103N NVP resistance mutation in HIV has been compared using plasmid-derived viral stocks.[13] In that study, two of three ASPCR-

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based methods and a yeast-based phenotypic assay (the TyHRT assay) were able to detect and quantify low-level HIV variants with K103N.[13] Another study found that the LigAmp and TyHRT assays provided consistent results for detection of K103N.[11] In this report, we compare an ASPCR assay to the LigAmp assay in terms of detection and quantification of the K103N mutation in plasma HIV from women who received SD NVP in a clinical trial to prevent HIV mother-to-child transmission. Both the LigAmp assay and ASPCR assays have been previously used in different studies to analyze K103N in women and infants who received SD NVP.[4,11,12,14,19]

MATERIALS AND METHODS

Source of samples

Plasma samples were collected from Malawian women enrolled in the NVAZ trial[20] 6 weeks after SD NVP administration. Human experimentation guidelines of the U.S Department of Health and Human Services and the authors' institutions were followed in conducting this research. Informed consent was obtained from all women prior to enrollment in the NVAZ trial.

HIV genotyping and subtyping

Samples were analyzed previously using the ViroSeq HIV-1 Genotyping System (Celera Diagnostics, Alameda, CA), an FDA-cleared assay based on population (bulk) sequencing.[21] HIV subtype was determined by phylogenetic analysis of *pol* region sequences; all samples were identified as HIV subtype C.[21] PCR products generated in the ViroSeq system were used as DNA templates for analysis with the LigAmp and ASPCR assays.

Analysis of K103N using the LigAmp assay

Samples were analyzed using the LigAmp assay, as described.[8] DNA templates were quantified prior to analysis using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE), and 50 pg of template DNA was added to each LigAmp reaction. In the first step of the LigAmp assay, upstream and downstream oligonucleotides are hybridized adjacent to one another on the antisense strand of the HIV DNA template. The LigAmp oligonucleotides include tails with sequences that serve as binding sites for M13 primers and a lacZ real-time PCR probe used in the second step of the LigAmp reaction (see below). The LigAmp oligonucleotides used in the ligation step were upstream 5′-**ACTGTAAAACGACGGCCAGTGTTCCCCTCAAACTGGCAGATGCACG**AGGAATACCACACCCAGCAGGGTTAAAAAAGGAC-3′ and downstream 5′-Phos-AAATCAGTGACAGTACTGGATGTGGGGG**TGGTCATAGCTGTTTCCTGCA**-3′. Oligonucleotide tails are shown in bold. The 3′ end of the upstream oligonucleotide corresponds to the third position (C) of the AAC codon in the K103N mutation. If the AAC codon is present in the HIV template, the two oligonucleotides are ligated to one another. An intentional sequence mismatch is included near the 3′ end of the upstream oligonucleotide (G, underlined) to enhance the specificity of the ligation reaction. The HIV binding sequences of LigAmp oligonucleotides are shown in Fig. 1A. In the second step of the LigAmp assay, the ligated DNA product is detected and quantified using a universal real-time PCR method.[8] All samples were analyzed in duplicate, and the results (threshold cycle, Ct) were averaged. A plasmid-derived standard curve was analyzed in each assay run, using methods identical to those used for test samples (see below). Results from individual samples were compared to results from the standard curve to quantify the percentage K103N.

Analysis of K103N using the ASPCR assay

DNA templates were quantified for the ASPCR assay using a Nanodrop spectrophotometer, as described above. We performed two reactions for each sample: a mutation-specific reaction and a total copy reaction. The mutation-specific reaction included an ASPCR mutant primer, an ASPCR reverse primer, and a Taqman probe (5′-FAM/TGTGGGGGAYGCATATTTTTCAGTTCCTTTAG BHQ-3′) (Integrated DNA Technologies, Coralville, IA). The total copy reaction included an ASPCR total copy primer, the ASCPR reverse primer, and the probe. The sequences of the ASPCR primers and probe are shown in Fig. 1A. Real-time PCR was performed in a 25 μl reaction with 1× Universal Taqman PCR mix (Applied Biosystems, Foster City, CA), 0.2 μM of each primer, 0.2 μM Taqman probe, and 100 pg of DNA template (ViroSeq PCR product). Samples were analyzed using a 7500 Real-Time PCR System (Applied Biosystems), with the following cycling conditions: 50°C for 2 min, 95°C for 10 min, and 45 three-step cycles of 95°C for 1 min, 50°C for 30 sec, and 72°C for 1 min. The differences between the threshold cycle (Ct) values for the mutant and total copy reactions were calculated (ΔCt) and were compared to a standard curve to determine the percentage K103N. Each sample was analyzed in duplicate, and the ΔCt values were averaged.

Preparation of plasmid-derived controls used in the LigAmp and ASPCR assays

Standard curves used in the LigAmp and ASPCR assays were generated by cloning ViroSeq PCR products from three women with subtype C HIV who had received SD NVP. Terminal adenosine residues were added to the 3′ end of the PCR products to facilitate T/A cloning and the PCR products were then ligated into the TOPO-TA vector PCR 2.1 (Invitrogen Corp., Carlsbad, CA). Because ViroSeq PCR products contain dUTP, plasmids resulting from ligation were transformed into an *Escherichia coli* strain that lacks the enzyme uracil-*N*-glycosylase (ung-negative strain CGSC #6799).[22] Plasmids containing HIV inserts were isolated, and were sequenced using the ViroSeq sequencing module. Paired plasmids were selected from each of the three samples: one with the K103N mutation (AAC, mutant) and one without the K103N mutation (AAA, WT, Fig. 1B). The HIV *pol* region of each plasmid was amplified by PCR using ViroSeq reagents, as follows: 1 μl of plasmid DNA was mixed with 9 μl of RNA diluent, 10 μl of reverse transcriptase mix, and 30 μl of PCR mix. PCR amplification was performed according to the ViroSeq manufacturer's protocol. PCR products were purified using spin columns, quantified using a Nanodrop spectrophotometer, and diluted with distilled water to 20 pg/μl. The wild-type (WT) and mutant control PCR products derived from each of the three samples were mixed together to

generate 20 pg/μl stocks containing different concentrations of mutant DNA (100% mutant, 10% mutant, 1% mutant, 0.1% mutant, 0.01% mutant, and 100% WT). One set of the six control stocks was analyzed in parallel with test samples in each assay run to generate the standard curve used for K103N quantification.

Analysis of HIV clones from test samples

ViroSeq PCR products derived from selected test samples were cloned as described above. *Pol* region sequences of HIV clones were determined directly from bacterial colonies using a single step amplification-sequencing method (AmpliSeq).[23] A small inoculum of each bacterial colony was added directly to a 20 μl reaction containing 4 μl Reaction Ready Mix (Applied Biosystems), 1× Sequencing Buffer (Applied Biosystems), 0.125 mM dNTPs (Invitrogen), 0.05 μM forward primer (5′-AACTCAATAAAAGAACTCAAGACTT-3′), and 0.25 μM reverse primer (5′-CATTGTTTRTACTAGGTATGGTGAA-3′). The AmpliSeq reaction was performed as follows: 95°C for 5 min, followed by 40 three-step cycles of 95°C for 15 sec, 50°C for 15 sec, and 60°C for 4 min. The reactions were ethanol precipitated, resuspended in 20 μl of HiDi formamide (Applied Biosystems), and then sequenced on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

Statistical methods

Measures of percentage K103N were log transformed to improve symmetry. Samples with LigAmp and ASPCR results below the 0.5% K103N assay cutoff (negative results) were assigned a value of 0.25% K103N for data analysis, to allow log transformation of results. Summary measures including mean, median, and standard deviation and two way plots were obtained. Spearman's and Pearson's correlation coefficients between log-transformed LigAmp and ASPCR were calculated. Exact fiducial two-sided 95% confidence intervals for population proportions were calculated.

RESULTS

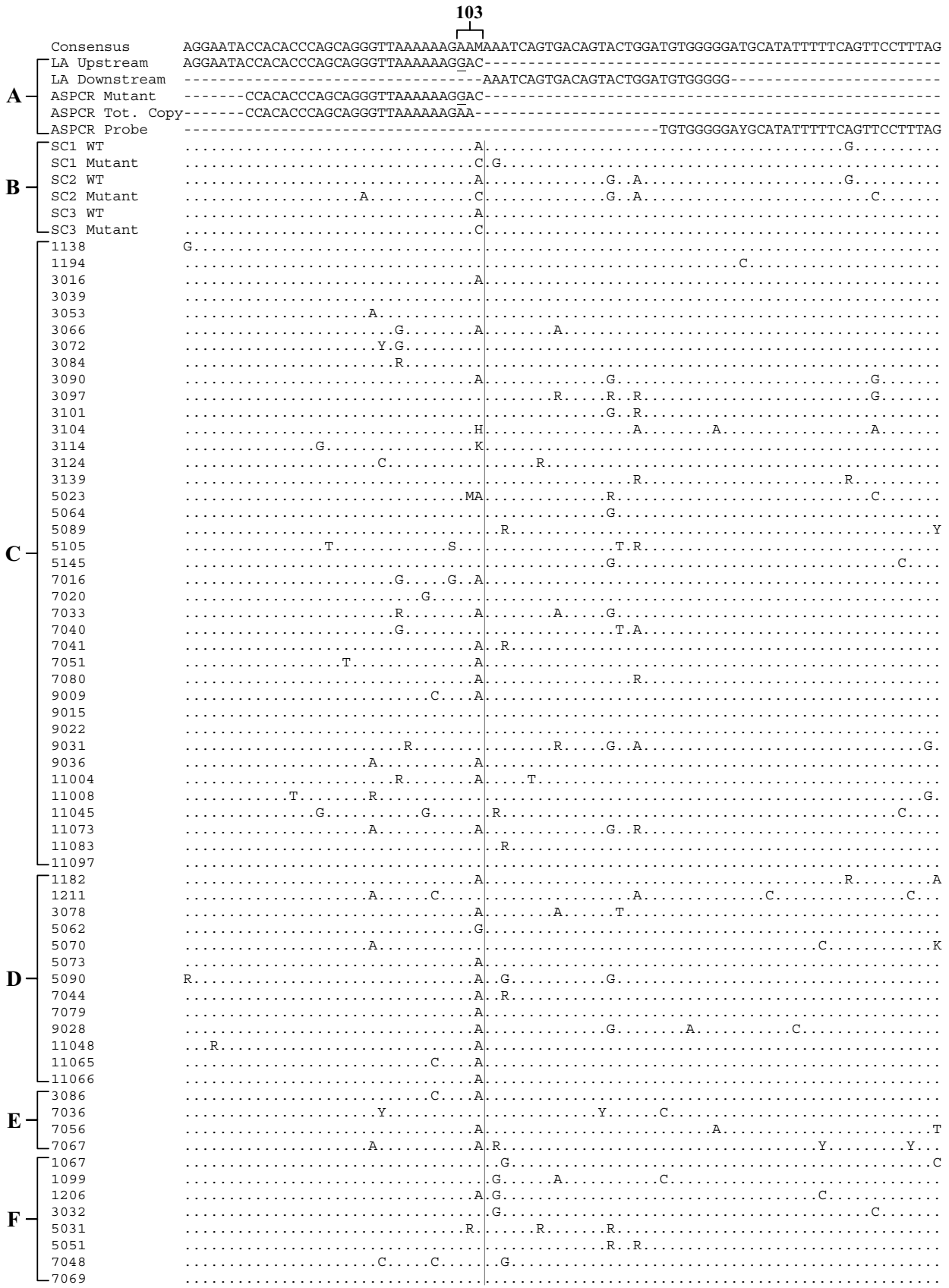
This study compared the performance of two point mutation assays for detection and quantification of the K103N (AAC) mutation: the LigAmp assay and an ASPCR assay (see Materials and Methods). We first compared results obtained from analysis of plasmid-derived controls containing known amounts of mutant (AAC) and WT (AAA) HIV DNA (see Materials and Methods). The plasmids used to generate these stocks contained one or more nucleotide differences at the binding sites of oligonucleotides, primers, and probes (oligonucleotide reagents) used in the two assays (Fig. 1). Sets of samples containing 10-fold dilutions of mutant DNA in WT DNA (standard curve sample sets) were prepared (SC1, SC2, SC3, Fig. 1B). Results obtained by analyzing the three standard curves with the LigAmp and ASPCR assays are shown (Fig. 2A). The assay cutoff for K103N detection was set at 0.5% K103N for both assays. A single standard curve was used for the analysis of test samples (SC2). Results obtained from analysis of that standard curve for each assay run performed in this project (four assay runs for LigAmp, five assay runs for ASPCR) are shown in Fig. 2B. This analysis shows that run-to-run variation of results obtained with both assays was minimal (R^2 for LigAmp: 0.9984; R^2 for ASPCR: 0.9890).

The LigAmp and ASPCR assays were used to analyze the K103N mutation in subtype C HIV from plasma samples collected from Malawian women who received SD NVP (see Materials and Methods). The samples were previously analyzed using the ViroSeq HIV Genotyping System.[21] PCR products from the ViroSeq system were then analyzed using the LigAmp assay and an ASPCR assay for detection and quantification of K103N. Results were obtained for both the LigAmp and ASPCR assays for 63 (96.9%) of 65 available samples. An alignment of *pol* region sequences from all 63 samples is shown (Fig. 1C–F). For most samples, the percentage K103N measured by the LigAmp and ASPCR assays was similar (R^2 = 0.92 Pearson, R^2 = 0.88 Spearman, Fig. 3). However, using a logarithmic base 10 scale, the ASPCR assay on average measured the percentage K103N 0.25 units higher than did the LigAmp assay ($p < 0.001$). When this value (0.25 $\log_{10}$ units) is transformed back to a nonlogarithmic scale, it corresponds to 1.78 (i.e., the percentage K103N values measured by the ASPCR assay were are on average 1.78 times higher than those measured by the LigAmp assay).

Forty-six samples (73.0%) were positive for K103N by both assays (Fig. 1C and F) and 13 samples (20.6%) were negative by both assays (Fig. 1D). None of the samples was positive by LigAmp and negative by ASPCR. However, four (6.3%) of 63 samples were positive for K103N by ASPCR and negative by LigAmp (percentage K103N by ASPCR: 0.6%, 0.6%, 1.2%, 1.5%, Fig. 1E and Fig. 3). K103N was detected by ViroSeq in one of those four samples (the sample with 1.5% K103N by ASPCR). It is not practical to use clones to determine which of these results is correct [i.e., to distinguish between a negative result by LigAmp (<0.5% K103N) and a positive result by ASPCR in this range (0.6–1.5% K103N)]. For example, over 2200 HIV variants from a single sample would have to be cloned and analyzed to distinguish between <0.5% K103N and ≥1% K103N with 80% power. Because of limitations in sample availability and the complexity of precisely quantifying the percentage K103N at very low levels, we did not perform any additional analysis of these four samples.

We selected eight samples with very discordant LigAmp and ASPCR results for further analysis (samples with data points far from the line of identify, Fig. 3). HIV variants from these eight selected discordant samples were cloned (40 clones/sample) and the percentages of the 40 clones isolated from each sample that had K103N were calculated. For each sample, we also calculated the 95% confidence intervals (CI) for the underlying percentage K103N in that sample based on the portion of 40 clones observed with K103N (Fig. 4).

The ViroSeq system does not provide quantitative information about the portion of different HIV variants in a mixed viral population. However, the relative peak heights of wild-type and mutant sequences in the electropherograms do provide some indication of the portion of the mutant viral population in the mixture (i.e., low, medium, or high, relative to wild-type). Figure 4 shows the ViroSeq electropherograms for the eight selected samples. In all eight cases, the percentage K103N determined by analysis of HIV clones was consistent with the qualitative ViroSeq electropherogram result.


103
Consensus AGGAATACCACACCCAGCAGGGTTAAAAAAGAAMAAATCAGTGACAGTACTGGATGTGGGGGATGCATATTTTTCAGTTCCTTTAG
LA Upstream AGGAATACCACACCCAGCAGGGTTAAAAAAGGAC
LA Downstream AAATCAGTGACAGTACTGGATGTGGGGG
ASPCR Mutant -------CCACACCCAGCAGGGTTAAAAAAGGAC
ASPCR Tot. Copy-------CCACACCCAGCAGGGTTAAAAAAGAA
ASPCR Probe TGTGGGGGAYGCATATTTTTCAGTTCCTTTAG
A
SC1 WT
SC1 Mutant
SC2 WT
SC2 Mutant
SC3 WT
SC3 Mutant
B
1138
1194
3016
3039
3053
3066
3072
3084
3090
3097
3101
3104
3114
3124
3139
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5105
5145
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D
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F

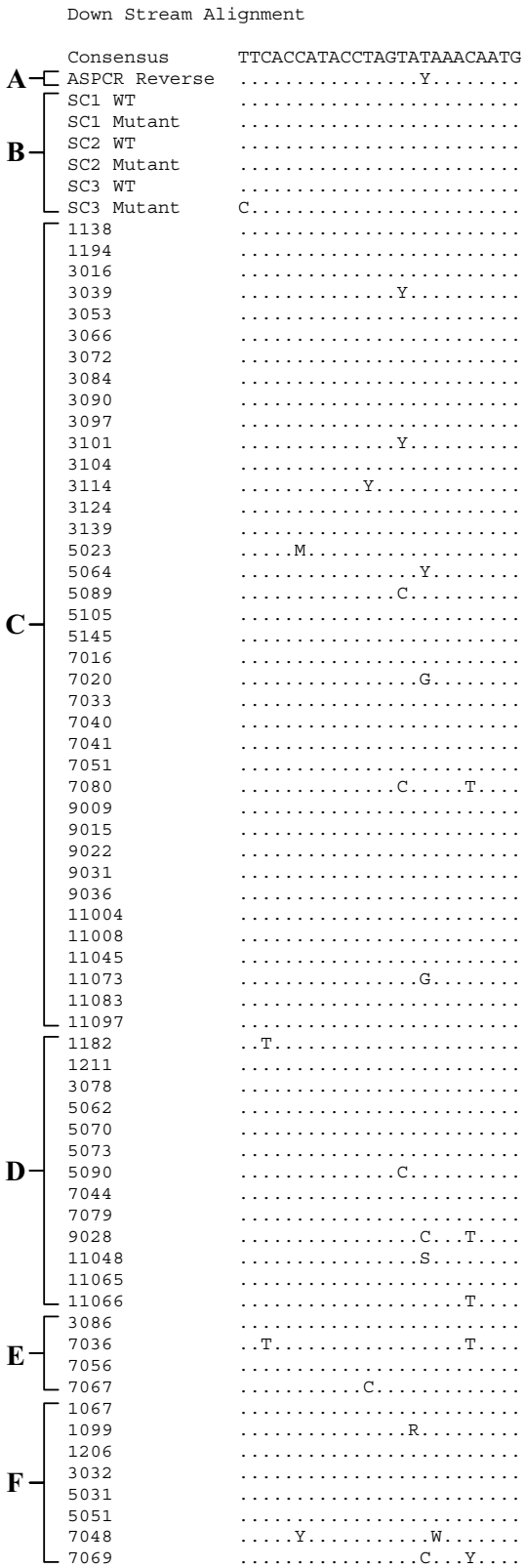


FIG. 1. Population sequences obtained using the ViroSeq system were aligned with the sequences of plasmid-derived controls, LigAmp oligonucleotides, ASPCR primers, and the ASPCR probe. A consensus sequence is shown at the top. The codon at position 103 in HIV reverse transcriptase is noted. Dots in the alignment indicate identity to the consensus sequence. The single letter codes for nucleotide mixtures are R = A + G, Y = C + T, M = A + C, K = T + G, S = C + G, W = A + T, H = A + C + T. Brackets on the left identify sets of sequences (**A–F**) as described below. (**A**) Sequences of the LigAmp (LA) upstream and downstream oligonucleotides, the ASPCR mutant primer, the ASPCR total copy primer, the ASPCR reverse primer, and the ASPCR probe. There is an intentional mismatch (G) in the LigAmp upstream oligonucleotide and the ASPCR mutant primer (underlined). LigAmp oligonucleotides include tails with binding sites for M13 primers and a LacZ PCR probe used in the detection step of the LigAmp assay (not shown); the oligonucleotide tails are described in the Materials and Methods section. (**B**) Sequences of plasmid-derived controls used in the LigAmp and ASPCR assays (see Materials and Methods); pairs of wild-type (WT) and mutant controls are designated as standard control panels 1–3 (SC1, SC2, SC3). (**C–F**) Population sequences from the 63 test samples, as follows. (**C**) Sequences from samples positive for K103N by both LigAmp and ASPCR. (**D**) Sequences from samples negative for K103N by both LigAmp and ASPCR. (**E**) Sequences from samples positive for K103N by ASPCR and negative for K103N by LigAmp. (**F**) Sequences from samples with discordant ASPCR and LigAmp results.

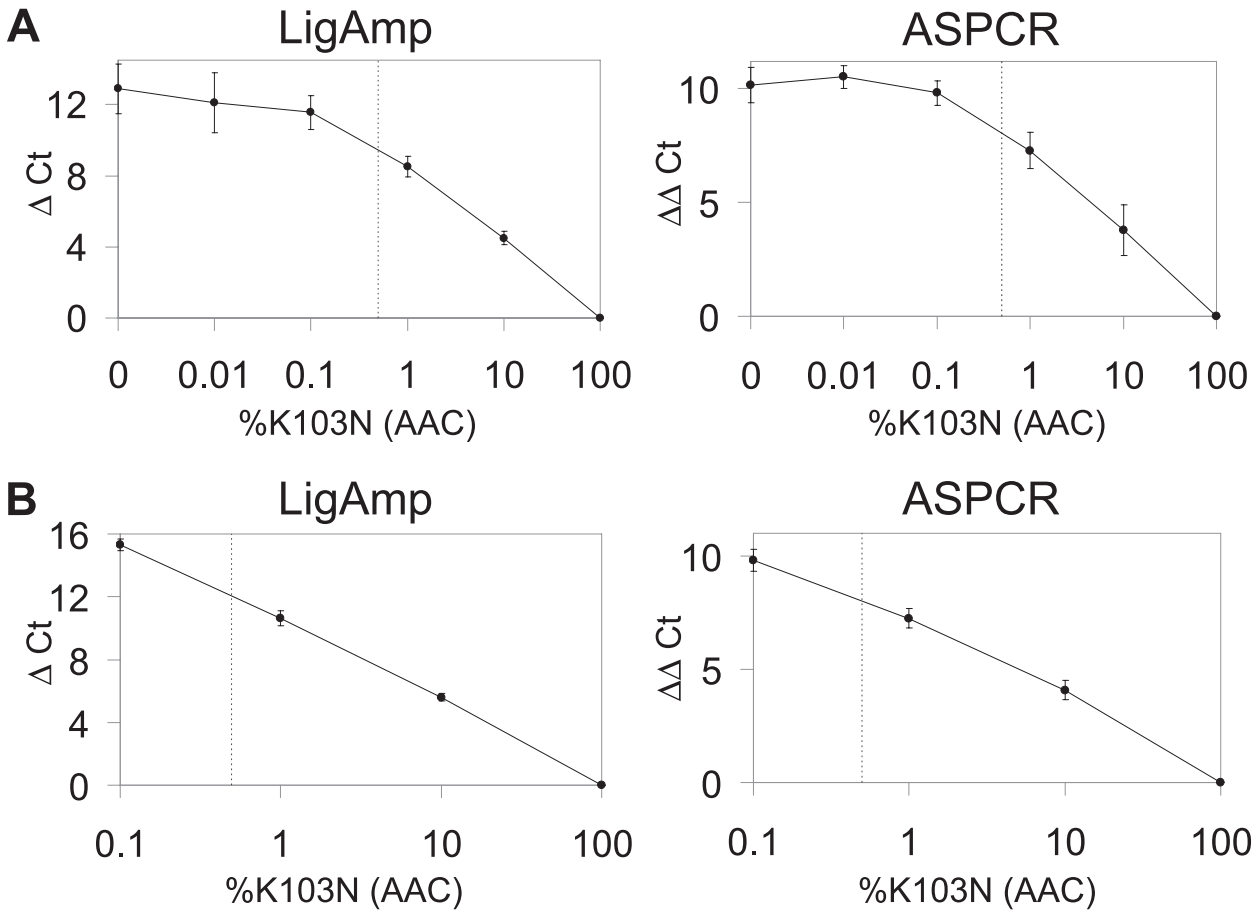


FIG. 2. Analysis of plasmid-derived control samples using the LigAmp and ASCPR assays. The LigAmp and ASPCR assays were used to analyze control samples derived from plasmid clones with and without the K103N mutation. Samples contained K103N at 0%, 0.01%, 0.1%, 1%, 10%, and 100% (see Materials and Methods). Raw data from the LigAmp assay (Ct = threshold cycle) were normalized so that the result obtained from the 100% K103N sample was assigned a ΔCt value of zero. For the ASPCR assay, the mutant reaction Ct was subtracted from the total copy reaction Ct to yield a single ΔCt value for each sample. The ΔCt values were then normalized, so that the result obtained from the 100% K103N sample was assigned a ΔCt value of zero. The 0.5% K103N assay cutoff used for both assays is shown (dotted lines). (**A**) The plots show the mean of results obtained with LigAmp (left) and ASPCR (right) using control samples derived from three different individuals (three standard curves, SC1, SC2, and SC3; Fig. 1B). Whiskers representing one standard deviation (SD) of the results obtained for each percentage K103N are shown. (**B**) The plots show the mean of results obtained from repeat analysis of a single standard curve (all assay runs in this study); this includes results from four LigAmp assay runs (left) and five ASPCR assay runs (right). Whiskers representing one standard deviation (SD) of the results obtained for each percentage K103N are shown.

We next compared the percentage K103N result from the clonal analysis to the percentage K103N results obtained using the LigAmp and ASPCR assays (Fig. 4). For one of the eight samples, the percentage K103N determined by both the LigAmp and the ASPCR assays was within the 95% CI for percentage K103N determined by clonal analysis (sample 1067). For one sample, the results from both the LigAmp and ASPCR assays were outside of the 95% CI for the clonal analysis (sample 1099); compared to the percentage K103N result from clonal analysis of this sample, the LigAmp result was low and the ASPCR result was high. For four of the remaining six samples, the percentage K103N from the LigAmp assay was within the 95% CI, while the percentage K103N from the ASPCR assay was above the upper CI (samples 1206, 5031, 5051, 7069; overestimation of the percentage K103N by ASPCR). For the remaining two samples, the percentage K103N from the ASPCR assay was within the 95% CI, while the percentage K103N from the LigAmp assay was below the lower CI (samples 3032, 7048; underestimation by LigAmp).

We compared the population sequence from each of the eight selected discordant samples (ViroSeq sequence) to the sequences of the primers and oligonucleotides used in the two assays, and to the sequences of other samples that had concordant results (Fig. 1). Most of the samples contained one or more nucleotide mismatches when compared to the sequence consensus and the sequences of reagent oligonucleotides (Fig. 1). However, it is possible that the type and position of mismatches, specific combinations of mismatches, or the presence of low-level mismatch variants (not represented in the population sequences) could affect the performance of either the LigAmp or

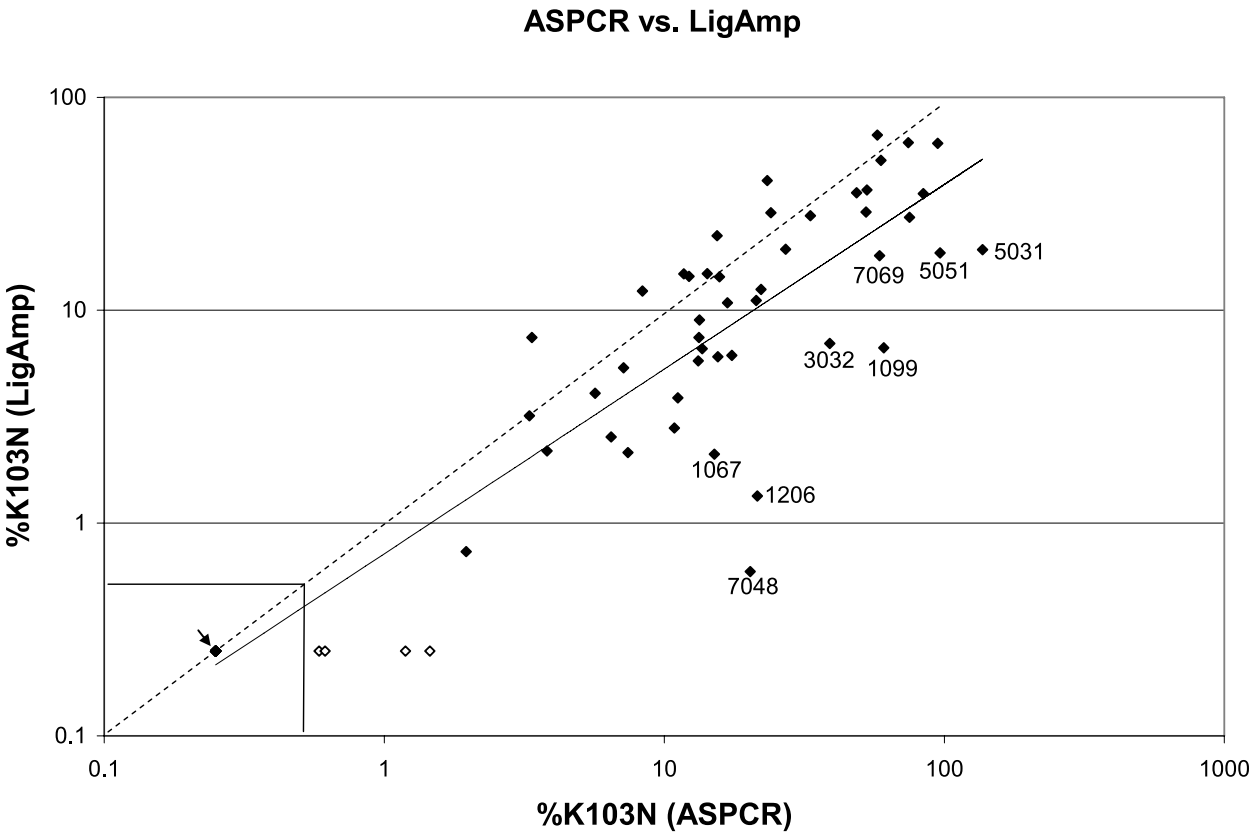
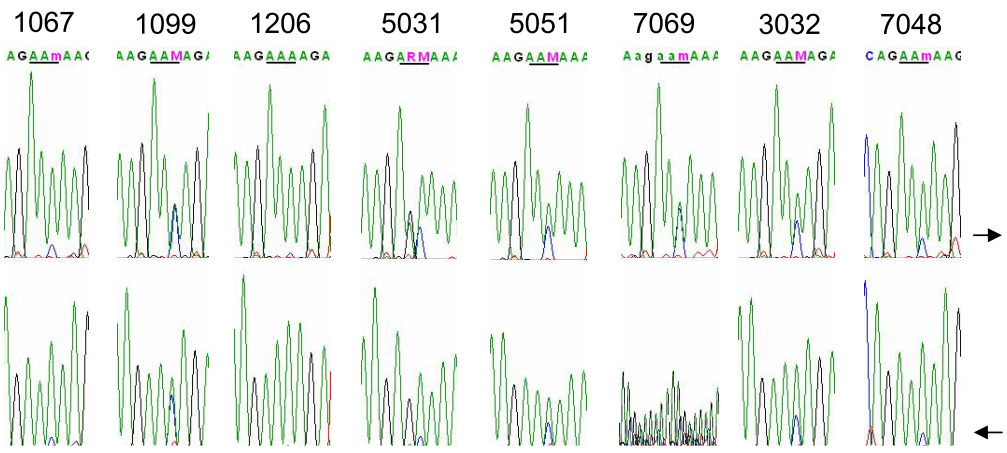


FIG. 3. Comparison of the percentage K103N measured using the LigAmp assay and the ASPCR assay. The percentage K103N in 63 clinical samples was measured using the LigAmp and ASPCR assays. Results from both assays were log transformed. Results from 13 samples that had values below the 0.5% K103N detection cutoff for the LigAmp and ASPCR assays were assigned a value of 0.25 to allow log transformation, and are shown as a single data point (arrow). The line of identity (dashed) and the trendline (solid) are shown. Sample numbers indicate the data points for eight discordant samples selected for further analysis (see text). Samples that had K103N detected with the ASPCR assay only are indicated (open symbols).

ASPCR assay. We analyzed the discordant samples in two groups: (1) the five samples for which ASPCR appeared to overestimate the percentage K103N (samples 1099. 1206, 5031, 5051, 7069), and (2) the three samples for which LigAmp appeared to underestimate the percentage K103N (samples 1099, 3032, 7048).

Overestimation of the percentage K103N by ASPCR could be explained by sequence mismatches that hinder hybridization or extension of the total copy primer. Mismatches at the ASPCR reverse primer binding site or the ASPCR probe binding site would be expected to decrease amplification in both the mutant and total copy reactions, and would therefore not be expected to influence quantification of the K103N mutation. Analysis of population sequences provided a possible explanation for overestimation of the percentage K103N in only one of the five samples with high ASPCR results. Sample 5031 contained a mixture of viruses with different nucleotides at the 3′ end of the ASPCR total copy primer binding site (R = A + G); inefficient binding of the total copy primer to DNA from that sample may have impaired amplification in the total copy reaction. This mismatch was not detected in any of the other samples tested. No explanation for overestimation of the percentage K103N was found for the other four samples (1099, 1206, 5051, 7069) by examining the population sequences or the sequences of clones derived from these samples.

Underestimation of the percentage K103N by LigAmp could be explained by mismatches that hinder either hybridization or ligation of the LigAmp oligonucleotides. Analysis of the population sequences provided a possible explanation for overestimation of the percentage K103N in all three of the samples with low LigAmp results. Samples 1099 and 3032 had a mismatch near the 5′ end of the LigAmp downstream oligonucleotide (G vs. A), which may have impaired ligation. This mismatch was also present in sample 1206, which had a LigAmp result near the lower 95% CI for the clonal result. The same mismatch was present as a mixture (R = A + G) in one of the four samples that was negative by LigAmp for K103N, but positive by ASPCR (Fig. 1E, sample 7067). Only one of the 38 concordant samples that was positive by both LigAmp and ASPCR had the same mismatch (Fig. 1C, mixture of A and G = R, sample 11045). Of note, this mismatch was also present in the mutant plasmid used to generate one of the standard curves (SC1), but did not appear to affect K103N quantification. In sample 1099, hybridization or ligation of the LigAmp oligonucleotides may have been hindered further by a combination of genetically linked mismatches in the oligonucleotide



	1067	1099	1206	5031	5051	7069	3032	7048
Clonal Analysis	5	35	5	20	32.5	27.5	30.0	12.5
95% CI	0.6-17	21-52	0.6-17	9.1-36	19-49	15-44	17-47	4.1-27
LigAmp	**2**	7	**1**	**19**	**19**	**27**	7	0.6
ASPCR	**15**	61	22	137	97	75	**39**	**20**

FIG. 4. ViroSeq electropherograms, clonal data, ASPCR results, and LigAmp results for selected samples (see text). ViroSeq electropherograms obtained using forward and reverse primers (arrows) are shown for the region near codon 103. The consensus sequence from the viral population of each sample is shown above the electropherogram; codon 103 is underlined. The percentage of clones with K103N (from among 40 clones analyzed per sample) is shown (Clonal Analysis). The 95% exact fiducial confidence intervals (CI) for percentage K103N calculated from the clonal data are shown. The percentage K103N detected in each sample using the LigAmp and ASPCR assays is shown; results from those assays that fall within the 95% confidence intervals of the clonal data are in bold.

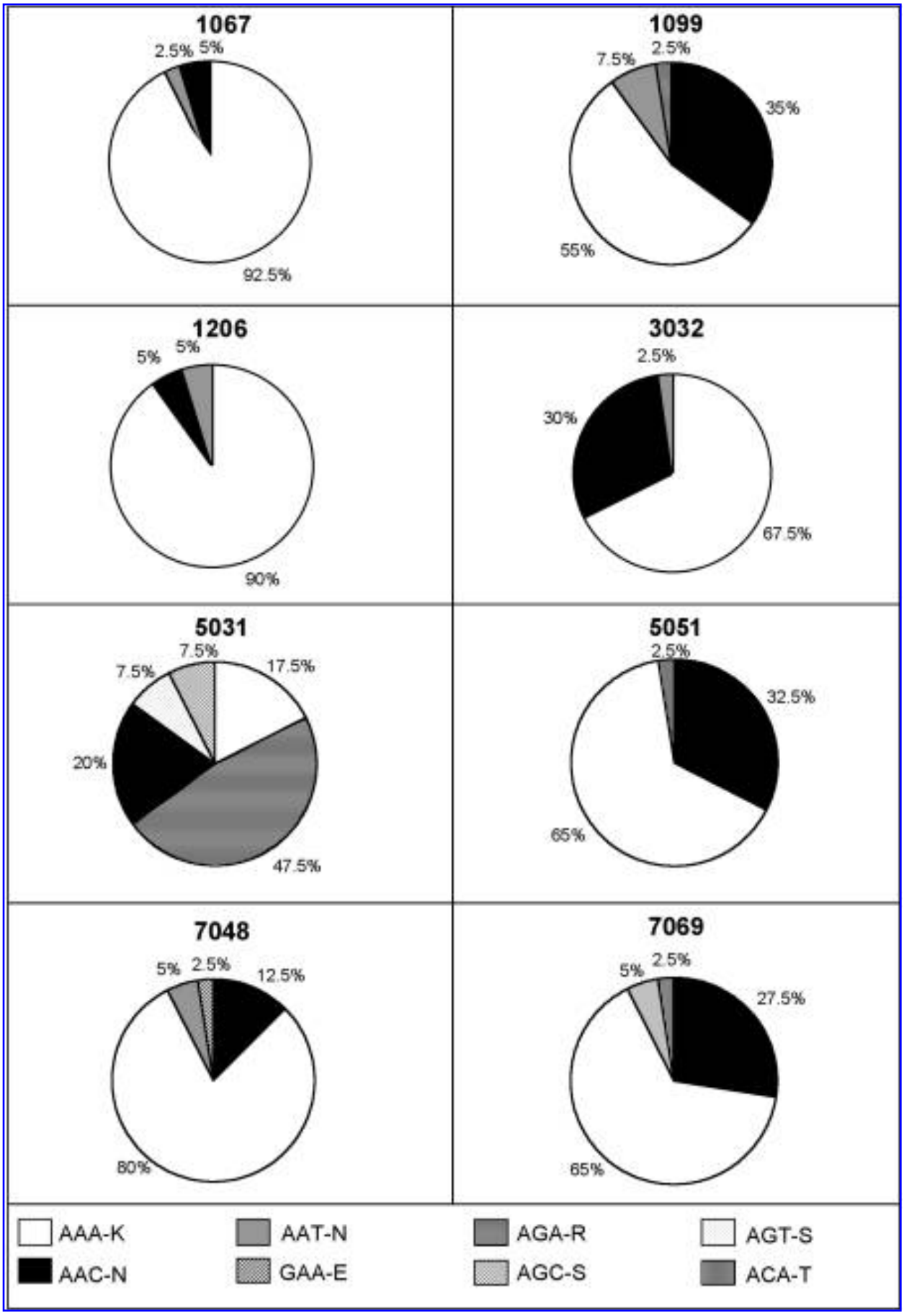
binding regions; 31 of the 40 clones from this sample had three mismatches at the LigAmp downstream oligonucleotide binding region, and the other 9 clones from this sample had four mismatches in this region. Underestimation of the percentage K103N by LigAmp in sample 7048 may also have resulted from a combination of mismatches in the LigAmp oligonucleotide binding regions. This sample had three mismatches in the LigAmp oligonucleotide binding regions (Fig. 1F); all three mismatches were present in all 40 clones derived from this sample. Among the 38 concordant samples positive for K103N by both assays (Fig. 1C), only one sample had three mismatches that were not detected as mixtures by ViroSeq (sample 7040).

Figure 5 provides a detailed analysis of the codons at position 103 in the 320 clones derived from the eight samples with discordant LigAmp and ASPCR results. This analysis underscores the complexity of mutation detection in HIV. A total of eight different codons were detected at position 103 in the clones (mean = 3.8 different codons within each sample). Two of these codons encode the K103N mutation: AAC and AAT. The AAT codon is relatively uncommon in women after SD NVP[11] and was identified in the population sequences from only two of the 63 women in this study. AAT was detected by clonal analysis in five of the eight samples, at levels ranging from 2.5% to 7.5% of the viral population. Other amino acid substitutions detected at codon 103 in the clones included K103R (AGA, two samples, at 2.5% and 47.5%), K103E (GAA, one sample at 2.5%), K103S (AGC and AGT, each present at 7.5% in one sample), and K103T (ACA, two samples at 2.5%). In sample 5031, alternate codons were present in more than half of the viral population (AGA at 47.5%, AGC at 7.5%, and AGT at 7.5%; Figs. 3 and 4). As described above, the presence of a nucleotide polymorphism at the second position of codon 103 in this sample (A to G change) was most likely the cause of overestimation of percentage K103N by the ASPCR assay. Other samples had clones with alternate codons at position 103 that would be expected to reduce amplification in the total copy ASPCR reaction (e.g., 1099, 5051, and 7069). However, because those alternate codons were present in only a small portion of the viral population (2.5% of the clones), they would not be expected to have had a significant impact on K103N quantification in those samples.

DISCUSSION

In the majority of clinical samples analyzed in this study, similar results were obtained for the percentage K103N using the LigAmp assay and an ASPCR assay. However, discordant

FIG. 5. Analysis of codon 103 in clones isolated from selected samples (see text). Each graph shows the results obtained from analysis of 40 clones from each sample. The percentage of clones in each sample with each type of codon is shown (see legend). K, lysine; N, asparagine; E, glutamate; R, arginine; S, serine; T, threonine.


1067
2.5%
5%
92.5%
1099
7.5%
2.5%
35%
55%
1206
5%
5%
90%
3032
2.5%
30%
67.5%
5031
7.5%
7.5%
17.5%
20%
47.5%
5051
2.5%
32.5%
65%
7048
5%
2.5%
12.5%
80%
7069
5%
2.5%
27.5%
65%
AAA-K
AAC-N
AAT-N
GAA-E
AGA-R
AGC-S
AGT-S
ACA-T

results were obtained for some samples. The LigAmp assay underestimated the percentage K103N in three (4.8%) of the samples, and the ASPCR assay overestimated the percentage K103N in five (7.9%) of the samples. Assay discordance was explained by variation at primer/oligonucleotide binding sites in some, but not all, of the samples with discordant results. In addition, there were four samples that tested negative by LigAmp (<0.5% K103N) and positive by ASPCR (0.6–1.5% K103N). In another study, an ASPCR assay with a similar primer design also overestimated the level of antiretroviral drug resistance mutations in some cases.[18] This trend (underestimation of K103N in some samples by LigAmp vs. overestimation of K103N in some samples by ASPCR) can be explained by differences in the design of the two types of assays. (1) In the LigAmp assay, each ligation reaction requires hybridization of the oligonucleotides to the original HIV template. Because the ligation oligonucleotides are single-stranded and all have the same polarity (either sense or antisense), ligated oligonucleotide pairs cannot serve as templates in subsequent ligation reactions. Therefore, nucleotide differences between the test template and the oligonucleotides could potentially hinder ligation in all of the ligation cycles, leading to underestimation of a mutation. In contrast, amplified PCR products become templates in subsequent PCR cycles. Because the PCR products incorporate the PCR primers at their terminal ends, nucleotide differences between the original HIV template and the primers are likely to hinder amplification only during the earliest cycles of the PCR reaction. (2) In ASPCR, if the 3′ nucleotide were missing from some mutant primer molecules (e.g., due to nuclease degradation or incomplete primer synthesis), those primers would lack any specificity for the mutation of interest. This could potentially lead to overestimation of the level of the mutation. In contrast, if terminal nucleotides were missing from some of the ligation oligonucleotides in the LigAmp assay, this would leave a gap between the two oligonucleotides, and no ligation would occur. This could potentially lead to underestimation of the level of a mutation.

Both the LigAmp and ASPCR assays can be designed to detect AAC, AAT, or both. In this study, we chose to compare the ability of the two assays to detect AAC, which is by far the most common NVP resistance mutation seen in women after SD NVP. In our previous studies, when AAT was detected, it was present at a low level, along with AAC.[11] In this study, AAT was detected in six of the eight discordant samples, but was detected by ViroSeq in only one of the 63 samples tested. In the clones derived from the discordant samples, AAT was detected only in samples that also had AAC, and AAC was generally detected in a higher number of clones (Fig. 5).

The high level of genetic diversity of HIV viruses was considered in the design of both assays used in this study. Both assays were able to tolerate nucleotide mismatches in the majority of samples tested. In the LigAmp assay, the specificity for mutation detection lies in the ligation step of the assay. Ligation oligonucleotides are long (typically 28–34 nucleotides in the hybridizing region of each oligonucleotide), and are hybridized to the DNA template at a low temperature (50°C). This minimizes the effect of sequence heterogeneity near the mutation.[10,19] However, the presence of numerous mismatches between the DNA template and the ligation oligonucleotides can interfere with oligonucleotide hybridization in some cases, and sequence variation very close to the ligation site may interfere with ligation. In both cases, sequence variation would tend to underestimate the level of the mutation in the sample, or to produce a false-negative result. In the ASPCR assay, sequence variation around position 103 can influence binding of the mutant-specific primer. This can be addressed by performing a second amplification reaction with a primer that overlaps the mutation-specific primer binding region, but is one or two nucleotides shorter (e.g., total copy primer; see Fig. 1).[4,14] Sequence variation at the ASPCR reverse primer and probe sites is less likely to influence mutation detection because the same reverse primer and probe are used in the mutation-specific and total copy reactions, and because the reverse primer and probe can be designed to hybridize to more conserved regions of the HIV template.

The amount of HIV RNA in clinical samples can vary over several orders of magnitude. For this reason, it may be desirable to adjust for the amount of DNA template in test samples. In the LigAmp assay, we measure DNA template concentration by spectroscopy, and add an equal amount of DNA to each reaction. An advantage of this approach is that the determination of template concentration is not influenced by differences in the HIV sequence. It is also possible to perform LigAmp for both mutant and wild-type sequences, either in two separate reactions or in a multiplexed reaction,[10] to adjust for differences in template concentration. However, because a spectrum of viral variants may be present in clinical samples (e.g., variants with codons other than wild-type AAA = K and mutant AAC = N; see Fig. 3), this approach may not always give an accurate reflection of the percentage K103N in the viral population. Different approaches have been used to adjust for DNA template concentration in ASPCR assays for HIV mutation detection. In our ASPCR assay, we quantify the amount of DNA template by spectroscopy prior to analysis, and add an equal amount of template DNA to each reaction. In another ASPCR assay, a real-time RT-PCR reaction is performed using primers directed toward a more highly conserved region of the HIV sequence to assess the concentration of the HIV template and adjust the amount of template before mutant quantification.[4,14]

This report compares detection and quantification of the K103N NVP resistance mutation using the LigAmp assay and an ASPCR assay using clinical samples. A variety of different ASPCR assays have been used to detect and quantify K103N in HIV samples. The performance of those assays, and the comparability of their results to results obtained by LigAmp and other methods, is expected to vary based on the assay design. Additional studies are needed to evaluate the performance of different methods used to detect and quantify drug resistance mutations in clinical samples from patients with HIV infection. The high genetic complexity of HIV that can exist even at a single codon underscores the importance of using clinical samples, rather than plasmid-derived viral stocks, for evaluating HIV point mutation assays.

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