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Quality Control Trial for Human Immunodeficiency Virus Type 1 Drug Resistance Testing Using Clinical Samples Reveals Problems with Detecting Minority Species and Interpretation of Test Results

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Between January and March 2000, a quality control panel for human immunodeficiency virus (HIV) drug resistance testing was analyzed by 20 laboratories in five countries. The panel consisted of three clinical samples with different drug resistance genotypes and phenotypes and one HIV-negative plasma. Participants were asked to report the methods used for amplification and sequencing, a list of drug resistance-associated mutations that were detected in the protease and reverse transcriptase of each sample, and an interpretation concerning the susceptibility or resistance to 14 antiretroviral drugs. A total of 22 genotypic data sets were generated, which showed an overall good technical quality except for three participants, who failed to report key mutations for drug resistance. Problems were encountered in three respects: (i) resistant minorities of L90M in the protease, which were determined to about 12% by real-time amplification, were only detected by one-fourth of the participants; (ii) newly described resistance mutations were frequently not reported; and (iii) interpretations of drug resistance-associated mutations varied widely, in particular for protease inhibitors. In some cases, different interpretations were caused by differences in the detection of resistant minorities, but even for the same genotypic profile, interpretations varied considerably. Similar discrepancies were revealed if current Web-based interpretation systems were used to predict drug resistance for samples of the proficiency panel. This indicates that a consensus for the interpretation of drug resistance-associated mutations is urgently needed.

The clinical benefit of drug resistance testing in the antiretroviral treatment of human immunodeficiency virus type 1 (HIV-1)-infected patients is becoming more and more obvious. Therefore, drug resistance testing is now considered the standard-of-care in the management of treatment failure (2, 19, 21). This is based on several retrospective and prospective studies, which showed that the decline in viral load and the number of patients with undetectable viremia significantly increased if antiretroviral therapy was changed according to the results of drug resistance testing (1).

Drug resistance can either be determined phenotypically by cultivating (recombinant) viruses in the presence of increasing drug concentrations or genotypically by searching for mutations that are known to be associated with drug resistance from in vitro or in vivo data. Whereas the laborious and time-consuming phenotypic assays will remain restricted to specialized laboratories, genotypic assays are now performed by many laboratories with in-house techniques or commercial kits which are offered for routine virological diagnostics. Two commercial kits for HIV genotyping (Viroseq HIV Genotyping System [Applied Biosystems, Foster City, Calif.] and TruGene HIV-1 Genotyping Kit [Bayer Diagnostics, Fernwald, Germany]) have meanwhile been approved by the U.S. Food and Drug Administration.

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In the last few years, multicenter quality control trials using the ENVA-1 and ENVA-2 proficiency panels have shown that the technical quality still has to be improved (13, 14). This was particularly true for the detection of minority species, which was evaluated by using well-defined mixtures of resistant and wild-type clones. Another major challenge lies in the interpretation of drug resistance. Lists of drug resistance-associated mutations have been published as regular updates (10) or electronically (e.g., at the Stanford University website [http://hivdb.stanford.edu/] and in the Los Alamos HIV database [http://hiv-web.lanl.gov/content/index], respectively) (15). However, the effect of mutations cannot be considered independently, because the effect of some mutations can be enhanced (9) or reduced (6, 16, 22) by others.

The quality control trial presented here was initiated to address the question of interpreting genotypic drug resistance. Furthermore, we wanted to investigate the suitability of clinical

TABLE 1. Precharacterization of quality control samples

Sample	Original viral load (copies/ml)	Sample dilution	Pretreatment[a] with:		
			NRTI	NNRTI	PI
1	500,000	1:50	Yes	Yes	Yes
2	180,000	1:16	Yes	No	Yes
3	45,000	1:10	Yes	Yes	Yes

[a] Patient 1 had received multiple antiretroviral drugs. Patient 2 had been treated with stavudine, lamivudine, saquinavir, and nelfinavir but was suspected to be noncompliant. Patient 3 had been treated with zidovudine, zalcitabine, didanosine, stavudine, lamivudine, nevirapine, indinavir, saquinavir, and nelfinavir.

TABLE 2. Determination of drug resistance-associated mutations by the participants of the quality control trial (including the precharacterization results)

Sample	Analyzed gene	Drug resistance-associated mutations[a] (% reported[b])
1	PR	10I (100), 20I (60), 46I (100), 63P (100), 71V (100), 73T (45), 77I (91), 83V (95), 90M (100)
	RT	41L (100), 67N (100), 69D (100), 74V (100), 98S (43), 100I (96), 103N (91), 108I (87), 118I (27), 184V (100), 210W (100), 211K (48), 214F (30), 215Y (96), 219R (52)
2	PR	10I (89), 63N (53), 71T (95), V771 (5)
	RT	181C[c] (5), 214F (32)
3	PR	36V[d] (9), 46I[d] (91), 63P (100), 77I (91), 88S (73), 90M[d] (23)
	RT	62V (95), 69D (100), 75I (100), 77L (91), 151M (95), 184V (100), 211K (50)

[a] Drug resistance-associated mutations in the protease (PR) and RT are given with reference to Schinazi et al. (10).
[b] In some instances, mutations were detected (as seen in the software printouts) but not scored as resistance associated; in others they were called atypical or unusual mutations. If mixtures of wild-type and drug resistance-associated mutations were indicated, the mutation was counted. Polymorphisms at positions 60 and 93 of the protease were not included in the evaluation.
[c] For sample 2, 0 of 8 analyzed clones presented with 181C.
[d] For sample 3, 0 of 11 analyzed clones presented with 36V, whereas 46I was detected in 8 of 11 and 90M was detected in 1 of 15 clones.

samples, since this allows to mimic routine diagnostic procedures more realistically than the use of viral clones.

MATERIALS AND METHODS

Quality control panel. The samples were prepared from the plasma of three HIV-1-infected patients with known drug resistance genotypes and phenotypes by dilution with an HIV-negative plasma to viral loads between 5,000 and 10,000 copies/ml (b-DNA 3.0; Bayer Diagnostics, Fernwald, Germany). The samples were prepared separately in a biosafety cabinet and dispensed into 1.8-ml sterile tubes with screw caps at a final volume of 1.0 ml. The samples were stored at −80°C until they were shipped on dry ice to the participating laboratories. Samples 1 and 2 each contained one positive plasma specimen; for sample 3, two plasma specimens from the same patient obtained 3 weeks apart were pooled and then diluted. Sample 4 consisted of the negative plasma used for dilution of samples 1 to 3. Details about sample preparation and drug histories are shown in Table 1.

Resistance analysis of the panel samples. We precharacterized the original and the diluted samples for genotypic (included in Table 2) and phenotypic (Table 3) drug resistance. Resistance testing was performed by a recombinant virus assay as described previously (18). In brief, the genes for the protease and the first 900 bp of the reverse transcriptase (RT) were amplified from patient plasma by nested PCR and cloned into a matched deletion mutant of the proviral HIV-1 clone NL4-3. Recombinant viruses were obtained by transient transfection of 293T cells. A CEMx174-derived cell line containing the gene for the secreted alkaline phosphatase under the control of the simian immunodeficiency virus long terminal repeat (7) was used as indicator cell line for drug susceptibility testing. The fold reduced susceptibility was calculated by dividing the 50% inhibitory concentration (IC_{50}) of the respective recombinant virus by the IC_{50} of the nonresistant reference strain determined in parallel. Genotyping was performed by direct sequencing of the amplification product described above. Sequences were aligned with the Wisconsin Package version 10.0 (Genetics Computer Group, Madison, Wis.). The estimated detection limit for minority species was ca. 30%. Sequences were screened for resistance-associated mutations compiled by Schinazi et al. (10).

Mutation-specific real-time amplification. Resistant minorities at position 90 of the protease were determined quantitatively by combining the amplification refractory mutation system (ARMS), which was first described by Newton et al. (8), with the real-time PCR technique. Dilutions of the first-round PCR product described above were amplified simultaneously in two parallel reactions, by using a common upstream primer (5′-GGAAGCTCTATTGGATACAGG-3′) and several sets of downstream primers with 3′ ends matching either the wild-type or the mutant sequence (Fig. 1A). The primers were used at a concentration of 600 nM. The amplification was performed with the SybrGreen PCR Mastermix (Applied Biosystems, Weiterstadt, Germany) for 40 cycles at 95°C for 15 min, 61°C for 30 min, and 72°C for 30 min after an initial denaturation of 10 min. Specific amplification of the 226-bp product was verified by subsequent gel electrophoresis. Fluorescent detection of amplicons was mediated by using the SDS 7700 (Applied Biosystems, Weiterstadt, Germany). The cycle number at which the fluorescence passed a fixed threshold line was defined as the threshold cycle number (C_t). Copy numbers were calculated for 90M and 90L variants by interpolation of the experimentally determined threshold cycle onto standard regression curves. To ensure an accurate quantification for both wild type and mutant, a separate standard curve was generated for each template. The distance between the C_t observed with authentic and inauthentic priming was called ΔC_t (Fig. 1A and B) and reflects the discriminatory ability.

Data analysis. All participants were asked to report their methods for RNA extraction, amplification, and sequencing; the regions analyzed; and the estimated detection limit for minorities. Additionally, participants were asked to report the drug resistance-associated mutations that had been detected in the protease and RT of each sample, the results of phenotypic resistance testing (if applicable), and an interpretation of susceptibility or resistance to 14 antiretroviral drugs. The results were to be returned within 3 months upon receipt of the quality control panel.

(This study was presented in part at the 8th Conference on Retroviruses and Opportunistic Infections, Chicago, Ill., February 2001 [abstr. 252], and at the Annual Meeting of the German Society for Virology, Dresden, Germany, March 2001 [abstr. 337].)

TABLE 3. Phenotypic results for the proficiency panel, obtained during precharacterization (Lab 0) and provided by two participants (Lab A and B)

Sample	Lab	Fold reduced susceptibility[a] to (classification of resistance[b]): NRTI						NNRTI			PI				
		ZDV	ddC	ddI	d4T	3TC	ABC	NVP	DLV	EFV	IDV	SQV	RTV	NFV	APV
1[c]	0	>268 (R)	12 (**R**)	15 (R)	19 (**R**)	>444 (R)	29 (R)	49 (R)	>190 (R)	>131 (R)	230 (R)	>143 (R)	59 (R)	24 (R)	17 (**R**)
	A	20 (R)	1 (**S**)	8 (R)	4 (**I**)	>12 (R)	9 (R)	141 (R)	>350 (R)	>7,500 (R)	51 (R)	42 (R)	26 (R)	46 (R)	2 (**S**)
2	0	5 (S)	1 (S)	1 (S)	1 (**S**)	2 (S)	2 (S)	1 (S)	1 (S)	2 (S)	1 (S)	1 (S)	1 (S)	1 (S)	3 (S)
	A	1 (S)	1 (S)	2 (S)	3 (**I**)	1 (S)	1 (S)	1 (S)	10 (S)	3 (S)	2 (S)	1 (S)	1 (S)	2 (S)	1 (S)
	B	6.0 (S)	1.9 (S)	1.1 (S)	3.4 (**S**)	3.1 (S)	NA	0.7 (S)	2.1 (S)	NA	0.6 (S)	0.4 (S)	0.2 (S)	NA	NA
3	0	>333 (R)	33 (R)	83 (R)	13 (R)	>156 (R)	22 (R)	1 (S)	1 (S)	1 (S)	11 (**R**)	3 (S)	1 (S)	15 (R)	1 (S)
	A	95 (R)	8 (R)	>22 (R)	27 (R)	>12 (R)	10 (R)	3 (S)	6 (S)	4 (S)	3 (**I**)	1 (S)	1 (S)	21 (R)	0.3 (S)
	B	>755 (R)	>31.8 (R)	12.8 (R)	>31.3 (R)	>47.6 (R)	NA	2.0 (S)	2.6 (S)	NA	1.3 (**S**)	1.9 (S)	0.5 (S)	14.5 (R)	NA

[a] S, susceptible; I, intermediately resistant; R, resistant; ZDV, zidovudine; ddC, zalcitabine; ddI, didanosine; d4T, stavudine; 3TC, lamivudine; ABC, abacavir; NVP, nevirapine; DLV, delavirdine; EFV, efavirenz; IDV, indinavir; SQV, saquinavir; RTV, ritonavir; NFV, nelfinavir; APV, amprenavir; NA, not available. Drug resistance is given as the reduction in susceptibility to the indicated antiretroviral drug.
[b] Discrepant results are marked in boldface.
[c] Participant B did not provide results for sample 1.

A

Primer sequence	ΔC_t	Detection limit of minority
5′-AGT-GCA-CCC-AAG-TTG-AGT-CAA-3′ WT	3-4.5	
5′-AGT-GCA-CCC-AAG-TTG-AGT-CA***T***-3′ Mut	5-6.5	5– 10%
5′-AGT-GCA-CCC-AAG-***C***TG-AGT-CAA-3′ WT1	8-10	
5′-AGT-GCA-CCC-AAG-***C***TG-AGT-CA***T***-3′ Mut1	10-12	0.5–1%
5′-AGT-GCA-***G***CC-AA***T***-***C***TG-AGT-CAA-3′ WT3	12-16	
5′-AGT-GCA-***G***CC-AA***T***-***C***TG-AGT-CA***T***-3′ Mut3	15-19	0.1%

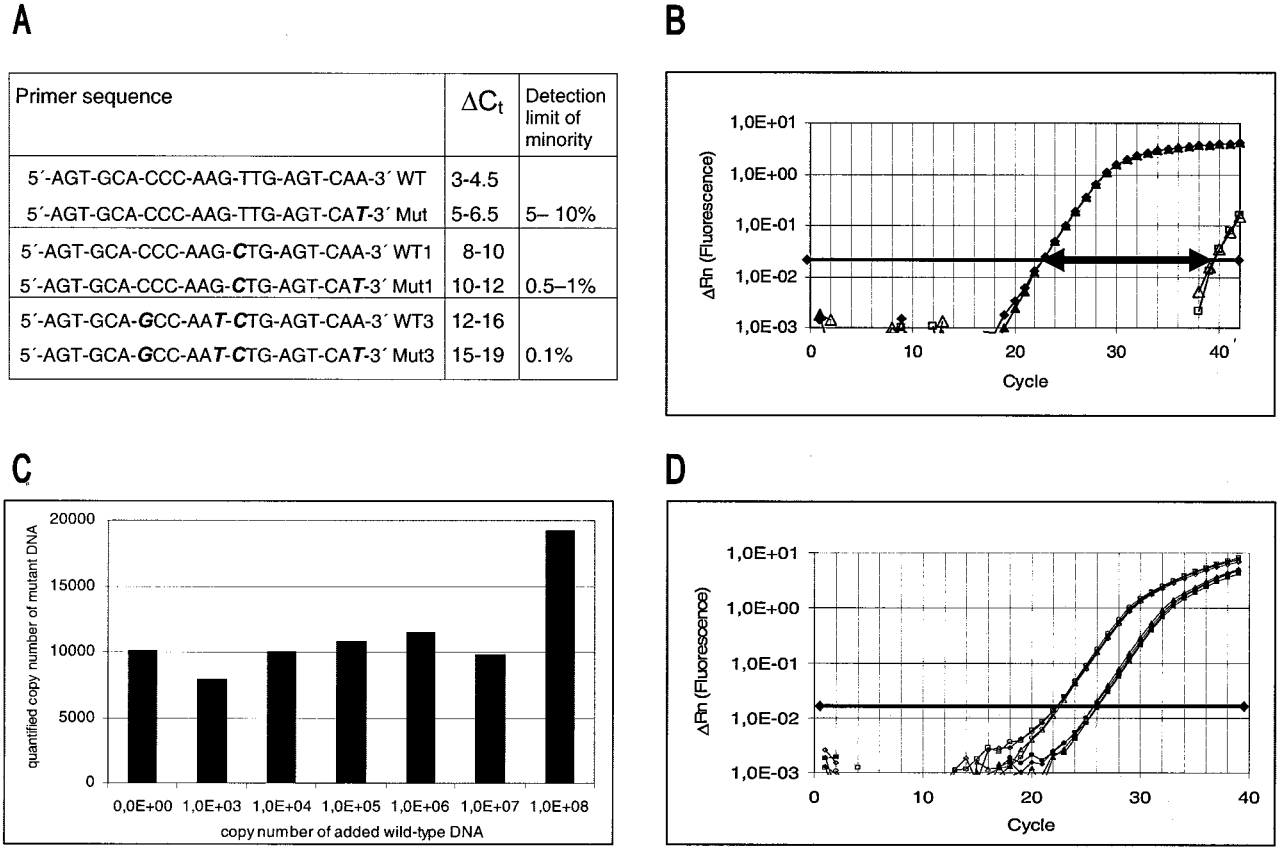

FIG. 1. Results of the real-time ARMS for 90L and 90M in the protease. (A) Downstream primer sets for the amplification of wild-type 90L (WT) and mutant 90M (Mut) sequences for sample 3 of the quality control trial. The mismatch at the 3′ end is responsible for the differences in threshold cycles (ΔC_t), which result from authentic amplification (e.g., WT template and WT primers) compared to amplification based on mispriming (e.g., WT template and MUT primers). The addition of one (WT1/Mut1) or three (WT3/Mut3) internal destabilizing mutations resulted in a higher discriminatory window, which consequently improved the detection limit for minorities. (B) Results of the real-time amplification of 10^6 copies of WT sequence per reaction, with WT3 or Mut3 as downstream primers (assay run in duplicate; black arrow indicates ΔC_t). The corresponding upstream primer was identical for WT and Mut amplification. The amplification was monitored through the binding of SybrGreen to double-stranded DNA. ΔRn, difference between the normalized reporter fluorescence and baseline. (C) Mixture of 10^4 copies of mutant template with increasing amounts of WT template DNA (10^3 to 10^8 copies/reaction). The first column shows quantitation of Mut template alone. Up to a 1,000-fold excess of WT template did not influence the accuracy of quantitation. (D) Real-time amplification of sample 3. The relation between 90L and 90M was calculated as 88% versus 12%, after the exact copy numbers had been determined by generating specific standard curves for wild-type and mutant template (see Results).

RESULTS

Participating laboratories and technologies used for sequence analysis. In January 2000, the panel of four samples was distributed to 24 laboratories in five countries (Germany, Austria, Belgium, the United States, and Luxembourg). Results were finally reported by 20 participants at universities (n = 12), private laboratories (n = 5), municipal hospitals (n = 1), public health authorities (n = 1), and commercial companies (n = 1). A total of 22 genotypic data sets were generated, because one participant reported the results of two operators with the same commercial kit and another participant provided the results from two different kits. Five data sets were incomplete; one laboratory analyzed only sample 1, in three cases data for sample 2 were missing, and in one case the protease from sample 1 could not be analyzed. Nine laboratories used in-house techniques for amplification and sequencing, whereas the Viroseq HIV Genotyping system (HIV Genotyping Software, v.2.1 [released October 1999 until April 2000]) and the TruGene HIV-1 Genotyping Kit (operating with the GeneObjects and GeneLibrary Software, v.3.1 [released July 1999 and October 1999], respectively, until July 2001) were used by seven and five laboratories, respectively. Eight laboratories identified drug resistance-associated mutations manually, nine laboratories used the Viroseq software, and five laboratories used the TruGene software. Seven laboratories also used the Stanford or Los Alamos database. Two participants also provided phenotypic results for at least two samples of the quality control panel.

Reporting of drug resistance-associated mutations. The majority of participants detected most of the drug resistance-associated mutations. Primary mutations that play a key role in mediating resistance to antiretroviral drugs (4) were reported for >90% of data sets (Table 2). A higher failure rate was observed for unusual mutations at drug resistance-associated positions: e.g., K20I, L63N, and G73T in the protease and A98S and K219R in the RT. The same was true for mutations V118I in the RT and N88S in the protease, for which information had been available only as meeting abstracts at the time

of the quality control trial and was published thereafter (3, 22). Finally, mutations R211K and L214F, which are associated with dual resistance to zidovudine and lamivudine (10), were only reported by a minority of participants.

Interestingly, 5 of 21 laboratories reported a mixture of wild-type and drug resistance-associated mutations at position 90 in the protease of sample 3 (Table 2). This had not been detected during the precharacterization of samples. It is noteworthy that the results that were provided by two operators from the same lab using the Viroseq kit differed exactly at this position. Since the presence of L90M considerably influenced the interpretation of drug resistance, further efforts were made to clarify the percentage of resistant minorities at this position.

Detection of minority species at position 90 in the protease. To further analyze the composition of sample 3 at position 90 in the protease, individual bacterial clones that were obtained during the recombinant virus assay were sequenced. Of 15 clones analyzed, 14 showed wild-type sequence TTG, and only one showed the mutated sequence ATG. To determine the exact proportion of 90M in this sample, the clone with the mutant sequence and one of the clones with wild-type sequence at position 90 were used to develop a real-time PCR assay with wild-type- and mutation-specific primers (see Materials and Methods). The differences in amplification efficiency for authentic and inauthentic priming (e.g., wild-type template and wild-type and/or mutant primer) were clearly shown (Fig. 1A and B). The introduction of three internal destabilizing mismatches in the mutant- and wild-type-specific primers increased the discriminatory ability, as shown by the increase in the differences of threshold cycles (ΔC_t) from 3 to 6.5 cycles to 12 to 19 cycles (Fig. 1A). This reduced the detection limit for minorities of 90M to ca. 0.1%. To ensure accurate quantification, two separate standard curves were generated for wild-type and mutant templates, taking into account possible differences in amplification efficiencies. Analyses of well-defined mixtures of mutant and wild-type template showed that an excess of wild-type DNA did not influence the accuracy of quantification unless a 10,000-fold excess was added (Fig. 1C). After specific standard curves had been generated for wild-type and mutant template, the first-round PCR product of sample 3 generated for genotyping was subjected to real-time amplification (Fig. 1D). Using three different dilutions (1:10 to 1:1,000), the relation between 90L and 90M was calculated as 87.8% versus 12.2%. The accuracy of the value was evaluated in seven duplicate determinations, which showed a coefficient of variation of 15.6%.

Determination of phenotypes. One laboratory provided phenotypic results for all samples; another provided phenotypic results for samples 2 and 3 (Table 3). Both laboratories used the homologous recombination assay described by Kellam and Larder (5). There was an agreement in the classification of resistance with our phenotypic precharacterization for most of the drugs. Major discrepancies were only observed for zalcitabine and amprenavir in sample 1 and indinavir in sample 3. In all of these cases, results from the homologous recombination assays showed a lower degree of resistance (1.3- to 3-fold-reduced susceptibility) compared to our ligation-based recombinant virus assay (11- to 17-fold-reduced susceptibility).

Interpretation of drug resistance. All participants except for one who produced two data sets provided an interpretation of the genotypic data with respect to susceptibility or resistance to 14 antiretroviral drugs (Table 4). For nucleoside and nonnucleoside RT inhibitors (NRTI and NNRTI, respectively), a high degree of consistency in interpretations was observed except for the prediction of resistance to stavudine in sample 1. Some obvious discrepancies were explained by the failure to identify primary mutations: the two laboratories that had not reported mutation K103N in the RT of sample 1 classified NNRTI as susceptible and the failure to identify Q151M in the RT of sample 3 led to an underestimation of resistance to zidovudine, stavudine, and abacavir by one participant. In another case, reporting of minorities of Y181C in the RT of sample 2 resulted in the prediction of intermediate resistance to nevirapine.

A wide range of interpretations was reported for resistance to protease inhibitors (PIs), in particular if mutations did not indicate broad cross-resistance, such as in sample 1. Although only secondary mutations were reported for sample 2, interpretations varied from susceptibility to all PIs to resistance to indinavir and nelfinavir plus reduced susceptibility to ritonavir. The interpretation of PI resistance in sample 3 was obviously influenced by the detection of L90M: the 15 participants who had only detected 90L at this position predicted susceptibility to any PI in 65% compared to 20% for the four participants who had detected 90M. A less pronounced effect was observed for N88S: resistance to nelfinavir was predicted in 67% by the six participants who had not reported this mutation compared to 77% for the 13 laboratories that had detected N88S. It was noteworthy that even the interpretation of the same mutational pattern (M46I, L63P, V77I, and N88S) varied from intermediate resistance to nelfinavir only to resistance against indinavir, ritonavir, and nelfinavir.

DISCUSSION

This quality control trial successfully addressed three important aspects of drug resistance testing: the technical quality, including the detection of minority species; the usefulness of clinical samples for quality control purposes; and the interpretation of drug resistance. Compared to previous quality control trials in which mixtures of well-defined viral clones were used (13, 14), the overall technical quality was good: most of the laboratories were able to amplify the samples of the quality control panel and to identify drug resistance-associated mutations. Serious problems were mainly observed in one laboratory that used in-house methods for sequencing and identification of drug resistance-associated mutations and did not report three key mutations. Another participant using in-house systems, but also one TruGene user, failed to report a key mutation. Since the original sequences generated by these users were not available, it remains unclear whether these mutations were not detected during sequencing or not identified as drug resistance-associated mutations during subsequent analysis. Some of the secondary mutations, and in particular unusual mutations at these positions, may not have been reported because their effect on drug resistance was either considered minimal or unclear. This indicates that a consensus is necessary as to which mutations should be considered truly drug resistance-associated and therefore be reported and which should be classified as polymorphisms. An updated ver-

TABLE 4. Interpretation of the genotypic drug resistance profiles for the quality control samples[a]

Sample	No. of samples with resistance predicted against:													
	NRTI						NNRTI			PI				
	ZDV	ddC	ddI	d4T	3TC	ABC	NVP	DLV	EFV	IDV	SQV	RTV	NFV	APV
1														
S			1	4			1	2	1				1	2
S/I									1					
I	1		1	4		1	2			1	1	3	1	4
I/R	1		3	1		3		1			1	1	1	1
R	18	20	15	6	20	16	16	16	17	18	17	14	16	11
NA	2	2	2	7	2	2	3	3	3	3	3	4	3	4
2														
S	17	17	17	17	17	17	16	17	17	11	13	13	15	16
S/I										2	2	2		1
I/R							1			2	2	2		
I/R										1			1	
R										1			1	
NA	5	5	5	5	5	5	5	5	5	5	5	5	5	5
3														
S	1			1		1	19	19	19	7	16	10	5	15
S/I										2		1	1	
I	1		1			1				4		6	6	3
I/R										2	1	1		
R	17	19	18	18	19	17				4	2	1	7	1
NA	3	3	3	3	3	3	3	3	3	3	3	3	3	3

[a] Abbreviations are as defined in Table 3. Some data were not available due to missing sequences, and for two data sets no interpretation was given at all. Two laboratories had not detected K103N in the RT of sample 1, and therefore, NNRTI were falsely predicted to be susceptible. For sample 2, one laboratory which had detected a mixture of 181C and 181Y in the RT reported intermediate resistance to nevirapine. For sample 3, four of the five laboratories which had detected mixtures at position 90 of the protease provided an interpretation. They predicted resistance to IDV-SQV-RTV-NFV-APV as I/R-R-I/R-R-I, R-R-R-R-R, I/R-I/R-I-R-I, and S-S-S-I-S, respectively.

sion of a consensus achieved in 2000 (4) has recently been published (http://www.iasusa.org).

Another problem that became obvious from this quality control trial was the dissemination of information about new resistance mutations. In the quality control samples, two mutations (N88S in the protease and V118I in the RT) were present about which information was only available via congress abstracts at that time. Mutation N88S, which is associated with resistance to nelfinavir and hypersusceptibility to amprenavir (22), was not reported by four TruGene users and two laboratories identifying drug resistance-associated mutations manually. Obviously, mutation N88S was not identified by the TruGene software at that time. This indicates that software programs for the interpretation of drug resistance have to be updated in short intervals, which may be easier for Web-based systems than kit-based software. Interestingly, mutation V118I was reported by three users of the Viroseq software but also by two laboratories using in-house techniques. Thus, good results can be achieved irrespective of the system used, if current knowledge is incorporated (17).

A further important aspect of quality control trials for HIV drug resistance is the investigation of the performance in detecting minorities of resistant virus. Clearly, this is much easier to control with viral clones than with clinical samples because exact mixtures of clones with mutant and wild-type sequences at certain positions can be generated. Clinical samples cannot be checked in advance in such detail. This may lead to unwanted surprises, as was the case for the 90M minority in the protease of sample 3. The proportion of the 90M minority in this sample was retrospectively quantified by a newly developed sensitive real-time PCR assay. However, this method cannot be performed on patient samples routinely. The high sequence variability of HIV-1 makes it necessary to adjust the primer sequences for each individual isolate and to establish standard curves for correct quantitation with wild-type and mutant template from the sample under investigation. The percentage of 90M was calculated as 12%, which is below the level of detection reported by Schuurman et al. (13, 14) and also lower than the estimates of minority detection given by the laboratories that participated in this quality control trial (15 to 50%). Nonetheless, five participants detected the resistant minority at this position. These were two of five TruGene users (40%), one of eight Viroseq users (13%), and two of nine (22%) participants using in-house techniques. Due to the low numbers, no valid conclusions can be drawn as to which of the systems is superior in detecting minorities. However, in a recently presented international quality control trial, kit-based systems seemed to be superior to homebrew assays (20). The clinical relevance of detecting 90M in this sample could be seen in the follow-up of patient 3, since 7 months later 90M was clearly present in the protease. It seems to be advisable to further precharacterize clinical samples for future proficiency panels either by analyzing several clones (R. M. Grant et al., 9th Conf. Retrovir. Opportunistic Infect., abstr. 595, 2002) or by using the LiPA genotyping system, for which a detection limit of 5 to 10% was reported (12).

This leads to the third problem to be addressed, the interpretation of mutational patterns. The use of clinical samples

has the advantage that it mimics the situation in routine practice, whereas a meaningful interpretation is almost impossible for mixtures of clones. The results of this trial show that the interpretation for resistance to NRTI and NNRTI was quite homogeneous except for stavudine in sample 1, which may be due to the fact that the influence of thymidine analogue mutations on stavudine resistance was unclear at the time the trial was performed. A much greater variability of interpretations was seen for the PIs, particularly for samples 2 and 3. This was in part due to the detection of 90M, which considerably influenced the interpretation of PI resistance for sample 3. Three of the four participants who identified this mutation and provided an interpretation predicted at least intermediate resistance to all PIs, whereas the majority of participants that did not detect 90M predicted susceptibility to all PIs except for nelfinavir.

But which is the correct interpretation of PI resistance for this sample? The phenotypes indicate that the sample has to be classified at least resistant to nelfinavir. Various susceptibilities to indinavir may be explained by the loss of resistant L90M variants during the cultivation period of the homologous recombination assays. Still, PI resistance may also be underestimated in the recombinant virus assay that was used for the precharacterization of the panel. If the genotypic profile of mutations M46I, L63P, V77I, and N88S is submitted to current genotypic drug resistance interpretation systems (11), interpretations vary from low-level resistance against indinavir and nelfinavir to resistance against indinavir, saquinavir, ritonavir, nelfinavir, and amprenavir. Contrary to the interpretations provided by the participants of the quality control trial, the incorporation of L90M does not change the prediction of PI resistance for the majority of systems. Importantly, predictions in this quality control trial were not only influenced by the detection of different mutational patterns, but also varied considerably for the same genotypic profile, which was particularly true for the PI resistance of sample 2. Thus, a consensus for interpretation of drug resistance-associated mutations and more information about the importance of drug resistance-associated mutations on clinical outcome are urgently needed. A first important step will be the construction of large retrospective clinical databases that allow testing for the performance of different algorithms and interpretation systems.

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REFERENCES

1. **De Mendoza, C., O. Gallego, L. Valer, and J. Gonzalez-Lahoz.** 2001. Update on genotype-guided antiretroviral therapy. AIDS Rev. **3:**208–215.
2. **EuroGuidelines Group for HIV Resistance.** 2001. Clinical and laboratory guidelines for the use of HIV-1 drug resistance testing as part of treatment management: recommendations for the European setting. AIDS **15:**309–320.
3. **Hertogs, K., S. Bloor, V. De Vroey, C. van Den Eynde, P. Dehertogh, A. van Cauwenberge, M. Sturmer, T. Alcorn, S. Wegner, M. van Houtte, V. Miller, and B. A. Larder.** 2000. A novel human immunodeficiency virus type 1 reverse transcriptase mutational pattern confers phenotypic lamivudine resistance in the absence of mutation 184V. Antimicrob. Agents Chemother. **44:**568–573.
4. **Hirsch, M. S., F. Brun-Vezinet, R. T. D'Aquila, S. M. Hammer, V. A. Johnson, D. R. Kuritzkes, C. Loveday, J. W. Mellors, B. Clotet, B. Conway, L. M. Demeter, S. Vella, D. M. Jacobsen, and D. D. Richman.** 2000. Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society–USA Panel. JAMA **283:**2417–2426.
5. **Kellam, P., and B. A. Larder.** 1994. Recombinant virus assay: a rapid, phenotypic assay for assessment of drug susceptibility of human immunodeficiency virus type 1 isolates. Antimicrob. Agents Chemother. **38:**23–30.
6. **Larder, B. A., S. D. Kemp, and R. Harrigan.** 1995. Potential mechanism of sustained antiretroviral efficacy of AZT-3TC combination therapy. Science **269:**696–699.
7. **Means, R. E., T. Greenough, and R. C. Desrosiers.** 1997. Neutralization sensitivity of cell culture-passaged simian immunodeficiency virus. J. Virol. **69:**5431–5436.
8. **Newton, C. R., A. Graham, L. E. Heptinstall, S. J. Powell, C. Summers, N. Kalsheker, J. C. Smith, and A. F. Markham.** 1989. Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). Nucleic Acids Res. **17:**2503–2516.
9. **Race, E., E. Dam, V. Obry, S. Paulous, and F. Clavel.** 1999. Analysis of HIV cross-resistance to protease inhibitors using a rapid single-cycle recombinant virus assay for patients failing on combination therapies. AIDS **13:**2061–2068.
10. **Schinazi, R. F., B. Larder, and J. W. Mellors.** 2000. Mutations in retroviral genes associated with drug resistance: 2000–2001 update. Int. Antiviral News **8:**65–92.
11. **Schmidt, B., H. Walter, and K. Korn.** 2002. Genotypic drug resistance interpretation systems: the cutting edge of antiretroviral therapy. AIDS Rev. **4:**148–156.
12. **Schmit, J. C., L. Ruiz, L. Stuyver, K. Van Laethem, I. Vanderlinden, T. Puig, R. Rossau, J. Desmyter, E. De Clercq, B. Clotet, and A. M. Vandamme.** 1998. Comparison of the LiPA HIV-1 RT test, selective PCR and direct solid phase sequencing for the detection of HIV-1 drug resistance mutations. J. Virol. Methods **73:**77–82.
13. **Schuurman, R., D. Brambilla, T. de Groot, D. Huang, S. Land, J. Bremer, I. Benders, C. A. Boucher, et al.** 2002. Underestimation of HIV type 1 drug resistance mutations: results from the ENVA-2 genotyping proficiency program. AIDS Res. Hum. Retrovir. **18:**243–248.
14. **Schuurman, R., L. Demeter, P. Reichelderfer, J. Tijnagel, T. de Groot, and C. Boucher.** 1999. Worldwide evaluation of DNA sequencing approaches for identification of drug resistance mutations in the human immunodeficiency virus type 1 reverse transcriptase. J. Clin. Microbiol. **37:**2291–2296.
15. **Shafer, R. W., D. R. Jung, and B. J. Betts.** 2000. Human immunodeficiency virus type 1 reverse transcriptase and protease mutation search engine for queries. Nat. Med. **6:**1290–1292.
16. **Tisdale, M., S. D. Kemp, N. R. Parry, and B. A. Larder.** 1993. Rapid in vitro selection of human immunodeficiency virus type 1 resistant to 3′-thiacytidine inhibitors due to a mutation in the YMDD region of reverse transcriptase. Proc. Natl. Acad. Sci. USA **90:**5653–5656.
17. **Vandamme, A. M., F. Houyez, D. Banhegyi, B. Clotet, C. De Schrijver, K. A. De Smet, W. W. Hall, R. Harrigan, N. Hellmann, K. Hertogs, C. Holtzer, B. Larder, D. Pillay, E. Race, J. C. Schmit, R. Schuurman, E. Schulse, A.**

Sonnerborg, and V. Miller. 2001. Laboratory guidelines for the practical use of HIV drug resistance tests in patient follow-up. Antiviral Ther. **6:**21–39.
18. **Walter, H., B. Schmidt, K. Korn, A. M. Vandamme, T. Harrer, and K. Überla.** 1999. Rapid, phenotypic HIV-1 drug sensitivity assay for protease and reverse transcriptase inhibitors. J. Clin. Virol. **13:**71–80.
19. **Weinstein, M. C., S. J. Goldie, E. Losina, C. J. Cohen, J. D. Baxter, H. Zhang, A. D. Kimmel, and K. A. Freedberg.** 2001. Use of genotypic resistance testing to guide HIV therapy: clinical impact and cost-effectiveness. Ann. Intern. Med. **134:**440–450.
20. **Wensing, A. M., W. Keulen, M. Buimer, D. Brambilla, R. Schuurman, and C. Boucher.** 2002. Analysis of the world-wide evaluation study on HIV-1 genotype interpretation; ENVA-3. Antiviral Ther. **6**(Suppl. 1)**:**101.
21. **Yeni, P. G., S. M. Hammer, C. C. Carpenter, D. A. Cooper, M. A. Fischl, J. M. Gatell, B. G. Gazzard, M. S. Hirsch, D. M. Jacobsen, D. A. Katzenstein, J. S. Montaner, D. D. Richman, M. S. Saag, M. Schechter, R. T. Schooley, M. A. Thompson, S. Vella, and P. A. Volberding.** 2002. Antiretroviral treatment for adult HIV infection in 2002: updated recommendations of the International AIDS Society-USA Panel. JAMA **288:**222–235.
22. **Ziermann, R., K. Limoli, K. Das, E. Arnold, C. J. Petropoulos, and N. T. Parkin.** 2000. A mutation in human immunodeficiency virus type 1 protease, N88S, that causes in vitro hypersensitivity to amprenavir. J. Virol. **74:**4414–4419.