

ORIGINAL RESEARCH

Detection of drug-resistant HIV minorities in clinical specimens and therapy failure

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Objective

Particularly for therapy-experienced patients, resistance assessment by genotypic or phenotypic methods produces discordances. This study seeks proof that differences may arise from the fact that genotyping produces a single summary sequence whereas replicative phenotyping (rPhenotyping) functionally detects and assigns resistances in mixed HIV populations.

Methods

For validation, defined mixes of wild-type and M184V mutant were analysed by rPhenotyping or standard genotyping. Allele-specific and quantitative polymerase chain reaction (PCR) set detection and quantification limits for minor virus populations *in vitro* and in authentic clinical samples showing geno-/pheno-discrepant lamivudine resistance.

Results

Allele-specific and real-time PCR methods detected down to 0.3% of mutant M184V. The functional assessment was sensitive enough to reveal < 1% of mutant M184V in mixed samples. Also in discordant samples from the diagnostic routine, in which rPhenotyping had identified drug resistance, real-time PCR confirmed minute amounts of mutant M184V.

Conclusion

By utilizing the replication dynamics of HIV under drug pressure, a rPhenotyping format potently reveals relevant therapy-resistant minority species, even of HIV known to possess reduced replicative fitness. With its rapid turnaround of 8 days and its high sensitivity, our rPhenotyping system may be a valuable diagnostic tool for detecting the early emergence of therapy-threatening HIV minorities or the persistence of residual resistant virus.

Keywords: allele-specific PCR, minor viral population, real-time PCR, replicative phenotyping, viral fitness

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Introduction

The ability of HIV to rapidly change its genome, particularly under drug pressure, is well established as a major cause of escape from antiretroviral therapy followed by clinical treatment failure [1–3]. In some cases, full resistance is achieved by single amino acid substitutions [4,5], whereas in others the virus has to undergo several genetic changes in a target gene in order to evade drug

pressure [6,7]. The former is exemplified by the methionine to valine change at position 184 of the reverse transcriptase gene, leading to high-level resistance (>500 fold), for example to the two drugs lamivudine (3TC) and emtricitabine [8–10].

Most *in vitro* studies find the dynamics of viral replication greatly affected by this mutation [11–13]. However, clinical and animal data are still somewhat conflicting on the impact on the course of infection *in vivo*, but it may be expected, in accord with the 'survival of the fittest' principle, that drug pressure will drive resistant variants, even when initially present only at small proportions, to outgrow a drug-susceptible wild type. A

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number of reports [14–18] have, mostly retrospectively, shown this for clinical situations, where individual mutations could be associated with loss of drug efficacy [such as nonnucleoside reverse transcriptase inhibitor (NNRTI) failure following a K103N change in reverse transcriptase]. Treatment failure for an inhibitor with the identification of low percentages of the mutant virus was taken as an indication for retrospectively searching for the respective mutation in plasma samples prior to overt clinical therapy failure. At present, such minority species cannot be detected early enough through conventional techniques such as population-sequencing analysis (detection limit 15–20%) [19,20]. A recent report was able to demonstrate direct detection of a resistant virus by real-time polymerase chain reaction (PCR) of the protease mutant D30N in clinical specimens that had remained undetectable by genotyping [21]. Highly sensitive detection, for example by allele-specific PCR, is therefore achievable in principle but the essential design of the discriminating primer requires precise knowledge of the mutation to be found. However, the large number of possible relevant mutations in the target genes of interest (the protease and reverse transcriptase genes, and soon integrase and envelope) renders the methodology out of reach for routine diagnostics. Nevertheless, it would be desirable for optimal clinical management of therapy resistance and for optimal treatment effectiveness to overcome these limitations, which are possibly reflected also in discrepancies between certain genotypic profiles (i.e. apparent absence of mutation) and accompanying clinical courses (i.e. early viral escape and eventually treatment failure).

As a refinement of the diagnostics services of InPheno, both genotyping and replicative phenotyping (rPhenotyping) have been provided since 2002. Plasma from HIV-infected patients, submitted for the determination of potential drug resistance, was routinely subjected to both methodologies in parallel. Resulting data pairs were submitted to a matched database, PhenoBase[®], to identify and investigate possible causes of discordance between sequence- and function-based determination. Interrogating analyses from 2005 linked discordant data sets with corresponding clinical cases. A possible link to a very early phase virological and eventually clinical failure was then to be prospectively confirmed with a subsequent determination in a later sample from the same patient. In this way, we examined the properties of rPhenotyping as a sensitive, direct methodology for the detection of viral minorities in clinical samples; where viral minorities were detected, we confirmed the result by profiling the same original samples using the most sensitive adaptations of genotyping methods, such as allele-specific PCR.

Methods

Sample preparation and amplification

Virus was pelleted by centrifugation of 1 mL of human ethylenediaminetetraacetic acid plasma at 50 000 *g* and 4 °C for 80 min. Pellets were re-dissolved in 600 µL of guanidinium isothiocyanate lysis buffer and RNA was extracted according to the Cobas Amplicor HIV-1 Monitor[™] v1.5 protocol (Roche Molecular Diagnostics, Indianapolis, IN, USA). Reverse transcription was performed using the ViroSeq HIV-1 Genotyping kit (Abbott Molecular, Des Plaines, IL, USA); protease and reverse transcriptase sequences were amplified with the Platinum PCR kit (Invitrogen, Paisley, UK) with a forward primer containing the restriction site *Apa*I and a reverse primer containing a restriction site such as the *Pin*AI site. The amplification product spans the p7-p1-p6 protease cleavage sites in the *gag* polyprotein, the entire protease coding region, and the reverse transcriptase gene up to codon 335.

Genotypic analysis by sequencing

Genotyping and alignment with the HXB2 reference were performed using the ViroSeq system (Abbott Molecular). Resistance-associated mutations were identified using version 4.2.6 of the STANFORD algorithm (<http://hivdb.stanford.edu/>).

Recombinant virus (RV) construction

A retroviral vector cassette designed to assess antiretroviral (ARV) drug susceptibility was constructed based on the laboratory isolate NL4-3, carrying a deletion between the *Apa*I (RE1) and *Pin*AI (RE2) sites. RVs were prepared from human plasma without clonal selection (pools) in order to capture and preserve the heterogeneity of the protease and reverse transcriptase sequences of a patient's virus population. Primers for amplifying the corresponding regions were designed to contain the same restriction sites. Amplicons were digested with RE1 and RE2, purified by agarose gel electrophoresis, and ligated to RE1- and RE2-digested vector DNA. Natural occurrence of internal RE1 and RE2 sites was infrequent (between 0.1 and 2%) in the Swiss collection (PhenoBase, 2006). Ligation reactions were used to transform competent *HB101*/λ (Promega GmbH, Mannheim, Germany). RV plasmid DNA was purified using the NucleoSpin[®] Plasmid kit (Macherey-Nagel AG, Oensingen, Switzerland).

Drug susceptibility assay

Human epitheloid cells (HeLa) were transfected with RV or a wild-type reference and dispensed into 96-well plates

containing serial dilutions of various protease and reverse transcriptase inhibitors. Cells were incubated for virus propagation. For amplification, progeny virus was then used to initiate an infection of the human lymphocyte line CEM-SS in 96-well plates in the presence of ARV drugs. Under enhancing conditions, virus from these cells readily transmitted to a third reporter cell containing a *lacZ* gene (encoding the bacterial beta-galactosidase) driven by the long terminal repeat (LTR) of HIV. A colorimetric assay readout (at 405 nm) was normalized as percentage viral inhibition using positive and negative control wells included in each 96-well plate and a curve was fitted using suitable software (XLFIT v 4.0.1; IDBS, Guildford, UK). The fold change in drug susceptibility was determined by comparing, for each sample, the 50% inhibitory concentration (IC₅₀) for the virus to the IC₅₀ for the drug-sensitive reference virus NL4-3 [22].

ARV drugs

The drugs used in the study, zidovudine (ZDV; GlaxoSmithKline, Brentford, UK), lamivudine (3TC; GlaxoSmithKline) and lopinavir (LPV; Abbott Laboratories, Abbott Park, IL, USA), were all processed directly from clinical preparations, and compounds were dissolved in DMSO as 10 mM stock solutions.

Site-directed mutagenesis

The mutation M184V was introduced into the reverse transcriptase gene of pNL4-3 using the QuikChange[®] Site-Directed Mutagenesis Kit (Stratagene, Edinburgh, UK) according to the manufacturer's instructions.

Allele-specific PCR

Resistant HIV minorities with mutations at position 184 of the reverse transcriptase gene were determined qualitatively by PCR with mutated primer specific for the region of interest. Plasmid DNA of the patient-derived RVs was amplified simultaneously in two parallel reactions using a common upstream primer (5'-AGCAGGATCCGATAGA CAAGGA-3') and one of two downstream primers, for which the 3' ends matched either the wild-type or the mutant sequence. For higher stringency, according to the literature, they contained three additional mutations (underlined: RT184M3, 5'-CAGATCGTACATATGAATCATC CAT-3'; RT184V3, 5'-CAGATCGTACATATGAATCATCCAC-3') [23]. Primers were used at concentrations of 10 µM. Amplification was performed using the Platinum PCR kit (Invitrogen) with 27 cycles at 95 °C for 15 s, 62 °C for 15 s, and 72 °C for 90 s after initial denaturation for 2 min.

Specific amplification of the 911-bp product was verified by subsequent gel electrophoresis. For each specific PCR reaction, wild-type pNL4-3 and point-mutated 184V were used as the respective negative and positive controls for specificity of amplification.

Quantitative mutation-specific PCR

The amplification was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) with 50 cycles at 95 °C for 10 s, 57 °C for 15 s, and 72 °C for 20 s after an initial 2-min step at 50 °C and denaturation for 10 min at 95 °C. Five microlitres of the cDNA products, generated with the ViroSeq HIV-1 kit (Abbott Diagnostics, Baar, Switzerland), was added to 5 µL of the master mix containing 2 × QuantiTect SYBR Green PCR Master Mix (Qiagen), RNase-free water, and 7.5 pmol of each primer designed to quantify the total amount of virus as well as the percentage of mutant 184V. The forward primer was designed to bind to a conserved region of polymerase (*pol*) (primer D3005: 5'-ATGGAAAGGATCACCAGCAA-3') and reverse primers were designed to equally quantify wild-type and mutant templates (primer R3123: 5'-CAGATCG TACATATGAATCATCCA-3', ending 5' to the mutant position at codon 184) and to match specifically the mutant sequence at codon 184 in the reverse transcriptase gene (primer RT184V3). Fluorescent quantification of amplicons was mediated using the Rotor-Gene[™] 3000 system (Corbett Life Science, Sydney, Australia). The cycle number at which the fluorescence passed a fixed threshold line was defined as the threshold cycle number (C_t). The number of cycles between the C_t values observed with authentic and inauthentic priming (ΔC_t) reflects the discriminatory ability of a given primer system.

Precise dilutions of cDNA were prepared in duplicate in five independent repeat experiments. All duplicates analysed yielded identical curves. In order to validate the method prior to its use on patient-derived samples, these experiments were performed on pre-prepared mixes of cDNA preparations of the 184M and 184V virus variants. As previously described, the relative amount of mutated sequences was calculated as the ratio between the quantity of mutated sequences in the sample and the quantity of total sequences in the sample [24].

Results

Parallel diagnostics for HIV resistance using genotypic and phenotypic methods

All HIV resistance tests performed at the Basel centre, Institute for Medical Microbiology/InPheno AG, on behalf

of the Swiss HIV Cohort Study were independently assessed using the same clinical specimen and two different systems: (i) standard population-based genotyping, which models viral resistance via algorithmic analysis of one apparent consensus sequence, and (ii) the rPhenotyping system PhenoTecT (InPheno AG, Basel, Switzerland), which functionally assesses viral amplification and replication in the presence of each respective drug individually. The sets then entered a database with currently >30 000 matched resistance profiles. The statistics for concordances/discordances between genotypic and phenotypic evaluations for the twenty available ARV drugs have previously been reported [25].

For the present study, we focused on one subset of virus samples from therapy-experienced patients for the year 2005. Profiles with genotype/phenotype discordance for the prediction of resistance to 3TC were analysed. The most common cause of resistance to this drug is the presence of a single methionine to valine substitution at position 184 of the reverse transcriptase gene. We hypothesized that the limited detection of underrepresented virus by conventional sequencing should, for populations below 15–20%, yield a different result from the replicative format of PhenoTecT. Using *in vitro* mixtures of several distinct virus mutants commonly found, the latter system has been demonstrated to reveal such virus populations within 4 days of *in vitro* exposure of the replicating virus to the respective drugs (examined for mutation-based resistances to protease inhibitors and several reverse transcriptase inhibitors; data not shown).

Limit of sensitivity of the replicative format of PhenoTecT

Analogous to reports on NNRTI mutants 103N and 181C, we determined the respective limit of detection for the rPhenotyping system for the fitness-affecting mutant M184V. A wild-type reference plasmid was point-mutagenized, and defined mixes of wild-type and mutated plasmids were evaluated for their sensitivity to the study drug 3TC. Susceptibility to the two unrelated drugs ZDV and LPV as controls remained unaffected by mutation M184V. Mixes were examined in half-logarithmic steps from 0% (pure pNL4-3 reference) to 0.1% ... to 100% (solely the M184V mutant).

Figure 1a shows that, whereas for the nonresponsive control drugs ZDV and LPV increasing amounts of mutant M184V had no impact on inhibition (graphs on the right), the inhibition curve for 3TC was significantly altered as soon as $\geq 1\%$ of the viral population was contributed by the resistant mutant. Figure 1b summarizes the respective resistance factors (RFs) as the ratio between the IC_{50} of the

sample and the wild-type IC_{50} . The link of the RF to an established clinically diagnosed resistance establishes a threshold (cut-off) value for this RF, above which a virus is statistically nonsusceptible. This cut-off was 3.0 for 3TC (Fig. 1b, dashed line), indicating that in the PhenoTecT system 1% of mutant M184V in the mixed virus population yielded a relevant decrease in susceptibility to 3TC (intermediate). Full resistance was demonstrable for 184V samples when $\geq 30\%$ of the viral population was contributed by the resistant variant. The RFs for the control drugs ZDV and LPV remained unchanged across the entire range of mixtures. Of concern was a potential bias arising from possible effects of an altered replication capacity of the virus; however, this could be excluded as in the assay format of PhenoTecT a reduced replication (lower viral fitness) results only in a lower absolute optical density and not in a shift in the IC_{50} as the parameter of inhibition. (In PhenoTecT the absolute readout for 184V was 80% of the wild-type value.)

Limit of detection of viral minority species mutated at reverse transcriptase position 184

To define the lower limit of detection for mutant virus within a mix, an allele-specific PCR method was employed using two oligonucleotides designed to carry at their respective 3' termini a nucleotide specific for one or the other variant according to a method described by Korn *et al.* [23], as follows. Using three additional noncritical mutations in their sequence, the two primer sets were optimized for selective amplification of one of the two fragments containing 184M or 184V in the above-defined proviral mixes. The amplification products depicted in Fig. 2a demonstrate such primer-specific amplification and detection down to 0.3% in the viral mixture.

Subsequently, patients' samples identified by discordant genotype and rPhenotype for 3TC were tested under the same conditions. The underlying hypothesis was that they might contain minor variants behind a dominant wild-type sequence. Figure 2b clearly demonstrates, for the original viral populations isolated from each of five discordant patient samples, that the expected mutant fragment could be selectively amplified with the specific primer. In contrast, initial genotyping had provided no evidence of the alteration. This strongly suggests that, in the authentic clinical plasma samples, the HIV-1 mutant 184V had already been established. Further support for the relevance of this amplification was provided by applying the same PCR amplification protocol to three randomly selected specimens from patients diagnosed as 3TC-susceptible by genotype and PhenoTecT. In all cases the protocol failed to produce a mutant-specific band on these RNAs (data not shown).

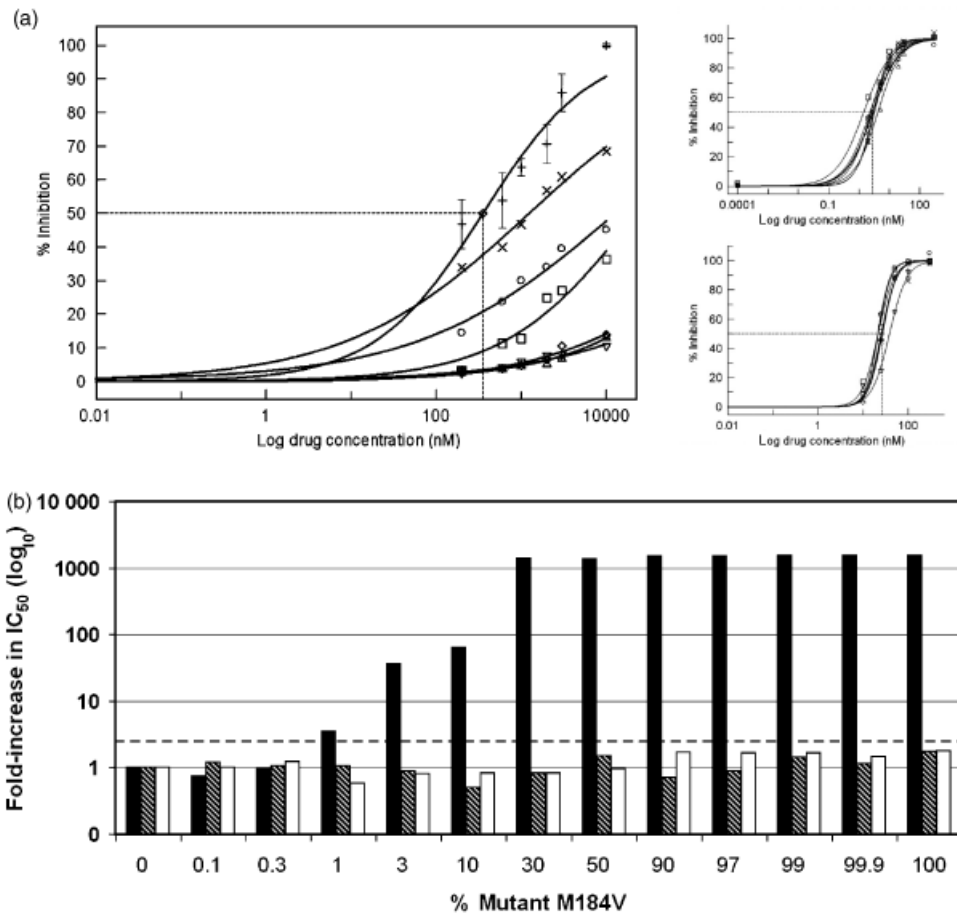


Fig. 1 Drug susceptibility of mixed populations of replicating HIV-1. (a) Wild-type (pNL4-3) and mutant (M184V) proviral plasmids were used to obtain defined mixed sequences ranging from 100% wild type to 100% mutant M184V, and were functionally analysed using replicative phenotyping (rPhenotyping). Inhibition of viral activity is expressed as a function of drug concentration. The main graph shows inhibition curves of viral replication for wild type alone (+), and 1% (x), 3% (o), 10% (□), 30% (◇), 50% (△) and 100% of 184V (▽) under lamivudine. The 50% inhibitory concentration (IC₅₀) for wild type alone is indicated as a dashed line. Inhibition curves for the control inhibitors zidovudine (top) and lopinavir (bottom) are shown on the right. (b) Assessment of the sensitivity of rPhenotyping (PhenoTect). Resistance factors for wild type (0%) and mixes with M184V (x% as indicated) are plotted for lamivudine (black bars) and for two unaffected controls, the reverse transcriptase inhibitor zidovudine (hatched bars) and the protease inhibitor lopinavir (white bars). The calculated cut-off value for resistance to lamivudine is indicated as a dotted line.

Quantitative detection of minority populations in clinical samples expressing mutated sequences

For a quantitative estimation of the proportion of mutant M184V in patients' blood, the allele-specific real-time PCR protocol was used to distinguish between the wild type and the 184V mutant and a subsequent SYBR Green assay designed for overall quantification. A set of primers was utilized, for which the ΔC_t between specific and nonspecific annealing was maximal. Three additional noncritical internal mismatches in the primer significantly improved discrimination and increased the number of threshold cycles from six to 13.

The respective threshold cycle for the first detection of a PCR amplicon for the 184V mutant plotted as a function of the relative proportion of mutant 184V in a defined mix (Fig. 3) shows that the ΔC_t between 0.1 and 0% mutant was still more than two cycles and therefore discernible. Each calculation was adjusted for the absolute quantity of a viral population present in the reaction by means of a second, parallel PCR using primers and target isogenic for both proviruses. These results demonstrate reliable and quantitative identification even of small subpopulations in a mixed HIV sample. These data were conversely verified further using a primer pair specific for detecting and quantifying small amounts of wild type in a mixed sample

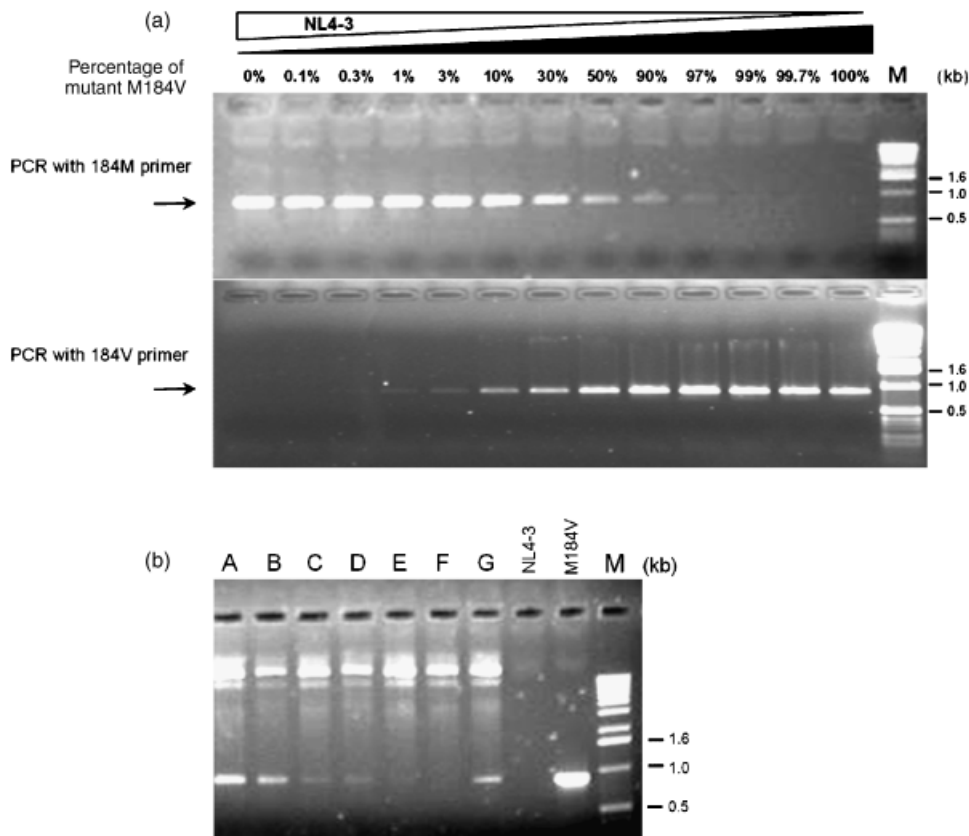


Fig. 2 Discrimination of HIV populations by mutation-specific polymerase chain reaction (PCR). (a) Aliquots of precise *in vitro* mixes of wild type and M184V (percentages as in Fig. 1) were amplified using primer sets for wild type (184M, top gel) or mutant (184V, bottom gel) and analysed by gel electrophoresis. Specific bands (arrow head) correspond to the expected product, visible for $\geq 0.3\%$ with both primer pairs. Lack of signal ($\leq 0.1\%$) defines the limit of detection. Absence of amplification of wild type with mutant primer or mutant with wild type primer served as validation. M, size standard (1-kb ladder). (b) The PCR products from seven geno-/pheno-discordant patient-derived viruses (lanes A–G) were electrophoretically analysed after amplification with the 184V-mutant primer set. Control lanes: WT, NL4-3; mutant, M184V; M, size standard (1-kb ladder).

with mutant (not shown). Using multiple repeats of duplicate determinations, as described by Hance *et al.* [24], a lower limit of sensitivity was determined using pure wild type (0% mutant) with the mutation-specific primer pair: wild type and mutant could no longer be discriminated in mixes with $< 0.02 \pm 0.03\%$ mutant (mean ± 2 standard deviations; $n = 9$). These values correlate well with the cut-off value of 0.05% previously reported for protease [24], above which a sample was considered positive for a mutant.

The established settings of this *in vitro* validation were now applied to the discordant patients' samples (Fig. 3). The percentages of mutated sequences in the circulating pool of viruses in the studied patients were calculated by duplicate determinations in four independent experiments. Analysis of plasma samples from patients A, C, D and G verified the presence of mutant 184V in the original specimen (Table 1).

A low abundance of mutant in a patient's blood may indicate either an early phase of emerging resistance or persisting residual resistant virus after cessation of drug treatment. For the patient samples in this study, the relative abundance of mutant behind a wild-type background was 0.25, 0.53, 0.85 and 1.5% (average of four experiments in duplicate) for patients A, D, C and G, respectively. For a fifth sample, patient B, no 184V mutant could be detected: C_t curves for quantification (nonmutant primers) were shifted to the far right and were shallower in shape, and the mutant-specific amplification curve did not separate from the curve for $< 0.01\%$ mutant. As a result, the data could not be interpreted for resistance determination.

Discussion

Certain discordances between diagnostic methodologies are not infrequent, the testing of HIV resistances to specific

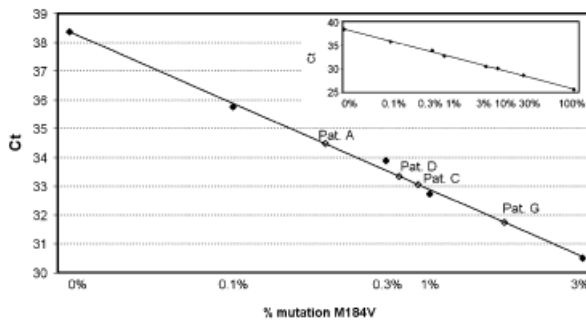


Fig. 3 Quantification of virus in clinical samples. Results from real-time polymerase chain reaction (PCR) amplification plotted as relative amount of mutant (%mutation) as a function of the threshold cycle (C_t) for detection of the M184V mutant. As standards (black diamonds), defined precise percentages of 184V were used for titration. Amounts of mutant virus present in patient-derived samples A, C, D and G (open diamonds) were determined after adjustment for viral load. The main graph depicts the lower end of the titration curve; the titration curve across the entire range is shown in the inset graph. Pat, patient.

Table 1 Detection by real-time polymerase chain reaction (PCR) of mutant sequences in deliberate HIV mixes and in patient samples

Samples	Threshold cycle (C_t)		% M184V
	Template-specific primer	Mutant-specific primer	
100%	15.85	24.85	100.00
30%	15.68	28.12	24.88
10%	14.96	28.95	9.97
3%	14.21	30.02	3.02
1%	15.44	32.26	0.91
0.3%	14.74	33.38	0.28
0.1%	15.79	35.28	0.10
0%	16.41	37.82	0.02
G	17.12	32.63	1.51
C	19.97	35.34	0.61
D	17.84	34.45	0.50
A	15.72	34.16	0.23

From the titration curve depicted in Fig. 3, key values were extracted as expected vs. measured percentages of mutant virus in mixed populations (top part above the dotted line), or to quantify the relative amounts of mutant virus in clinical samples (G, C, D and A).

therapeutics being a case in point. Even when the same initial methodology is used, for example viral sequences obtained in a clinical sample, opposite diagnostic recommendations may be produced, depending on the algorithm used for interpretation [26–28]. The replicative HIV-phenotyping format used in this study demonstrates low-level detection of relevant, drug-resistant virus variants directly in clinical specimens. The example of the 3TC-resistant variant 184V was used, because an independent technical proof was available through the application of a highly sensitive allele-specific real-time PCR protocol. This highly sensitive rPhenotyping may be of general utility for diagnostic use as the methodology does not require an

Table 2 Clinical parameters of the seven patients in this study

Patient	Viral load (copies/mL)	CD4 (cells/ μ L)	Lamivudine treatment
A	100 500	42	Current
B	16 900	800	No information
C	69 854	ND*	Current
D	690 000	61	Current
E	> 100 000	195	Never
F	2461	ND	Current
G	30 585	ND	In past*

*Previous therapy regimen, not currently. ND, not determined.

algorithmic interpretation and is thereby principally applicable to any of the viral targets or drug classes. For such special cases, the substantial gain in sensitivity might merit in principle, a more complex format of phenotyping.

Linking of the findings of this study to treatment history, as shown in Table 2, further confirmed that a resistance to 3TC had arisen only recently, or was currently in *statu nascendi*, or was conferred by nonprominent virus representatives, which were for example (still) restricted to a putative small compartment (patients A, C, D and F). In contrast, previous therapy episodes with 3TC, for example for patient G, suggest that resistant viruses had been selected during this time and were still detectable months after discontinuation.

As an indication of therapeutic failure, patients presented with detectable plasma virus loads between 2400 (patient F) and > 100 000 copies/mL (patient A). The persisting mutant viruses were therefore likely to in part contribute to the unsuccessful viral suppression. In contrast, for the sample from patient E, no 184V virus was detected, and, according to the treatment record, this patient had never received 3TC. This case might illustrate the limits of a PCR-based verification, as such a protocol is only suitable for perfectly matching sequences. M184V-based resistance to 3TC is mainly caused by an ATG to GTG transition (for >90% of all matching geno-R/pheno-R patients in the database), but a small proportion of patient-derived resistant viruses carry a GTA codon (Val). This double mutated codon (ATG to GTA) will be missed by the chosen strategy. Similarly, mismatches in forward or reverse amplification primers will prevent PCR amplification. For the sample from patient B, amplification by allele-specific PCR was successful, but real-time PCR repeatedly failed. Subtype analysis revealed that the virus was of CRF02_AG subtype. As the consensus of this HIV recombinant shows three mismatches in the forward quantification primer, the subtype would explain the observed failure to quantify (this was not genotypically verified for the virus minority in sample B).

Minor virus variants arising in a patient's circulation could provide an explanation for the onset of progression

towards therapeutic failure, as their early presence may allow viral optimization, for example optimization of the replicative capacity of the virus [29], which could occur unnoticed in the background. It seems logical that, in clinical settings, such virus populations might be particularly prevalent in noncompliant patients, during drug holidays, under structured treatment interruption strategies [24,30,31], or as early events in the context of transmitted mutant HIV variants with suboptimal replicative fitness [32,33]. A significant fitness cost would lead to a more or less stable balance with wild-type viruses and in the absence of drug pressure prevent them from easily becoming the predominant virus form in a patient.

It will be interesting to apply the technology (and hypothesis) to studies on transient therapies, for example the short-term administration of nevirapine to pregnant women for prevention of mother-to-child transmission, for further investigation of the presence and persistence of viral minorities with replication properties inferior to those of wild type [34–37].

The retrovirus HIV has an intrinsic ability to rapidly mutate and thereby change in a more or less random way, which could reflect an initial broadening of the genetic diversity towards rapid viral response to and escape from drug pressure. The genetic complexity of viruses tends to increase over the course of long treatment periods and with an increasing number of regimen changes (Swiss HIV Cohort Study, manuscript in preparation).

Genotypic algorithms, as self-optimizing systems, improve their performance over time. Nevertheless, a few intrinsic shortcomings remain that necessitate the use of alternative dissecting methods. For new drugs with little resistance information available, they principally fail to assign new mutations or novel resistance patterns. Moreover, they have a limited ability to detect and monitor viral speciation during extended treatment periods, as these favour the evolution of mixed virus populations. The clinical cases presented in this report suggest that a functional assessment, for example by rPhenotyping, possesses certain properties superior to those of population-based genotyping and may compensate in these cases for the higher degree of technical sophistication and cost of phenotypic tests. Nevertheless, further studies are needed to examine how this advantage will translate into clinical utility towards improved disease management of the therapy-experienced patient.

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