HUMAN IMMUNODEFICIENCY VIRUS 1 STRAINS RESISTANT TO NUCLEOSIDE INHIBITORS OF REVERSE TRANSCRIPTASE IN ISOLATES FROM THE CZECH REPUBLIC AS MONITORED BY LINE PROBE ASSAY AND NUCLEOTIDE SEQUENCING

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Summary. – The genetic resistance to nucleoside inhibitors of the reverse transcriptase (RT) of human immunodeficiency virus 1 (HIV-1) isolates in the Czech Republic was examined by a line probe assay (LiPA) and nucleotide sequencing. The results of LiPA analysis of 294 blood specimens obtained from 156 patients revealed a high incidence of mutations in the RT gene related to resistance to various drugs (67.3%) in various combinations. Mutations in RT gene (M41L, K70R and T215Y/F) conferring the resistance to zidovudine (ZDV) were most frequent (62.6%), that (M184V) responsible for the resistance to lamivudine (3TC) was less frequent (33.7%), while those linked to the resistance to dideoxyinosine (ddI) and dideoxyinosine together with dideoxycytidine (ddI/ddC) were rather rare (6.5% and 5.1%, respectively). LiPA gave a high rate of uninterpretable results due to codon hybridization failure, especially in HIV-1 isolates of non-B subtype. Thirty-two specimens were analyzed also by direct sequencing of a part of RT gene. The results obtained by LiPA and the sequencing were highly concordant for codons successfully analyzed by both methods, but the sequencing provided information also about the codons that could not be analyzed by LiPA. A high prevalence of resistant strains in the Czech Republic and their heterogeneity justifies a regular HIV-1 resistance testing. LiPA turned out as a fast, powerful and most reliable tool for such a purpose. However, due to an increasing diversity of HIV-1 strains circulating in the Czech Republic, LiPA cannot replace the nucleotide sequence analysis.

Key words: human immunodeficiency virus 1; reverse transcriptase; antiviral drugs; resistant mutants; line probe assay; nucleotide sequencing

Introduction

Appearance of HIV-1 mutants resistant to antiretroviral drugs during therapy is a frequent reason for a treatment failure. A number of mutations in the *pol* gene have been related to the resistance to particular drugs (reviewed by Hammond *et al.*, 1999). The HIV-1 drug resistance testing and the genotyping of HIV-1 strains have been shown to result in a more complete virological evidence and in a therapeutic benefit, respectively (Durant *et al.*, 1999; Baxter *et al.*, 2000). However, a genetic diversity of HIV-1 strains, an increasing number of antiviral drugs used in various combinations, and a resistance to many drugs makes selection of a convenient method for resistance testing and interpretation of results very difficult.

It has been shown that, despite a relatively low number of HIV-1-positive individuals, the HIV-1 population in the

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Abbreviations: ddC = dideoxycytidine; ddI = dideoxyinosine; HIV-1 = human immunodeficiency virus 1; LiPA = line probe assay; RT = reverse transcriptase; RT-PCR = reverse transcription-polymerase chain reaction; 3TC = lamivudine; ZDV = zidovudine

Czech Republic became very diverse. HIV-1 subtype B, a major subtype circulating in Europe and the USA, still predominates, but the presence of other subtypes, namely A, C, CRF_01, AE and F has been documented and tends to increase (Mayer *et al.*, 1998; Quinone-Mateu *et al.*, 1999; Reiniš *et al.*, 2001). The resistance to antiretroviral treatment has been studied mainly on HIV-1 subtype B strains, also because the majority of laboratory strains cluster with the subtype B, while the genotype to phenotype relationship in the subtype non-B isolates is much less understood. Also, little is known about the sensitivity and specificity of genotyping diagnostic assays in the analysis of subtype non-B strains. The majority of infected individuals in the Czech Republic have received antiviral therapy under diverse therapeutic schemes. Taken together, all these facts make the resistance testing and the diagnostic method optimizing in the Czech Republic a complex problem of particular importance.

Recently, the laboratory guidelines for HIV-1 drug resistance testing in Europe have been specified (The Euro Guidelines Group for HIV Resistance, 2001). However, there is at present no recommendation of a specific assay. Although a number of genotyping methods have been recently developed the nucleotide sequencing of RT and protease genes remains the major one. One of the methods for the genetic screening of HIV-1 isolates resistant to nucleoside inhibitors of RT is LiPA. (Stuyver *et al.*, 1997). This method is based on RT and amplification of viral genetic material and reverse hybridization of the amplified products with specific probes. LiPA could detect mutations in RT codons 41, 69, 70, 184 and 215 resulting in a change in the encoded amino acid, and these mutations were found to be linked to the resistance to ZDV, 3TC, ddC, and ddI, respectively.

The aim of this study was (i) the examination of prevalence of HIV-1 strains isolated in the Czech Republic and resistant to nucleoside inhibitors of RT, (ii) the comparison of efficacy of LiPA in the analysis of HIV-1 strains of B and non-B subtype, and (iii) the comparison of the results of LiPA with those of nucleotide sequencing. Our experience showing that the PCR products from LiPA can be used directly in nucleotide sequencing may be regarded as an important methodical amendment.

Materials and Methods

Blood specimens obtained from HIV-1-positive patients in 1997–2000 were coded for this study to keep the patient names unrevealed. The patients had a diverse clinical status, mode of transmission, age and sex. Most of them were undergoing various therapeutic schemes and all of them were participating in a diagnostic program at the National Institute of Public Health, Prague, the Czech Republic. Viral load was determined in all specimens by quantitative RT-PCR using a commercial kit (HIV Monitor, Roche Diagnostic Systems, Rotkreuz, Switzerland). Only the specimens with viral load over 4,000 genome copies/ml blood were enrolled in the study. The genetic subtype of 165 isolates obtained from 71 patients was known as determined earlier by a phylogenetic analysis of *env* and *gag* fragments (Quinone-Mateu *et al.*, 1999; Reiniš *et al.*, 2001; Reiniš, unpublished results) and by the analysis of nucleotide sequences of RT gene made in this study. Most isolates, 132, were of B subtype, while 33 were of non-B subtype.

LiPA. Total RNA was extracted from plasma using the QIAamp Viral RNA Kit (Qiagen, Germany). RT-PCR was performed as described previously (Stuyver *et al.*, 1997). Reverse hybridization and detection were carried out using the commercial LiPA HIV-1 RT Kit (Innogenetics, Belgium) according to the manufacturer's protocol. Briefly, biotinylated PCR fragments were reversibly hybridized with short immobilized oligonucleotide probes and the resulting hybrids were detected via biotin-streptavidin coupling by use of an enzymatic color system.

Nucleotide sequence analysis. The RT-PCR products from LiPA were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Germany). Both strands of the amplified fragments were directly sequenced. Alternatively, products of the first step of the nested PCR were amplified by a second PCR using RT4 and RT1 primers (Stuyver *et al.*, 1997), and these products were purified as described above. Then a cycle sequencing with AmpliTaq FS Polymerase, fluorescent dye-labeled dideoxy terminators (BigDye™; PE Biosystems, Great Britain) and RT4 and RT1 primers was carried out. Electrophoretic analysis of the sequence fragments and data collection were performed in an automated ABI 310 Capillary Genetic Analyzer (PE Biosystems, Great Britain). DNA translation, determination of resistance-related codons, and determination of the B or non-B subtype based on fragments covering RT codons 40–220 was performed using the HIV-SEQ software deposited in the Stanford HIV RT and Protease Sequence database (accessible at http://hivdb.stanford.edu/hiv/; Schafer *et al.*, 1999). Ambiguities occurred in the sequences only when no base was clearly dominating. The sequences were deposited in the GenBank database under Acc. Nos. AF454512-AF454543.

Terminology. Mutation of an amino acid constituting a codon reflected in change of the encoded amino acid is described in three different ways. (i) A mutation without specifying the codon and the encoded amino acid before and after mutation. (ii) A mutation with specifying the codon and the encoded amino acid before and after mutation. For example M184V means that codon 184 encoded amino acid M in the wild type virus but amino acid V in the mutant. (iii) A mutation which can be deduced from two results describing the situation before and after mutation. For example M184 for the wild type virus means that codon 184 encoded amino acid M, while V184 for the mutant means that codon 184 encoded amino acid M.

Phylogenetic analysis. Genetic subtypes of all sequenced isolates were further confirmed by phylogenetic analysis. The sequences were aligned with the reference sequences obtained from the Los Alamos HIV Database (available at http://hiv-web.lanl.gov) using the Clustal W method. Pairwise genetic distances were calculated by the Kimura two-parameter method, and tree topologies were inferred by a neighbor-joining method using the Treecon Phylogenetic Software package (Van de Peer and De Wachter, 1994).

Results

A panel of 294 specimens from 156 HIV-1-positive patients was successfully analyzed by LiPA (Table 1). Mutations in codons of RT gene linked to any RT inhibitor tested were found in 67.3% of specimens. A higher proportion of resistant strains was found in specimens obtained from patients whose seropositivity was identified before 1998 (78.4%) than after 1998 (25.8%).

The resistance to ZDV linked to the mutations M41L, T215F, and K70R was most abundant (62.6%). It was detected alone or in combination with resistance to other inhibitors. The resistance to 3TC linked to mutation M184V was less abundant (33.7%), while the resistance to ddI and ddI/ddC was quite rare (6.5% and 5.1%, respectively). The resistance to 3TC, ddC and ddI was detected mainly in combination with the resistance to ZDV. Wild type non-resistant strains were found in 32.7% of all specimens.

LiPA gave uninterpretable results for 11.6% (codon 184) to 24.1 % (codon 41) of the analyzed codons (Table 2). Further, the proportion of uninterpretable results was determined in specimens in which the genetic subtype was known. In this case, all specimens, for which RT-PCR was successful, were evaluated. The percentage of uninterpretable results was dramatically higher for non-B subtype isolates in comparison with subtype B isolates for all the codons tested, except for codons 214/215.

Sequence analysis of RT gene was performed on 32 selected specimens. Firstly, the genetic subtype was determined by phylogenetic analysis (Fig. 1). Secondly, the mutations linked to the resistance to the RT inhibitors tested were studied and the results were compared to those obtained by LiPA (Tables 3 and 4). The nucleotide sequences at the critical positions where the hybridization probes were bound to PCR product are presented in Fig. 2. In general, the sequencing confirmed interpretable LiPA results with both wild type and resistant strains. Discordant results were obtained mainly as a result of the ability of LiPA to detect the mixed virus populations (minor discrepancies).

Major discrepancies were found only in a few codons. In two cases (specimens 19183XVIII and 60196IX), the mutation determined by sequencing was L74I (the mutation in the codon from TTA to ATA). There was no specific probe in LiPA detecting such a mutation. However, in the specimen 19183XVIII, LiPA detected a wild type L74. By chance, the results of both the sequencing and LiPA analysis of the same specimen obtained two years ago were available, confirming the presence of the wild-type L74 by both methods.

In the second specimen (60196IX), L74I was obtained by sequencing, while L74V was obtained by LiPA.

The second codon for which a major discrepancy was observed was 215. In the specimens 18568XV and 53486XII the sequencing revealed the mutations T215C and T215F (transitions from ACC to TTC), while LiPA gave the mutation T215Y. We looked for a mutation in the vicinity of this codon and found the mutation from GGA to GGG in codon 213 (Fig. 2). This type of mutation was observed also

Table 1. Results of LiPA analysis

		All patients	Seropositive patients before end 1997	Seropositive patients in 1998–2000
No. of successfully analyzed specimens		294 (100%)	232 (100%)	62 (100%)
No. of analyzed individuals		156	108	48
Resistance not detected		32.7%	21.6%	74.2%
Resistance detected		67.3%	78.4%	25.8%
ZDV resistance	Alone	29,3%	33.6%	12.9%
In combination with resistance to:	3TC	21.8%	26.7%	3.2%
	3TC, ddC	4.1%	5.2%	0%
	3TC, ddI/ddC	3.1%	3.9%	0%
	ddC	2.4%	2.6%	1.6%
	ddI/ddC	2.0%	2.6%	0%
	Total	62.6%	74.6%	17.7%
3TC resistance	Alone	4.8%	3.9%	8%
	Total	33.7%	39.7%	11.3%
ddC resistance	Total	6.5%	7.8%	1.6%
ddI/ddC resistance	Total	5.1%	7.5%	0%

For the abbreviations of inhibitors see the list of abbreviations on the front page.
Number of HIV-1 strains with mutations conferring primary or secondary resistance to ZDV (mutations M41L, T215F, K70R), 3TC (M184V), ddI and ddC (L74V) and ddC (T69R).

Table 2. Specimens with uninterpretable codons

	All specimens analyzed n=294[a]	Subtype B isolates n=132[b]	Subtype non-B isolates n=33[b]
Codon	Percentage		
41	24.1	14.4	81.2
69/70	15.6	12.1	54.6
74	15.6	18.2	45.5
184	11.6	5.3	57.6
214/215	17	15.2	15.1

Subtype B: 132 specimens from 55 patients, Subtype non-B: 33 specimens from 16 patients (subtype A or CRF_01AE: 11 specimens from 7 patients; subtype C: 17 specimens from 5 patients; subtype F: 2 specimens from 2 patients; subtype G: 1 specimen from 1 patient). Two specimens originated from 1 patient infected with a recombinant strain (subtype D in RT).
[a]Specimens evaluated in this study had not more than 3 uninterpretable codons.
[b]All specimens in which the amplification control worked were evaluated in this study.

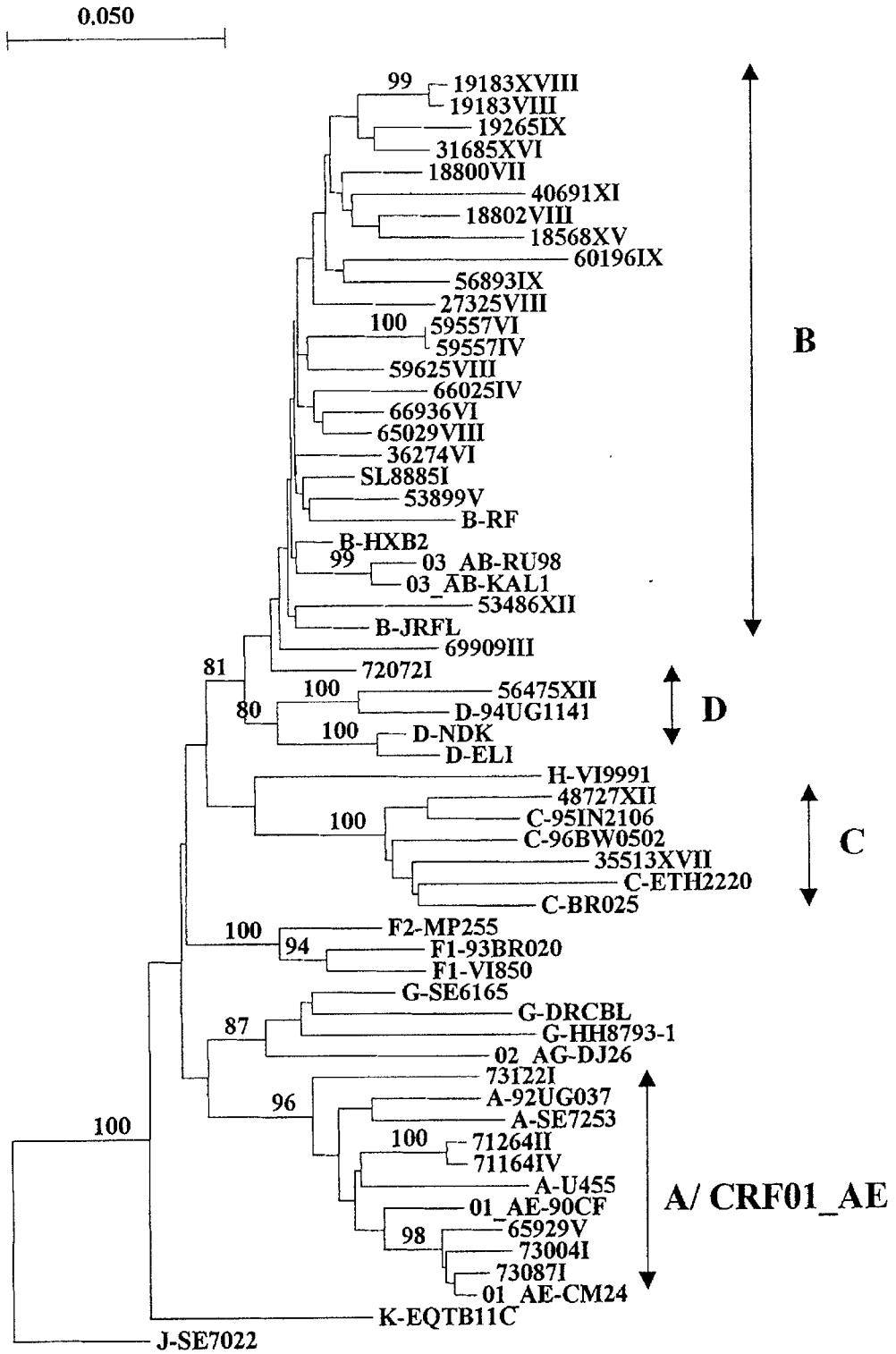

Fig. 1

Phylogenetic tree based on *pol* fragment nucleotide sequences including RT region corresponding to codons 40–210

The tree was constructed by the neighbor-joining method. The sequences of the Czech isolates (presented under their code numbers) and the reference sequences of the M group of HIV-1 strains obtained from the Los Alamos HIV-1 database were used for analysis. The tree was rooted with the SE7022 (subtype J) sequence. The reliability of the topology was determined by bootstrap analysis (500 resamplings). The bootstrap values over 80% are displayed.

Table 3. Comparison of results of LiPA and sequence analysis of subtype B isolates

Specimen ID	Sequencing results	LiPA results	Discrepancies
18568XV	L41, D44, N67, N103, I108, I118, V184, W210, **C215**, R219	41n, T69/K70, L74,V74, M184,V184, F214/**Y215**	74, 184, 215
18800VII	L41, I118, V184, W210, Y215	L41, T69/K70, L74, V184, F214/Y215	
18802VIII	L41, N67, V184, W210, Y215	41n, T69/K70, L74, V184, F214/Y215	
19183VIII	N67, R70, F215, Q219	M41, R70, L74, M184, 214/215n	
19183XVIII	L41, N67, AT69, R70, **I74**, N103, CY181, F215, Q219	M41 L41, R70, R69/R70, 69/70N69/R70, **L74**, M184,V184, 214/215n	41, 69, 74,184
19265IX	L41, N67, R70, M75, W210, Y215, Q219	L41, R70, 74n, M184, F214/Y215	
27325VIII	L41, I118, Y215	L41, T69/K70, 74n, M184, F214/Y215	
31685XVI	L41, N67, R70, N103, C181, AG190, F215, Q219	L41, R70, L74, M184, F214/F215	
36274VI	Wild type	M41, T69/K70, L74, M184, F214/T215	
40691XI	L41,D44,D69,V74,I100,N103, I118, W210, Y215, R219	L41, D69, V74, M184, F214/Y215	
53486XII	L41, V184, **F215**	L41, 69/70n, L74, V184, F214/Y215	215
53899V	Wild type	M41, 69/70n, 74n, M184, 214/215n	
56893IX	L41, N67, R70, V184, Y215, E219	L41, R70, L74,V184, F214/**Y215**	70
59557V	Wild type	M41, T69/K70, L74, M184, F214/T215	
59557VI	Wild type	M41, T69/K70, L74, M184, F214/T215	
59625VIII	L41, DE44, I118, W210	L41, T69/K70, 74n, 184n, F214/Y215	
60196IX	L41, G67, N69, R70, **I74**, I118, V184, F215	L41, 69/70n, **V74**, V184, 214/215n	74
65029VIII	Wild type	M41, T69/K70, L74, M184, F214/T215	
66025IV	Wild type	M41, 69/70n, L74, M184, F214/T215,L214/T215	214
66936VI	Wild type	M41, T69/K70, L74, M184, F214/T215	
69909III	E179	M41, T69/K70, L74, M184, F214/T215	
72072I	Wild type	M41, T69/K70, L74, M184, F214/T215	
SL8885I	Wild type	M41, T69/K70, L74, M184, F214/T215	

Mutations within RT codons 40–220 are presented. A result represents characteristic of a codon: the encoded amino acid (a letter) and the codon position (a number). Major discrepancies are in bold, *n* means a non-interpretable LiPA result. A sample ID consists of identification of a patient (an Arabic number) and a number of plasma specimens obtained (a Roman number).

in specimens 19183VIII and 19183XVIII for which the sequencing showed mutation T215F, while LiPA failed. The mutation T215F was detected also in the specimen 31685XVI, but this time by both methods and codon 213 was a consensus GGA.

For all 9 isolates that were determined as non-B subtype strains and analyzed by sequencing LiPA gave uninterpretable results for at least two codons. The lower number of mutations, related to the resistance in the non-B subtype isolates, could be explained, in general, by more recent infections.

Discussion

The data obtained revealed that a majority (67.3%) of the HIV-1 isolates tested harbored at least one mutation concerning the codons determining the resistance to RT inhibitors. Although this number is probably lower in the entire HIV-1 population in the Czech Republic, as (i) only the specimens with medium or high viral load were analyzed and (ii) the specimens from patients for whom the therapy was highly efficient were excluded, a significant portion of HIV-1 strains circulating in the Czech Republic are resistant to RT inhibitors. This is in agreement with the fact that a majority of infected individuals have been intensively treated with antiretroviral drugs. Resistant HIV-1 strains were found also in the specimens originating from individuals whose seropositivity has been identified within last two years after introduction of combination therapy, although to a lesser extent. This suggests that the resistance to RT inhibitors must be considered also in newly detected HIV-1 infections.

In the majority of HIV-1 resistant strains one or more mutations concerning the codons linked to the resistance to ZDV were identified. This is in agreement with the fact that ZDV is the major and first RT inhibitor used in the Czech Republic. Furthermore, a number of HIV-1 strains with several mutations concerning the codons linked to the ZDV resistance including the mutation M184V were found. The latter mutation conferred resistance to 3TC and a moderate cross-resistance to ddI and ddC. On the other hand, Nijhuis *et al.* (1997) have reported that the mutation M184V suppresses the resistance to ZDV and multiple amino acid substitutions are required for establishment of the co-resistance to ZDV. Therefore, the determination of susceptibility to 3TC and ZDV combination treatment should be evaluated individually for each patient.

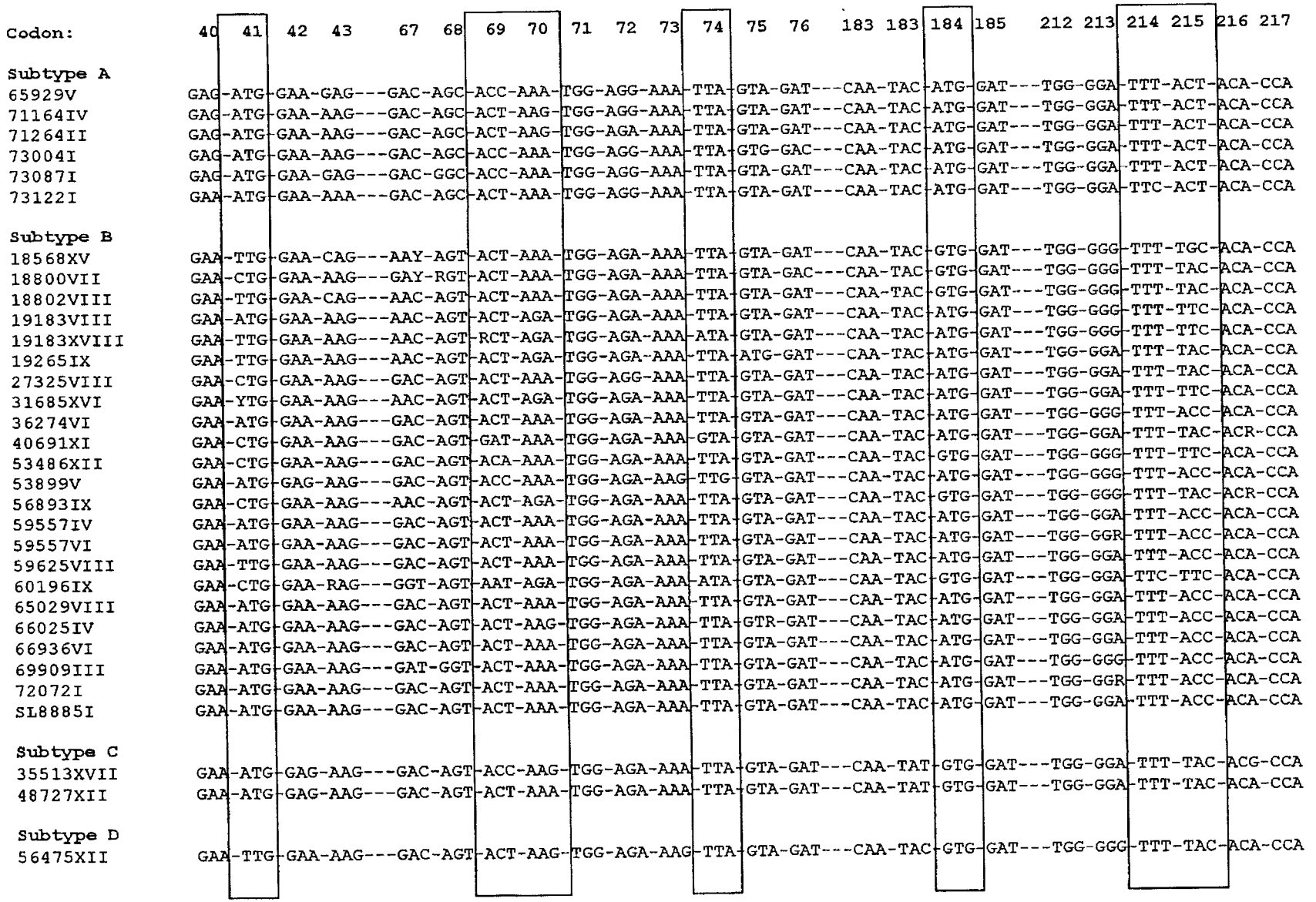

Fig. 2
Nucleotide sequences of the selected codons in RT region
The codons related to RT resistance and investigated by LiPA are boxed.

Table 4. Comparison of results of LiPA and sequence analysis of non-subtype B isolates

Specimen ID	Subtype	Sequencing results	LiPA results
65929V	A	Wild type	41n, 69/70n, 74n, M184, T215
71164IV	A	Wild type	41n, 69/70n, 74n, M184, T215
71264II	A	Wild type	41n, 69/70n, L74, M184, T215
73004I	A	Wild type	41n, 69/70n, 74n, M184, 214/215n
73087I	A	Wild type	41n, 69/70n, 74n, M184, T215
73122I	A	Wild type	41n, T69/K70, M184, T215
35513XVIII	C	N103, V184, Y215, K219	41n, 69/70n, L74, 184n, F214/Y215
48727XII	C	V184, Y215	41n, T69/K70, L74, 184n, F214/Y215
56475IX	D	L41, V184, W210, Y215	L41, 69/70n, 74n, V184, F214/Y215

Specimens 65929V, 73004I and 73087I were determined as CRF_01 AE. As these recombinants were of subtype B in *pol* but of subtype A in RT, they were classified as of subtype non-B in this study.

LiPA has been earlier shown to give concordant results with other genotyping methods and its major advantage is the ability to determine semiquantitatively a mixed virus population in the isolate (Descamps *et al.*, 1998; Wilson *et al.*, 2000; Schmit *et al.*, 1998). However, a limited number of specific probes and a frequent failure of hybridization, caused mainly by sequence polymorphism in the regions adjacent to the codons related to resistance (Koch *et al.*, 1999; Puchhammer-Stöckl *et al.*, 1999), limit the efficiency of this method. Our experience confirmed these postulates and, moreover, showed that especially the analysis of the non-B subtype isolates did not yield interpretable results.

We have proposed that LiPA should be complemented with nucleotide sequence analysis of those specimens, which gave uninterpretable results. A principal cause of the LiPA analysis problems is a hybridization failure, while RT-PCR is usually successful. Therefore we used already prepared amplicons as a genetic material for direct sequencing or reamplification. Thus, the information obtained by LiPA could be easily supplemented by the results of sequencing of a principal region of RT to avoid repeating the RNA extraction and RT-PCR. This approach is not only time and material saving but might be useful in case the original specimen is not more available.

Comparison of LiPA and sequencing results showed a number of minor discrepancies due to the ability of LiPA to detect minor quasispecies. We have tried to cast more light on the causes of several major discrepancies. All of them appeared in the analysis of the strains in which the sequence analysis identified a sequence that could not be detected by LiPA due to the lack of a specific probe.

Interestingly, a mutation from L74 (wild type) to V74 through a temporary appearance of I74 has been described earlier in a patient who was receiving ZDV but had switched to ddI therapy (St Clair *et al.*, 1991). It is plausible that a mixture of strains was present also in the analyzed specimens. In codon 215, exactly the same sequence motif led one time to hybridization failure and another time to a major discrepancy. Taken together, these data suggest that the major discrepancies between the results of LiPA and those of sequencing arise because LiPA detects a minor viral quasispecies in a mixed virus population, while a major one cannot be detected because of lack of a specific probe rather than because of hybridization failure.

We have shown a prevalence of the HIV-1 strains resistant to various nucleoside inhibitors of RT in various combinations in the Czech Republic. Furthermore, the analytical methods should yield molecular-epidemiological data on the HIV-1 population, especially on the emerging proportion of non-B subtype strains in circulation in the country. A combination of several genotyping methods like nucleotide sequence analysis and LiPA would be beneficial for obtaining reliable results. However, the expected increase of heterogeneity of HIV-1 population, introduction of new drugs, and a rapid progress in sequencing techniques promote the nucleotide sequence analysis for a method of choice in future.

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