Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Virological Methods 132 (2006) 181–186
Journal of Virological Methods
www.elsevier.com/locate/jviromet

A genotypic assay for the amplification and sequencing of *gag* and protease from diverse human immunodeficiency virus type 1 group M subtypes

Kristel Van Laethem [a,b,*], Yoeri Schrooten [a,b], Sarah Dedecker [a], Lies Van Heeswijck [a], Koen Deforche [a], Eric Van Wijngaerden [c], Marc Van Ranst [a,b], Anne-Mieke Vandamme [a]

[a] *Rega Institute for Medical Research, Katholieke Universiteit Leuven, Leuven, Belgium*
[b] *Aids Reference Laboratory, University Hospitals Leuven, Leuven, Belgium*
[c] *Internal Medicine, University Hospitals Leuven, Leuven, Belgium*

Received 6 July 2005; received in revised form 13 September 2005; accepted 3 October 2005
Available online 4 November 2005

Abstract

In human immunodeficiency virus type 1 (HIV-1), an interaction exists between the in vivo evolution of Gag protein and protease to escape from antiretroviral drug selective pressure. Therefore, it was decided to develop a genotypic assay for the amplification and sequencing of HIV-1 *gag* and protease. As the HIV-1 pandemic is characterised by a large genetic diversity, the assay developed was evaluated on a panel of 28 genetically divergent samples belonging to the following subtypes A1, B, C, D, F1, F2, G, H, J, CRF01-AE, CRF02-AG and CRF13-cpx. The assay displayed a detection limit ranging between 500 RNA copies/ml and 5000 RNA copies/ml plasma. Full-length sequences could be obtained for 25 samples. The population sequences of the three other samples lacked a part of the sequence because of heterogeneous signal, probably due to the presence of quasispecies with insertions/deletions of a different length.

Keywords: RNA extraction; cDNA synthesis; PCR; HIV-1 subtypes; Sensitivity

1. Introduction

Since the discovery of human immunodeficiency virus type (HIV) as the causative agent for acquired immune deficiency syndrome (AIDS), research efforts led to the development and clinical use of several drugs for the inhibition of the retroviral replication cycle. The FDA currently approved drugs target three steps of the replication cycle, i.e. the fusion between viral and cellular membranes, the reverse transcription of viral RNA into cDNA and the maturation of newly synthesized virus particles. The use of drug combinations has lead to a dramatic decrease in morbidity and mortality within the patient population. However, lack of complete therapy adherence or limited therapy potency can lead to therapy failure which often results in development of viral resistance and ultimately might result in subsequent failure to following therapies due to cross-resistance. Viral drug resistance is mostly characterised by amino acid changes within the target site of the respective drug. Resistance mutations towards the fusion inhibitor are found mainly in the transmembrane glycoprotein gp41 of which the N-terminal heptad repeat region is targeted by enfuvirtide (Rimsky et al., 1998; Sista et al., 2004). Resistance towards non-nucleoside reverse transcriptase inhibitors is associated with mutations within a hydrophobic pocket that is targeted by this class of inhibitors (Balzarini, 2004). Resistance towards nucleoside reverse transcriptase inhibitors is a more complex issue. Some mutations impair the incorporation of the nucleoside reverse transcriptase inhibitors, others improve the removal of the incorporated nucleoside analogue from the terminated DNA chain, but all of them are located within the reverse transcriptase (Clavel and Hance, 2004). Therapy failure to protease inhibitors are characterised by mutations within the protease. These mutant proteases often display a reduced activity resulting in a decreased replication capacity of the virus. However, mutant viruses have the oppor-

* Corresponding author at: Rega Institute for Medical Research and University Hospitals Leuven, Microbiology and Immunology, Clinical and Epidemiological Virology, AIDS Reference Laboratory, Minderbroedersstraat 10, 3000 Leuven, Belgium. Tel.: +32 16 332177; fax: +32 16 332131.
E-mail address: kristel.vanlaethem@uz.kuleuven.ac.be (K. Van Laethem).

0166-0934/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.jviromet.2005.10.008

tunity to evolve further in the presence of suboptimal therapy resulting in the selection of mutations that recover the impaired protease activity. These compensatory mutations are located within the protease itself but also within and outside the natural substrate of the protease, i.e. the Gag cleavage sites (Doyon et al., 1996; Mammano et al., 2000; Myint et al., 2004). Recently, the impact of Gag mutations on drug susceptibilities has also been reported (Prado et al., 2002; van Maarseveen et al., 2005). The aim of this study was to develop an assay for the amplification and sequencing of *gag* and protease. HIV-1 displays an enormous genetic diversity that is reflected by the presence of 9 subtypes, 16 circulating recombinant forms and a multitude of unique recombinant forms within group M that is responsible for the pandemic (Takebe et al., 2004). The assay was designed to detect all these variants.

2. Materials and methods

2.1. Samples

Dilution series of ACH2 cells (containing one HIV-1 subtype B provirus per cell) in a background of HUT78 cells were used to optimise the cycling conditions. After the optimisation at the DNA level, dilution series of HIV-1 (III_B) virus (subtype B) were used (kindly provided by R.C. Gallo when at the National Institutes of Health, Bethesda, MD) to fine-tune conditions for RNA extraction, cDNA synthesis and PCR. In addition to this laboratory strain, plasma samples were included from 28 patients who had attended the University Hospitals in Leuven and for whom a genotypic resistance analysis towards protease and reverse transcriptase inhibitors had been performed as part of their routine management. The samples were selected based on the subtype that was determined by submitting the *pol* sequences to the Rega HIV-1 subtyping tool (beta version) (de Oliveira et al., 2005) (Table 1). The plasma viral load was determined using VERSANT HIV-1 RNA 3.0 Assay (bDNA, Bayer HealthCare, Brussel, Belgium). Plasma was obtained from a HIV-1 seronegative blood donor and was subsequently used for the serial dilution of HIV-1 positive plasma samples.

Table 1
Panel composition

Patient	Subtype[a]	Sample	Viral load[b] (RNA copies/ml)
1	A1	AR02-358	3609 (1000)
2	A1	AR02-025	94885
3	A1	AR02-852	17390
4	B	AR01-273	12492 (5000)
5	B	AR02-477	221109 (1000)
6	B	AR01-405	198797
7	B	H97-3457	61722
8	B	P98-334	500000
9	B	P00-200	41032
10	C	AR01-096	292949 (5000)
11	C	P99-137	111873
12	C	AR00-001	170937
13	C	AR04-789	26224
14	D	AR02-080	22138 (1000)
15	D	AR04-342	14921
16	F1	AR04-538	9422
17	F2	AR05-661	13714
18	G	AR01-177	93289 (5000)
19	G	AR02-436	167506
20	G	AR04-834	65167
21	H	P99-048	234615 (1000)
22	A1/H	AR04-301	907
23	J	AR01-485	419945
24	J	AR01-461	7071
25	CRF01-AE	AR02-480	2149 (1000)
26	CRF02-AG	AR02-032	78310 (5000)
27	CRF13-cpx	AR01-989	9640 (1000)
28	CRF13-cpx	P99-496	318922

[a] As obtained by submitting the *gag* and *pol* sequences to the Rega HIV-1 subtyping tool (beta version)(de Oliveira et al., 2005).
[b] All samples could be amplified with the *gag*-protease PCR. Some samples were subjected to a dilution procedure and their detection limit is displayed between brackets.

2.2. Primer development and synthesis

The PCR and sequencing primers were designed using the *gag–pol* gene alignment of several HIV-1 group M strains (Kuiken et al., 2002). The primer sequences are given in Table 2. Primers were developed and analysed using the Oligo software (Medprobe, Oslo, Norway). The primers were synthesised by Invitrogen (Merelbeke, Belgium).

2.3. Extraction

For HIV-1 proviral DNA detection, 1 ml cell suspensions containing 10^7 cells were extracted using QIAamp DNA Blood Mini Kit (Westburg, Leusden, The Netherlands). For HIV-1 viral RNA detection, 1 ml plasma samples or 1 ml virus III_B supernatant were ultracentrifuged at 37 100 × *g* for 1 h to pellet the virus and they were extracted subsequently using QIAamp Viral RNA Mini Kit (Westburg, Leusden, The Netherlands).

2.4. cDNA synthesis and amplification of gag-PR region

HIV-1 RNA was reverse transcribed into cDNA using the following conditions: 1× Expand Buffer, 10 mM DTT, 1 mM dNTPs, 20 U RNase inhibitor, 50 U Expand Reverse Transcriptase (Roche Diagnostics, Mannheim, Germany) and 2 μM antisense primer KVL065. RNA template and primer were preincubated for 10 min at 65 °C and the cDNA synthesis was performed by an incubation step at 42 °C for 1 h. A 2259 bp nucleotide fragment (cDNA or DNA), encompassing the *gag*-protease region, was amplified in an outer PCR using the Expand High Fidelity PCR System (Roche Diagnostics, Mannheim, Germany) and the following conditions: 1× Expand HF Buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM primer KVL064, 0.4 μM primer KVL065 and 2.625 U Expand High Fidelity PCR System enzyme mix. Cycling conditions were: a denaturation step of 2 min at 95 °C, 10 cycles of 15 s at 95 °C, 30 s at 57 °C, 2 min 30 s at 68 °C, 30 cycles of 15 s at 95 °C, 30 s at 57 °C, 2 min 30 s at 68 °C + cycle elongation of 5 s for each cycle and a final extension step of 10 min at 72 °C. A 1998 bp nucleotide frag-

Table 2
Primers for the amplification and sequencing of the HIV-1 *gag*-protease region

Primers[a]	Sequence 5′–3′	Position[b]	Description
KVL064	GTT GTG TGA CTC TGG TAA CTA GAG ATC CCT CAG A	570–603	Sense outer primer for amplification
KVL065	TCC TAA TTG AAC YTC CCA RAA RTC YTG AGT TC	2797–2828	Antisense outer primer for amplification
KVL066	TCT CTA GCA GTG GCG CCC GAA CAG	626–649	Sense inner primer for amplification
KVL067	GGC CAT TGT TTA ACY TTT GGD CCA TCC	2597–2623	Antisense inner primer for amplification
GA1	GAC GCA GGA CTC GGC TTG CT	688–707	Sense primer for sequencing
AV103	GCC ATA TCA CCT AGA ACT TT	1225–1244	Sense primer for sequencing
AV159	GGG GTT AAA TAA AAT AGT AAG	1593–1613	Sense primer for sequencing
G110	AGG CTA ATT TTT TAG GGA	2078–2095	Sense primer for sequencing
KVL078	CAT TCC TGG CTT TAA TTT TAC TG	2575–2597	Antisense primer for sequencing
KVL079	GTG TCC TTC CTT YCC ACA TTT CC	2030–2052	Antisense primer for sequencing
KVL080	CCC ATT CTG CAG CTT CCT CAT TG	1404–1426	Antisense primer for sequencing
KVL081	AAT CKT TCY AGC TCC CTG CTT GC	898–920	Antisense primer for sequencing

[a] Primers GA1 and G110 were previously published (Salminen et al., 1995).
[b] Positions according to pNL4.3 (AF324493).

ment of *gag*-protease was amplified in an inner PCR using the Expand High Fidelity PCR System and the following conditions: 1× Expand HF Buffer, 2 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM primer KVL066, 0.4 μM primer KVL067 and 2.625 U Expand High Fidelity PCR System enzyme mix. Cycling conditions were: a denaturation step of 2 min at 95 °C, 10 cycles of 15 s at 95 °C, 30 s at 58 °C, 2 min 30 s at 68 °C, 30 cycles of 15 s at 95 °C, 30 s at 58 °C, 2 min 30 s at 68 °C + cycle elongation of 5 s for each cycle and a final extension step of 10 min at 72 °C. Amplification products were separated on a 1% agarose gel and visualized by ethidium bromide staining. The images were processed on a videoimager (ImageMaster VDS, Amersham Biosciences, Roosendaal, The Netherlands).

2.5. Sequencing

PCR products for population sequencing were purified with Microspin S-400 (Amersham Biosciences, Roosendaal, The Netherlands). Sequencing was performed using the ABI PRISM BigDye Terminator v3.1 Ready Reaction Cycle Sequencing Kit and the reactions were run on an ABI3100 Genetic Analyzer (Applera, Nieuwerkerk a/d Ijssel, The Netherlands).

2.6. Data analysis

The sequences were analysed using Sequence Analysis version 3.7 and SeqScape version 2.0 (Applera, Nieuwerkerk a/d Ijssel, The Netherlands). The subtype of the newly obtained *gag*-protease fragments was obtained by submitting the sequence to the Rega HIV-1 subtyping tool (beta version) (de Oliveira et al., 2005).

3. Results

3.1. Performance of the PCR assays

HIV-1 infected ACH2 cells were diluted in HIV-1 negative HUT78 cells for use in the optimisation steps of the cycling conditions for the amplification of the *gag*-protease region. The dilution series ranged from 1 000 000 DNA copies/ml (i.e. 10 000 DNA copies/PCR) until 100 DNA copies/ml (i.e. 1 DNA copy/PCR). The optimised nested protocol reached a detection limit of 1000 DNA copies/ml (i.e. 10 DNA copies/PCR). No cross-reactivity was seen with genomic DNA (tested on HIV-1 negative HUT 78 cells).

Dilution series of HIV-1 (III_B) virus in PBS, ranging from 1 000 000 RNA copies/ml until 500 RNA copies/ml, were used to fine-tune the experimental conditions for the extraction, cDNA synthesis and the amplification of the *gag*-protease region. The nested protocol reached twice a detection limit of 500 HIV-1 (III_B) RNA copies/ml and once a detection limit of 1000 HIV-1 (III_B) RNA copies/ml (i.e. 50–100 cDNA copies/PCR).

A panel of patient plasma samples was selected. It reflected the HIV-1 subtypes within group M that were present in our patient population. All patient samples were detected by PCR. Ten-fold dilution series of a few patient samples were made in HIV-1 negative plasma, taking care that for patient samples with a sufficiently high viral load, at least 1000 RNA copies/ml could be tested as the final dilutions. The detection limit ranged between 5000 and 1000 RNA copies/ml for all tested subtypes (Table 1).

3.2. Performance of the sequencing assays

For each patient sample, population sequencing was undertaken on the purified amplification product from the last positive sample of the dilution series or from the original sample in case no dilution series was carried out on that particular sample. A total of eight primers were used for the sequencing of the *gag*-protease region (Tables 2 and 3 and Fig. 1). The sequencing success rate ranged from 23/28 (82%) to 28/28 (100%): 23/28 (82%) for primer AV103, 24/28 (86%) for primer AV159, 27/28 (96%) for primers G110 and KVL078, 27/27 or 28/28 (100%) for primers GA1, KVL079, KVL080 and KVL081. The unsuccessful sequencing reactions were due to mismatches between primer and template at the 3′ end of the primer sequence. Some sequencing fragments displayed heterogeneous signal due to the presence of a variety of nucleotide insertions and deletions in the quasispecies of the virus population. Therefore, not all primers could generate a useable population sequence. The

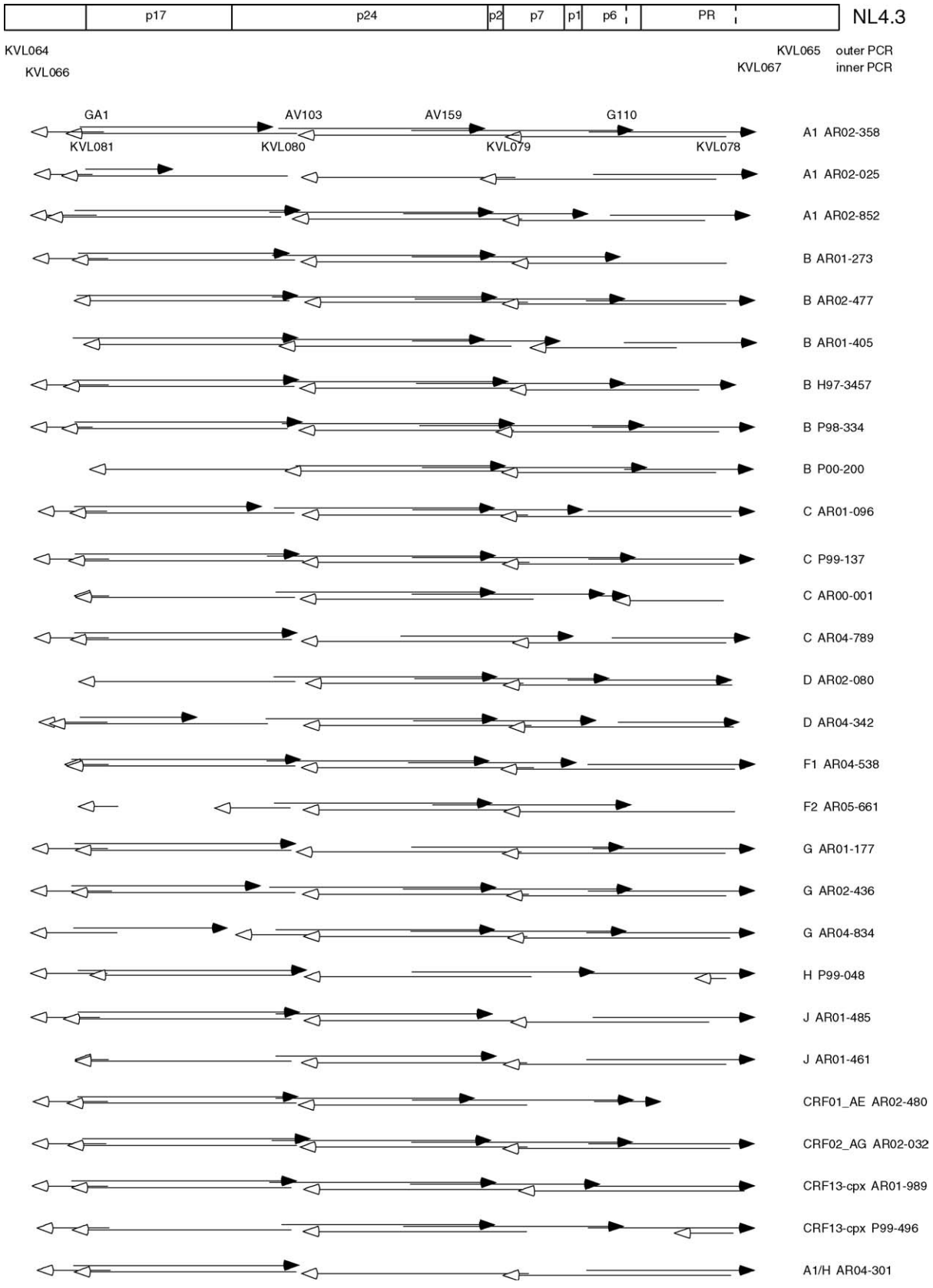

Fig. 1. Performance of sequencing primers: (——►) segment covered with sense sequencing primer and (◄——) segment covered with antisense sequencing primer.

Table 3
Qualitative scoring of the sequencing primers of the HIV-1 *gag*-protease region

Subtype	Sample	GA1		AV103		AV159		G110		KVL078		KVL079		KVL080		KVL081	
A1	AR02-358	+	524	+	560	+	601	+	600	+	600	+	607	+	626	+	198
A1	AR02-025	+/–	239	–	0	–	0	+	445	+	640	+	582	+	614	+	160
A1	AR02-852	+	612	+	609	+	502	+	381	+	549	+	623	+	635	+	180
B	AR01-273	+	573	+	606	+	567	–	0	+	583	+	620	+	604	+	206
B	AR02-477	+	601	+	610	+	609	+	458	+	608	+	606	+	584	Nd	0
B	AR01-405	+	611	+	552	+	403	+	361	+	399	+	630	+	563	Nd	0
B	H97-3457	+	613	+	606	+	568	+	336	+	513	+	607	+	627	+	479
B	P98-334	+	618	+	630	+	610	+	442	+	604	+	584	+	614	+	168
B	P00-200	0	0	+	570	+	610	+	351	+	583	+	611	+	540	Nd	0
C	AR01-096	+	510	+	600	+	463	+	453	+	626	+	612	+	610	+	188
C	P99-137	+	609	+	620	+	607	+	452	+	629	+	617	+	638	+	205
C	AR00-001	+/0	0	+	597	+	523	+/0	90	+/0	298	+	633	+	595	+/0	95
C	AR04-789	+	606	–	0	+	470	+	374	+	580	+	618	+	601	+	202
D	AR02-080	0	0	+	607	+	529	+	446	+	621	+	592	+	588	+/0	0
D	AR04-342	+	319	+	628	+	496	+	331	+	610	+	619	+	593	+	187
F1	AR04-538	+	622	+	605	+	457	+	454	+	635	+	631	+	619	+/–	120
F2	AR05-661	0	0	+	593	+	539	0	0	+	628	+	591	+/0	207	+/0	110
G	AR01-177	+	601	–	0	+	578	+	440	+	606	+	612	+	586	+	207
G	AR02-436	+	514	+	616	+	621	+	452	+/0	597	+	614	+	607	+	220
G	AR04-834	+/0	419	+	596	+	586	+	456	+	603	+	607	+/0	222	+	237
H	P99-048	+	621	–	0	+	497	+	460	+/–	86	+	617	+	555	+	210
A1/H	AR04-301	+	612	–	0	–	0	+	457	+	618	+	617	+	603	+	201
J	AR01-485	+	602	+	588	–	0	+	440	+	539	+	570	+	618	+	188
J	AR01-461	+/0	0	+	600	–	0	+	457	+	604	+	613	+	586	+/0	92
CRF01_AE	AR02-480	+	600	+	541	+	601	+/0	183	–	0	+	621	+	623	+	205
CRF02-AG	AR02-032	+	619	+	595	+	601	+	452	+	617	+	618	+	622	+	202
CRF13-cpx	AR01-989	+	612	+	616	+	516	+	452	+	622	+	621	+	623	+	205
CRF13-cpx	P99-496	+/0/+	0	+	579	+	598	+	453	+/0/+	161	+	607	+	594	+	198

Nd: sequencing was not done; +: sequencing was successful; 0: sequencing was successful but a heterogeneous signal was obtained; –: sequencing was not successful; the range that was covered with each sequencing primer is displayed in number of nucleotides.

final success rate dropped to the range of 19/28 (68%)–28/28 (100%) depending on which region within *gag* should have been covered with the particular primer: 19/28 (68%) for primer GA1, 20/27 (74%) for primer KVL081, 22/28 (79%) for primer AV159, 23/28 (82%) for primers AV103 and KVL078, 24/28 (79%) for primer G110, 26/28 (93%) for primer KVL080 and 28/28 (100%) for primer KVL079. Population sequencing resulted nevertheless in full-length bi-directional *gag*-protease sequences for nine samples (AR02-358, AR02-477, P98-334, AR01-096, P99-137, AR04-538, AR02-436, AR02-032 and AR01-989). Full-length but only partly bi-directional sequences were obtained for the remaining samples, with the exception of samples AR00-001, AR05-661 and AR02-480. The first and second sample lacked a part of the sequence in the protease and *gag* region respectively and both failures in obtaining full-length sequence were due to the presence of heterogeneous signal. The last sample missed the protease sequence almost completely due to the combined effect of a heterogeneous signal with primer G110 and a negative reaction with primer KVL078.

Almost all samples were classified within the *gag* gene to the same subtype as within the *pol* gene. Only one sample, AR04-301, displayed a recombination event between two subtypes (subtypes A1 and H).

4. Discussion

This study describes an assay for the genotypic characterisation of the HIV-1 *gag*-protease region. It could be used for therapy monitoring studies for the determination of amino acid changes in Gag and protease or in epidemiological studies for mapping the circulating subtypes and recombinants.

The amplification of HIV-1 *gag* template is more challenging than the amplification of HIV-1 *pol* sequences, currently undertaken for drug resistance testing, due to the higher nucleotide variability in the *gag* region (Kuiken et al., 2002). The primers were chosen at the most conserved sites surrounding or within the target region to make the amplification and sequencing possible of all HIV-1 genetic variants within group M. In addition, ambiguity positions within the primers were implemented to take into account the genetic diversity at the primer binding sites. This approach resulted in a successful amplification of 28 genetically divergent samples belonging to subtypes A1, B, C, D, F1, F2, G, H, J, CRF01-AE, CRF02-AG and CRF13-cpx.

As the detection of genotypic drug resistance could be one of the purposes of this assay, it is important to be able to monitor the active replicating population responsible for therapy failure. After viral failure, it is desirable to change therapy as soon as possible so as not to compromise the immune status and future therapy options of the patient. Resistance testing has to be performed on plasma samples before stopping or changing therapy and often displaying low viral loads. Therefore, a nested PCR approach was chosen to increase the sensitivity of the assay. The amplification of long RNA templates displays, in comparison to the amplification of DNA templates, a lower sensitivity due to the higher fragility of the RNA template and the limited efficiency of cDNA synthesis. Nevertheless, the assay described displayed a sensitivity of 500–1000 RNA copies/ml supernatant which corresponds to 50–100 cDNA copies/PCR. If one takes into account an average cDNA synthesis efficiency of approximately 10%, the detection limit is in fact 5–10 cDNA copies/PCR. This sensitivity is comparable to that obtained with the assays that are used currently for the monitoring of genotypic resistance in protease and reverse transcriptase. When using genetically diverse HIV-1 patient samples this nested protocol showed a detection limit of 1000–5000 RNA copies/ml plasma for all tested samples.

The performance of the sequencing primers was checked on the entire test panel. In addition, the highest dilutions for some samples were sequenced to check the performance on divergent samples with a low viral load (1000 or 5000 RNA copies/ml). The sequencing success rate ranged from 82 to 100%. As most of the regions within *gag* are characterised by insertions and/or deletions, the presence of quasispecies displaying insertions and/or deletions of different length resulted into a shift of the electropherograms and no interpretable population sequence when these quasispecies were present at a concentration that is high enough for detection. Nevertheless, full-length sequences were obtained for 25 out of 28 samples.

In conclusion, the *gag*-protease assay developed has been shown to be a useful tool for the genotypic characterisation of diverse HIV-1 group M subtypes.

Acknowledgements

This work was supported by the AIDS Reference Laboratory of Leuven that receives support from the Belgian Ministry of Social Affairs through a fund within the Health Insurance System and by the Fonds voor Wetenschappelijk Onderzoek Vlaanderen (G.0266.04).

References

Balzarini, J., 2004. Current status of the non-nucleoside reverse transcriptase inhibitors of human immunodeficiency virus type 1. Curr. Top. Med. Chem. 4, 921–944.

Clavel, F., Hance, A.J., 2004. HIV drug resistance. N. Engl. J. Med. 350, 1023–1035.

de Oliveira, T., Deforche, K., Cassol, S., Salminen, M., Paraskevis, D., Seebregts, C., Snoeck, J., van Rensburg, E.J., Wensing, A.M.J., van de Vijver, D.A., Boucher, C.A., Camacho, R., Vandamme, A.-M., 2005. An automated genotyping system for analysis of HIV-1 and other microbial sequences. Bioinformatics 21 (19), 3797–3800.

Doyon, L., Croteau, G., Thibeault, D., Poulin, F., Pilote, L., Lamarre, D., 1996. Second locus involved in human immunodeficiency virus type 1 resistance to protease inhibitors. J. Virol. 70, 3763–3769.

Kuiken, C., Foley, B., Freed, E., Hahn, B., Marx, P., McCutchan, F., Mellors, J., Wolinsky, S., Korber, B., 2002. HIV sequence compendium. In: Theoretical Biology and Biophysics. Los Alamos National Laboratory, Los Alamos, p. 611.

Mammano, F., Trouplin, V., Zennou, V., Clavel, F., 2000. Retracing the evolutionary pathways of human immunodeficiency virus type 1 resistance to protease inhibitors: virus fitness in the absence and in the presence of drug. J. Virol. 74, 8524–8531.

Myint, L., Matsuda, M., Matsuda, Z., Yokomaku, Y., Chiba, T., Okano, A., Yamada, K., Sugiura, W., 2004. Gag non-cleavage site mutations contribute to full recovery of viral fitness in protease inhibitor-resistant human immunodeficiency virus type 1. Antimicrob. Agents Chemother. 48, 444–452.

Prado, J.G., Wrin, T., Beauchaine, J., Ruiz, L., Petropoulos, C.J., Frost, S.D., Clotet, B., D'Aquila, R.T., Martinez-Picado, J., 2002. Amprenavir-resistant HIV-1 exhibits lopinavir cross-resistance and reduced replication capacity. AIDS 16, 1009–1017.

Rimsky, L.T., Shugars, D.C., Matthews, T.J., 1998. Determinants of human immunodeficiency virus type 1 resistance to gp41-derived inhibitory peptides. J. Virol. 72, 986–993.

Salminen, M.O., Carr, J.K., Burke, D.S., McCutchan, F.E., 1995. Genotyping of HIV-1. In: Myers, G., Korber, B., WainHobson, S., Jeang, K.T., Henderson, L.E., Pavlakis, G.N. (Eds.), Human retroviruses and AIDS: a compilation and analysis of nucleic acid and amino acid sequences. Los Alamos National Laboratory, Los Alamos, N. Mexico, pp. III-30–III-34.

Sista, P.R., Melby, T., Davison, D., Jin, L., Mosier, S., Mink, M., Nelson, E.L., DeMasi, R., Cammack, N., Salgo, M.P., Matthews, T.J., Greenberg, M.L., 2004. Characterization of determinants of genotypic and phenotypic resistance to enfuvirtide in baseline and on-treatment HIV-1 isolates. AIDS 18, 1787–1794.

Takebe, Y., Kusagawa, S., Motomura, K., 2004. Molecular epidemiology of HIV: tracking AIDS pandemic. Pediatr. Int. 46, 236–244.

van Maarseveen, N., Nijhuis, M., Schipper, P., Goededebuure, I., Dulude, D., Brakier-Gingras, L., Heilek-Snyder, G., Cammack, N., Boucher, C., 2005. Novel HIV drug resistance mechanism leading to protease inhibitor (PI) resistance in response to a high genetic barrier PI in vitro. In: Proceedings of the Third European HIV Drug Resistance Workshop, Abstract 94, Athens, Greece.