Short communication

HIV contamination of commercial PCR enzymes raises the importance of quality control of low-cost in-house genotypic HIV drug resistance tests

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Background: Low-cost in-house technologies for genotypic drug resistance testing use reagents with quality labels for research only. Here, we report on the results of PCR amplifications in negative-controls that were observed in two independent laboratories.
Methods: Positive PCR amplifications of protease and reverse transcriptase fragments for genotypic drug resistance testing of HIV on dried blood and/or plasma spots were observed on negative-control samples and were analysed in detail by PCR and sequence and phylogenetic analyses to identify the origin of the PCR contamination.
Results: Detailed analysis revealed that the RT-PCR enzymes were contaminated with an HIV-based vector commercialized by the same company.
Conclusions: These observations show the need to implement quality control steps that verify for the absence of HIV in new reagent batches because this can significantly compromise molecular diagnosis of HIV and genotypic drug resistance tests using in-house protocols.

Introduction

The number of people receiving antiretroviral therapy in resource-limited countries has increased over the past years [1]. However, viral load and genotypic drug resistance tests are not yet available at affordable costs in these countries; therefore, low-cost technologies for viral load measurement [2] have been developed and in-house tests are commonly used for drug resistance testing [3–6].

In the framework of a study to optimize genotypic drug resistance testing on dried blood and/or plasma spots [7], we observed PCR amplifications in negative-controls and investigated, in detail, the origin of these contaminations. Here, we report on the cause of PCR amplifications in negative-controls that were observed in two independent laboratories, which were found to be related to the reverse transcriptase (RT)-PCR enzymes that were contaminated with an HIV-based vector commercialized by the same company.

Methods

Nested RT-PCR was used to amplify the protease (PR) and the RT regions according to the validated in-house method for genotypic HIV drug resistance testing protocol from the Agence Nationale de Recherches sur le Sida et les Hépatites Virales (ANRS) working group on HIV drug resistance, which was adapted for dried spots [7,8]. Briefly, 10 µl of RNA samples were used for RT-PCR using the Superscript One-Step RT-PCR method for long templates (product reference 11922-028; Invitrogen Life Technologies, Cergy Pontoise, France). An aliquot of 5 µl of the first-round product was then used for nested PCR using the HotStar Taq Master Mix kit (product reference 203205; Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. Amplification products were visualized by 1% agarose gel electrophoresis with ethidium bromide staining. The amplified fragments were purified and directly sequenced without an intermediary cloning step using the BigDye Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems, Courtaboeuf, France). Electrophoresis and data collection were performed on an Applied Biosystems 3130XL genetic analyzer. Sequences were obtained for both strands of DNA, assembled and edited using SeqMan from Lasergene (Madison, WI, USA).

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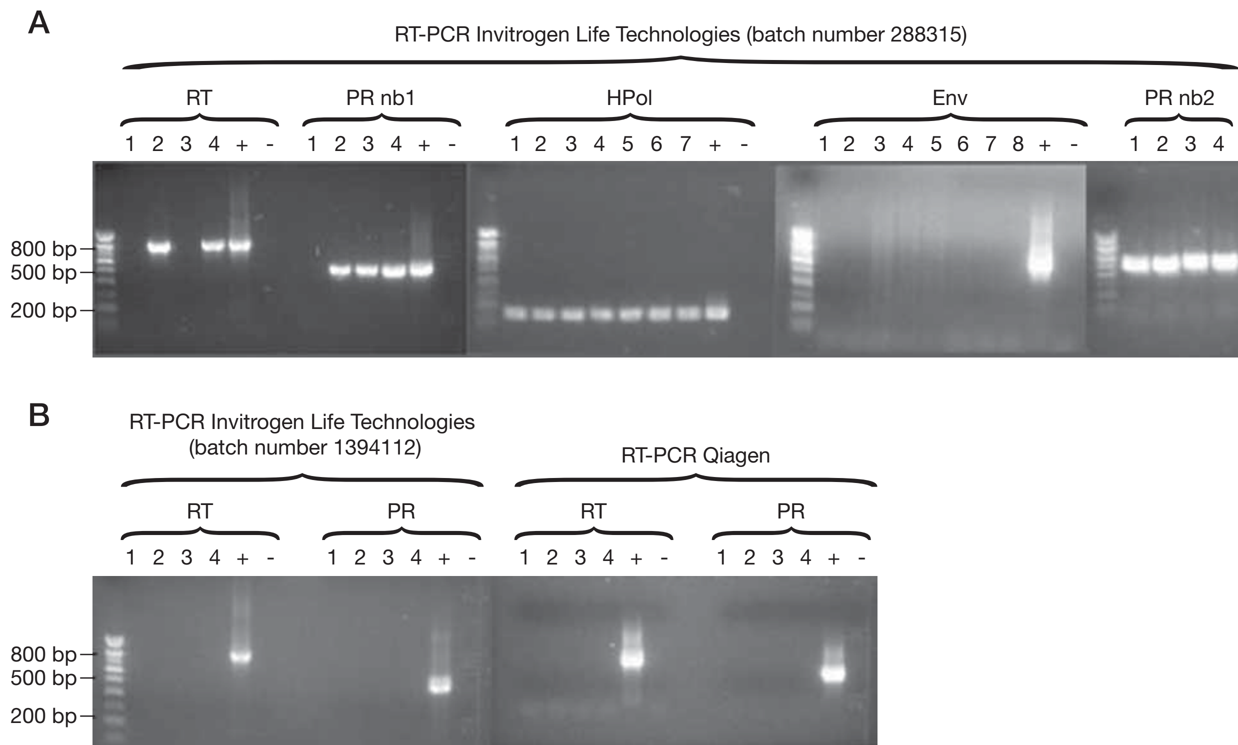
Figure 1. Amplification results of RT, PR, integrase region of *pol* and *env* fragments of HIV



The nested reverse transcriptase (RT)-PCR protocol used different One-Step RT-PCR enzymes and, as second round PCR, the HotStar Taq Master Mix kit was used (Qiagen, Courtaboeuf, France) for all experiments. **(A)** RT-PCR using the enzymes from the Superscript One-Step RT-PCR kit (Invitrogen Life Technologies, Cergy Pontoise, France) for long templates (batch number 288315). The same enzyme was used for nested PCR only, without a reverse transcription cycle, to amplify protease (PR) fragments (noted as PR nb2). **(B)** RT-PCR using enzymes from the Superscript One-Step RT-PCR kit (batch number 1394112) and the One-Step RT-PCR kit from Qiagen. Numbered samples are from water samples. Hpol, fragment in the integrase region of *pol*; +, positive-control samples (mp642 HIV type-1 strain CRF02); –, negative-control of the second round only.

Results

In February 2007, we obtained positive PCR amplifications for PR and RT fragments on negative-control samples with water using, for the RT-PCR first round, the Superscript One-Step RT-PCR method for long templates from Invitrogen Life Technologies (product reference 11922-028, batch number 288315 with enzyme batch number 1411037 and buffer batch number 1403096) and, for the second round PCR enzyme, the HotStar Taq enzyme from Qiagen (batch number 127131610). After changing all the reagents not included in the enzyme kits, such as water, oligonucleotides and $MgCl_2$, PCR results on negative-controls were still positive. We carried out additional RT-PCRs in the integrase region of the *pol* (identified as Hpol) [9] and the V3–V5 region in *env* [10]. RT, PR and Hpol fragments were amplified from water samples contrary to the *env* fragment as shown in Figure 1A. Moreover, we repeated the same nested PCR reactions to amplify the PR fragment but without the reverse transcription step and also observed positive amplifications, supporting that the contaminant was a DNA molecule (Figure 1A). Not all the PCR attempts on water were positive in the RT and PR PCR reactions indicating that this contamination could go unnoticed when only one negative-control sample is included per PCR run. Interestingly, the negative-control of the second PCR round was always negative, suggesting that the contamination occurred in the first PCR round. We then tested other RT-PCR enzymes: another batch of the RT-PCR enzyme from Invitrogen Life Technologies (enzyme batch number 1389921 from the kit batch number 1394112) and the One-Step RT-PCR kit from Qiagen (product reference 210210). As shown in Figure 1B, no amplification from water samples was observed. Finally, we directly sequenced the PR amplification products from water samples and performed phylogenetic tree analysis using the neighbour-joining method and bootstrap approach implemented in ClustalX version 2.0 [11,12]. This analysis showed that the amplified product corresponds to an HIV type-1 (HIV-1) subtype B sequence and a subsequent BLAST search, followed by sequence alignment, showed clearly

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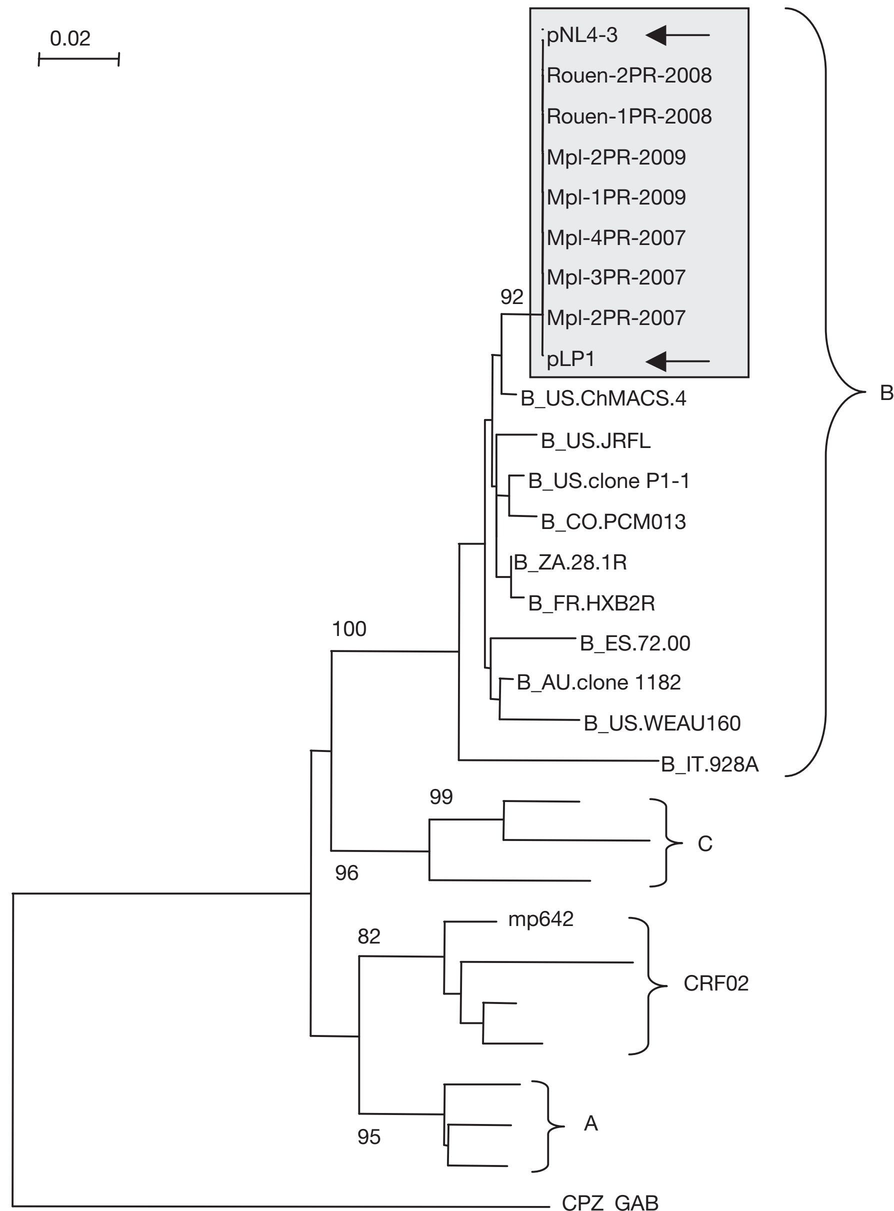
Figure 2. Phylogenetic tree analysis of the HIV-1 protease sequences



Sequences are derived from PCR products obtained from water samples using the Superscript One-Step reverse transcriptase (RT)-PCR method for long templates (Invitrogen Life Technologies, Cergy Pontoise, France; batch number 288315) in 2007 and in 2009 by the Montpellier Laboratory (Mpl; Monpellier, France) and obtained with the Superscript III One-Step RT-PCR system with Platinum Taq High Fidelity (Invitrogen Life Technologies; batch number 433415) by the Rouen Laboratory (Rouen, France). Only the bootstrap values >80% were indicated. mp642 corresponds to the sequence of the HIV strain used as positive-control in the PCR experiments. Scale bar represents substitutions per site. HIV-1, HIV type-1.

that the sequence was identical to the well-known HIV-1 vector NL4-3 (accession number AF324493), from which the pLP1 plasmid, an HIV-1 lentiviral vector system commercialized by Invitrogen Life Technologies, was derived (Figure 2). However, these systems have never been used in our laboratory. Interestingly, the pLP1 construct encodes only the HIV-1 Gag, Pol and Rev proteins and not the Env protein, thereby, explaining the negative PCRs for *env*.

The same problems occurred again in 2009 with the same enzyme and surprisingly with the same enzyme batch number (288315) as in 2007, showing

Figure 3. Alignment of sequences

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pLP1     AGCTAGGCCCTTTTGCTAATCATGTTCATACCTCTTATCTTCCTCCCACAGCTCCTGGGC
Seq1     AGCTAGGCCCTTTTGCTAATCATGTTCATACCTCTTATCTTCCTCCCACAGCTCCTGGGC
Seq2     AGCTAGGCCCTTTTGCTAATCATGTTCATACCTCTTATCTTCCTCCCACAGCTCCTGGGC
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pLP1     AACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGCACGTGAGATCTGAATTCGA
Seq1     AACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGCACGTGAGATCTGAATTCGA
Seq2     AACGTGCTGGTCTGTGTGCTGGCCCATCACTTTGGCAAAGCACGTGAGATCTGAATTCGA
         ************************************************************

NL4-3                  ATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGATAGAT
pLP1     GATCTGCCGCCGCCATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGATCGAT
Seq1     GATCTGCCGCCGCCATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGATCGAT
Seq2     GATCTGCCGCCGCCATGGGTGCGAGAGCGTCAGTATTAAGCGGGGGAGAATTAGATCGAT
         ******************************************************** ***

NL4-3    GGGAAAAAATTCGGCTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAACATATAGTAT
pLP1     GGGAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAACATATAGTAT
Seq1     GGGAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAACATATAGTAT
Seq2     GGGAAAAAATTCGGTTAAGGCCAGGGGGAAAGAAAAAATATAAATTAAAACATATAGTAT
         ************** *********************************************

NL4-3    GGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACATCAGAAG
pLP1     GGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACATCAGAAG
Seq1     GGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACATCAGAAG
Seq2     GGGCAAGCAGGGAGCTAGAACGATTCGCAGTTAATCCTGGCCTGTTAGAAACATCAGAAG
         ************************************************************

NL4-3    GCTGTAGACAAATACTGGGACAGCTACAACCAGCCCTCCAGACAGGATCAGAAGAACTTA
pLP1     GCTGTAGACAAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTA
Seq1     GCTGTAGACAAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTA
Seq2     GCTGTAGACAAATACTGGGACAGCTACAACCATCCCTTCAGACAGGATCAGAAGAACTTA
         ******************************** **** **********************

NL4-3    GATCATTACATAATACAGTAGCAGTCCTCTATTGTGTGCATCAAAGGATAGAGGTAAAAG
pLP1     GATCATTATATAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG
Seq1     GATCATTATATAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG
Seq2     GATCATTATATAATACAGTAGCAACCCTCTATTGTGTGCATCAAAGGATAGAGATAAAAG
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NL4-3    ACACCAAGGAAGCTTTAGAGAAAATAGAGGAAGAGCAAAACAAAAGTAAGAAAACAGCAC
pLP1     ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAAGCAC
Seq1     ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAAGCAC
Seq2     ACACCAAGGAAGCTTTAGACAAGATAGAGGAAGAGCAAAACAAAAGTAAGAAAAAAGCAC
         ******************* ** ******************************* *****

NL4-3    AGCAAGCAGCAGCTGACACAGGAAACAGCAACAAGGT
pLP1     AGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGT
Seq1     AGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGT
Seq2     AGCAAGCAGCAGCTGACACAGGACACAGCAATCAGGT
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Fragments from the human β-globulin intron (grey) to HIV type-1 gag derived from PCR products, Seq1 and Seq2, obtained from water samples using the Superscript One-Step reverse transcriptase PCR method for long templates (Invitrogen Life Technologies, Cergy Pontoise; batch number 288315) and the NL4-3 (AF324493) and pLP1 vectors.

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that this batch was still commercially available after we had informed the company in 2007. PR amplification products from water samples were sequenced and were indeed identical to the contamination from 2007 (Figure 2). In order to prove that the PCR contamination was derived from the pLP1 plasmid commercialized by Invitrogen Life Technologies, we amplified a 565 base pair fragment using forward primers in the human β-globulin intron (first round PCR 5′-AGCAGCTACAATCCAG CTACCAT-3′ and second round PCR 5′-AAGGCTGGATTATTCTGAGTCCA-3′) and reverse primers in HIV gag (first round PCR 5′-GGCTCCYTCTGATAATGCTG-3′ and second round PCR 5′-CTGCACTATAGGGTAATTTTGGCT G-3′). We obtained two positive PCR products on 10 water samples using, for the first round, the enzyme batch number (288315), which proves that we amplified a plasmid construct. Moreover, the sequence analysis showed clearly that our contamination is identical to the pLP1 plasmid and not to the NL4-3 vector in the *gag* HIV region (Figure 3).

In early 2008, we communicated our findings to the members from the ANRS working group on drug resistance in resource-limited countries. Interestingly, at the end of 2008, the laboratory in Rouen, France, had a similar problem but with another enzyme from the same company: the Superscript III One-Step RT-PCR system with Platinum Taq High Fidelity (product reference 12574-035 and batch number 433415; Invitrogen Life Technologies). Sequence and phylogenetic analysis of the contaminations in Rouen's laboratory showed that the sequences were also identical to the pLP1 plasmid (Figure 2). According to the manufacturer's product description, the Invitrogen Life Technologies RT-PCR kits (product references 11922-028 and 12574-035) are composed each by a different RT enzyme (SuperScript II and SuperScript III, respectively), but seem to have the Platinum Taq DNA high-fidelity polymerase enzyme in common.

Discussion

In-house methods use many individual reagents from multiple companies, which are commercialized for research applications only and thus have different quality control labels from those used for commercial genotyping assays with clinical implications. Surprisingly, this study showed that, in addition to validation of new enzyme batches for potential differences in their performance, contamination of the PCR enzymes with HIV-based vectors should also be considered as possible. Contamination of the PCR enzymes with HIV-based vectors can lead to erroneous results of molecular diagnosis or genotypic drug resistance testing for HIV. Moreover, using such reagents can compromise findings and conclusions of research studies on HIV. These observations show the need to implement quality control steps that also verify for the absence of HIV in new reagent batches. These findings also illustrate the importance of systematic phylogenetic analysis to detect possible contaminations.

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Disclosure statement

The authors declare no competing interests.

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