JAC
antiviral

Detection and quantification of minority HIV isolates harbouring the D30N mutation by real-time PCR amplification

Maria G. Detsika[1], Becky Chandler[1], Saye H. Khoo[1], Craig Winstanley[2], Pat Cane[3], David J. Back[1] and Andrew Owen[1]*

[1]*Department of Pharmacology and Therapeutics, University of Liverpool, 70 Pembroke Place, Liverpool L69 3GF, UK;* [2]*Department of Medical Microbiology and Genitourinary Medicine, University of Liverpool, Daulby Street, Liverpool L69 3GA, UK;* [3]*UK Health Protection Agency, Centre for Infections, Porton Down, Salisbury SP4 0JG, UK*

Objectives: **HIV drug resistance is a major concern as the emergence of resistant strains of virus results in failure of first-line therapies with an associated increase in the cost of subsequent regimens. Genotypic resistance is currently assessed by direct sequencing and cannot detect resistant species below 20%. Real-time PCR amplification was assessed for its ability to detect the signature mutation for nelfinavir, D30N.**

Methods: **A real-time PCR assay was optimized for detection of low levels of D30N and tested on *in vitro*-generated nelfinavir-resistant isolates as well as 10 clinical isolates (which were also characterized by sequencing).**

Results: **The sensitivity of the assay was 1% and quantification was possible as low as 4% of the total viral population. Furthermore, this methodology enabled quantification of the 30N mutation in two isolates shown to be negative by sequencing.**

Conclusions: **Real-time PCR is a promising tool for the detection of minority species of HIV but further studies are required to determine the specificity of the assay in a larger and thus more diverse set of clinical isolates.**

Keywords: resistance, protease, allelic discrimination

Introduction

HIV drug resistance is a complex and multifactorial phenomenon. It is the consequence of mutations that emerge in the viral proteins targeted by the current antiretroviral agents[1] and is a major cause of therapy failure leading to elimination of therapy options,[2] with an associated increase in cost of therapy. Worryingly, recent evidence suggests that 1 in 10 new infections are with resistant strains of virus.[3]

Genotypic resistance is currently assessed by sequencing techniques, which allow detection of mutations in the viral genome associated with decreased sensitivity to antiretroviral compounds.[4] However, conventional sequencing techniques do not detect resistant minority species that circulate at low levels.[5] Determining the role of minority species in the development of drug resistance is essential.

Real-time PCR has previously been used for the detection of HIV reverse transcriptase and protease resistance mutations such

as K103N and V82A, respectively.[6,7] In this study, we describe the development of a sensitive real-time PCR assay for the detection and quantification of the D30N mutation.

Materials and methods

Clinical isolates

Ten PCR amplicons of known sequence derived from patient samples were obtained from HPA, Birmingham, UK. Nine were 30N mutants and one was wild-type (WT).

Generation of nelfinavir-resistant HIV-IIIB species by in vitro passage

MT-4 cells (0.5×10^6) were infected with HIV-IIIB at a multiplicity of infection of 0.001 (2 h), washed and resuspended in the presence of nelfinavir (a gift from Roche Pharmaceuticals, Welwyn, UK) at

*Corresponding author. Tel: +44-151-794-5919; Fax: +44-151-794-5656; E-mail: aowen@liv.ac.uk

Table 1. Oligonucleotide sequences (numbering from HIV-IIIB)

Name	Sequence	Position (HIV-IIIB)
Site-directed mutagenesis		
D30NF	5′-CTCTATTAGATACAGGAGCAGATAATACAGTATTAGAAGAAATGAGTT-3′	1899–1946
D30NR	5′-AACTCATTTCTTCTAATACTGTATTATCTGCTCCTGTATCTAATAGAG-3′	1946–1899
Nested protease gene PCR amplification		
OUTF	5′-AAGGGCTGTTGGAAATGTGG-3′	1566–1585
OUTR	5′-CTGTATGTCATTGACAGTCC-3′	2886–2905
INF	5′-CAGAGCCAACAGCCCCACCAG-3′	1729–1749
INR	5′-GGGCCATCCATTCCTGGCTT-3′	2167–2187
Real-time PCR amplification		
total viral DNA probe	TTTGGCAACGACCCCTC	1860–1885
total DNA forward primer	5′-AAGCAGGAGCCGATAGACAAG-3′	1794–1814
total DNA reverse primer	5′-GTTGCCCCCCTATCTTTATTGTG-3′	1867–1889
D30N probe	CAGGAGCAGATAATACAGTATT	1911–1932
D30N forward primer	5′-TCACTCTTTGGCAACGACCC-3′	1842–1861
D30N reverse primer	5′-TGGTTTCCATCTTCCTGGCAAA-3′	1945–1965
D30N probe-blocking primer	5′-CAGGAGCAGATGATACAGTATT-3′	1911–1932

an initial concentration of 20 nM. Cells were incubated (37°C, 5% CO_2, 6 days), and successful infection verified by observation of cytopathic effects. Viral supernatants were used to infect fresh cells in the presence of increasing concentrations of nelfinavir (final concentration 180 nM). Following each incubation, culture supernatants were frozen (-80°C) and stored for subsidiary analysis.

Reverse transcription-nested PCR amplification and sequencing of the protease gene

RNA was extracted from the viral supernatants using the QIAgen viral RNA extraction mini kit as described by the manufacturer. RNA was treated using the amplification grade DNase I kit (Sigma, Suffolk, UK) prior to reverse transcription using Taqman reverse transcription reagents (Applied Biosystems, Warrington, UK) and standard methodology. Nested PCR amplification was conducted in 50 μL volumes containing 1× *Taq* buffer, dNTPs (100 μM each) (Invitrogen, Carlbad, CA, USA), $MgCl_2$ (2.5 mM), *Taq* DNA polymerase (2.5 U, Eppendorf AG, Hamburg, Germany) and nuclease-free water. In addition, for first-round PCR amplification, INF and INR primers were included and for second-round PCR amplification, OUTF and OUTR primers were included (200 nM; Table 1). Following an initial denaturation step (5 min), samples were subjected to 30 cycles of denaturation at 95°C (1 min), annealing at 65°C (1 min) and extension at 72°C (2 min). PCR amplicons were purified using the Amersham S-400 purification spin columns as described by the manufacturer prior to sequencing. All sequencing was conducted by Lark Technologies Ltd using an ABI 3730*xl* DNA Analyzer.

Construction of a standard curve for real-time PCR amplification of the 30N mutation

In order to create a positive control, WT-PCR amplicon (HPA) was cloned into the pGEM-T vector (Promega), according to the manufacturer's instructions to generate pGEM-T-WT. The D30N mutation was then introduced using a Quick Change Site-Directed Mutagenesis Kit (Stratagene) and the primers are shown in Table 1. Successful mutagenesis was confirmed by direct sequencing using the INR primer (Lark Technologies Ltd). The mutated plasmid was purified using a QIAgen Mini Prep kit and quantified using PicoGreen. The concentration was standardized to 0.2 ng/μL and a standard curve containing 1%, 2%, 3%, 4%, 5%, 10%, 15% and 20% mutant protease was constructed by dilution with the WT construct. This standard curve was co-amplified in parallel to each real-time PCR amplification for quantification of the D30N mutant.

Real-time PCR detection of minority strains

The WT sequence and sequences from clinical samples carrying the D30N and other mutations were aligned in order to identify relatively highly conserved regions of the protease gene for primer design. Primers and allele-specific probes were synthesized by Applied Biosystems (Table 1). In order to avoid non-specific binding of the 30N probe to the WT DNA, a probe-blocking primer was added to the 30N amplification reaction that had the same sequence as the WT probe (unlabelled). Probe-blocking primer was added at a higher concentration to that of the probe for two reasons. First, for minority species WT DNA was by definition present at higher concentrations in the sample and second, enhanced specificity allowing greater sensitivity was attained (data not shown). Primers and probes were also designed for conserved regions of the *gag-pol* gene (avoiding D30N and other mutations) in order to quantify total viral copy number in the samples.

Real-time PCRs were carried out on the Opticon Monitor thermal cycler (Bio-Rad, CA, USA). Each reaction (25 μL) contained 1× Universal Master-mix (Abgene, Surrey, UK), 54 μM primers, 15 μM probe, 54 μM probe-blocking primer, nuclease-free water and DNA (5 μL). For amplification of total DNA, a probe-blocking primer was not required. All amplifications were carried out in duplicate for standards and samples. Following annealing at 94°C for 10 min, 40 cycles (30 s at 94°C, 30 s at 68.5°C and 30 s at 72°C) of amplification were performed. Quantification was allowed by plotting the ΔC_t of the standard curve mixtures [cycle threshold $C(t)D30N - C(t)total$]
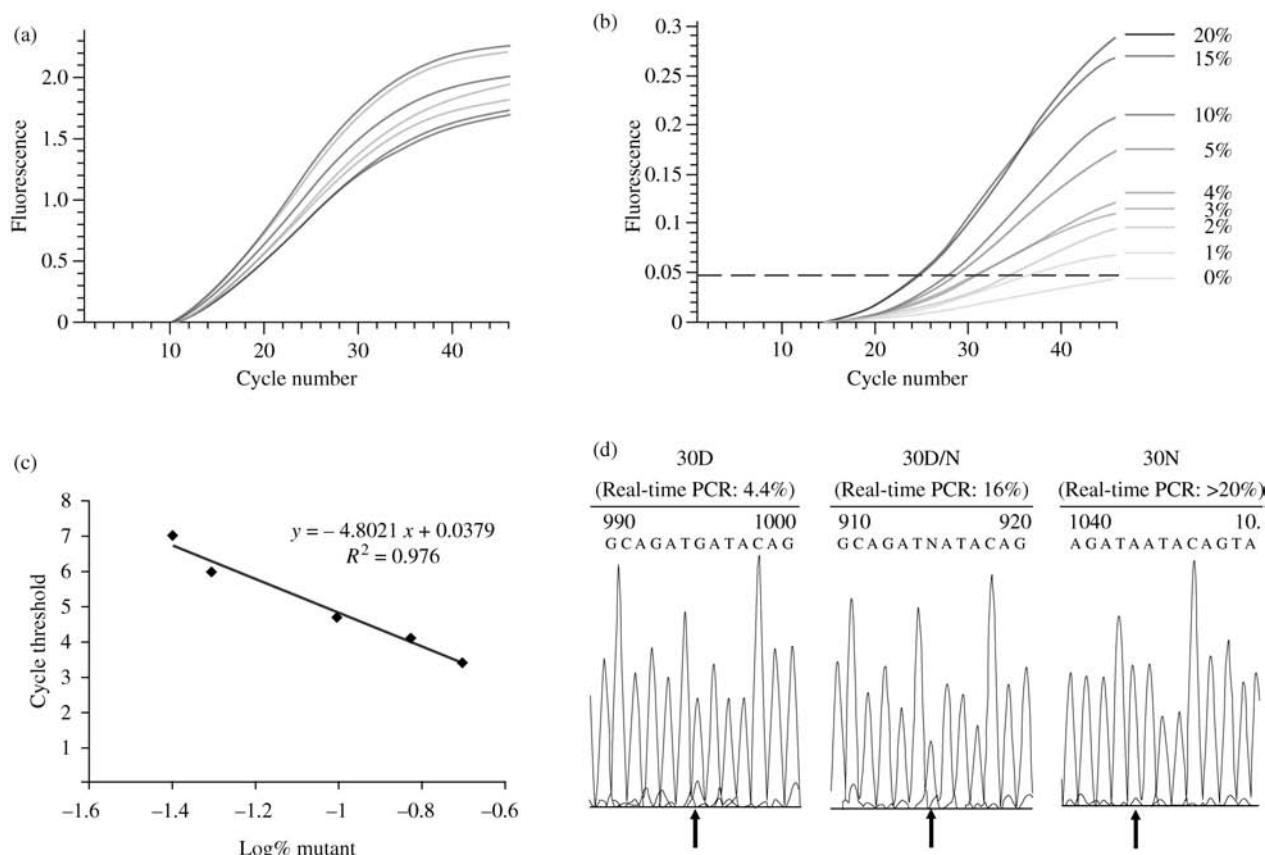
Figure 1. (a) Amplification of total DNA by the Taqman assay. (b) Detection of the D30N mutation in the mixtures of the standard curve. (c) Representative standard curve for the mixtures. (d) Representative sequence chromatograms for WT (30D), mixture (30D/N) and mutant (30N) isolates. The percentage of the mutation quantified in each sample by real-time PCR is also shown. A colour version of this figure is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

against log D30N percentage. The intra-assay variability was assessed using 10 replicates of high and low D30N concentrations (20% and 4%). For inter-assay variation, five of each sample were also assayed in three distinct reactions.

Results

Curves for D30N standards are shown for total and 30N amplifications in Figure 1(a) and Figure 1(b), respectively. The limit of quantification of the mutation, estimated from the standard curve (Figure 1c), was 4%, whereas the limit of detection as observed from the real-time PCR trace (Figure 1b) was 1%.

Coefficients of variation (CVs) for intra-assay variability of the 20% mixture were 13.1% and 0.2% for 30N and total amplification, respectively. For the 4% sample, CVs for intra-assay variability of 30N and total amplification were 12.9% and 2.4%, respectively. For inter-assay variability, the CV for 30N amplification was 1.9% and the CV for total amplification was 5.7%.

Of the 33 isolates derived from serial passage, 20 were found to contain the mutation (30N), 4 contained a mixture of the WT and the mutant (30D/N) and 9 were WT (30D) by sequencing (Figure 1d). When analysed using real-time PCR, 22 30N, 3 30D/N and 8 30D isolates were detected. Of the nine isolates that were negative for 30N by sequencing, one was found to contain the mutation at 4.4% by real-time PCR. Of the four

isolates designated 30D/N by sequencing, two were shown to contain the mutation at 12% and 16% and two at levels above 20%. Furthermore, all nine 30N amplicons derived from patient samples were found to contain the mutation (30N) when tested by real-time PCR.

Discussion

Real-time PCR assays have previously been developed for the detection of M184V, K103N, L90M and V82A.[6,7] However, an assay for detection of D30N, a mutation associated with nelfinavir treatment and resistance[8,9] has not been reported previously. In clinical and *in vitro* studies,[10,11] detection of 30N has been carried out by direct population sequencing, the detection limit of which is estimated at ~20%,[12,13] precluding the detection of minority species.

In this study, real-time PCR was assessed for its ability to detect and quantify the D30N mutation. A number of sequences of the *gag-pol* gene were aligned, in order to design primer and probe sets. Primers (specific to conserved regions of the gene) and a 30N-specific probe allowed the detection of the mutation as low as 1% and quantification as low as 4% of total viral DNA. This methodology is based upon allelic discrimination, which is routinely used for identification of host single nucleotide polymorphisms.[14–16] However, since humans are diploid,

the ratio of one allele to another at any given position will never be lower than 50% (one copy of each allele per chromosome). Therefore, in order to utilize this same chemistry to detect minority species of virus as low as 1% of the viral population it was necessary to include a blocking primer in order to minimize binding of the mutant probe to WT sequence at low mutant sequence concentrations. This approach was found to maximize specificity and sensitivity of the assay without compromising predictive power.

When the assay was assessed for its ability to detect 30N minority species (generated by *in vitro* serial passage), the mutation was detected in one isolate (at 4.4%) that would have been missed by sequencing. Quantification in isolates reported as 30D/N by sequencing was possible revealing the presence of the mutant at 12% and 16%. Furthermore, two of the 30D isolates harboured the mutation at levels above 20% further highlighting the insensitivity and inability to quantify using sequencing as they should have been characterized as 30D/N.

Several studies have reported minority resistant species in the early phase of therapy failure and that the emergence of predominantly resistant quasispecies is seeded by pools of minor variants.[17–19] The inability of conventional sequencing methods to detect mutant populations at a frequency of less than ∼20% of the total viral population limits their utility in this important area of research. Here, we describe a sensitive assay for the detection and quantification of the 30N mutation at a frequency as low as 1% of the viral population providing a tool for further investigations into the development of nelfinavir resistance.

Funding

Transparency declarations

A. O., S. H. K. and D. J. B. have received research funding from Boehringer Ingelheim, GlaxoSmithKline, Abbott Laboratories, Pfizer, AstraZeneca, Tibotec and Roche Pharmaceuticals.

Supplementary data

A colour version of Figure 1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

References

1. Clavel F, Hance AJ. HIV drug resistance. *N Engl J Med* 2004; **350**: 1023–35.

2. DeGruttola V, Dix L, D'Aquila R *et al.* The relation between baseline HIV drug resistance and response to antiretroviral therapy: re-analysis of retrospective and prospective studies using a standardized data analysis plan. *Antivir Ther* 2000; **5**: 41–8.

3. Cane P, Chrystie I, Dunn D *et al.* Time trends in primary resistance to HIV drugs in the United Kingdom: multicentre observational study. *BMJ* 2005; **331**: 1368.

4. Hirsch MS, Brun-Vezinet F, D'Aquila RT *et al.* Antiretroviral drug resistance testing in adult HIV-1 infection: recommendations of an International AIDS Society-USA Panel. *JAMA* 2000; **283**: 2417–26.

5. Gunthard HF, Wong JK, Ignacio CC *et al.* Comparative performance of high-density oligonucleotide sequencing and dideoxynucleotide sequencing of HIV type 1 pol from clinical samples. *AIDS Res Hum Retroviruses* 1998; **14**: 869–76.

6. Hance AJ, Lemiale V, Izopet J *et al.* Changes in human immunodeficiency virus type 1 populations after treatment interruption in patients failing antiretroviral therapy. *J Virol* 2001; **75**: 6410–7.

7. Metzner KJ, Bonhoeffer S, Fischer M *et al.* Emergence of minor populations of human immunodeficiency virus type 1 carrying the M184V and L90M mutations in subjects undergoing structured treatment interruptions. *J Infect Dis* 2003; **188**: 1433–43.

8. Sugiura W, Matsuda Z, Yokomaku Y *et al.* Interference between D30N and L90M in selection and development of protease inhibitor-resistant human immunodeficiency virus type 1. *Antimicrob Agents Chemother* 2002; **46**: 708–15.

9. Johnson VA, Brun-Vezinet F, Clotet B *et al.* Update of the drug resistance mutations in HIV-1: 2005. *Top HIV Med* 2005; **13**: 51–7.

10. Svedhem V, Lindkvist A, Bergroth T *et al.* Diverse pattern of protease inhibitor resistance mutations in HIV-1 infected patients failing nelfinavir. *J Med Virol* 2005; **76**: 447–51.

11. Patick AK, Mo H, Markowitz M *et al.* Antiviral and resistance studies of AG1343, an orally bioavailable inhibitor of human immunodeficiency virus protease. *Antimicrob Agents Chemother* 1996; **40**: 292–7.

12. Schuurman R, Brambilla D, de Groot T *et al.* Underestimation of HIV type 1 drug resistance mutations: results from the ENVA-2 genotyping proficiency program. *AIDS Res Hum Retroviruses* 2002; **18**: 243–8.

13. Schuurman R, Demeter L, Reichelderfer P *et al.* Worldwide evaluation of DNA sequencing approaches for identification of drug resistance mutations in the human immunodeficiency virus type 1 reverse transcriptase. *J Clin Microbiol* 1999; **37**: 2291–6.

14. Owen A, Chandler B, Bray PG *et al.* Functional correlation of P-glycoprotein expression and genotype with expression of the human immunodeficiency virus type 1 coreceptor CXCR4. *J Virol* 2004; **78**: 12022–9.

15. Leschziner G, Zabaneh D, Pirmohamed M *et al.* Exon sequencing and high resolution haplotype analysis of ABC transporter genes implicated in drug resistance. *Pharmacogenet Genomics* 2006; **16**: 439–50.

16. Kitteringham NR, Palmer L, Owen A *et al.* Detection and biochemical characterisation of a novel polymorphism in the human GSTP1 gene. *Biochim Biophys Acta* 2007; **1770**: 1240–7.

17. Charpentier C, Dwyer DE, Mammano F *et al.* Role of minority populations of human immunodeficiency virus type 1 in the evolution of viral resistance to protease inhibitors. *J Virol* 2004; **78**: 4234–47.

18. Dykes C, Najjar J, Bosch RJ *et al.* Detection of drug-resistant minority variants of HIV-1 during virologic failure of indinavir, lamivudine, and zidovudine. *J Infect Dis* 2004; **189**: 1091–6.

19. Lecossier D, Bouchonnet F, Clavel F *et al.* Hypermutation of HIV-1 DNA in the absence of the Vif protein. *Science* 2003; **300**: 1112.