**Are subtype differences important in HIV drug resistance?**

Lessells RJ[1,2], Katzenstein DK[3], de Oliveira T[1,4*]

[1] Africa Centre for Health and Population Studies, University of KwaZulu-Natal, Somkhele, South Africa

[2] Department of Clinical Research, London School of Hygiene and Tropical Medicine, Keppel Street, London, WC1E 7HT, United Kingdom

[3] Stanford University Medical Center, Department of Medicine, Stanford, California 94305, USA

[4] Department of Research of Infection, University College London, London, W1T 4JF, United Kingdom

*Corresponding author: Tulio de Oliveira

Africa Centre for Health and Population Studies

PO Box 198, Mtubatuba

South Africa

Email: tdeoliveira@africacentre.ac.za

Phone: +27-35-550-7500

Fax: +27-35-550-7565

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**Abstract**

The diversity of human immunodeficiency virus type 1 (HIV-1) has given rise to multiple subtypes and recombinant strains. The majority of research into antiretroviral agents and drug resistance has been performed on subtype B viruses, yet non-subtype B strains are responsible for 90% of global infections. Although it seems that combination antiretroviral regimens are effective against all HIV-1 subtypes, there is emerging evidence of subtype differences in drug resistance, relevant to antiretroviral strategies in different parts of the world. For this purpose, extensive sampling of HIV genetic diversity, curation and analyses are required to inform antiretroviral strategies in different parts of the world.

## Introduction

The last decade has seen substantial global scale-up of antiretroviral therapy (ART) for HIV infection and more than six million people are receiving ART in low- and middle-income countries [1]. Antiretroviral drug resistance is one of the main threats to global control of HIV [2]. The majority of persons living with HIV infection are infected with non-subtype B variants of HIV type 1 (HIV-1) [3]. There is increasing evidence that polymorphisms that occur naturally in different HIV-1 subtypes impact on drug resistance and susceptibility to antiretroviral drugs.

Here, we outline the latest developments in subtyping tools, drug resistance databases and review recent evidence from *in vitro* and clinical studies regarding drug resistance among HIV-1 subtypes (Box 1).

## HIV-1 origin, subtypes and recombinants

HIV-1 main group (group M) originated in West-Central Africa approximately 100 years ago [4,5]. It has since diversified into a large number of variants, including nine subtypes (A-D, F-H, J-K), six sub-subtypes (A1-A4, F1-F2), multiple (>48) circulating recombinants forms (CRFs) and thousands of unique recombinant forms (URFs) (Los Alamos HIV Sequence Database; URL: http://www.hiv.lanl.gov) [5,6]. The classification of recombinant viruses is based on complete genome analysis: CRFs are widespread, whereas URFs are restricted to a limited number of individuals [6]. The high number of existing HIV-1 variants is caused by both biological and epidemiological factors, which have been recently reviewed [4,5,7].

HIV-1 variants are continually introduced into new populations by mobility and migration [3,5-7]. As HIV-1 variants intermix in different part of the world, the likelihood of generating new recombinant viruses increases [6]. For example, a recent study in Quebec, Canada identified four subtypes, three CRFs and two new URFs. One of the new URFs is a recombinant of A/B (the RT/protease region was largely of subtype A, the integrase was subtype B) is spreading and may be classified as a new CRF once complete genomes are sequenced [8]. Studies in London have detected all HIV-1 subtypes, the majority of CRFs and many previously undetected URFs [9,10]. Identification of individuals infected with different subtypes is increasing in metropolitan areas [8,11].

**Subtyping tools and drug resistance databases**

HIV-1 subtyping can be achieved by automated subtyping tools. At the time of this review, over 400,000 isolates have been subtyped using the Rega HIV-1 subtyping tool using phylogenetic analysis to identify subtypes and CRFs. A recent upgrade has allowed the identification of many new CRFs and, for the first time, the classification of URFs [Rega HIV Subtyping Tool V3; URL: http://www.bioafrica.net]. Figure 1 shows a new feature of Rega Subtyping Tool V3, which is the phylogenetic identification of recombinant segments. A large comparison study of over 6,000 sequences, carefully subtyped by phylogenetic methods, was conducted to evaluate the accuracy of REGAv3 and six other subtyping tools (ACP Pena *et al*. 17th International Bioinformatics Workshop on Virus Evolution and Molecular Epidemiology, Belgrade, Serbia, August 2012). The comparison tools included two new, sophisticated tools: SCUEL [12] and COMET V2 (D Struck *et al*. 8th European HIV Drug Resistance Workshop, Sorrento, Italy, March 2010). The three tools identified most of the pure subtypes in *pol* with high sensitivity and specificity (>95%). COMETv2 and REGAv3 identify the two most important CRFs (CRF01_AE and CRF02_AG) in more than 95%. Given that the great majority (>90%) of the infections in the world are due to subtypes A, B C, CRF01_AE and CRF02_AG [3,5,7], these recent subtyping tools can accurately identify most of the epidemiologically important HIV-1 variants and classify new recombinants.

International and country specific drug resistance databases are important repositories of HIV-1 genetic data [13]. The UK Drug Resistance Database contains over 10,000 non-B subtype isolates and proposals can be submitted for the use of data [UK HIV Drug Resistance Database; URL: http://www.hivrdb.org.uk]. The Stanford HIV Drug Resistance Database (HIVDB) curates all published data and contains nearly 150,000 sequences. This is presented in many statistical and graphical formats [Stanford HIV Drug Resistance Database; URL: http://hivdb.stanford.edu]. HIVDB data and analyses have shown that, in spite of the large genetic variation found within subtypes, no major drug resistance mutation naturally occurs in naïve sequences [14,15]. However, a number of treatment-experienced mutation differences have been highlighted in the literature and are stored and curated in HIVDB. Table 1 summarises HIV drug resistance mutations associated to subtypes and CRFs in treatment experienced samples.

**Nucleoside and nucleotide reverse transcriptase inhibitors**

The lysine to arginine mutation at position 65 (K65R) is a major mutation which confers broad high-level resistance to most nucleoside- and nucleotide-reverse transcriptase inhibitors (NRTI/NtRTIs), except zidovudine. There is a substantial body of evidence that K65R emerges more frequently and more rapidly in subtype C viruses than in subtype B.

It has now been demonstrated that the difference in selection of K65R between subtypes B and C is related to the template nucleotide sequence and preferential pausing of reverse transcription at position 65. The nucleotide sequence at codons 64-65-66 differs between subtypes B and C and subtype C viruses contain a homopolymeric stretch of adenine bases. This leads to RT pausing during the synthesis of double-stranded DNA from the single-stranded DNA intermediate template, a process which is template-specific but independent of the RT enzyme [16]. Subsequent misalignment of the template and primer leads to the AAG to AGG change responsible for the K65R mutation [17].

Several recent studies have used ultra-deep pyrosequencing (UDPS) or allele-specific PCR (AS-PCR) to explore the frequency of low-level K65R mutants in both ART-naïve and ART-experienced individuals [18-22]. In one study using UDPS, the frequency of K65R at both >1% and >0.4% levels was higher in subtype C compared to subtype B and non-B/non-C subtypes [18]. In another study, the K65R mutation was detected at a higher frequency in ART-naïve subtype C individuals compared to those infected with subtype CRF01_AE (6% vs. 1%) and subtype B [19]. In ART-experienced patients with virological failure and without K65R on conventional Sanger sequencing, one study detected the presence of K65R by AS-PCR in 13% (4/30) of patients [20]; conversely other groups using UDPS detected no additional mutations in those without K65R on Sanger sequencing [21]. It is important to note the limitations of these highly sensitive sequencing techniques and spurious detection of the K65R mutation through PCR-induced mutation has been demonstrated [22].

There is recent clinical evidence demonstrating frequent and early emergence of K65R on tenofovir-based first-line ART regimens in South Africa[23]. Recent analysis of large scale implementation of TDF, 3TC and NVP indicates a higher rate of virological failure with this regimen[24]. In addition, a case report has documented the presence of low-level K65R mutants pre-treatment by clonal analysis, with enhancement of K65R variants within two months of treatment [25]. There is an urgent need for further research to determine the prevalence and impact of low-level K65R mutants, especially in settings where subtype C predominates and where tenofovir is now a component of first-line ART regimens.

**Non-nucleoside reverse transcriptase inhibitors**

The emergence of NNRTI resistance mutations occurs after single dose nevirapine (sdNVP) for the prevention of mother-to-child HIV transmission. Previous work has suggested that this occurs more frequently with subtype C viruses [26,27]. One more recent study showed that resistance mutations could be demonstrated using allele-specific PCR in 25% of patients more than 24 months after sdNVP exposure (C Yang *et al*. 17th Conference on Retroviruses and Opportunistic Infections, San Francisco, California, February 2010). However, the clinical significance of this is uncertain as the same study demonstrated no association between the presence of resistance mutations and virological failure on a subsequent NNRTI-based regimen (PJ Weidle *et al*. 17th Conference on Retroviruses and Opportunistic Infections, San Francisco, California, February 2010).

Etravirine is a second-generation NNRTI which retains activity against strains with some resistance to nevirapine and efavirenz and which might therefore be an option as a component of a salvage regimen for antiretroviral-experienced patients. It has demonstrated good efficacy across subtypes [28]. There are a number of etravirine resistance-associated mutations (RAMs) which reduce the response to etravirine. These mutations are commonly present as polymorphisms in ART-naïve individuals infected with non-B subtypes, especially CRF02_AG [29]. There is conflicting evidence on the resistance pathways selected by etravirine therapy. One study found E138K the first mutation to emerge in subtypes B, C and CRF02_AG [30]. A separate study found the same for subtype C but demonstrated preferential selection of Y181C for subtype B virus [31].

A novel mutation in the C-terminal domain of RT (N348I) has recently been reported to reduce susceptibility to etravirine in subtypes A, B and C [32]. One clinical trial in South Africa found the N348I mutation present in 24% of patients failing first-line NNRTI regimens with subtype C virus, most commonly with nevirapine[33]. This mutation is not included in standard mutation lists or algorithms but more data are urgently required to determine clinical relevance.

Rilpivirine (RPV) is another second-generation NNRTI with equal efficacy and similar patterns of resistance across subtypes.[34,35]. However, regardless of subtype, RPV has suboptimal efficacy compared to efavirenz in ART-naïve individuals with HIV RNA >100,000 copies/ml [35].

**Protease inhibitors**

Non-polymorphic mutations in the protease gene have a greater impact on baseline susceptibility to protease inhibitors than polymorphic mutations [15]. However, recent evidence has suggested that

the polymorphism at codon 36 in the protease gene (M36 in subtype B and I36 in most other subtypes) affects both the patterns of resistance that emerge under drug pressure and viral replication capacity [36]. Similarly, the M89 polymorphism in subtypes A, C, and CRF01_AE (L89 in subtype B) preferentially leads to the emergence under drug pressure of the M89T mutation, which confers high-level resistance to nelfinavir, atazanavir and lopinavir [37]. There is also *in vitro* evidence that CRF2_AG viruses with the 17E/64M polymorphisms demonstrate hypersusceptibility to certain protease inhibitors (nelfinavir, atazanavir and indinavir) [38].

Mutations in the *gag* cleavage sites and gag matrix protein are known to contribute to protease inhibitor resistance and polymorphisms in this region may be more common in non-B subtypes [39,40]. Baseline polymorphisms in this region have been shown to affect virological outcomes with lopinavir/ritonavir monotherapy [41]. The importance of the subtype differences in *gag* are not well defined but may be more important in boosted PI monotherapy, which is under investigation for second-line therapy in resource-limited settings [42].

**Integrase inhibitors**

Raltegravir, a first-generation integrase inhibitor (INI), has demonstrated good efficacy in ART-naïve and ART-experienced individuals infected with different HIV subtypes [43]. However, both raltegravir and the other first-generation INI elvitegravir have a relatively low genetic barrier to resistance. The primary mutations in the integrase gene associated with INI resistance are E92Q, Y143R/C, Q148K/R/H and N155H. The residues associated with primary resistance seem to be highly conserved across subtypes, but polymorphisms at the sites of secondary mutations are more common in non-B subtypes [8,44-46]. There is some evidence that the effect of certain integrase mutations might differ according to subtype. Subtype B integrase enzyme with the N155H mutation (±E92Q) exhibited increased resistance to raltegravir compared to the subtype C enzyme [47].

Dolutegravir and MK-2048 are second-generation integrase inhibitors in development that have higher genetic barriers to resistance and retain activity against viruses with resistance to raltegravir or elvitegravir. There is some early evidence to suggest subtype differences could modulate the emergence of resistance to these drugs. In both INI naïve and raltegravir-experienced individuals, polymorphisms at codons 101 and 124 were more frequent in non-B subtypes than subtype B; these mutations were particularly prevalent in subtypes C and CRF02_AG [48]. Whilst the R263K mutation seems to be the most common mutation selected during dolutegravir therapy in subtype B, the

G118R mutation previously associated with MK-2048 resistance might be a more common pathway in subtype C [49,50].

**Clinical efficacy of new antiretroviral agents by HIV-1 subtype**

Clinical evidence of different subtype responses to antiretroviral therapy (ART) might be the first indicator of subtype differences in the development of drug resistance. Inclusion of individuals infected with different subtypes is increasingly the norm in clinical trials of new antiretroviral agents, although the numbers infected with some non-B subtypes are quite low. Table 2 shows the virological responses by HIV-1 subtype in recently published clinical trials of new antiretroviral agents.

Cohort studies can also provide evidence of subtype differences in ART responses, although this is complicated by the use of different ART regimens. A collaborative group in the UK found that virological outcomes were broadly similar between subtypes A, B and C [51], while a more recent study from the Swiss HIV Cohort Study, which restricted analysis to white Caucasians, infected with non-B subtypes, had a lower risk of virological failure which was particularly apparent for subtypes A and CRF02_AG [52].

**Conclusions**

There is no compelling evidence that HIV-1 subtype needs be considered in the choice of ART regimens for first- or second-line therapy, and other considerations of cost, effectiveness, toxicities and tolerability are more important in low- and middle-income countries. However, recent evidence of subtype differences in drug resistance could potentially impact on antiretroviral strategies. The large amount of resistance data produced as part of surveillance studies and clinical care can be used to explore the differences in drug resistance between HIV-1 subtypes. National HIV drug resistance databases such as the ones in the UK, Switzerland and, recently, the Southern African Treatment and Resistance Network (SATuRN) Stanford and Rega public drug resistance databases are very useful national strategic resources to tackle the spread of drug resistance. The appreciation of subtype differences is also important to the development of new drugs, treatment strategies, drug sequencing, assessing response to treatment and surveillance for the transmission of resistance. In each of these areas, and in tracking the evolution of the HIV pandemic, differences among subtypes continue to play an important role.

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**Table 1** Recently described drug resistance mutations between HIV-1 subtypes and CRFs and impact on antiretroviral drug resistance and susceptibility

| Position | Mutation | Comments | Reference |
|---|---|---|---|
| Reverse transcriptase | | | |
| RT65 | K65R | Subtype C - AAG (K); subtype B - AAA (K): preferential pausing of reverse transcription, related to homopolymeric stretch of adenine bases | [16,17] |
| RT138 | E138K | E138K the first mutation to emerge in subtype C during etravirine therapy | [30] |
| RT181 | Y181C | Preferential selection of Y181C for subtype A and B during etravirine therapy | [31] |
| RT348 | N348I | Reduces susceptibility to etravirine in subtypes A, B and C. High prevalence in subtype C samples from patients failing first-generation NNRTIs | [32,33] |
| Protease | | | |
| PR17 | 17E | CRF2_AG hypersusceptibility to nelfinavir, atazanavir and indinavir | [38] |
| PR36 | - | Subtype C - ATA (I); subtype B - ATG (M): affects susceptibility to protease inhibitors and viral replication capacity | [36] |
| PR64 | 64M | CRF2_AG hypersusceptibility to nelfinavir, atazanavir and indinavir | [38] |
| PR89 | M89T | Subtype C - ATG (M); subtype B - CTG (L): leads to preferential emergence of M89T in subtype C | [37] |
| Integrase | | | |
| IN92 | E92Q | N155H/E92Q double mutant 10-fold more resistant to raltegravir and elvitegravir in subtype B versus subtype C | [47] |
| IN101 | L101I | Present more frequently in non-B subtypes compared to subtype B (both INI-naïve and RAL-experienced) | [48] |
| IN118 | G118R | Most common resistance pathway during dolutegravir therapy in subtype C | [50] |
| IN124 | T124A | Present more frequently in INI-naïve non-B subtypes compared to subtype B | [48] |
| IN155 | N155H | Subtype B with this mutation more resistant to raltegravir (and elvitegravir) than subtype C | [47] |
| IN263 | R263K | Most common resistance pathway during dolutegravir therapy in subtype B | [49] |

**Table 2** Virological outcomes with specific antiretroviral drugs across different HIV-1 subtypes
*Virological outcomes reported only for regimen including investigational drug, except for ARTEMIS where lopinavir and darunavir both reported*

| **Clinical trials** | | | | | |
|---|---|---|---|---|---|
| **Drug name** | **Clinical trial(s)** | **Patient population** | **Participants (by subtype)** | **Virological outcomes[a]** | **Reference** |
| Lopinavir | ARTEMIS | ART naïve | 208 subtype B<br>50 subtype C<br>87 other subtypes | 78% subtype B<br>82% subtype C<br>78% other subtypes | Dierynck 2010 [53] |
| Darunavir | ARTEMIS | ART naïve | 210 subtype B<br>39 subtype C<br>93 other subtypes | 81% subtype B<br>87% subtype C<br>88% other subtypes | Dierynck 2010 [53] |
| Etravirine | DUET-1, DUET-2 | ART experienced | 561 subtype B<br>33 non-B subtypes (no subtype C) | 60% subtype B<br>73% non-B subtypes | Vingerhouts 2010 [28] |
| Rilpivirine | ECHO, THRIVE | ART naïve | 485 subtype B<br>76 subtype C<br>125 other subtypes | 84% subtype B<br>86% subtype C<br>86% other subtypes | Cohen 2012 [34] |
| Maraviroc | MERIT | ART naïve | Not reported | 70% subtype B<br>61% subtype C<br>57% other subtypes | Cooper 2010 [54] |
| Raltegravir | STARTMRK | ART naïve | 219 subtype B<br>19 subtype C<br>32 other subtypes | 89% subtype B[b]<br>95% non-B subtypes[b] | Rockstroh 2011 [43] |
| Raltegravir | BENCHMRK-1, BENCHMRK-2 | ART experienced | 416 subtype B<br>3 subtype C<br>33 other subtypes | 61% subtype B[b]<br>67% non-B subtypes[b] | Rockstroh 2011 [43] |
| **Cohort studies** | | | | | |
| **Cohort** | **Drug regimens** | **Patient population** | **Participants (by subtype)** | **Virological outcomes** | **Reference** |

| | | | | | |
|---|---|---|---|---|---|
| UK CHIC | 2 NRTI + NNRTI<br>2 NRTI + boosted PI | ART naïve | 1550 subtype B<br>272 subtype C<br>66 subtype A | 89% subtype B[c]<br>94% subtype C[c]<br>97% subtype A[c] | Geretti 2009 [51] |
| Swiss HIV cohort | Various | ART naïve and mono/dual-NRTI experienced | 2166 subtype B<br>383 non-B subtypes | 89% subtype B[d]<br>90% non-B subtypes[d] | Scherrer 2011 [52] |

a Outcome HIV RNA <50 copies/ml at week 48 unless otherwise stated

b HIV RNA <50 copies/ml at week 96; data not reported separately for subtype C

c HIV RNA <50 copies/ml at 12 months

d HIV RNA <50 copies/ml 90-365 days after ART initiation; results only shown for those who started ART 1999-2009

**Box 1 Summary of main concepts**

- HIV-1 diversity has given rise to numerous subtypes and recombinant forms
- New subtyping tools (e.g. Rega HIV-1 Subtyping Tool version 3, SCUEL and COMET) can accurately identify the most important HIV-1 variants
- National and international public drug resistance databases are useful resources to trace the evolution of drug resistance in different subtypes
- HIV-1 subtype genetic variation can influence the development of drug resistance and the susceptibility to certain antiretroviral drugs
- K65R is an example of a clinically relevant mutation that emerges more frequently and more rapidly in subtype C viruses compared to subtype B; this has been shown to be related to the different template nucleotide sequence
- Evidence from recent clinical trials and cohort studies suggests that response to combination antiretroviral regimens does not differ substantially by HIV-1 subtype
- Appreciation of subtype differences is important in the development of new drugs and in the formulation of antiretroviral strategies

**Figure 1** Recombination profile and phylogenies of recombinant regions of a CRF03_AB isolate [Rega HIV Subtyping Tool V3; URL: http://www.bioafrica.net].

One of the new features of Rega Subtyping Tool version 3.0 is that it can perform detailed recombination analyses. The tool detects recombination, identifies the recombinant fragments and creates a phylogenetic tree for each of the fragment. This figure show a CRF recombinant A/B sequence (CRF03_AB, Genbank accession number: ). The subtype A region is from position 2252 to 2782 (Protease amino acid position 1-99 and RT 1-78) and subtype B from 2782 to 4822 (RT amino acid position 79-440 and Integrase amino acid position 1-198). Numbering is performed based on the complete genome reference sequence HXB2.