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Subtypes A1 and D, and recombinant HIV-1 natural polymorphisms associated with lenacapavir drug resistance in Uganda

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Background: Lenacapavir, a novel HIV-1 capsid inhibitor, shows promise for treating MDR HIV-1, as well as for pre-exposure prophylaxis (PrEP) in prevention of HIV infection. Its unique mechanism and lack of cross-resistance with other antiretroviral classes make lenacapavir a significant addition to HIV therapy. The clinical trials CALIBRATE and CAPELLA have demonstrated high viral suppression rates in both ART-naive individuals and individuals with MDR HIV-1. Lenacapavir-associated resistance mutations, such as M66I and Q67H, rarely seen as natural polymorphisms in lenacapavir-naive populations, are predominantly studied in subtype B HIV-1.

Objectives: Our study aimed to investigate the prevalence of lenacapavir resistance-associated mutations in HIV-1 subtypes A1 and D in a cohort of individuals living with HIV-1 from southwestern Uganda.

Methods: Utilizing plasma samples from ART-naive adults living in Uganda, HIV-1 Gag p24 (capsid) sequences were analysed for lenacapavir resistance mutations.

Results: Among 546 lenacapavir-naive participants, no major lenacapavir resistance-associated mutations were found. Minor mutations were present in 1.6% of participants, with T107A being the most common. Longitudinal data indicated the persistence of T107A for at least 3 years post-ART initiation in one participant. Phylogenetic analysis indicated individuals carrying T107A were found independently in distinct locations within the tree, suggesting that T107A might have arisen from multiple distinct base substitution events. Shannon entropy analysis showed high variability in certain capsid sites, but none overlapped with known lenacapavir resistance sites.

Conclusions: These findings suggest a low prevalence of naturally occurring lenacapavir resistance mutations in Uganda, supporting lenacapavir's potential efficacy in this region.

Introduction

Lenacapavir is a HIV-1 capsid inhibitor recently FDA-approved for the treatment of heavily treatment-experienced individuals with MDR HIV-1 and has shown to be efficacious as pre-exposure prophylaxis (PrEP) in prevention of HIV infection.[1–3] Lenacapavir is long-acting and can be administered as either an injection or as tablets, or can be given in combination with other antiretrovirals.[4] Given its novel mechanism and viral gene target, no cross-resistance between lenacapavir and other ART drug classes has been reported.[5]

Clinical trials that have studied the efficacy of lenacapavir for treatment of HIV-1 have included people living with HIV (PLWH)

who were previously ART-naive (CALIBRATE), as well as individuals with MDR HIV-1 (CAPELLA), with both studies demonstrating high rates of viral suppression (92% at 54 weeks and 83% at 26 weeks, respectively).[6,7] These clinical trials, in addition to data from *in vitro* studies,[8,9] have identified lenacapavir-associated substitution mutations in the capsid associated with decreased lenacapavir susceptibility, such as M66I and Q67H.[7–9]

Reassuringly, studies among PLWH who are lenacapavir-naive have shown these mutations are rarely observed as natural viral polymorphisms.[10] However, these data mostly originate from populations in which subtype B HIV-1 is most common,[10,11] whereas other genetically distinct HIV-1 subtypes are the predominant strains circulating in sub-Saharan Africa where HIV prevalence is highest.[12] An analysis of sequences from the Los Alamos National Laboratory database previously reported a 0.19% prevalence of polymorphisms associated with lenacapavir resistance and, notably, sequences were predominantly (42.07%) HIV-1 subtype B, with subtypes commonly found in East Africa (subtypes A1 and D) accounting for <10% of sequences analysed.[11] Similarly, a study analysing specimens from 1500 PLWH who were either ART-naive or ART-experienced, but all naive to lenacapavir, did not identify polymorphisms associated with lenacapavir resistance in any of the samples analysed. However, as with the first study, HIV-1 subtypes that are most common in East Africa were under-represented, accounting for only <5% of the study population.[10] A recent study of 10057 sequences from clinical trials and publicly available datasets (5% A1 and 4% D subtypes), reported a 0% prevalence of polymorphisms associated with lenacapavir resistance, except for T107A.[13] This study reported that T107A was present at 1.8% and 0% among the 489 subtype A1 and 430 subtype D genomes analysed, respectively.

Importantly, a clinical trial on the use of twice-yearly lenacapavir as PrEP among women and adolescent girls in South Africa and Uganda (PURPOSE 1) recently reported no incidence of HIV infections, showing promise for the use of lenacapavir in sub-Saharan Africa.[3] Thus, in light of HIV-1's genetic diversity, it is important to survey whether non-B HIV-1 subtypes contain natural polymorphic mutations that are associated with lenacapavir resistance before this drug is considered for broad implementation in regions affected by non-B subtypes. In this study, we examined HIV-1 Gag p24 (capsid) sequences from a cohort of PLWH in southwestern Uganda living with predominantly subtype A1 and D HIV-1 to estimate the prevalence of naturally occurring polymorphisms associated with lenacapavir resistance in this geographic region.

Methods

Ethics

The study was approved by the Mbarara University of Science and Technology Human Subjects Committee (14/01-03), the Uganda Council of Science and Technology (HS 938), Partners Healthcare Human Subjects Committee (2011P000522), the University of British Columbia/Providence Healthcare Research Ethics Board (H11-01642), the University of California Human Research Subjects Committee (10-03457), and the Weill Cornell Medical College Institutional Review Board (19-12021173). All participants provided signed consent forms.

Cohort description

The Uganda AIDS Rural Treatment Outcomes (UARTO) cohort enrolled ART-naive adults aged 18 years and above who were living with HIV and who were in the care of the Mbarara Regional Referral Hospital Immune Suppression Syndrome Clinic in Mbarara, Uganda, between 2002 and 2010. Participants were followed longitudinally after ART initiation with study visits approximately every 3–4 months until 2015.[14] No participants had exposure to lenacapavir. Plasma samples were collected at study entry prior to ART initiation and at all follow-up visits.

HIV-1 gag sequencing

Archived plasma samples collected at any timepoints with detectable viral load (defined as >40 copies/mL) pre- and post-ART initiation were subjected to Sanger sequencing of HIV-1 *gag*. Briefly, total nucleic acids were extracted from plasma samples using NucliSENS® easyMAG, and were subjected to nested PCR amplification targeting HXB2 coordinate 680–2724[15] followed by Sanger sequencing. In cases of PCR and/or sequencing failure in the first attempt, samples were processed using two additional sets of alternative PCR primers and eight additional sequencing primers (Figure S1, available as Supplementary data at *JAC* Online). All primers used in this study are listed in Table S1. HIV-1 *gag* nucleotide sequences were aligned using the in-house software ReCALL, which uses a modified Needleman–Wunsch algorithm for alignment.[16]

HIV-1 subtyping

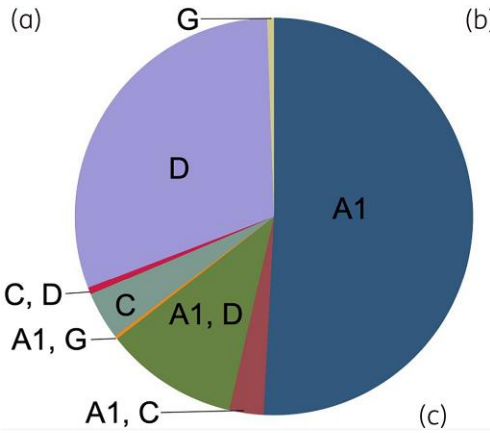
Viral subtypes were determined using RIP 3.0 (primary subtyping method), which was then compared against results obtained from REGA 3.0 and an in-house Hamming distance method written in Pearl language (Figure 1). For the in-house Hamming distance method, we pairwise-aligned each *gag* nucleotide sequence against HXB2 using a modified GOTOH algorithm.[17] Deletions are padded with '-' characters and are forced to obey codon boundaries, and insertions relative to HXB2 are stripped out. Insertions that are 'close' to positions where insertions are commonly observed (i.e. *gag* codons 111, 119, 125, 374, 388, 428, 441, 452, 457, 471, 481) are forced to be at those positions. Each sequence is then compared with a set of 40 subtype reference sequences downloaded from the Los Alamos HIV Sequence Database subtype reference that have each also undergone the same alignment, gap-padding and insertion-stripping procedure as above, and pairwise concordance is calculated. Any nucleotide mismatch between the query and reference sequence is counted as a difference (i.e. mixtures, indels are considered mismatches). The reference sequence with the highest concordance with the query is considered the best match, and the subtype of that reference is assigned to the query.

Gag sequence analyses

Nucleotide sequence alignment was performed using MUSCLE against reference sequence HXB2 and then the p24 (capsid) region was translated into amino acids to locate lenacapavir resistance mutations listed below. Phylogenetic analysis was performed using PhyML, available in the Los Alamos HIV Sequence Database.[18] Shannon entropy was performed using the Entropy tool (option: Entropy-One) in the Los Alamos HIV Sequence Database.[19] All nucleotide sequences corresponding to Gag p24 (capsid) in this study were uploaded to GenBank (accession numbers MH971615–MH971951[20] and PP575129–PP575532).

Definition of lenacapavir resistance mutations

We defined lenacapavir-associated resistance mutations according to the 2022 IAS–USA drug resistance mutations list,[21] including major mutations M66I and Q67H, and minor mutations L56I, K70N/S/R, N74D/S, A105T and T107N.[8] We also examined mutations associated with resistance reported in other studies[22–25] and in the Stanford HIVdb version 9.6[26] including Q67K/N/Y, N57S, K70H, N74H, A105E/S and T107A/C.



(b)

HIV-1 Subtype by RIP 3.0	Counts	Percentage
A1	278	50.9%
A1, C	15	2.7%
A1, D	59	10.8%
A1, G	2	0.4%
C	20	3.7%
C, D	3	0.5%
D	166	30.4%
G	3	0.5%
Total	546	100.0%

(c)

Subtyping Tools	Count Concordance	Total	Percentage
Hamming-RIP	630	741	85%
Hamming-REGA	604	741	82%
RIP-REGA	694	741	94%
Hamming-RIP-REGA	604	741	82%

Figure 1. UARTO cohort HIV-1 subtype distribution. The primary subtyping method used in this study was RIP 3.0 (Los Alamos HIV Sequence Database). (a) and (b) The predominant circulating HIV-1 subtypes in the UARTO cohort were subtype A1 and D. (c) RIP results were compared with subtyping by Hamming distances against subtype references and REGA. Overall, the three subtyping methods were 82% concordant. All discordant cases between Hamming distances and RIP were associated with RIP calling a sequence 'recombinant' while Hamming distance could not identify recombinants. Likewise, all discordance cases between Hamming and REGA were associated with recombinants in REGA but not in Hamming. In 27 of the 47 discordant cases between RIP and REGA, RIP called non-recombinants in 10/27 subtype A1, 1/27 subtype C, 13/27 subtype D and 3/27 subtype G sequences, whereas REGA labelled them as intersubtype recombinants, and in the rest of the 20/47 discordant cases, both algorithms concluded intersubtype recombinants but differed in the subtype compositions. Since RIP is an algorithm designed specifically for predicting recombinants, we have selected it as the primary subtyping method used in our final reporting in this study.

Statistical analyses

All statistical analyses were performed using STATA15. Statistical significance was defined as $P < 0.05$.

Results

Cohort characteristics

A total of 609 treatment-naive individuals were enrolled in the UARTO cohort over a period of 9 years between 2002 and 2010 (Figure S2). At enrolment, the median age was 34 (IQR 29–39) years, and 68% of the participants were female. The median viral load prior to ART initiation was 5.15 $\log_{10}$ copies/mL (IQR 4.63–5.64), and the median CD4 count was 124 cells/mm^3 (IQR 59–197) (Table 1). All participants went on to receive an NNRTI-containing ART regimen. Median follow-up time was 5.8 years (IQR 0.3–7.0). From the 609 study participants, 741 *gag* sequences were obtained from 546 individuals (90%). Of the 546 individuals, 461 only had pre-ART samples (461 *gag* sequences), 81 had both pre- and post-ART samples (272 *gag* sequences) and 4 had only post-ART samples (8 *gag* sequences). Cohort viral subtype distribution by RIP was 50.9% (A1), 3.7% (C), 30.4% (D), 0.5% (G) and 14.5% intersubtype recombinants by RIP 3.0 (Figure 1a and b). RIP subtyping results were 85% concordant with subtyping by Hamming distances against subtype references and 94% concordant with subtype calls generated by REGA (Figure 1c). In this study, we have selected RIP as the primary subtyping method used in our final reporting since it is an algorithm designed specifically for predicting intersubtype recombinants, which are prevalent in this geographical region.

HIV-1 gag sequencing

We were unable to obtain *gag* sequences from 63/609 UARTO cohort participants despite multiple PCR attempts with multiple primers or due to sample unavailability (Figures S1 and S2). Thus, we compared demographic and pre-treatment initiation baseline characteristics between individuals who did and did not yield *gag* sequence data to examine for bias in sampling. We observed that there was no statistically significant difference in age (Mann–Whitney test *P* value 0.150), viral load (Mann–Whitney test *P* value 0.098) and sex (χ^2 test *P* value 0.699) between participants with and without *gag* sequence data. However, participants with no *gag* sequence data had significantly lower median CD4 count/mm^3 (Mann–Whitney test *P* value 0.005) and were enrolled in earlier years of the study (Mann–Whitney test *P* value <0.001) compared with those with *gag* sequence data (Table 1).

Lenacapavir-resistance associated mutations in UARTO

Our cohort was lenacapavir-naive, and thus sequences represent naturally occurring capsid polymorphisms detected in non-subtype B HIV-1 strains circulating in southwestern Uganda. Of our 546 participants, none had natural viral polymorphisms associated with major mutations conferring decreased susceptibility to lenacapavir as defined by the 2022 IAS–USA drug resistance mutations list. Minor mutations were detected in 9/546

Table 1. Participant pre-therapy initiation baseline characteristics (UARTO cohort, $n=609$)

	Participants with *gag* sequence data ($n=546$)	Participants without *gag* sequence data ($n=63$)	*P* value	All participants ($n=609$)	Statistical test
Age, years, median (IQR)	34 (29–39)	36 (31–40)	0.150	34 (29–39)	Mann–Whitney
CD4 count/mm^3, median (IQR)	127 (65–196)[a]	73 (9–204)[b]	0.005	124 (59–197)	Mann–Whitney
Viral load (log copies/mL), median (IQR)	5.16 (4.67–5.65)	5.12 (4.14–5.64)	0.098	5.15 (4.63–5.64)	Mann–Whitney
Enrolment year, median (IQR)	2007 (2006–07)	2004 (2003–07)	<0.001	2007 (2005–07)	Mann–Whitney
Sex female, *n* (%)	377 (69)	38 (60)	0.699	415 (68)	Chi-squared
Initial ART, *n* (%)					
3TC/ZDV/NVP	314 (58)	16 (25)	<0.001	330 (54)	Chi-squared
3TC/d4T/NVP	170 (31)	42 (67)		212 (35)	
3TC/ZDV/EFV	50 (9)	1 (2)		51 (8)	
Others	8 (1)	0 (0)		8 (1)	
Missing	4 (1)	4 (6)		8 (1)	

Statistical significance was defined as a *P* value of <0.05. 3TC, lamivudine; ZDV, zidovudine; NVP, nevirapine; d4T, stavudine; EFZ, efavirenz.
[a]In the group with *gag* sequence data ($n=546$), CD4 count information was unavailable from two individuals (MBA1007, MBA1476) and were excluded from the statistical analysis.
[b]In the group without *gag* sequence data ($n=63$), CD4 count information was unavailable from four individuals (MBA1200, MBA1254, MBA1424, MBA1032) and were excluded from the statistical analysis.

participants (1.6%; 95% CI 0.8%–3.1%). Of these nine individuals, the most common capsid polymorphism was T107A, found in seven participants prior to ART initiation (four individuals with subtype A1 and three with subtype D). Longitudinal *gag* sequence data were available for one of the seven individuals, and showed that T107A persisted for at least 3 years post-ART initiation. Other polymorphisms identified in this cohort were T107T/C/S, identified in one participant (subtype A1) prior to ART initiation, and K70R, identified in one participant (C/D recombinant subtype) 6 months after ART initiation despite having WT K70 prior to ART initiation.

Since T107A was the only resistance-associated mutation observed in multiple individuals, we next investigated whether there were any phylogenetic links between viral genomes containing this mutation. A maximum-likelihood phylogenetic tree of these viral sequences revealed that capsid sequences containing T107A were found independently in distinct locations within the phylogenetic tree and neighbouring sequences did not contain T107A, suggesting a likelihood that T107A might have arisen from multiple distinct base substitution events (Figure 2). Next, we examined whether highly polymorphic sites in Gag p24 were associated with sites linked to lenacapavir resistance. Shannon entropy analyses of p24 amino acid sequences revealed that the top-three least-conserved sites were A14, P207 and T171 (subtype A1) and I91, G116 and A14 (subtype D), none of which overlapped with previously reported resistance mutation sites. Table 2 summarizes all non-WT mutations observed in this cohort.

Additional polymorphisms that are not currently defined as lenacapavir-associated mutations on the 2022 IAS drug resistance mutations list were identified (Table 2). The most common of these additional polymorphisms were L56M ($n=258$) and T107S ($n=10$), both of which were found across multiple subtypes.

Discussion

In this analysis of Gag p24 sequences from PWH in Uganda with diverse HIV-1 subtypes and no history of exposure to lenacapavir, we did not identify any naturally occurring viral polymorphisms associated with major lenacapavir resistance. We did identify polymorphisms associated with minor resistance to lenacapavir in 1.6% of participants ($n=9/546$), with T107A identified most commonly. Reassuringly, the low prevalence of lenacapavir-associated resistance mutations identified in this cohort is consistent with previous reports from cohorts that included fewer participants with subtypes A and D.[10,11,13] We also identified polymorphisms that are not currently included in the 2022 IAS–USA drug resistance mutations list, with L56M and T107S identified most frequently.

Regarding mutations at position 107, in the CAPELLA study, one participant was found to have T107S alone, which was determined to have no impact on lenacapavir susceptibility given a fold change of only 1.3.[27] However, phenotypic data from site-directed mutagenesis studies suggest that T107S may restore replication capacity when occurring in combination with M66I or Q67H, and may also reduce lenacapavir susceptibility by 10-fold when occurring with Q67H and K70R.[28] Similarly, the effect of T107A alone on susceptibility to lenacapavir has been reported to be minimal, with a fold change of 0.6 compared to WT, and T107A may dampen the effect of an isolated M66I.[28] In

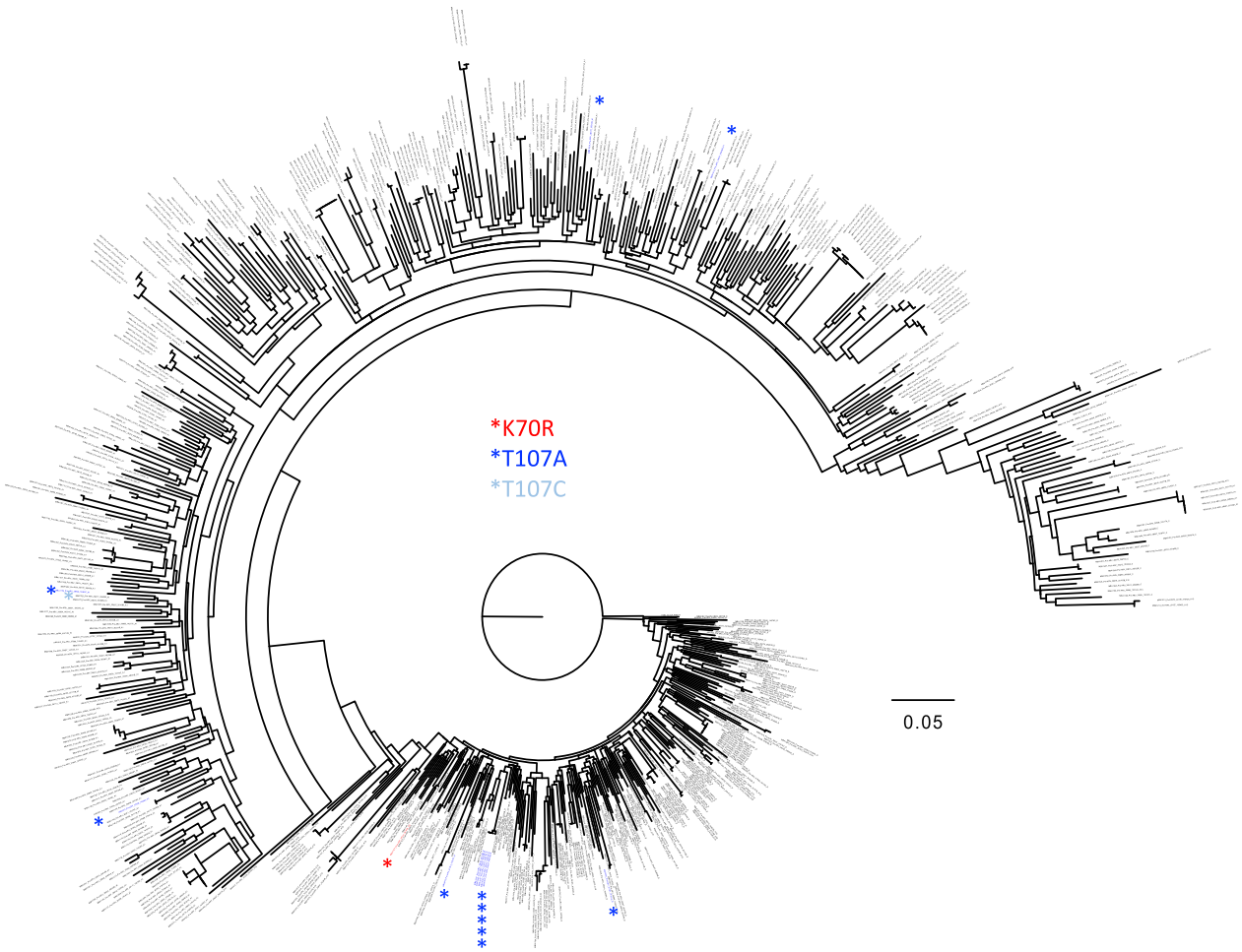


Figure 2. Phylogenetic analysis of *gag* by PhyML. Phylogenetic analysis of all 741 *gag* p24 sequences in this study shows the lack of clustering of T107A, suggesting T107A did not occur as transmission clusters in this cohort.

contrast, when occurring with Q67H, T107A decreases lenacapavir susceptibility, leading to high-level resistance (resistance score of 60), compared with low-level resistance for Q67H alone (resistance score 30).[29] In addition to T107A, T107C was identified as T107T/C/S in one of our study participants. While the effect of T107C alone on lenacapavir susceptibility has not been reported, T107C has been detected along with M66I, Q67H and K70R in one individual who experienced virological failure in the CAPELLA trial, resulting in a 12.2-fold change to lenacapavir susceptibility.[28] Thus, the contribution of mutations at position 107 in capsid to lenacapavir resistance should be further investigated, particularly given their presence among lenacapavir-naive individuals in this study population. Of note, because T107A is not a known HLA-associated immune escape mutation,[30] it is unlikely that this mutation has emerged due to immune selection. We also observed that viral genomes containing T107A did not fall into monophyletic clusters in the phylogenetic tree, which suggests the presence of T107A in multiple individuals could have occurred from independent base substitution mutation events.

We also identified one participant with K70R, which when occurring alone is associated with 1.2-fold reduced lenacapavir susceptibility but has been associated with 15–20-fold reductions in lenacapavir susceptibility when combined with Q67H.[26]

Consistent with three other studies that have examined the prevalence of naturally occurring polymorphisms associated with lenacapavir resistance among PLWH who are lenacapavir-naive,[10,11,13] our results show a low prevalence of naturally occurring HIV variants associated with lenacapavir resistance. Our study further adds to the existing literature by providing data from a cohort exclusively compromised of participants in East Africa with non-B subtypes. Our inclusion of sequences with recombinant HIV-1 subtypes is also novel.

Results of this study should be interpreted in light of the following limitations. Firstly, this study summarizes sequence data derived from a single site in Uganda, and thus results may not be generalizable to regions with different HIV-1 subtype distributions. Still, our results are in line with results of other studies showing that naturally occurring polymorphisms associated with lenacapavir resistance are rare, regardless of the viral subtype. Furthermore, the cohort analysed in this study was followed between 2002 and 2014. However, even though the cohort is not contemporary, we would not expect the baseline prevalence of

Table 2. List of Gag p24 mutations evaluated and detected in this study

IAS–USA 2022 list of resistance mutations	Other mutations examined[a]	Frequency (%) detected in this cohort ($n=546$)	Other non-WT mutations detected (%)
L56I	None	0/546 (0)	L56M: 258/546 (47.3)
	N57S[26]	0/546 (0)	
M66I	None	0/546 (0)	None
Q67H	Q67K/N[23]	0/546 (0)	Q67Q/R: 1/546 (0.2)
	Q67Y[26]		
K70N/S/R	K70H[23]	K70R: 1/546 (0.2)	None
N74D/S	N74H[23]	0/546 (0)	N74N/K: 1/546 (0.2)
A105T	A105S[23]	0/546 (0)	A105I: 1/546 (0.2)
	A105E[26]		A105V: 1/546 (0.2)
			A105A/V: 2/546 (0.4)
T107N	T107A/C[23]	T107N: 0/546 (0)	T107I: 1/546 (0.2)
		T107A: 5/546 (0.9)[b]	T107S or T/S: 9/546 (1.6)
		T107T/A: 2/546 (0.4)	
		T107T/S/C: 1/546 (0.2)	
Any of the above		9/546 (1.6)	

L56M ($n=258$): 87.2% subtype A1, 0.4% subtype C, 3.1% subtype D, 9.3% intersubtype; Q67Q/R ($n=1$): 100% subtype A1; K70R ($n=1$): 100% subtype C, D; N74N/K ($n=1$): 100% subtype A1; A105I ($n=1$): 100% subtype A1; A105V ($n=1$): 100% subtype D; A105A/V ($n=2$): 50% subtype A1, 50% subtype D; T107A ($n=5$): 60% subtype A1, 40% subtype D; T107T/A ($n=2$): 50% subtype A1, 50% subtype D; T107I ($n=1$): MBA1418 100% subtype A1; T107S ($n=7$): 42.9% subtype A1, 14.3% subtype C, 14.3% subtype D, 28.6% intersubtype; T107T/S ($n=2$): 50% subtype A1, 50% subtype D; T107T/S/C ($n=1$): MBA1418 100% subtype A1.

[a]These mutations have been associated with resistance in published studies but were not included into the IAS–USA 2022 list of drug resistance mutations in HIV-1.

[b]T107A has not been associated with subtype-specific polymorphisms.

Gag p24 polymorphisms associated with lenacapavir resistance to change over time given the current lack of capsid inhibitor availability in the region. Secondly, in this study, we used terminologies 'major' and 'minor' mutations to describe mutations listed as major and minor mutations, respectively, according to the 2022 IAS–USA drug resistance mutations list.[21] However, it should be noted that the words 'major' and 'minor' reflect scientific knowledge at the time of the mutation list curation. Furthermore, the definition of lenacapavir resistance employed for this study only incorporates mutations that are listed in the IAS–USA 2022 list of resistance mutations[21] and the Stanford HIVdb version 9.6,[26] as well as recently identified mutations presented at major academic conferences; however, new resistance mutations may be identified in the future. Especially in the case of a new class of antiretroviral drug such as lenacapavir, it is possible that the definitions of which mutations are 'major' and which are 'minor' may change as more research is conducted, and new mutations relevant to resistance may be added. Thirdly, despite our relatively high PCR success rate at 90%, we were unable to capture 10% of the cohort's *gag* genotype, likely due to viral sequence diversity and primer mismatch. There is a possibility that relevant mutations may exist in this group. Finally, another limitation of our study is the lack of phenotypic data, meaning that we were unable to evaluate how any of these mutations observed in our cohort affect lenacapavir's susceptibility and viral replication capacity in the genetic context of other polymorphisms common to this cohort. Future studies should also evaluate treatment responses of individuals receiving lenacapavir who host viruses with any of these mutations.

Conclusions

Among individuals in Uganda living with subtype A1, D and intersubtype recombinant HIV-1, we observed a 1.6% prevalence of naturally occurring polymorphisms associated with lenacapavir resistance and an absence of major lenacapavir-associated mutations. Our findings provide preliminary evidence that lenacapavir is likely to be active against circulating HIV-1 viruses in East Africa. As lenacapavir is introduced, future studies should monitor for the emergence of resistance mutations in this region.

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Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH.

Transparency declarations

None to declare.

Supplementary data

Figures S1 and S2 and Table S1 are available as Supplementary data at *JAC* Online.

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