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Research article

Genetic landscape for majority and minority HIV-1 drug resistance mutations in antiretroviral therapy naive patients in Accra, Ghana

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ABSTRACT

Background: The successful detection of drug-resistance mutations (DRMs) in HIV-1 infected patients has improved the management of HIV infection. Next-generation sequencing (NGS) to detect low-frequency mutations is predicted to be useful for efficiently testing minority drug resistance mutations, which could contribute to virological failure. This study employed Sanger sequencing and NGS to detect and compare minority and majority drug resistance mutations in HIV-1 strains in treatment-naive patients from Ghana.

Method: From a previous study, 20 antiretroviral therapy (ART)-naive participants were selected for a cross-sectional study. Sanger sequencing and NGS techniques were used to detect the majority and minority HIV drug resistance (HIVDR) mutations, respectively, in the protease (PR) and partial reverse transcriptase (RT) genes. NGS detected mutations at 1 % and 5 % frequencies and Sanger sequencing at ≥20 % frequencies. The sequences obtained from NGS and Sanger sequencing platforms were submitted to the Stanford HIV drug resistance database for subtyping, mutation identification, and interpretations.

Results: Sequences from the twenty participants where the CRF02_AG was the predominant strain (16, 80 %) were analyzed. NGS detected 25 mutations in the RT and PR genes, compared to 21 mutations by Sanger sequencing. Minority DRMs were detected at the prevalence of 55.0 % with NGS against 35 % DRMs by Sanger sequencing. One of the patients had eight different HIVDR variants, with two minority variants. These mutations were directed against PI (K20I and D30DN), NNRTI (Y181C, M23LM and V108I) and NRTI (K65R, M184I, and D67N).

Conclusion: The study affirms the usefulness of genomic sequencing for drug resistance testing in HIV. It further shows that Sanger sequencing alone may not be adequate to detect mutations and that NGS capacity should be developed and deployed in the Ghanaian clinical settings for patients living with HIV.

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1. Introduction

With the advent of antiretroviral therapies (ARTs), the Human Immunodeficiency Virus (HIV) infection has become a manageable chronic infection. It has led to a significant reduction in the rate of HIV-related mortality [1–4]. The UNAIDS 95-95-95 fast-track target estimates that by 2025, 95 % of people living with HIV should know their status, whereas 95 % of known HIV-positive patients should be on ART, out of which 95 % should have viral suppression, to end the AIDs epidemic by 2030 [5]. However, achieving this target in low- and middle-income countries is challenging. ARTs target and block replication phases of HIV with a consequent reduction in viral load. However, the effectiveness of ARTs is threatened by HIV drug resistance (HIVDR), particularly in low- and middle-income countries, where treatment monitoring is most challenging [6,7]. HIVDR may stem from several causes, such as high genetic variability of the virus, low genetic drug barrier, and adherence-related factors. While some HIV variants may have primary resistance to antiretrovirals, the majority of drug resistance arise via drug exposure [8,9]. A person infected with HIV experiences a rapid formation and spread of several HIV-1 variants [10,11].

The quasispecies nature of HIV-1 challenges the detection of drug resistance mutations (DRMs) [12]. Minority variants found in fewer than 20 % of quasispecies may contain drug-resistant mutations [13,14]. However, detecting these variants with DRMs at a frequency of less than 20 % of the viral population is challenging using the often-employed Sanger sequencing [15]. The mutations found in low frequencies could result in virological failure (VF), particularly when using regimens based on non-nucleoside reverse transcriptase inhibitors (NNRTIs) [13,14]. The low-frequency DRMs have been associated with VF in ART treatment naïve patients with current diagnosis, especially in patients receiving NNRTIs [16,17]. In a study by Moreno et al. (2023), thirteen out of twenty patients with VF had at least one drug resistance mutation related to reverse transcriptase inhibitors and protease inhibitors (PI) at a frequency level of $\leq$1, which were not detected in the previous genotyping using Sanger sequencing method [18].

Furthermore, different studies have recently demonstrated that particular HIVDR variants are clinically significant at 1 % of the viral population, as the low-frequency variants can swiftly replicate and become the predominant viral population through the selective pressure of antiretroviral drugs, causing treatment failure [12,16,19]. However, their relation to HIV-1 subtype diversity has not been extensively studied. Recent studies indicate that next generation sequencing (NGS) can detect about 100 % of all mutations detected using the Sanger technique [13,15,20,21]. Baseline minority mutations in treatment-naive patients could pose significant threat to treatment outcomes. In this study, we sought to determine minority and majority mutations in treatment naive patients predominantly infected with CRF02_AG strain from Ghana selected from a previous study which examined the prevalence and impact of Hepatitis B and C Virus Co-infection in antiretroviral treatment naïve-patients with HIV infection [22].

2. Methods

2.1. Study participants

The study was cross-sectional, using twenty patients from a previous study [22]. These patients were treatment-naïve and had reported not using any antiretroviral drug in the period preceding the study. Informed written consent was obtained from the patients.

2.2. RT-PCR and sanger sequencing

The High Pure Viral RNA kit (Roche Diagnostics GmbH, Mannheim, Germany) was used to isolate RNA from stored plasma samples. Partial polymerase genes (1065-1372bp) were amplified with a similar nested PCR protocol described previously [23]. Reverse transcription was done using the Titan One tube RT-PCR system (Roche Diagnostics GmbH, Mannheim, Germany). The original protocol was modified to amplify the polymerase (*pol*) gene fragment spanning the protease (PR) and up to 230 amino acids in the reverse transcriptase gene (RT). Generation of PCR products and sequencing was done as described previously [24]. Briefly, the PCR products were cleaned using the High Pure PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany). The Big Dye Terminator cycle sequencing kit v3.1 and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems International Incorporated, Foster City, USA) were used for forward and reverse sequencing. Furthermore, a third sequencing primer (RT-sec-1-S: 5′ CAA AAA TTG GGC CTG AAA ATC CAT A 3′) was employed for ensuring that the nucleotides towards the end of the RT region were acquired, which is essential for the first 227 amino acids. To sequence the *pol* genes of HIV-1 strains that were challenging to amplify using the in-house assay, another technique called the ViroSeq HIV-1 Genotyping System v2 (Celera Diagnostics, Foster City, CA, USA) was employed [23].

2.3. Next generation sequencing

Reverse transcribed DNA samples were fragmented and sequencing libraries were prepared using the NEBNext® Ultra™ II FS DNA Library Prep Kit for Illumina. Briefly, the fragmented DNA were size selected (>300 bp) using AMPure XP beads (Beckman Coulter, United States). The fragments were end-repaired and Illumina-specific adapter sequences were ligated to each fragment. Each sample was individually indexed, quantified using a fluorometric method (NEBNext Library Quant Kit for Illumina), diluted to a standard concentration (4 nM) and then sequenced on Illumina's MiSeq platform, using the MiSeq v3 kit (600 cycles), following a standard protocol as described by the manufacturer. For each sample, 100 Mb of data (2x300 bp paired-end reads) was produced. PASeq, a free automated HIV drug resistance analysis pipeline, was used to de-novo assemble MiSeq sequence reads and subsequently assessed the read quality (IrsiCaixa; Barcelona, Spain) [20]. The Stanford HIV Drug Resistance Database (https://hivdb.stanford.edu/) evaluated

amino acid variants produced via PASeq (HIVDB; version 8.4) [20,25,26]. Twenty [20] samples with successful sequences from both Sanger sequencing and NGS platforms were analyzed.

2.4. Phylogenetic analysis and drug resistance testing

Phylogenetic relationships were inferred using a partial *pol* region containing 867 nucleotides as described [24,27]. MEGA version 11 was used to create Neighbour-joining trees based on Kimura's 2-parameter distance. The sequences were submitted to the Stanford University HIV Drug Resistance Database (https://hivdb.stanford.edu/) for interpretation of resistance and assignment of subtype. All mutations commented on by the Stanford database were reported, and the level of resistance indicated. The GenBank accession numbers of the sequences used in this study are OR133351-OR133370.

3. Results

3.1. Demographic and clinical characteristics

Most participants were female (60.0 %), with an average age of 40 (22–69) years. All the participants had less than 350 $CD4^+$ cells/μL. Most participants were within the World Health Organization (WHO) stage 3 (75.0 %) (Table 1).

3.2. Phylogenetic inference and drug resistance mutations

Majority (16/20, 80 %) were CRF02_AG strains with subtypes C (1, 5 %), G (1, 5 %) and 06cpx (1, 5 %) in the minority (Fig. 1). However, the subtype for one sample could not be inferred due to an unreadable chromatogram from Sanger sequencing (Table 2).

In the RT and PR genes, Sanger sequencing identified 21 mutation events at ≥20 % frequency of the viral population whereas NGS identified 16 and 9 mutation events at 1 % and 5 % frequencies, respectively (Table 2). The NGS aspect of the work focused only on low-level frequencies since it is indicated that NGS can detect about 100 % of all mutations detected by the Sanger platform [13,15,20,21]. The prevalence of resistance mutations among the patients as detected with NGS and Sanger sequencing were 11/20 (55 %) and 7/20 (35 %), respectively. Four participants had no mutations detected by NGS or Sanger. One of the participants had 8 HIVDR mutations, two of which were detected by NGS, and Sanger identified the remaining six mutations. These mutations were directed against PI (K20I and D30DN), NNRTI (Y181C, M23LM and V108I) and NRTI (K65R, M184I, and D67N). Nine patients with no detectable mutation from Sanger sequencing had DRM at low-level frequencies from NGS. Sanger platform also identified two of the minority mutations (K103 N and M184I) detected by NGS at 5 % frequency (Table 2). The most common minority resistance mutations found by NGS in the RT and PR genes, respectively, were D67 N (22 %) (Fig. 2a) and M46I (58 %) (Fig. 2b), whereas the most common mutation found by the Sanger sequencing in the PR gene was K20I (51 %) (Fig. 2c). Within the RT region, the mutations observed from Sanger sequencing, K70R, K65R, M184I, Y181C, M23LM, S68G, K103 N, V179I, and V179A were detected in single frequencies.

3.3. Antiretroviral drug susceptibility

The mutations detected by Sanger sequencing were generally susceptible (S) or had potential low-level resistance (PLLR) against PIs. However, one participant had high-level resistance (HLR) against NRTIs and NNRTIs, while another had HLR against some NNRTIs (Table 3). In contrast, the NGS platform detected a mix of S, PLLR, low-level resistance (LLR), intermediate resistance (IR) and HLR mutations against PIs. Additionally, three participants had HLR against NNRTIs, and two others had HLR against NRTIs. Some mutations detected by NGS had LLR/IR against NNRTIs or NRTIs (Table 4).

4. Discussion

This study sought to detect the majority and minority drug resistance mutations among HIV-1 strains in Ghana. The CRF02_AG strain was predominant. This is in line with previous reports on subtype diversity and drug resistance mutations in Ghana, which have

Table 1
Demographic and clinical characteristics of the study population.

Variables	Frequency
Mean Age (Years), (range)	40 (22–69)
Gender, n (%)	
Male	8 (40.0)
Female	12 (60.0)
$CD4^+$ count, n (%)	
≥350 cells/μL	0 (0.0)
≤350 cells/μL	20 (100.0)
WHO stage, n (%)	
Stage 3	15 (75.0)
Stage 4	5 (25.0)

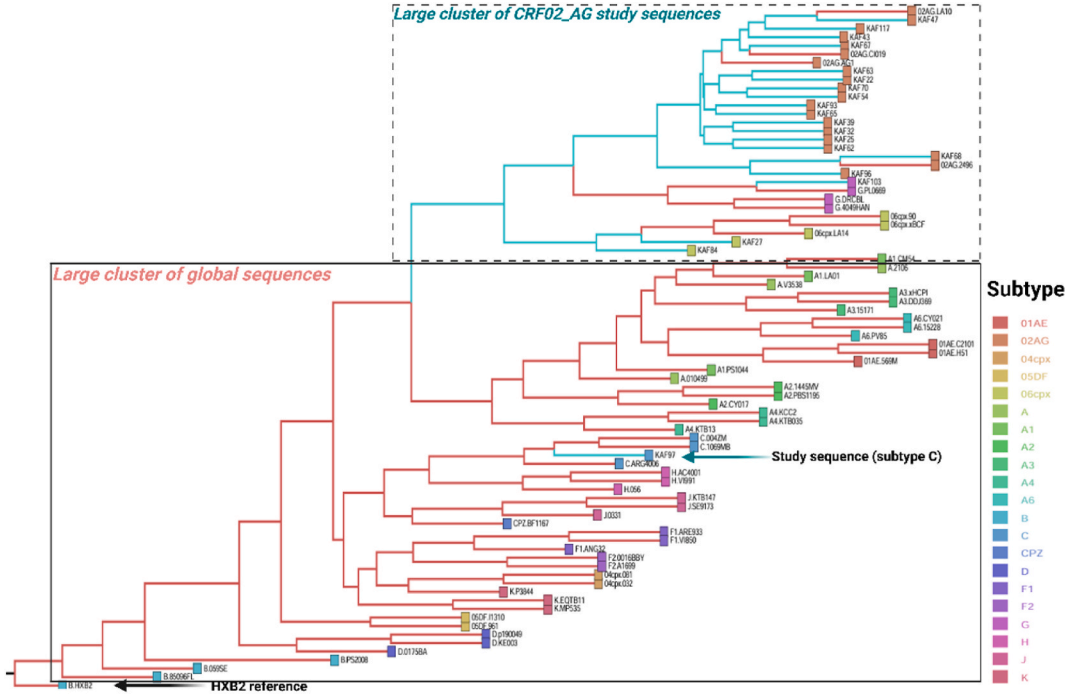

Fig. 1. Phylogenetic tree of the genotype diversity of the HIV-1 subtypes
MEGA version 11 was used to create a neighbour-joining tree based on Kimura's 2-parameter distance. Bootstrapping with 1000 replicates was used to examine the reliability of the tree topologies, with bootstrap support of ≥70 % necessary to establish a phylogenetic cluster. The majority of the sequences were clustered around the CRF02_AG strain.

Table 2
Comparison of HIV-1 drug resistance mutations detected by Sanger sequencing and NGS.

ID (KAF)		SANGER			NGS 5 %			NGS 1 %		
	Subtype	NRTI	NNRTI	PI	NRTI	NNRTI	PI	NRTI	NNRTI	PI
22	CRF02_AG	K70R		L10I/V, K20I						
38	CRF02_AG							D67N		M46I
39	CRF02_AG				D67N, F77L					
43	CRF02_AG									
47	CRF02_AG			L10I/V, V11I, K20I						
51	CRF02_AG									
52	CRF02_AG									
53	CRF02_AG				T215A					
54	CRF02_AG							D67N		I47V
63	CRF02_AG									
65	CRF02_AG				T215A			K219R, K70R		
68	CRF02_AG	K65R M184I	Y181C M23LM	K20I D30DN				D67N	V108I	
70	CRF02_AG							A62V		I50V, M46I
84	06cpx	S68G	K103N	K20I		K103N	M46I			L89V
93	CRF02_AG								V108I G190E	M46I
96	CRF02_AG		V179I	K20I						
97	C		V179A							
103	G			L10M, K20I, V82I						
108	a				M184I, G190A	K103N				
114	CRF02_AG									I47V

Key.
[a] KAF108 had no sequence from the Sanger sequencing platform and thus did not have any subtyping done.

estimated that about 70 % of the HIV-1 strains circulating in Ghana are CRF02_AG [7,27–34]. Again, the recombinant strain, CRF02_AG, has higher infectivity than the parental subtypes A and G [30].

Aside from the challenge of HIV-1 subtype diversity, achieving the UNAIDS 95-95-95 fast-track target has become difficult,

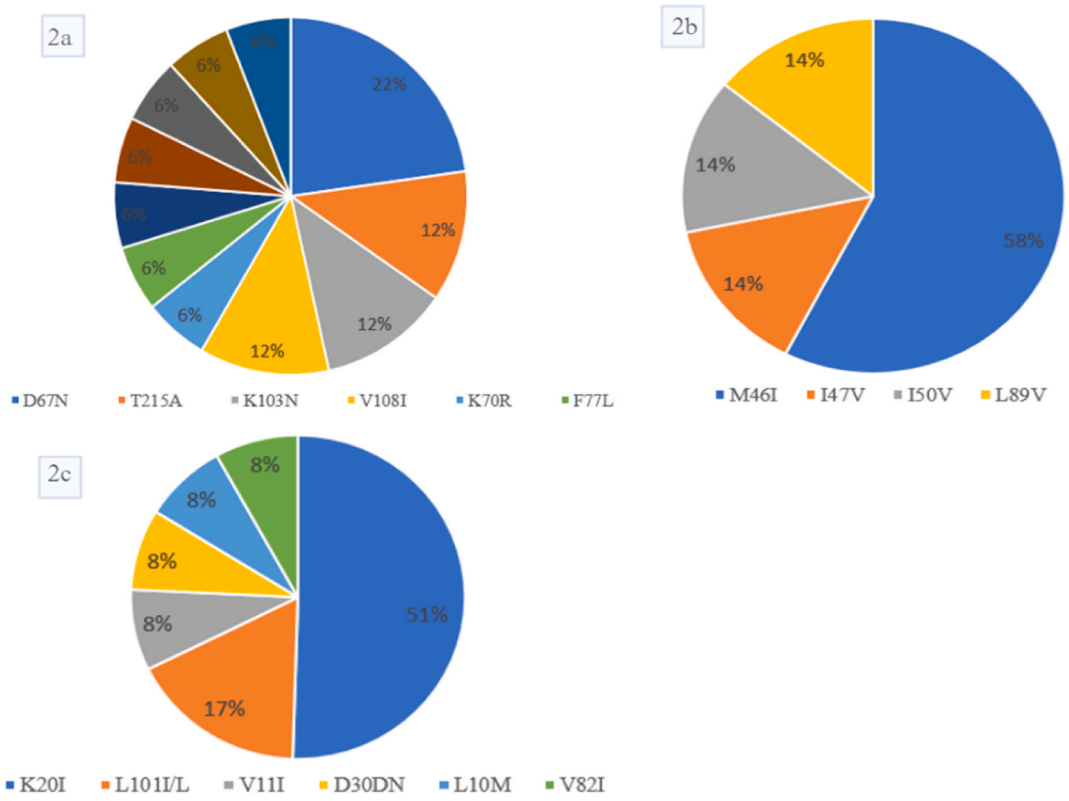

Fig. 2. The percentage occurrence of DRMs detected by NGS in the RT gene (Fig. 2a), PR gene (Fig. 2b) and also by Sanger sequencing (Fig. 2c).

especially in resource limited settings since more affordable and sensitive technologies are required to monitor the success of ARTs [5]. Over the years, Sanger sequencing has been the standard technique for HIVDR testing. However, it is limited by high sequencing costs and low throughput [35]. It cannot detect the minority HIV-1 variants with less than 20 % frequencies of viral quasispecies [12]. With the limitations observed in Sanger sequencing, some studies recommend using NGS, which can detect minority variants of the viral population [13,15]. Selection of minority drug resistance HIV-1 strains not detected by standard Sanger sequencing under ART pressure can shift the viral quasispecies distribution, becoming dominant members of the virus population and eventually causing virological failure [13].

In this study, minority drug resistance mutations were detected at a higher prevalence with NGS compared to the majority drug resistance detected by Sanger. This prevalence for low-frequency drug resistance was equally high compared to the prevalence (64.0 %) in another study conducted among thirty-three HIV-infected patients failing first-line therapy with no detectable drug resistance mutation by the Sanger platform [13]. Moreover, a higher prevalence of minority drug resistant mutations in ART-naïve and ART-experienced patients with virological failure were detected using a deep sequencing technique [12]. The high prevalence of drug resistant mutations observed in the current study was expected since most of the patients, even though ART-naïve were at WHO stages 3 and 4 with significantly lower $CD4^{+}$ cell count.

The most frequent minority drug-resistant mutation detected in the RT gene was D67N, followed by T215A, K103N, and V108I in equal occurrence, and the same frequency for F77L, K219R, K70R, A62V and G190E. Similarly, a study by Deletsu et al. (2020), which had CRF02_AG dominance of 68 %, detected the majority of these minority drug resistance mutations in high frequencies. Major nucleoside reverse transcriptase inhibitor resistance mutations (NRTIs) M184I, D67N, T215F and K70R and Non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance mutations, K103N were found in their study [36]. Similar to our study, D67N, K103N, and M184I were also detected in another study among HIV patients with virological failure [13]. The detection of drug resistance mutations as minority variants in this study and as majority variants in previous studies indicate how low-frequency mutations may swiftly become dominant. Hence early detection of the minority variants can significantly impact patient treatment outcomes.

D67 N is a non-polymorphic thymidine analog mutation (TAM) associated with low-level resistance to zidovudine and stavudine. It reduces susceptibility to abacavir (ABC), didanosine (ddl), and tenofovir disoproxil fumarate (TDF) when present with other TAMs. Again, D67 N has been associated with ART-naïve patients, confirming the current study's prevalence. Similarly, D67N was detected in high prevalence in other studies [29,36]. However, while the mutation was detected in the minority species from ART-naïve participants, their study participants were ART-experienced, and the mutations were detected as majority [29,36]. The presence of K103N mutation causes high-level reductions in nevirapine and efavirenz susceptibility, as observed in this study. The K103N mutation has also been detected in different studies as majority mutation in Ghana, with the CRF02_AG strain being the dominant subtype. This

Table 3
HIV-1 drug resistance mutations detected by Sanger sequencing and their drug susceptibilities.

ID* (KAF)	HIV-1 Drug Resistance Mutations			Drug Susceptibilities																			
				NRTI							NNRTI					PI							
	NRTI	NNRTI	PI	3TC	ABC	AZT	D4T	DDI	FTC	TDF	EFV	ETR	NVP	RPV	DOR	ATV/r	DRV/r	FPV/r	IDV/r	LPV/r	NFV	SQV/r	TPV/r
KAF 22	K70R	-	L10I/V, K20I																				
KAF 47	-	-	L10I/V, V11I, K20I																				
KAF 68	K65R, M184I	Y181C, M230LM	K20I, D30DN																				
KAF 84	S68G	K103N	K20I																				
KAF 96	-	V179I	K20I																				
KAF 97	-	V179A	-																				
KAF 103	-	-	L10M, K20I, V82I																				

Legend	
	Susceptible (S) Potential low-level resistance (PLLR)
	Low-level resistance (LLR) Intermediate resistance (IR)
	High-level resistance (HLR)

again, suggests how minority mutation can swiftly develop to become dominant in the viral population to cause virological failure [28,29,34,36–39].

M184I causes high-level *in-vitro* resistance to lamivudine (3 TC) and emtricitabine (FTC) and low-level resistance to ddI and ABC. Similar to the current study, another study detected M184I and K103N as majority mutation in one participant which confer resistance to 3 TC, ABC and DDI [36]. V108I is a relatively non-polymorphic accessory mutation selected *in vitro* and/or *in vivo* with each NNRTI. It causes low-level reductions in susceptibility to nevirapine (NVP) and doravirine (DOR). Alone, V108I does not appear to reduce susceptibility to efavirenz (EFV), etravirine (ETR), or rilpivirine (RPV) [40].

Interestingly, V108I in the current study was detected in two patients. One patient had a minority drug resistance combination of V108I, G190E and M46I, conferring high-level resistance to EFV, ETR, or RPV. Similarly, V1081, G190E and M46I were detected in a study as HIV-1 minority variants in ART naïve patients with virological failure after 12 months of follow-up in Panama [18]. Although Panama is dominated by HIV-1 subtype B [41–43], the trend in the detection of minority mutations and their association with virological failure is similar to the current study. Another participant in the present study had a single minority drug resistance mutation of V108I but had multiple majority mutations, Y181C and M230LM, which confers high-level resistance to EFV, ETR, or RPV (Table 3) [40]. Consistent with the current study, mutations V108I and Y181C were detected in different studies by Delgado et al. (2008), Nii-Trebi et al. (2013), and V108I, Y181C and M230L by Nii-Trebi et al. (2017). However, these mutations were detected as majority in treatment-experienced patients [27,28,37]. Notably, a participant in this study had multiple majority drug resistance mutations for the NRTIs, NNRTIs and the PIs classes with additional minority mutation against NNRTI and PI (Table 2). Also, a participant with multiple DRMs, including six majority and two minority mutations, showed high-level resistance against 3 TC, ABC, DDI, FTC, EFV, ETR, NVP, RPV, DOR and stavudine (D4T). However, there was no resistance against protease inhibitors (Tables 3 and 4).

Interestingly, NGS detected DRMs T215A and K103N at frequencies of 22.104 and 28.571, respectively, which Sanger sequencing did not detect as the majority DRMs (Table 4). The Sanger platform only identified K103N in another participant, which NGS also detected at a frequency of 98.419 (Table 4). Moreover, T215A was detected in another Ghanaian study as majority mutation [39]. This indicates that, even at frequencies greater than 20 %, Sanger sequencing platform, though very useful, could miss some critical mutations.

5. Conclusion

The study suggests the need to detect minority drug resistance mutations in patients living with HIV (PLWH) and receiving care using a more sensitive and advanced technology. Optimizing NGS for clinical monitoring of HIV drug resistance profiles in resource-

Table 4
HIV-1 drug resistance mutations detected by NGS at low frequencies and their drug susceptibilities.

Mutant[+]	*ID Mutation frequency (KAF)	Drug susceptibility																			
		NRTI							NNRTI					PI							
		3TC	ABC	AZT	D4T	DDI	FTC	TDF	EFV	ETR	NVP	RPV	DOR	ATV/r	DRV/r	FPV/r	IDV/r	LPV/r	NFV	SQV/r	TPV/r
M46I PR	$38^{2.059}$,																				
	$70^{1.443}$,																				
	$84^{9.804}$,																				
	$93^{1.059}$																				
I47V PR	$54^{1.017}$																				
	$114^{1.922}$																				
I50V RT	$70^{2.036}$																				
A62V RT	$70^{1.575}$																				
D67N RT	$38^{1.04}$																				
	$39^{2.381}$																				
	$54^{1.042}$																				
	$68^{4.11}$																				
K70R RT	$65^{1.322}$																				
F77L RT	$39^{1.587}$																				
L89V	84																				
K103N RT	$84^{98.419}$																				
	$108^{28.571}$																				
V108I RT	$68^{2.222}$																				
	$93^{1.356}$																				
M184I RT	$108^{8.333}$																				
G190A RT	$108^{7.692}$																				
G190E RT	$93^{1.929}$																				
T215A RT	$53^{5.515}$																				
	$65^{22.104}$																				
K219R RT	$65^{1.449}$																				

*ID indicates patient ID with mutation frequency as a superscript; [+] indicates the gene on which the mutant was detected. Superscripts PR and RT are protease and reverse transcriptase genes, respectively.

Legend	
	Susceptible (S) Potential low-level resistance (PLLR)
	Low-level resistance (LLR) Intermediate resistance (IR)
	High-level resistance (HLR)

constraint settings such as in Ghana could better inform clinicians on effective treatment choices and will be critical in achieving the UNAIDS 95/95/95 targets.

Ethics

The study was approved by the University of Ghana Medical School Ethics and Protocol Review Committee (MS-Et/M.5.1 -P.7/2006-07), and written informed consent was obtained from patients before enrolment in the study.

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None.

Data availability

The sequence data used in this study are deposited in the GenBank repository with the accession numbers OR122251-OR133370.

CRediT authorship contribution statement

Pious Appiah: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation. **Gaspah Gbassana:** Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Data curation. **Mildred Adusei-Poku:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Methodology, Formal analysis, Data curation. **Billal Musah Obeng:** Writing – review & editing, Visualization, Validation,

Software, Formal analysis, Data curation. **Kwabena Obeng Duedu:** Writing – review & editing, Visualization, Validation, Supervision, Software, Data curation. **Kwamena William Coleman Sagoe:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33180.

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