Infection and Drug Resistance

Dovepress
Taylor & Francis Group

Open Access Full Text Article

ORIGINAL RESEARCH

Development and Evaluation of an Optimised Sanger-Based Assay for HIV-1 Drug Resistance Genotyping in Chinese Circulating Strains Across Subtypes and Viral Loads

Mengying Li[1,2,*], Fengting Yu[2,3,*], Fei Liu[4], Xi Chen[4], Di Mao[4], Haichao Xiao[4], Hanxi Zhang[3,5], Fujie Zhang[2,3]

[1]Medical School, University of Chinese Academy of Sciences, Beijing, People's Republic of China; [2]Beijing Ditan Hospital, Capital Medical University, Beijing, People's Republic of China; [3]Clinical Center for HIV/AIDS, Capital Medical University, Beijing, People's Republic of China; [4]Sailian Biotech Co., Ltd., Guangzhou, Guangdong, People's Republic of China; [5]WHO Collaborating Centre for Comprehensive Management of HIV Treatment and Care, Beijing Ditan Hospital, Capital Medical University, Beijing, People's Republic of China

*These authors contributed equally to this work

Correspondence: Fujie Zhang, Beijing Ditan Hospital, Capital Medical University, Beijing, 100015, People's Republic of China, Tel/Fax + 86 10 58900931, Email treatmentditan@163.com; treatment@chinaaids.cn

Purpose: HIV drug resistance is increasing globally, especially in resource-limited settings. NNRTIs, commonly used as first-line ART, have a low genetic barrier and are prone to resistance mutations. Lifelong ART contributes to the accumulation of drug resistance mutations (DRMs), compromising treatment efficacy. Genotypic resistance testing is essential before initiating or modifying ART. However, the Sanger sequencing method relies on successful PCR amplification, which is often suboptimal in low viral load (VL) samples, limiting sensitivity and coverage.

Patients and Methods: We developed an optimized primer system targeting conserved regions in the protease (PR), reverse transcriptase (RT), and integrase (IN) genes, covering PR aa 1–99, RT aa 1–410 (including NNRTI resistance sites Y318 and N348), and IN aa 1–288. A total of 2,071 HIV-positive plasma samples collected in China (Jan 2023–Dec 2024) were analyzed using a PCR-Sanger sequencing method. Subtyping was performed using BLAST, COMET 2.4, and the HIV-1 Gene Sequences Database (China). Amplification success rates and mutation detection were evaluated across VL levels.

Results: The overall amplification success rates were 87.40% (1,810/2,071) for the PR/RT region and 87.06% (1,803/2,071) for the IN region. In samples with VLs of 50–200 copies/mL, the success rates remained above 80% for PR/RT and 78.10% for IN. For samples with VL ≥1000 copies/mL, both regions achieved amplification rates above 99%. Among eight samples harboring Y318 or N348 mutations, all were successfully amplified at 1000, 400, 200, and 100 copies/mL. Three of them consistently yielded detectable mutations across all gradients. Subtyping revealed CRF01_AE and CRF07_BC as the predominant strains, consistent with national epidemiological trends.

Conclusion: The optimized system improves amplification sensitivity and mutation coverage, especially in low-VL samples. It enables stable detection of key NNRTI resistance mutations and shows strong subtype compatibility, supporting its utility in clinical resistance surveillance and early detection.

Keywords: HIV, non-nucleoside reverse transcriptase inhibitors, NNRTIs, genotypic resistance testing, low viral load, sanger sequencing, primer optimisation

Introduction

The widespread implementation of antiretroviral therapy (ART) has transformed HIV/AIDS into a manageable chronic disease. When ART was initially introduced in 1996, standard regimens included two nucleoside/nucleotide reverse

Received: 26 August 2025
Accepted: 5 December 2025
Published: 11 December 2025

transcriptase inhibitors (NRTIs) and one non-nucleoside reverse transcriptase inhibitor (NNRTI).[1,2] However, certain antiretroviral drugs (ARVs) have relatively low genetic barriers to resistance. Combined with the high variability and frequent recombination of HIV, this can lead to the emergence of drug resistance mutations (DRMs), resulting in uncontrolled viral replication, treatment failure, and transmission of resistant strains.[3]

According to the latest World Health Organization (WHO) reports, the global prevalence of HIV drug resistance continues to increase (https://www.who.int/zh/news-room/fact-sheets/detail/hiv-drug-resistance). In China, the pre-treatment drug resistance (PDR) rate has reached 5.56%, with a consistent upward trend.[4] Many developing countries face even higher DRM rates, compounded by limited access to resistance testing.[5] In response, WHO recommends expanded resistance surveillance and capacity building for resistance testing.[6–8]

In recent years, technologies for detecting HIV-1 DRMs have advanced considerably. Next-generation sequencing (NGS) and whole-genome sequencing (WGS) provide enhanced sensitivity for identifying low-frequency variants and offer broader genomic coverage, with WGS addressing the short-read limitations inherent to NGS platforms.[8–13] Digital droplet PCR (ddPCR) has demonstrated excellent analytical sensitivity for quantitative applications;[14,15] however, its use in qualitative DRM detection remains exploratory. Despite the clear technical advantages of these emerging methods, their requirements for specialised laboratory infrastructure, advanced bioinformatics support, and comparatively high operational costs continue to limit their feasibility for routine clinical use, particularly in resource-limited settings.

By contrast, population-based Sanger sequencing remains the predominant approach for clinical HIV-1 resistance monitoring due to its robustness, reproducibility, accessibility, and cost-effectiveness. Nonetheless, current Sanger-based workflows exhibit two major limitations. First, the amplification range is often insufficient. Most in-house methods and commercial kits only cover PR aa 1–99, RT aa 1–300, and IN aa 1–288, missing key resistance sites such as Y318 and N348.[16,17] Second, sensitivity is suboptimal in samples with low viral loads (VLs), typically defined as <1,000–2,000 copies/mL. Under such conditions, the detection rate declines significantly,[16,17] limiting early identification of resistance mutations.

In addition, the pronounced genetic diversity of HIV-1 poses further challenges for molecular assay performance. Circulating strains in China are characterized by a complex subtype distribution, dominated by CRF01_AE, CRF07_BC, subtype B, and multiple recombinant forms.[18–20] Previous studies have indicated that sequence polymorphisms and subtype-specific variability may influence molecular diagnostic accuracy by altering primer or probe binding affinity, thereby reducing amplification success or overall assay sensitivity.[21–23] Given this diverse epidemiological landscape, primer systems tailored to locally circulating strains are essential for improving assay reliability and ensuring accurate genotypic resistance detection.

To address these challenges, we optimized a primer system targeting the pol region based on full-length and gag-pol sequences of HIV-1 strains circulating in China. A PCR-Sanger method was established with extended coverage to key NNRTI-associated resistance sites. The optimized PR/RT primers expanded the RT sequence coverage from 320 to 410 amino acids, enabling the detection of previously undercovered mutations, including Y318 and N348. This study systematically evaluated the method in terms of amplification performance, mutation detection stability, and subtype compatibility, aiming to support its potential use in clinical HIV drug resistance monitoring, especially for low-VL samples.

Materials and Methods

Primer Design and Screening

Primers targeting the HIV-1 PR/RT and integrase (IN) regions were designed based on full-length or full *gag-pol* sequences representative of HIV-1 strains circulating in China. Conserved regions encompassing known DRMs sites, including Y318 and N348, were selected as amplification targets. Primer design was performed using Primer Premier 5.0 software (Premier Biosoft). A total of 25 primer pairs were designed for the PR/RT region and 18 pairs for the IN region. Primer performance was optimized through three rounds of stepwise screening using pseudovirus constructs and 10 clinical plasma samples. Among these samples, 2 had VLs ranging from 9,000 to 200,000 copies/mL, and 8 had VLs ranging from 9,000 to 70,000 copies/mL (Figure 1A–F). The final selected primer sets were validated in 10 clinical samples with VLs of 1,000–2,000 copies/mL to

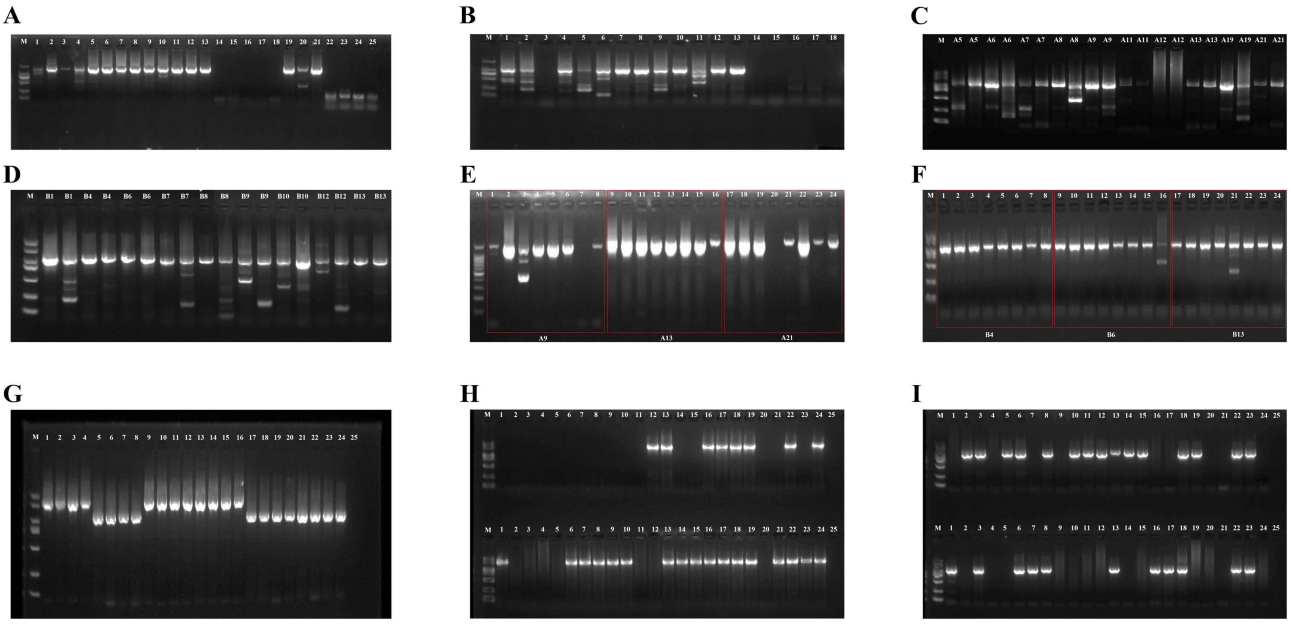

Figure 1 Primer screening and performance validation for amplification of HIV-1 PR/RT and IN regions. Panels A–B use a DNA molecular weight marker containing 2000, 1000, 750, 500, 250, and 100 bp bands. Panels C–I use a marker containing 2000, 1500, 1000, 750, 500, 250, and 100 bp bands. (**A** and **B**) Initial screening of 25 PR/RT primer pairs (**A**) and 18 IN primer pairs (**B**) using pseudovirus templates. (**C** and **D**) Based on initial results, selected PR/RT primers from lanes 5, 6, 7, 8, 9, 11, 12, 13, 19, and 21 (**C**) and IN primers from lanes 1, 4, 6, 7, 8, 9, 10, 12, and 13 (**D**) were subjected to second-round screening in two clinical samples per primer pair. (**E-F**) Further screening of PR/RT primers (lanes 9, 13, 21) (**E**) and IN primers (lanes 4, 6, 13) (**F**) was performed using eight clinical samples with VLs of 1,000–2,000 copies/mL. Each primer pair's amplification results are shown in adjacent gel lanes. (**G**) Validation of the final selected primer pair 13 (PR/RT) and primer pair 4 (IN) in 10 additional clinical samples. Lane 25 is the negative control. (**H**) Comparative amplification using the optimised PR/RT system versus a commercial genotyping kit: upper panel shows results from the commercial kit; lower panel shows results from the optimised system. Lane 25 is the negative control. (**I**) Comparison of the IN primer system developed in this study (upper panel) with a published in-house method (lower panel). Lane 25 is the negative control. The optimised primer system demonstrated improved amplification, especially in low-VL samples.

assess amplification consistency (Figure 1G). Subsequently, 8 clinical samples with initial VLs of 9,000–70,000 copies/mL were serially diluted to three VL strata (1,000–2,000, 500–1,000, and 50–500 copies/mL). Amplification performance of the optimised primer sets was then compared with that of commercial HIV-1 drug resistance genotyping kits[24–26] and an in-house method described by Liu et al[27](Figure 1H and 1I). Details of the 20 clinical samples used for primer screening and validation are provided in Supplementary Table 1. The primer design and its associated amplification system are protected under an authorized Chinese national invention patent (Patent No. 202310253125.4).

Study Subjects

In this retrospective, single-center study, a total of 2,071 plasma samples were collected from 2,050 people living with HIV (PLWH) who underwent HIV-1 confirmatory testing at Beijing Ditan Hospital between January 2023 and December 2024. The number of plasma samples exceeded the total number of participants because some individuals contributed more than one sample at different clinical timepoints (eg, during regimen changes due to side effects or financial constraints). All samples were stored at −80 °C prior to genotypic resistance testing. Of these, 2,046 samples yielded successful amplification of the PR/RT region, and 2,041 samples yielded amplification of the IN region. Among the successfully amplified samples, 8 were identified to harbor mutations at the Y318 or N348 loci. This study had been approved by the ethics committee of Beijing Ditan Hospital of Capital Medical University (Approval number: DTEC-KY2023-002-01) and complied with the Declaration of Helsinki.

HIV RNA Extraction

A total of 200 μL of plasma was processed for viral RNA purification using a standardized extraction kit (Guangzhou Life Technologies Daan Diagnostics Co., Ltd). All steps were carried out strictly in accordance with the manufacturer's recommended procedures.

Serial Dilution of Samples

Samples harboring Y318 or N348 mutations were serially diluted to final concentrations of 1,000, 400, 200, and 100 copies/mL. Due to an initial VL of 642 copies/mL, sample DR-GM1273 was only diluted to three concentrations: 400, 200, and 100 copies/mL. All diluted samples were stored at −80 °C prior to RNA extraction.

Amplification of the Pol Region

A PCR–Sanger detection method based on an optimised primer system was developed in-house, and the primer design together with the amplification strategy has been granted a Chinese national invention patent (Patent No. 202310253125.4). The method targets key drug resistance-associated regions within the HIV-1 *pol* gene, including PR, RT, and IN, with expanded coverage of critical NNRTI-related mutation sites such as Y318 and N348. The PCR reaction system and amplification procedure are detailed below (Tables 1–4).

Sanger Sequencing

Positive PCR products were purified and sequenced using the 3500XL Dx Genetic Analyzer (Applied Biosystems, USA). HIV-1 subtypes were determined using COMET HIV-1 v2.4 (http://comet.retrovirology.lu), the Los Alamos HIV BLAST tool (https://www.hiv.lanl.gov/content/sequence/BASIC_BLAST/basic_blast.html), and the HIV-1 Gene Sequences Database (China) (https://nmdc.cn/hiv/). DRMs and associated resistance levels were interpreted using the Stanford HIV Drug Resistance Database (HIVdb) version 9.6.[28]

Table 1 First-Round RT-PCR Reaction System for PR/RT and IN Regions

Component	Volume (μL)
RT-PCR reaction buffer for PR/RT or IN	14
RT enzyme mix for PR/RT or IN	1
RNA	10
Total volume	25

Table 2 First-Round RT-PCR Cycling Conditions

Temperature	Time	Cycles
50°C	30 min	1
94°C	5 min	1
94°C	30 s	35
53°C	45 s	
72°C	2 min	
72°C	10 min	1

Table 3 Second-Round PCR Reaction System for PR/RT and IN Regions

Component	Volume (μL)
PCR reaction buffer for PR/RT or IN	42
PCR enzyme mix for PR/RT or IN	3
DNA	5
Total volume	50

Table 4 Second-Round Nested PCR Cycling Conditions

Temperature	Time	Cycles
94°C	5 min	1
94°C	30 s	3
53°C	45 s	
72°C	2 min	
94°C	30 s	27
58°C	45 s	
72°C	90 s	
72°C	5 min	1

Next-Generation Sequencing

Purified PCR products (>50 ng) were end-repaired, phosphorylated at the 5′ end, and A-tailed at the 3′ end using the End Prep Enzyme Mix, followed by ligation with sequencing adapters. Libraries were constructed using the VAHTS Universal Pro DNA Library Prep Kit for Illumina (ND608, Vazyme, China), incorporating P5 (5′-AATGATACGGCGACCACCGAGATCTACAC-3′) and P7 (5′-CAAGCAGAAGACGGCATACGAGAT-3′) adapters. Adapter-ligated products were purified using magnetic beads, PCR-amplified, and re-purified to obtain the final library. Quality control was performed prior to sequencing. Paired-end (PE150) sequencing was performed on the Illumina NovaSeq platform according to the manufacturer's instructions. Raw data were processed with Trimmomatic v0.39 to remove adapter sequences and filter low-quality reads. Clean reads were aligned to the HIV-1 reference genome (HXB2) using Bowtie2 v2.2.5, and alignment files were processed and filtered using Samtools v1.6. Variant calling was performed at the amino acid level using Quasitools v0.7.0, and resulting variants were saved in Variant Call Format (VCF). DRMs were interpreted at multiple mutation detection thresholds (MDTs) using a customized Python pipeline incorporating the Stanford HIVdb algorithm version 9.6.

Statistical Analysis

Sociodemographic data were organized using Microsoft Excel. Continuous variables were presented as medians with interquartile ranges (IQRs), while categorical variables were reported as frequencies and percentages. HIV-1 genotyping was considered successful when both PCR amplification and Sanger sequencing were completed successfully. Amplification success rates were calculated across different VL strata. Additionally, the consistency of drug-resistant mutation (DRM) detection was evaluated by comparing diluted and undiluted samples. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Results

Primer Design and Validation

As shown in Figure 1A and F, a stepwise primer screening strategy was employed to identify primer pair 13 for the PR/RT region and primer pair 4 for the IN region as the most effective combinations for consistent amplification across various sample types. These primer pairs were initially validated using 10 clinical samples with viral loads ranging from 1,000 to 2,000 copies/mL (Figure 1G), demonstrating stable amplification with distinct electrophoretic bands. To further assess amplification performance, the optimized primer system was compared against commercially available HIV-1 drug resistance genotyping kits[24–26] and an in-house method previously described by Liu et al[27] in 8 clinical plasma samples (Table 5). These samples with initial viral loads of 9,000–70,000 copies/mL were serially diluted to generate three viral load tiers (1,000–2,000 copies/mL, 500–1,000 copies/mL, and 50–500 copies/mL). The optimized system showed superior amplification success compared to all reference methods, particularly in samples with low viral loads (50–500 copies/mL), including lanes 2 to 9 in Figure 1H and lanes 17 to 24 in Figure 1I. These results highlight the enhanced

Table 5 Comparison of Drug Resistance Mutations Detected by Commercial Assays and the Optimised PCR–Sanger Method Across Different Viral Load Groups

A. PR/RT Region				
Viral load (copies/mL)	**Sample ID**	**Commercial assay (PR/RT)**	**Optimised assay (PR/RT)**	**Mutation difference**
50–500	S2	Fail	K103N	New assay detected mutation; commercial failed
	S4	Fail	Fail	Concordant
	S5	Fail	Fail	Concordant
	S7	Fail	Fail	Concordant
	S6	Fail	Fail	Concordant
	S8	Fail	K65R, S68SN, Y115F, M184MV, L210LW, V106M, V179E	New assay detected mutation; commercial failed
	S9	Fail	M184V, V179E	New assay detected mutation; commercial failed
	S10	Fail	V179T, Y181C, G190A, K238T	New assay detected mutation; commercial failed
500–1000	S2	Fail	K103N	Concordant
	S4	Fail	M184V,V106I,Y188L	New assay detected mutation; commercial failed
	S5	Fail	M184V	New assay detected mutation; commercial failed
	S7	Wild-type	Fail	New assay failed
	S6	K65R, S68SG, M184V, K101P, K103N	K65R, S68SG, M184V, K101P, K103N,N348I	Broader detection by new assay
	S8	Fail	K65R, S68SN, Y115F, M184MV, L210LW, V106M, V179E	New assay detected mutation; commercial failed
	S9	Fail	M184V, V179E	New assay detected mutation; commercial failed
	S10	V179T, Y181C, G190A	V179T, Y181C, G190A, K238T	Broader detection by new assay
1000–2000	S2	K103N	K103N	Concordant
	S4	M184V,V106I,Y188L	M184V,V106I,Y188L	Concordant
	S5	M184V	M184V	Concordant
	S7	Fail	Fail	Concordant
	S6	Fail	K65R, S68SG, M184V, K101P, K103N, N348I	New assay detected mutation; commercial failed
	S8	K65R, S68SN, Y115F, M184MV, L210LW, V106M, V179E	K65R, S68SN, Y115F, M184MV, L210LW, V106M, V179E	Concordant
	S9	Fail	M184V, V179E	New assay detected mutation; commercial failed
	S10	V179T, Y181C, G190A	V179T, Y181C, G190A, K238T	Broader detection by new assay
B. IN Region				
Viral load (copies/mL)	**Sample ID**	**Commercial assay (IN)**	**Optimised method (IN)**	**Mutation difference**
50–500	S2–S10	Mostly WT or Fail	Mostly WT or Fail	No discrepancy
500–1000	S2–S10	Mostly WT or Fail	Mostly WT or Fail	No discrepancy
1000–2000	S2–S10	All WT	All WT	Concordant

Notes: This table summarises the serial dilutions of eight clinical samples with initial viral loads ranging from 9,000 to 70,000 copies/mL. Each sample was diluted to generate three viral load tiers: 1,000–2,000 copies/mL, 500–1,000 copies/mL, and 50–500 copies/mL. Table A presents resistance testing results for the PR/RT region, while Table B shows results for the IN region.

Abbreviations: Failed, indicates unsuccessful amplification; WT, wild-type sequence.

sensitivity and efficiency of the optimized assay in detecting HIV-1 sequences under low-viremia conditions (Figure 1H and I). A detailed comparison of detected drug resistance mutations is provided in Table 5.

For the PR/RT region, the optimised assay identified all mutations detected by the commercial methods and additionally recovered multiple mutations in low–viral-load samples (50–500 copies/mL), including K103N, M184V, V179E, Y181C and K238T, in cases where commercial assays failed to amplify. Enhanced detection was also observed at 500–2,000 copies/mL, with broader mutation profiles recovered, particularly in samples harbouring complex resistance patterns. For the IN region, the optimised assay demonstrated comparable performance to commercial assays across all viral load groups, with fully consistent results in the 1,000–2,000 copies/mL group. These findings indicate that the optimised primer system provides mutation detection profiles equivalent to or more comprehensive than commercial assays, particularly under low-viremia conditions where commercial amplification frequently fails.

Patient Characteristics

To further validate the application of the optimised PCR primer system and detection method in real-world clinical settings, a total of 2,071 plasma samples were retrospectively collected from 2,050 HIV-1–infected individuals between January 2023 and December 2024. Among these, eight samples were confirmed to harbor mutations at the Y318 or N348 sites by Sanger sequencing (Table 6). Of the eight individuals, 87.5% (7/8) were male, with a median age of 35.5 years (interquartile range [IQR], 29.75–40.75). The median VL of the undiluted samples was 31,387.5 copies/mL (IQR, 1,402.5–359,034.25 copies/mL). Subtype analysis identified CRF01_AE in 4 cases (50%), CRF07_BC in 3 (37.5%), and CRF08_BC in 1 (12.5%). Half of the patients (4/8) were treatment-naïve, and the other half had received ART for at least 1 year. All previously treated patients had initiated therapy with a regimen of tenofovir disoproxil fumarate (TDF), lamivudine (3TC), and efavirenz (EFV).

Amplification Success Rates Across Different HIV-1 Viral Load Levels

Between 2023 and 2024, the overall amplification success rates for the PR/RT and IN regions were 87.40% (1,810/2,071) and 87.06% (1,803/2,071), respectively. As shown in Figure 2, amplification efficiency varied by plasma VL. For the PR/RT region (Figure 2A), amplification success approached 100% in samples with VL >200 copies/mL. In the low VL category (50–200 copies/mL), success rates were 82.86% (29/35) in 2023 and 83.33% (25/30) in 2024, with minimal year-to-year variation. In contrast, the IN region (Figure 2B) showed reduced amplification efficiency in low VL samples, with success rates of 82.86% (29/35) in 2023 and 73.33% (22/30) in 2024. However, for samples with VL ≥1,000 copies/mL, both regions demonstrated amplification success rates exceeding 99% across both years. These

Table 6 Clinical Characteristics of 8 PLWHs Harboring Y318 or N348 Mutations

ID	Sex	Age	HIV VL (Copies/mL)[a]	HIV-1 Subtype	PIs	NRTIs	NNRTIs	INSTIs
DR-GM1273	M	40	642	07_BC	None	M184V	K103N, N348I	None
HIVDR1563	M	29	544,000	01_AE	None	K65R, T69del	K101E, G190S, Y181C, N348NI (N348IN) [b]	None
HIVDR1727	M	30	59,591	01_AE	None	D67N, K70E, M184V	V106M, H221Y, F227L, Y318F	None
HIVDR1999	F	58	3184	08_BC	None	None	(N348T) [b]	None
HIVDR2269	M	35	1470	07_BC	None	K65R, S68SG	K103N, Y181C, H221Y, Y188L (N348T) [b]	None
HIVDR3070	M	28	1200	07_BC	None	None	G190GE, N348NI (N348IN) [b]	None
HIVDR3324	M	36	297,379	01_AE	None	None	N348NI (N348IN) [b]	None
HIVDR 3451	M	43	1,625,789	01_AE	None	None	(N348T) [b]	None

Notes: [a]Data were collected at the time of sampling; [b]Mutations in parentheses indicate non-resistance-associated polymorphisms.
Abbreviations: VL, Viral Load; PI, Protease Inhibitors; NRTIs, Nucleoside Reverse Transcriptase Inhibitors; NNRTIs, Non-Nucleoside Reverse Transcriptase Inhibitors; INSTIs, Integrase Strand Transfer Inhibitors; M, Male; F, Female.

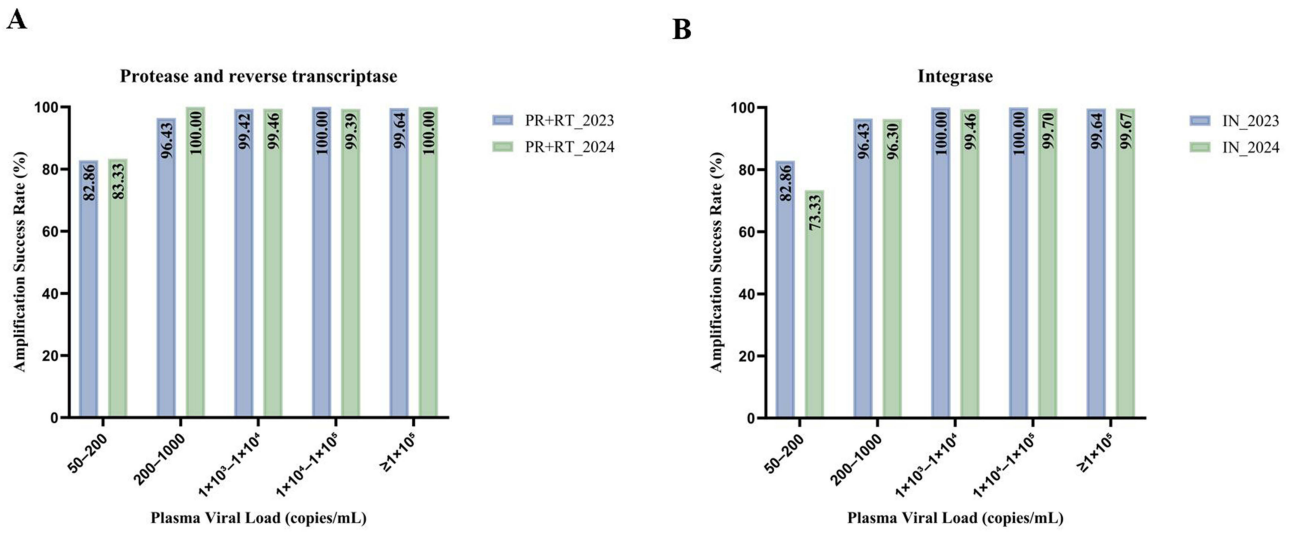

Figure 2 Amplification success rates for the PR+RT (**A**) and IN (**B**) regions across different HIV-1 viral load categories.

findings indicate that the system provides consistent and robust amplification for PR/RT and IN regions in moderate to high VL samples. Notably, amplification efficiency remained satisfactory even in low-level viremia, supporting its utility in drug resistance genotyping under challenging clinical conditions.

Detection Stability of Y318 and N348 Mutations Under Serial Dilution

To evaluate the detection stability of the optimised primers for key drug resistance sites following amplification extension, two NNRTI-associated mutations—Y318 and N348—were selected for analysis. The optimised primer system extended the sequencing coverage of the PR/RT region from 320 to 400 amino acids, enabling the inclusion of these critical sites. Among the successfully amplified and sequenced samples, eight were found to carry Y318 or N348 mutations (DR-GM1273, HIVDR1563, HIVDR1727, HIVDR1999, HIVDR2269, HIVDR3070, HIVDR3324, and HIVDR3451). These samples were serially diluted to viral load gradients of 1000, 400, 200, and 100 copies/mL for amplification testing, except DR-GM1273, which had an original viral load of 642 copies/mL and was diluted to three levels. Mutation detection at positions Y318 and N348 was then compared across gradients to assess detection consistency at varying viral loads.

All eight samples were successfully amplified. The detection results for the Y318 and N348 mutations at different dilution levels are summarized in Table 7. At all four VL levels, the Y318F and N348T mutations were consistently detected in HIVDR1727, HIVDR1999, and HIVDR2269. In contrast, detection rates in the remaining five samples were lower: 66.67% (2/3) for DR-GM1273, 50% (2/4) for both HIVDR1563 and HIVDR3324, 0% (0/4) for HIVDR3070, and 25% (1/4) for HIVDR3451. At the lowest VL of 100 copies/mL, the overall detection success rate for Y318 and N348 mutations was 37.5% (3/8).

NGS was performed on the samples with undetected mutations to investigate the potential reasons for detection failure. NGS was conducted on both undiluted and diluted samples of HIVDR1563, HIVDR3324, HIVDR3070, and HIVDR3451; however, the corresponding mutation sites were not identified (Table 8). Sample DR-GM1273 was not subjected to NGS under the original VL due to insufficient sample volume. Among the four samples analyzed, the undiluted samples of HIVDR1563 and HIVDR3324 showed N348I mutation frequencies of 27.35% and 28.31%, respectively. These results suggest that in highly diluted samples (200 and 100 copies/mL), the mutation frequency may have fallen below the detection threshold of the assay. This likely explains the undetected N348 mutation in DR-GM1273 at 100 copies/mL as well. In contrast, NGS failed to detect N348 mutations in the original VL samples of HIVDR3070 and HIVDR3451. This may be attributed to sample degradation or prolonged storage, potentially compromising RNA quality.

Table 7 Detection of Y318 and N348 Mutations in Serially Diluted Samples

	DR-GM1273	HIVDR1563	HIVDR1727	HIVDR1999	HIVDR2269	HIVDR3070	HIVDR3324	HIVDR 3451
Original VL (copies/mL)	642	544,000	59,591	3184	1470	1200	297,379	1625789
Mutation result (undiluted)	N348I	N348NI, N348IN	Y318F	N348T	N348T	N348NI, N348IN	N348NI, N348IN	N348T
Mutation result (1000 copies/mL)	None	N348NI, N348IN	Y318F	N348T	N348T	None	N348NI, N348IN	N348T
Mutation result (400 copies/mL)	N348I	N348NI, N348IN	Y318F	N348T	N348T	None	N348NI, N348IN	None
Mutation result (200 copies/mL)	N348I	None	Y318F	N348T	N348T	None	None	None
Mutation result (100 copies/mL)	None	None	Y318F	N348T	N348T	None	None	None

Notes: Only the Y318 and N348 mutation results are shown; the table does not represent the complete mutation profiles of the eight samples. None, Mutation not detected.
Abbreviation: VL, Viral Load.

Table 8 Confirmation of Mutation Detection by NGS

Mutation Result	DR-GM1273	HIVDR1563	HIVDR3070	HIVDR3324	HIVDR3451
SS (undiluted sample)	N348I	N348NI, N348IN	N348NI, N348IN	N348NI, N348IN	N348T
NGS (undiluted sample)	-	N348I (27.35%) [a]	None	N348I (28.31%) [a]	None
1000 copies/mL	-	-	None	-	-
400 copies/mL	-	-	None	-	None
200 copies/mL	-	None	None	None	None
100 copies/mL	None	None	None	None	None

Notes: "-": Not tested by next-generation sequencing (NGS); None: Mutation not detected. [a]Mutation frequency.
Abbreviation: SS, Sanger sequencing.

HIV-1 Subtyping Results

To further assess the subtyping performance of the optimised system, successfully genotyped samples from 2023 and 2024 were analysed using COMET HIV-1 2.4, BLAST, and the HIV-1 Gene Sequences Database (China). Subtypes consistent across all three tools were accepted as the final subtype assignment; samples with inconsistent results were classified as unique recombinant forms (URFs). As shown in Figure 3, the predominant subtypes identified in both years were CRF01_AE (351 in 2023; 352 in 2024) and CRF07_BC (346 in 2023; 334 in 2024), followed by subtype B (50 in both years), CRF55_01B (23 in 2023; 21 in 2024), and CRF117_0107 (18 in 2023; 49 in 2024). Unclassified subtypes were observed in 22 cases in 2023 and 19 cases in 2024. Overall, the subtype distribution remained stable over the two-year period.

Discussion

Given that PLWH require lifelong ART, the long-term selective pressure inevitably leads to the accumulation of DRMs, particularly in individuals with suboptimal treatment adherence. These drug-resistant strains may subsequently be transmitted to uninfected populations.[5,29] Therefore, HIV drug resistance genotyping prior to ART initiation or resumption is essential for optimising individualised treatment regimens.

In this study, the amplification efficiency and sensitivity of the optimised primer system were validated by comparing its performance with that of commercial HIV-1 drug resistance genotyping kits[24–26] and a previously reported in-house method.[27] The system demonstrated robust performance across a wide range of VLs and HIV-1 subtypes. In samples with viral loads ≥1,000 copies/mL, the amplification success rates for both PR/RT and IN regions approached 100%, indicating high stability and consistency. Even in low VL samples (50–200 copies/mL), success rates exceeded 80% for PR/RT and 70% for IN, suggesting improved sensitivity in challenging clinical conditions. This approach has the

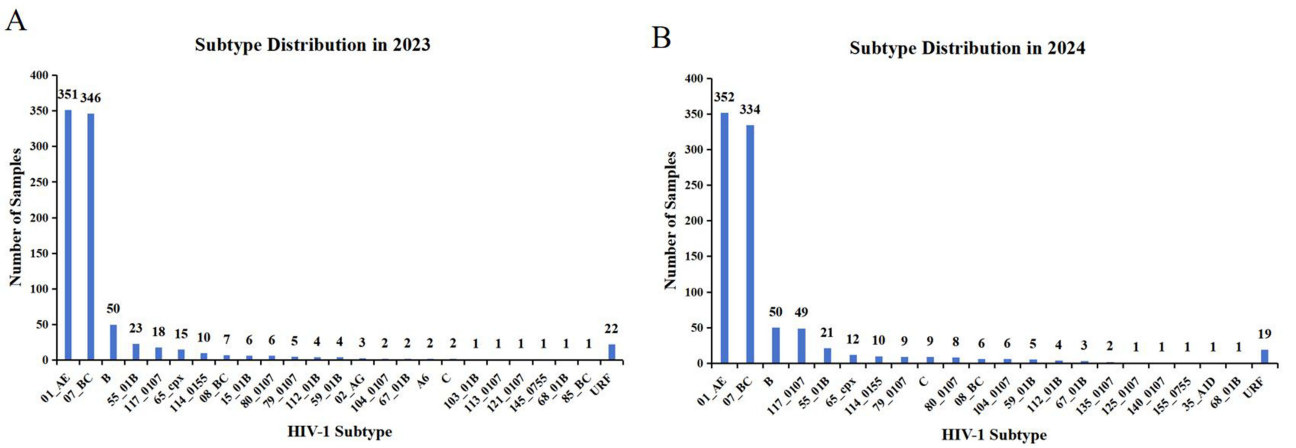

Figure 3 HIV-1 subtype distribution among successfully genotyped samples in 2023 (**A**) and 2024 (**B**).

potential to enhance the identification rate of drug-resistant mutations in individuals with low VLs, thereby reducing the risk of false negative results caused by HIV DNA contamination or nucleic acid degradation.[7]

To assess the detection stability of key NNRTI resistance mutations in the extended amplification region, gradient dilution experiments were performed on eight samples harbouring Y318 or N348 mutations. All samples were successfully amplified at viral load levels ranging from 1,000 to 100 copies/mL. Notably, three samples consistently yielded positive mutation detection across all gradients, suggesting good stability. However, overall detection efficiency declined with lower viral loads: only 3/8 (37.5%) samples retained detectable mutations at 100 copies/mL. NGS analysis revealed that failed detections were associated with lower mutation frequencies (eg, <30%), likely falling below the detection threshold after dilution. This pattern aligns with the well-recognised limitation of population-based Sanger sequencing, which generally requires variants to comprise approximately 15–20% of the viral population to be reliably visualised.

These findings highlight that, while the expanded primer system improves amplification coverage, its ability to detect low-frequency variants remains constrained by the intrinsic characteristics of Sanger sequencing rather than by the primer design itself. Nevertheless, within its amplification range, the assay can detect both previously reported and newly emerging mutations provided that their variant frequencies exceed the analytical threshold. Mixed viral populations can also be identified when the minority component is present at detectable levels. For samples requiring higher sensitivity—such as those with low-frequency variants or suspected mixed infections—NGS or ddPCR can serve as complementary approaches.

As HIV treatment strategies evolve toward dual-drug regimens and long-acting injectables, newer NNRTIs such as doravirine are increasingly being explored. Existing studies suggest that mutations such as Y318F and N348I can reduce susceptibility to NNRTIs, including doravirine, efavirenz, and nevirapine.[1,30] Therefore, expanding the amplification range to cover these clinically relevant resistance sites is crucial for improving the comprehensiveness of resistance monitoring and providing valuable data to guide both clinical treatment and new drug development.[1]

Furthermore, the optimised primer system demonstrated high subtype compatibility, showing consistent amplification success across multiple HIV subtypes, including CRF07_BC, CRF01_AE, subtype B, and their recombinant forms. The subtype distribution observed in this study was consistent with current HIV epidemiological patterns in China,[4,31] supporting its potential for broader application in routine clinical practice. Although the assay was designed based on Chinese circulating strains, the expanded coverage of conserved regions within the pol gene suggests the potential for broader applicability.

In addition to viral subtype diversity, demographic factors such as age may also influence the prevalence and detectability of drug-resistant mutations. Previous studies have shown that increasing age is often associated with longer infection duration and cumulative antiretroviral selective pressure, which may contribute to higher resistance rates.[32] In the present study, the median age of the eight individuals harbouring Y318 or N348 mutations was 35.5 years, consistent with the age distribution commonly reported among PLWH both domestically and internationally.[33–37] As half of these

individuals were treatment-naïve and all detected mutations corresponded to established NNRTI resistance pathways, the impact of age on the mutation patterns observed in this cohort is likely limited. Nevertheless, studies with larger cohorts encompassing a wider age range are warranted to further clarify whether age-related differences play a role in the emergence or detection of resistance mutations.

In recent years, advanced technologies such as NGS,[8–10] WGS,[11–13] and ddPCR[28] have markedly improved the sensitivity and genomic coverage of HIV drug resistance testing, particularly for identifying low-frequency variants and analyzing viral population diversity. NGS and WGS enable the detection of minority variants and generate comprehensive genomic information; however, their implementation requires specialized laboratory infrastructure, trained personnel, and extensive bioinformatics support, in addition to higher operational costs. These factors limit their routine use in many clinical settings. ddPCR is mainly applied for high-sensitivity quantitative analysis, and its role in qualitative resistance detection remains under investigation, requiring further methodological refinement.[38–41] In contrast, Sanger sequencing remains the predominant approach for HIV drug resistance surveillance in most regions due to its robustness, reproducibility, cost-effectiveness, and well-established workflow. The optimised primer system developed in this study —designed based on the sequence characteristics of HIV-1 strains circulating in China—successfully expands the coverage of the RT region and demonstrates reliable amplification across major subtypes. Notably, it maintains high amplification performance even in samples with low viral loads, supporting its suitability for large-scale clinical implementation. Nevertheless, emerging sequencing platforms continue to offer unique advantages for detecting low-abundance mutations, characterising viral quasispecies, and achieving more comprehensive resistance profiling. Future work may focus on adapting the current primer system for compatibility with NGS/WGS workflows or integrating it with high-sensitivity enrichment methods such as ddPCR to further enhance detection performance—particularly for low-viral -load samples and low-frequency drug resistance mutations.

Despite these promising results, this study has several limitations. First, amplification efficiency in the IN region was slightly lower than in the PR/RT region, possibly due to lower sequence conservation or suboptimal primer specificity. Second, amplification failure mechanisms were not fully explored; future studies should investigate contributing factors such as sample storage duration and RNA quality. Finally, for samples with low-frequency mutations or compromised RNA integrity, next-generation sequencing or repeat testing may still be necessary to ensure result reliability.

Conclusion

In conclusion, the optimised PCR-Sanger–based method demonstrated expanded amplification coverage and improved sensitivity, particularly for low viral load samples and key NNRTI resistance sites. It also exhibited strong performance across major circulating HIV subtypes in China. These attributes underscore its potential utility in resistance monitoring, especially in resource-limited or early screening settings, and support its further development for clinical application.

Abbreviations

HIV, Human Immunodeficiency Virus Type; ART, Antiretroviral Therapy; NNRTI, Non-Nucleoside Reverse Transcriptase Inhibitor; NRTI, Nucleoside/Nucleotide Reverse Transcriptase Inhibitor; PI, Protease Inhibitor; INSTI, Integrase Strand Transfer Inhibitor; DRM, Drug Resistance Mutation; PR, Protease; RT, Reverse Transcriptase; IN, Integrase; PCR, Polymerase Chain Reaction; VL, Viral Load; NGS, Next-Generation Sequencing; PLWH, People Living with HIV.

Ethics Approval and Informed Consent

This study was approved by the Ethics Committee of Beijing Ditan Hospital, Capital Medical University (Approval number: DTEC-KY2023-002-01) and complied with the Declaration of Helsinki. The requirement for informed consent was waived by the ethics committee (Waiver No. AF/SS-20/05.2), as the study involved the use of existing clinical records and stored plasma samples, posed minimal risk to participants, and fully protected personal privacy and confidentiality.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Acknowledgments

We acknowledge Sailian Biotech Co., Ltd. (Guangzhou, China) for their collaboration and technical support in developing the Human Immunodeficiency Virus Type 1 (HIV-1) Protease/Reverse Transcriptase/Integrase Region Drug Resistance Mutation Detection Kit (Sanger Sequencing Method), which is based on the primer system optimised in this study.

Funding

This work was supported by the Beijing Key Laboratory of Technology and Application of Antiinfective New Drugs Research and Development; Beijing Municipal Administration of Hospitals Incubating Program code:PX2023063; The science foundation of Beijing Ditan Hospital, Capital Medical University (DTQL202401).

Disclosure

The authors report no conflicts of interest in this work.

References

1. Brenner BG, Oliveira M, Ibanescu R-I, Routy J-P, Thomas R. Doravirine responses to HIV-1 viruses bearing mutations to NRTIs and NNRTIs under in vitro selective drug pressure. *J Antimicrob Chemother*. 2023;78(8):1921–1928. doi:10.1093/jac/dkad184
2. Sterne JA, Hernán MA, Ledergerber B, et al. Long-term effectiveness of potent antiretroviral therapy in preventing AIDS and death: a prospective cohort study. *Lancet*. 2005;366(9483):378–384. doi:10.1016/s0140-6736(05)67022-5
3. Zhang J, Sun B, Sheng Z, et al. Full-spectrum surveillance of pre-treatment HIV drug resistance in Southeastern China. *Pharmaceuticals*. 2024;17(7). doi:10.3390/ph17070900
4. Shi YZ, Huang HH, Wang XH, et al. Retrospective study on genetic diversity and drug resistance among people living with HIV at an AIDS clinic in Beijing. *Pharmaceuticals*. 2024;17(1). doi:10.3390/ph17010115
5. Khodadad N, Hashempour A, Nazar M, Ghasabi F. Evaluating HIV drug resistance in the Middle East and North Africa and its associated factors: a systematic review. *Virol J*. 2025;22(1):112. doi:10.1186/s12985-025-02740-8
6. Dauwe K, Staelens D, Vancoillie L, Mortier V, Verhofstede C. Deep sequencing of HIV-1 RNA and DNA in newly diagnosed patients with baseline drug resistance showed no indications for hidden resistance and is biased by strong interference of hypermutation. *J Clin Microbiol*. 2016;54(6):1605–1615. doi:10.1128/jcm.00030-16
7. Metzner KJ. Technologies for HIV-1 drug resistance testing: inventory and needs. *Curr Opin HIV AIDS*. 2022;17(4):222–228. doi:10.1097/coh.0000000000000737
8. Avidor B, Girshengorn S, Matus N, et al. Evaluation of a benchtop HIV ultradeep pyrosequencing drug resistance assay in the clinical laboratory. *J Clin Microbiol*. 2013;51(3):880–886. doi:10.1128/jcm.02652-12
9. Kemp SA, Kamelian K, Cuadros DF, et al. HIV transmission dynamics and population-wide drug resistance in rural South Africa. *Nat Commun*. 2024;15(1):3644. doi:10.1038/s41467-024-47254-z
10. Ouyang F, Yuan D, Zhai W, Liu S, Zhou Y, Yang H. HIV-1 drug resistance detected by next-generation sequencing among ART-naïve individuals: a systematic review and meta-analysis. *Viruses*. 2024;16(2). doi:10.3390/v16020239
11. Faraci G, Park SY, Dubé MP, Lee HY. Full-spectrum HIV drug resistance mutation detection by high-resolution complete pol gene sequencing. *J Clin Virol*. 2023;164:105491. doi:10.1016/j.jcv.2023.105491
12. Ng TT, Su J, Lao HY, et al. Long-Read sequencing with hierarchical clustering for antiretroviral resistance profiling of mixed human immunodeficiency virus quasispecies. *Clin Chem*. 2023;69(10):1174–1185. doi:10.1093/clinchem/hvad108
13. Delaney KE, Ngobeni T, Woods CK, et al. Nano-RECall provides an integrated pipeline for HIV-1 drug resistance testing from Oxford Nanopore sequence data. *Trop Med Int Health*. 2023;28(3):186–193. doi:10.1111/tmi.13851
14. Ishak A, AlRawashdeh MM, Esagian SM, Nikas IP. Diagnostic, prognostic, and therapeutic value of droplet digital PCR (ddPCR) in COVID-19 patients: a systematic review. *J Clin Med*. 2021;10(23). doi:10.3390/jcm10235712
15. Liu S, Huang X, Zhang S, et al. Droplet digital polymerase chain reaction (ddPCR) for bloodstream infections: a meta-analysis. *J Infect*. 2024;89(6):106329. doi:10.1016/j.jinf.2024.106329
16. Lan Y, Ling X, Deng X, et al. Drug resistance profile among HIV-1 infections experiencing ART with low-level viral load in Guangdong china during 2011-2022: a retrospective study. *Infect Drug Resist*. 2023;16:4953–4964. doi:10.2147/idr.S419610
17. Bareng OT, Choga WT, Maphorisa ST, et al. HIV-1C in-house RNA-based genotyping assay for detection of drug resistance mutations in samples with low-level viral loads. *Infect Drug Resist*. 2022;15:7565–7576. doi:10.2147/idr.S388816

18. He S, Song W, Guo G, et al. Multiple CRF01_AE/CRF07_BC recombinants enhanced the HIV-1 epidemic complexity among MSM in Shenyang City, Northeast China. *Front Microbiol*. 2022;13:855049. doi:10.3389/fmicb.2022.855049
19. Wang D, Feng Y, Hao J, et al. National and regional molecular epidemiology of HIV-1 - China, 2004-2023. *China CDC Wkly*. 2024;6 (48):1257–1263. doi:10.46234/ccdcw2024.252
20. Ye M, Chen X, Duo L, et al. Identification of two novel HIV-1 circulating recombinant forms of CRF111_01C and CRF116_0108 in southwestern Yunnan, China. *Virulence*. 2022;13(1):19–29. doi:10.1080/21505594.2021.2010399
21. Christopherson C, Sninsky J, Kwok S. The effects of internal primer-template mismatches on RT-PCR: HIV-1 model studies. *Nucleic Acids Res*. 1997;25(3):654–658. doi:10.1093/nar/25.3.654
22. Swanson P, de Mendoza C, Joshi Y, et al. Impact of human immunodeficiency virus type 1 (HIV-1) genetic diversity on performance of four commercial viral load assays: lCx HIV RNA Quantitative, AMPLICOR HIV-1 MONITOR v1.5, VERSANT HIV-1 RNA 3.0, and NucliSens HIV-1 QT. *J Clin Microbiol*. 2005;43(8):3860–3868. doi:10.1128/jcm.43.8.3860-3868.2005
23. Rowley CF, Boutwell CL, Lockman S, Essex M. Improvement in allele-specific PCR assay with the use of polymorphism-specific primers for the analysis of minor variant drug resistance in HIV-1 subtype C. *J Virol Methods*. 2008;149(1):69–75. doi:10.1016/j.jviromet.2008.01.005
24. Li Q, Yu F, Song C, et al. A concentration method for HIV drug resistance testing in low-level viremia samples. *Biomed Res Int*. 2022;2022:2100254. doi:10.1155/2022/2100254
25. Li Q, Yu F, Song C, et al. HIV-1 genotypic resistance testing using sanger and next-generation sequencing in adults with low-level Viremia in China. *Infect Drug Resist*. 2022;15:6711–6722. doi:10.2147/idr.S387215
26. Li Q, Chen M, Zhao H, et al. Persistent low-level viremia is an independent risk factor for virologic failure: a retrospective cohort study in China. *Infect Drug Resist*. 2021;14:4529–4537. doi:10.2147/idr.S332924
27. Liu L, Dai L, Yao J, et al. Lack of HIV-1 integrase inhibitor resistance among 392 antiretroviral-naïve individuals in a tertiary care hospital in Beijing, China. *Aids*. 2019;33(12):1945–1947. doi:10.1097/qad.0000000000002282
28. Yang X, Yan C, Li M, et al. Improved detection of HIV-1 drug resistance with droplet digital polymerase chain reaction: a comparative study and its implications. *Open Forum Infect Dis*. 2025;12(6):ofaf286. doi:10.1093/ofid/ofaf286
29. Yuan L, Chen K, Cai Y, et al. Analysis of ART effects and drug resistance in adult HIV/AIDS patients in Meigu County, Liangshan Prefecture, China. *BMC Infect Dis*. 2024;24(1):155. doi:10.1186/s12879-024-09048-y
30. Radzio J, Sluis-Cremer N. Subunit-specific mutational analysis of residue N348 in HIV-1 reverse transcriptase. *Retrovirology*. 2011;8:69. doi:10.1186/1742-4690-8-69
31. Xiao P, Li J, Fu G, Zhou Y, Huan X, Yang H. Geographic distribution and temporal trends of HIV-1 subtypes through heterosexual transmission in china: a systematic review and meta-analysis. *Int J Environ Res Public Health*. 2017;14(7). doi:10.3390/ijerph14070830
32. Pang X, He Q, Tang K, et al. Drug resistance and influencing factors in HIV-1-infected individuals under antiretroviral therapy in Guangxi, China. *J Antimicrob Chemother*. 2024;79(5):1142–1152. doi:10.1093/jac/dkae084
33. Fabeni L, Armenia D, Abbate I, et al. HIV-1 transmitted drug resistance in newly diagnosed individuals in Italy over the period 2015-21. *J Antimicrob Chemother*. 2024;79(9):2152–2162. doi:10.1093/jac/dkae189
34. Tan T, Bai C, Lu R, et al. HIV-1 molecular transmission network and drug resistance in Chongqing, China, among men who have sex with men (2018-2021). *Virol J*. 2023;20(1):147. doi:10.1186/s12985-023-02112-0
35. Áy É, Müller V, Mezei M, et al. Transmitted drug resistance in newly diagnosed and treatment-naïve HIV type 1-infected patients in Hungary. *J Glob Antimicrob Resist*. 2020;20:124–130. doi:10.1016/j.jgar.2019.07.014
36. Crowell TA, Danboise B, Parikh A, et al. Pretreatment and acquired antiretroviral drug resistance among persons living with HIV in Four African Countries. *Clin Infect Dis*. 2021;73(7):e2311–e2322. doi:10.1093/cid/ciaa1161
37. Yendewa GA, Sahr F, Lakoh S, et al. Prevalence of drug resistance mutations among ART-naive and -experienced HIV-infected patients in Sierra Leone. *J Antimicrob Chemother*. 2019;74(7):2024–2029. doi:10.1093/jac/dkz134
38. Cassidy NAJ, Fish CS, Levy CN, et al. HIV reservoir quantification using cross-subtype multiplex ddPCR. *iScience*. 2022;25(1):103615. doi:10.1016/j.isci.2021.103615
39. Scutari R, Galli L, Alteri C, et al. Evaluation of HIV-DNA and residual viremia levels through week 96 in HIV-infected individuals who continue a two-drug or switch to a three-drug integrase strand transfer inhibitor-based regimen. *Int J Antimicrob Agents*. 2023;61(5):106771. doi:10.1016/j.ijantimicag.2023.106771
40. Donadoni M, Cakir S, Bellizzi A, Swingler M, Sariyer IK. Modeling HIV-1 infection and NeuroHIV in hiPSCs-derived cerebral organoid cultures. *J Neurovirol*. 2024;30(4):362–379. doi:10.1007/s13365-024-01204-z
41. González-Navarro I, Urrea V, Gálvez C, et al. Assessing advances in three decades of clinical antiretroviral therapy on the HIV-1 reservoir. *J Clin Invest*. 2024;135(2). doi:10.1172/jci183952