

## Evaluation of a Novel In-house HIV-1 Genotype Drug Resistance Assay using Clinical Samples in China



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**Abstract: Background:** HIV drug resistance poses a major challenge for anti-retroviral treatment (ART) and the prevention and control of HIV epidemic.

**Objective:** The study aims to establish a novel in-house assay with high efficiency, named AP in-house method, that would be suitable for HIV-1 drug resistance detection in China.

**Methods:** An in-house HIV-1 genotyping method was used to sequence the partial pol gene from 60 clinical plasma samples; the results of our test were compared with a commercial ViroSeq HIV-1 genotyping system.

**Results:** Among sixty samples, 58(96.7%) were successfully amplified by AP in-house method, five of them harbored viral load below 1,000 copies/ml. The genotype distribution was 43.1% CR-F07\_BC (25/58), 39.7% CRF01\_AE (23/58), 6.9% CRF55\_01B (4/58), 5.2% subtype B (3/58) and 5.2% CRF08\_BC (3/58). Compared with that of the ViroSeq system, the consistent rate of these nucleotides and amino acids obtained by AP in-house method was up to  $99.5 \pm 0.4\%$  and  $99.5 \pm 0.4\%$ , respectively. A total of 290 HIV-1 drug resistance mutations were identified by two methods, including 126 nucleoside reverse transcriptase inhibitors (NRTIs), 145 non-nucleoside reverse transcriptase inhibitors (NNRTIs) and 19 protease inhibitors (PIs) resistance mutations. Out of them, 94.1% (273/290) were completely concordant between the AP in-house method and the ViroSeq system.

**Conclusion:** Overall, the evaluation of AP in-house method provided comparable results to those of the ViroSeq system on diversified HIV-1 subtypes in China.

**Keywords:** HIV-1, anti-retroviral treatment, genotype, drug resistance mutation, protease inhibitor, nucleoside reverse transcriptase inhibitors, non-Nucleoside reverse transcriptase inhibitors.

### 1. INTRODUCTION

Anti-retroviral treatment (ART) is one of the best treatments for HIV-infected individuals at present by suppressing virus replication and recovering CD4<sup>+</sup> cell counts effectively [1-5]. Moreover, the implementation of ART might be able to dramatically reduce HIV/AIDS-related morbidity and mortality [1-5]. Since the National Free ART program in 2003, over 59% of patients who met the criteria of ART initiation received the free ART by 2015 in China [6-10], and the coverage was expanded to respond to the “90-90-90” target [11-15]. However, the wide use of ART will lead to the continuous selective pressure of drugs and high variability of HIV-1 [16-22], which results in the emergency of drug resistance mutation and its spread, and consequently increases the risk of ART failure [23-25]. HIV drug

resistance is the major challenge for ART and the prevention and control of HIV epidemic [26-28]. As reported in a cross-sectional survey in China, the rate of patients with virological failure was 8.5% and the prevalence of HIV drug resistance mutations among patients receiving first-line ART was 4.3% [29]. Meanwhile, the prevalence of transmitted drug resistance was 3.6% shown in another nationwide cross-sectional survey in China [9]. Evidently, it is important to enhance the detection of HIV-1 drug resistance for guiding appropriate use of drugs and preventing the production of drug-resistant HIV strains [4, 30].

In addition to the development of drug resistance mutations, another consequence of the high variability of HIV is genetic diversity [31-33]. In Group M (HIV-1 pandemic globally), nine subtypes and 102 circulating recombinant forms (CRFs) have been identified so far (<http://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html>). Moreover, the distributions of HIV-1 genotypes worldwide were

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geographically different and dynamically changing [34-38]. The high variability of HIV brings a particular technological challenge to the local detection of drug-resistant mutations with the commercial kits, [39] such as ViroSeq system, most of which were designed and optimized for subtype B virus [31, 40]. In China, however, the predominant circulating strains were CRF07\_BC, CRF01\_AE, CRF08\_BC, subtype B', as well as special CRFs [40-45]. Not only that, the clinical application of commercial tests has been limited owing to the tedious manual operation and expensive cost. It is necessary to develop a cost-effective method that is readily adaptable for HIV-1 genotype resistance assay in China. Here, we designed the AP in-house method, a novel HIV-1 genotype drug resistance assay by optimizing the operation of viral RNA extraction and RT-PCR to minimize the error caused by manual operations and experimental procedures. The clinical performance characteristics of this HIV-1 drug resistance detection were also evaluated by comparing it with ViroSeq system.

## 2. MATERIALS AND METHODS

### 2.1. Clinical Specimens

Sixty stored plasma samples were obtained from patients failing ART (2016-2018) in the anti-HIV treatment program of the 13<sup>th</sup> Five-Year Plan in China. All these samples had been previously tested for HIV-1 drug resistance using the ViroSeq System and aliquots were stored at -80°C. The study was also approved by the Institutional Research Ethics Community of Beijing Ditan Hospital, Capital Medical University with reference No.2019(037)-002, and all participants provided written informed consent.

### 2.2. Viral Load Detection

The HIV-1 viral loads in these patients' plasma were re-tested with m2000sp Liquid Handler & m 2000 rt Real Time PCR System (Abbott) following the manufacturer's protocol, which could be used to evaluate amplification sensitivity for AP in-house method [46, 47].

### 2.3. HIV-1 Drug Resistance Genotyping by ViroSeq System

HIV-1 genotyping was performed with ViroSeq HIV-1 Genotyping System (Abbott molecular) [48, 49]. The analysis procedure followed was according to the manufacturer's instructions. In this system, HIV-1 RNA is extracted manually from 0.5 mL of plasma, followed by reverse transcription with MuLV reverse transcriptase. After the UNG enzyme destroys any species of DNA containing deoxyuridine and a single 40 cycles PCR with AmpliTaq Gold DNA Polymerase, the PCR yields a 1.8 kb DNA product. PCR products are then purified with PCR Cleanup Kit and sequenced by ABI 3130xl Genetic Analyzer according to product instruction for user issued by the manufacturer. The resulting sequences are assembled and analyzed using ViroSeq Genotyping System Software.

### 2.4. RNA Extraction and Amplification of HIV-1 Partial Pol Gene in AP In-House Method

Viral RNA for AP in-house method was extracted from 200 µL plasma using TGuideS32 automated nucleic acid extraction system (TIANGEN BIOTECH (Beijing) CO., LTD) according to the manufacturer's instructions. The process of RT-PCR of RNA and PCR was simplified into one tube using PrimeScript™ One StepOne RT-PCR kit, Ver.2(-TAKARA, Cat. RR055A), following the manufacturer's protocol. The primers for RT-PCR were Brf-F1 (forward, 5'-TCA CTC TTT GGC AAC GAC CC-3') and Brf-R2 (reverse, 5'-GGA GTC TTT CCC CAT ATT ACT ATG CTT TC-3'[-GAAAGCATAGTAATATGGGGAAAGACTCC]). Reverse transcription was performed at 50°C 40 min with primer Brf-R2, and the amplification started with an initial denaturation at 94°C for 3 min. 50 cycles of PCR were performed with PCR conditions of 94°C for 20 s, 59°C for 30 s, and 72°C for 1 min following an extension to 72°C for 10 min. The amplification products were identified with 1% agarose gel electrophoresis. For the positive products, DNA sequencing was performed on a 3730XL DNA genetic analyzer (Applied Biosystem, CA, USA), according to manufacturer's instructions.

### 2.5. Drug Resistance Mutation and Phylogenetic Analysis in AP In-house Method

The obtained sequences were assembled, aligned and edited by Sequencher 5.0 software with a sequencing electrogram height over 30% of the wild-type bases as mutations. The valid *pol* sequences were then submitted to the Stanford University HIV Drug Resistance Database (HIVdb version 8.9-1, <http://hivdb.stanford.edu>) for genotypic resistance interpretation. The HIV-1 subtypes of partial *pol* sequences were identified by submitting to the REGA HIV-1 Subtyping Tool Version 3.0 and then confirmed using phylogenetic trees, which was conducted by the neighbor-joining method with 1,000 bootstrap replicates in MEGA 5.0 software. The bootstrap values up to 70% were considered significant.

### 2.6. Sequence Analysis

The evaluation of AP in-house method was done by comparing it with ViroSeq system, which was considered as the gold standard. To assess its accuracy, the concordance rate of nucleotide or amino acid sequences generated was analyzed between the ViroSeq system and AP in-house method. Simply, the proportion (%) of partial discordance and discordance in all obtained nucleotide or amino acid sequences for each sample were calculated.

Drug resistance-associated mutations in protease and reverse transcriptase identified by two methods were compared to evaluate the feasibility and effectiveness of AP in-house method. The partial and complete discordance mutations among all identified HIV-1 drug resistance mutations were calculated as above. Then, the distinctions between AP in-house method and ViroSeq system in partial or complete discordance mutations were characterized.

### 3. RESULTS

#### 3.1. Confirmation of HIV-1 Infection and Viral Load Measurement

Sixty clinical specimens were collected from HIV-infected patients with antiviral treatment failure. To confirm HIV status of these specimen, their viral load was retested (Table 1). The median viral loads were 73,918 copies/ml (range from 211 to 3,073, 020 copies/ml). HIV-1 subtypes of 59 samples were identified with sequences obtained from the ViroSeq system by Phylogenetic analyses (Table 1). The HIV-1 subtype included 26 CRF07\_BC, 23 CRF01\_AE, 4 CRF55\_01B, 3 subtype B' and 3 CRF08\_BC, as shown in Table 1.

#### 3.2. Determination of the Sensitivity and Specificity of AP In-house Method

First, we evaluated the sensitivity of AP in-house method with 60 clinical specimens from patients failing ART in China, of which 58 (96.7%) were amplified and sequenced successfully, shown in Table 1. Of the two specimens that failed in amplification, one had a viral load of 6,800 copies/ml and the other one had a viral load of 211 copies/ml. The former could not be amplified with ViroSeq System, even at viral loads above 1,000 RNA copies/mL. The inability to be amplified could likely be attributed to the compromised quality of this specimen. The remaining specimens could be successfully amplified by using ViroSeq System. Nonetheless, it is worth noting that the AP in-house method was able to amplify 5 of 6 specimens with a viral load below 1,000 copies/ml (Table 1). The amplification sensitivity of AP in-house method reached a viral load of 1000 copies/ml, consistent with the ViroSeq System.

Next, the specificity of AP in-house to amplify different HIV-1 subtypes or CRF was evaluated on 58 clinical specimens. Phylogenetic analyses revealed that the subtype distribution

among these obtained sequences was CRF07\_BC (25/58), CRF01\_AE (23/58), CRF55\_01B (4/58), subtype B (3/58) and CRF08\_BC (3/58), as shown in (Table 1 and Fig. 1).

#### 3.3. Comparison of Nucleotide Sequences and Amino Acid Sequences Obtained from the AP In-house Method with that from the ViroSeq System

A total of 58 samples were amplified and sequenced successfully by both AP in-house method and ViroSeq system. Phylogenetic analysis showed that sequences from each sample obtained by two methods clustered monophyletically together (Fig. 1). The comparison of nucleotide sequences and amino acid sequences obtained by ViroSeq System and AP in-house method showed  $99.5 \pm 0.4\%$  and  $99.5 \pm 0.4\%$  (mean  $\pm$  SD) nucleotide and amino acid identity, respectively. Among 74,635 nucleotides obtained from 58 samples, 394 nucleotide differences were observed between the two methods which were in 80% of the instances caused by the difference in the detection of nucleotide mixtures (315/394). The other discordances (79/394) resulted from the different nucleotides detected. A total of 24,847 amino acids were identified from 58 samples by two methods. The amino acid discordances were observed at 133 positions (18 at drug resistance positions and others at non-drug resistance positions), and 110 amino acids were partial amino acid discordance.

#### 3.4. Comparison of Drug Resistance Mutations Identified by AP In-house Method with that by the ViroSeq System

There were a total of 290 HIV-1 drug resistance mutations in protease and reverse transcriptase identified by ViroSeq system and AP in-house method, including 126 nucleoside reverse transcriptase inhibitors (NRTIs), 145 non-nucleoside reverse transcriptase inhibitors (NNRTIs) and 19 protease inhibitors (PIs) resistance mutations, respectively

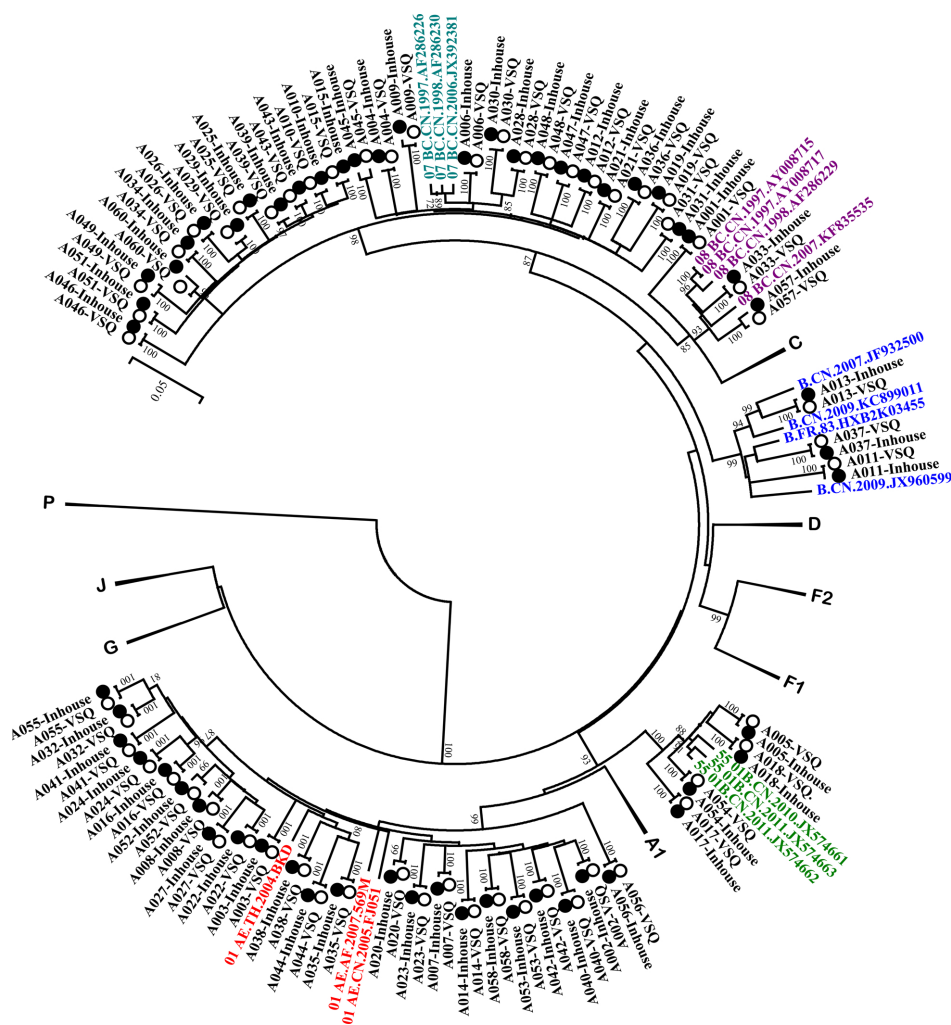
**Table 1. The amplification efficiency of AP In-house method compared to ViroSeq System.**

Sample Number	Viral Load (copies/ml)	HIV-1 Subtype or CRF	In-house Method	ViroSeq System
-	<1,000	-	-	-
A059	211	CRF07_BC	-	+
A007	225	CRF01_AE	+	+
A060	283	CRF07_BC	+	+
A040	430	CRF01_AE	+	+
A009	748	CRF07_BC	+	+
A034	796	CRF07_BC	+	+
-	>1,000-10,000		-	-
A020	1,289	CRF01_AE	+	+
A014	1,753	CRF01_AE	+	+
A053	2,016	CRF01_AE	+	+
A057	3,131	CRF08_BC	+	+
A015	3,729	CRF07_BC	+	+
A058	4,931	CRF01_AE	+	+
A010	6,081	CRF07_BC	+	+

(Table 1) contd....

Sample Number	Viral Load (copies/ml)	HIV-1 Subtype or CRF	In-house Method	ViroSeq System
A050	6,800	-	-	-
A051	6,800	CRF07_BC	+	+
A043	7,499	CRF07_BC	+	+
A033	7,765	CRF08_BC	+	+
A039	8,930	CRF07_BC	+	+
A008	9,056	CRF01_AE	+	+
-	>10,000-100,000		-	-
A038	10,634	CRF01_AE	+	+
A025	13,392	CRF07_BC	+	+
A019	13,965	CRF07_BC	+	+
A042	24,253	CRF01_AE	+	+
A005	29,700	CRF55_01B	+	+
A048	31,850	CRF07_BC	+	+
A013	41,826	B	+	+
A001	50,518	CRF08_BC	+	+
A011	56,880	B	+	+
A044	64,502	CRF01_AE	+	+
A017	73,659	CRF55_01B	+	+
A055	74,176	CRF01_AE	+	+
A052	83,851	CRF01_AE	+	+
A002	89,575	CRF01_AE	+	+
A024	92,114	CRF01_AE	+	+
A030	92,114	CRF07_BC	+	+
-	>100,00-1,000,000		-	-
A012	110,464	CRF07_BC	+	+
A046	124,395	CRF07_BC	+	+
A035	141,066	CRF01_AE	+	+
A037	154,478	B	+	+
A023	181,408	CRF01_AE	+	+
A031	182,680	CRF07_BC	+	+
A056	186,550	CRF01_AE	+	+
A036	190,501	CRF07_BC	+	+
A016	226,859	CRF01_AE	+	+
A027	268,275	CRF01_AE	+	+
A045	319,476	CRF07_BC	+	+
A021	323,972	CRF07_BC	+	+
A003	337,842	CRF01_AE	+	+
A006	362,290	CRF07_BC	+	+
A022	383,117	CRF01_AE	+	+
A047	482,464	CRF07_BC	+	+
A028	485,847	CRF07_BC	+	+
A054	517,378	CRF55_01B	+	+
A049	616,122	CRF07_BC	+	+
A029	738,856	CRF07_BC	+	+
A041	770,488	CRF01_AE	+	+
A004	855,620	CRF07_BC	+	+
A026	886,039	CRF07_BC	+	+
-	>1,000,000		-	-
A032	1,123,622	CRF01_AE	+	+
A018	3,073,020	CRF55_01B	+	+

- negative; + positive.



**Fig. (1).** Phylogenetic tree analyses of partial pol gene of HIV-1 obtained by AP in-house method and ViroSeq system. It was constructed with MEGA 5.0 using the neighbor-joining method with the Kimura two-parameter model and 1,000 bootstrap replication tests. The scale bars were shown as 0.05. Bootstrap values (>70) were shown at the corresponding nodes. Solid circle (●): sequences obtained by AP in-house method; Hollow circle (○): sequences obtained by ViroSeq system. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

**Table 2.** Comparison of drug resistance mutations detected by AP in-house method and ViroSeq system.

Region	-	Analyzed Cords	Concordant	Partial Discordant	Discordance
protease					
-	PIs	19	19(100%)	0	0
reverse transcriptase					
-	NRTIs	126	120(95.2%)	6(4.8%)	0
-	NNRTIs	145	134(92.4%)	10(6.9%)	1(0.7%)

ViroSeq was used as the reference standard.

PIs: Protease Inhibitors.

NRTIs: Nucleoside Reverse Transcriptase Inhibitors;

NNRTIs: Non-Nucleoside Reverse Transcriptase Inhibitors.

(Table 2). Among them, partial discordance mutations were observed at 16 positions, and one complete discordance mutation was observed in RT region (Table 3). Out of 16 partial discordance mutations, mixture bases in 12 positions were only identified by the ViroSeq system, and three cases

by AP in-house method. The other one at position 101 in RT region was identified with different mixture bases by two methods. Moreover, there was one NNRTI resistance mutation (V179D) that was only observed by AP in-house method but not by the ViroSeq system.

**Table 3. Detail of drug resistance mutations among partially discordant and discordant amino acid positions.**

Category	Position	ViroSeq System		In-house Method	
		Base <sup>a</sup>	Amino acid	Base <sup>a</sup>	Amino acid
<b>Partially discordant mutations</b>					
NRTIs Resistance	67	RAC	DN	AAC	N
-	74	WTA	LI	TTA	L
-	184	ATG	M	RTG	MV
-	184	RTR	MIV	ATA	I
-	219	AAM	KN	AAC	N
-	219	AAA	K	RAA	KE
NNRTIs Resistance	101	MAA	KQ	SAA	EQ
-	103	AGA	R	ARA	KR
-	179	GMA	AE	GMW	ADE
-	179	GWT	VD	GAT	D
-	181	TRT	YC	TGT	C
-	221	YAT	HY	TAT	Y
-	227	YTT	FL	CTT	L
-	227	YTT	FL	TTT	F
-	227	YTK	FL	TTG	L
-	230	HTG	ML	ATG	M
<b>Discordant mutations</b>					
NNRTIs Resistance	179	GTC	V	GAC	D

<sup>a</sup>IUPAC codes for sequence wildcard letters.

### 3.5. Comparison of the Testing Period between AP In-house Method and ViroSeq System

Automation nucleic acid extraction machine was used in our AP in-house method, substituting manual sample preparation in the ViroSeq system, which significantly reduced working time. As shown in Table 4, testing for 32 samples took only 4.5 h for AP in-house method to obtain the PCR products, while ViroSeq system took 10h.

**Table 4. Comparison turn around time (TAT) of AP in-house and ViroSeq system for 32 samples.**

Item	AP In-house Method	ViroSeq System
Sample Preparation	0.5 h	3 h
Reverse Transcription & PCR	4 h	7 h
Cycle Sequencing	5 h	5 h
Sequencing using 3130XL	14-15 h	14-15 h
Data analysis	1 h	1-2 h

h: hour.

## 4. DISCUSSION

In this study, a cost-effective HIV drug resistance mutation method was designed and optimized as AP in-house method. Compared with other reported in-house methods [50], the extraction of nucleic acid in AP in-house method was improved with instruments rejecting the approach chosen by hand. Moreover, two-step PCR was simplified into one-step, reducing the possibility of cross-contamination and increasing the detection efficiency. It was shown that 32

samples could be extracted and amplified within 4.5 hours by AP in-house method; however, it took 8 hours for the extraction and amplification of 12 samples by commercial kit and 7 hours for 24 samples by the traditional in-house method.

It was reported that HIV-1 circulating subtype in China was identified, including CRF01\_AE, subtype B, CRF07\_BC, and CRF08\_BC, [43, 51-58] as well as the novel HIV-1 CRFs [38, 59-64] (CRF55\_01B [65], CRF59\_01B [66], etc). So, the ability to detect mutations on diversified HIV-1 subtypes is necessary for HIV-1 drug resistance assays. Of the 58 samples detected by both methods, there were circulating subtypes in China, which suggested that the specificity of AP in-house method was similar to that of ViroSeq system and AP in-house method could be used for the testing of drug-resistant strains in China. And also, the lowest limit of amplification sensitivity of AP in-house method reached at the viral load of 225 copies/ml, higher than the 1000 copies/ml established by the ViroSeq system and traditional in-house method. All these indicated that this AP in-house method could be applied to HIV-1 drug resistance detection in China.

There is an advantage in the ViroSeq system [50], in which software is designed specifically for the analysis of drug resistance to PIs and RTIs. While for AP in-house method, the Stanford HIV Drug Resistance Database (HIVDB) was used to analyze drug resistance mutations. Although some of the nucleotide sequences obtained by the two methods were the same, the identified mutations might be a bit different depending on the particular database. For instance, H221Y [67], V106I [68] and K70T [69] were only known as

drug resistance mutations in HIVDB; however, L10I, K43T, A71T, A71V, T69N, K101Q and K103R were only identified in ViroSeq system v2.8. Therefore, multiple and updating databases should be used for the most comprehensive testing.

Different from the entire PR designed in the ViroSeq system, the AP in-house method was designed to detect drug resistance mutations (DRM) in part of PR (codons 4 to 99) according to HIV-1 subtype B in plasma samples. However, the major and minor drug resistance mutations in PR could be successfully detected by using this AP in-house method because the first common polymorphic/ non-polymorphic accessory PI-selected mutation was first present at position tenth in PR in the Stanford University HIV drug resistance database. Moreover, more than 51 amino acids (codons 336 to 386) in the first part of the RT gene were sequenced using AP in-house method compared to that obtained by using ViroSeq system, which could be used to detect more drug-resistant mutations in RT region. N348I [70-75], the additional miscellaneous mutation in the connection domain of the HIV-1 RT region, was detected in two plasma samples (A009 and A024) by using our AP in-house method in this study. Given the Stanford University HIV drug resistance database, N348I could reduce NRTIs Zidovudine susceptibility about 3-fold and NNRTIs Nevirapine and Efavirenz susceptibility by 3-fold and 2-fold, respectively [76]. Furthermore, N348I could enhance the resistance to Etravirine and Rilpivirine [70]. As a result, more positions of drug resistance mutations could be detected by AP in-house method than that in the ViroSeq system, which made it more extensive applications in HIV-1 drug resistance detection.

## CONCLUSION

We designed and evaluated an efficient in-house method for the identification of HIV-1 drug resistance mutations. The sensitivity and specificity of AP in-house method were comparable to the ViroSeq system and other assays published previously [50]. In addition, AP in-house method had the advantage of identifying novel drug resistance mutations located beyond the detectable regions of the ViroSeq system. The validated AP in-house method could serve as a powerful tool to effectively test patients failing ART and monitor the emergence and transmission of HIV-1 drug resistance in China.

## LIST OF ABBREVIATIONS

HIV-1	= Human Immunodeficiency Virus- 1
ART	= Anti- Retroviral Treatment
CRFs	= Circulating Recombinant Forms
PIs	= Protease Inhibitors
NRTIs	= Nucleoside Reverse Transcriptase Inhibitors
NNRTIs	= Non-Nucleoside Reverse Transcriptase Inhibitors

## ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

This study was reviewed by the Institutional Research Ethics Community of Beijing Ditan Hospital, Capital Medical University [No.2019(037)-002], China.

## HUMAN AND ANIMAL RIGHTS

No animals were used in this research. All humans research procedures were in accordance with the standards set forth in the Declaration of Helsinki principles of 1975, as revised in 2013 (<http://ethics.iit.edu/ecodes/node/3931>).

## CONSENT FOR PUBLICATION

Written informed consents were obtained from all recruited individuals.

## STANDARDS OF REPORTING

The study conforms to the STARD guidelines.

## AVAILABILITY OF DATA AND MATERIALS

Stanford University HIV Drug Resistance Database (HIVdb version 8.9-1, <http://hivdb.stanford.edu>). We confirm that the data supporting the results and findings of this study are available within the article.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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## REFERENCES

- [1] Bandera A, Gori A, Clerici M, Sironi M. Phylogenies in ART: HIV reservoirs, HIV latency and drug resistance. *Curr Opin Pharmacol* 2019; 48: 24-32. <http://dx.doi.org/10.1016/j.coph.2019.03.003> PMID: 31029861
- [2] Churchill MJ, Deeks SG, Margolis DM, Siliciano RF, Swanstrom R. HIV reservoirs: what, where and how to target them. *Nat Rev Microbiol* 2016; 14(1): 55-60. <http://dx.doi.org/10.1038/nrmicro.2015.5> PMID: 26616417
- [3] Kumi Smith M, Jewell BL, Hallett TB, Cohen MS. Treatment of HIV for the prevention of transmission in discordant couples and at the population level. *Adv Exp Med Biol* 2018; 1075: 125-62. [http://dx.doi.org/10.1007/978-981-13-0484-2\\_6](http://dx.doi.org/10.1007/978-981-13-0484-2_6) PMID: 30030792
- [4] Lu DY, Wu HY, Yarla NS, Xu B, Ding J, Lu TR. HAART in HIV/AIDS Treatments: Future Trends. *Infect Disord Drug Targets* 2018; 18(1): 15-22.

- <http://dx.doi.org/10.2174/1871526517666170505122800> PMID: 28474549
- [5] Pace M, Frater J. A cure for HIV: is it in sight? *Expert Rev Anti Infect Ther* 2014; 12(7): 783-91. <http://dx.doi.org/10.1586/14787210.2014.910112> PMID: 24745361
- [6] Cao W, Hsieh E, Li T. Optimizing treatment for adults with HIV/AIDS in China: Successes over two decades and remaining challenges. *Curr HIV/AIDS Rep* 2020; 17(1): 26-34. <http://dx.doi.org/10.1007/s11904-019-00478-x> PMID: 31939111
- [7] Peng Z, Wang S, Xu B, Wang W. Barriers and enablers of the prevention of mother-to-child transmission of HIV/AIDS program in China: A systematic review and policy implications. *Int J Infect Dis* 2017; 55: 72-80.
- [8] Wen Y, Bar KJ, Li JZ. Lessons learned from HIV antiretroviral treatment interruption trials. *Curr Opin HIV AIDS* 2018; 13(5): 416-21. <http://dx.doi.org/10.1097/COH.0000000000000484> PMID: 29878912
- [9] Zhao S, Feng Y, Hu J, *et al.* Prevalence of Transmitted HIV drug resistance in antiretroviral treatment naïve newly diagnosed individuals in China. *Sci Rep* 2018; 8(1): 12273. <http://dx.doi.org/10.1038/s41598-018-29202-2> PMID: 30115986
- [10] Zhao Y, Han MJ, Gan XM, Ma Y, Zhao DC. Characteristics and viral suppression among people living with HIV from the National Free Antiretroviral Therapy Programme, 2019. *HIV Med* 2020; 21(11): 701-7. <http://dx.doi.org/10.1111/hiv.13020> PMID: 33369034
- [11] Zhu Q, Fang P, Zhao Y, Dai D, Luo X. How about the quality and recommendation on prevention, diagnosis, and treatment of HIV/AIDS guidelines developed by WHO: A protocol for systematic review. *Medicine (Baltimore)* 2020; 99(52): e23638. <http://dx.doi.org/10.1097/MD.00000000000023638> PMID: 33350740
- [12] Wang G, Lu C, Qin S, *et al.* 90-90-90 cascade analysis on reported CLHIV infected by mother-to-child transmission in Guangxi, China: a modeling study. *Sci Rep* 2020; 10(1): 5295. <http://dx.doi.org/10.1038/s41598-020-62281-8> PMID: 32210333
- [13] Le Guillou A, Pugliese P, Raffi F, *et al.* Reaching the second and third joint united nations programme on HIV/AIDS 90-90-90 targets is accompanied by a dramatic reduction in primary human immunodeficiency virus (HIV) infection and in recent HIV infections in a large french nationwide HIV cohort. *Clin Infect Dis* 2020; 71(2): 293-300. <http://dx.doi.org/10.1093/cid/ciz800> PMID: 31612225
- [14] Zhang N, Huang T, Yang XG, *et al.* A cross-sectional study on HIV/AIDS "90-90-90" treatment target in Shandong province, 2015. *Zhonghua liu xing bing xue za zhi = Zhonghua liuxingbingxue zazhi* 2017; 38(10): 1367-71.
- [15] Levi J, Raymond A, Pozniak A, Vernazza P, Kohler P, Hill A. Can the UNAIDS 90-90-90 target be achieved? A systematic analysis of national HIV treatment cascades. *BMJ Glob Health* 2016; 1(2): e000010. <http://dx.doi.org/10.1136/bmjgh-2015-000010> PMID: 28588933
- [16] Namale G, Kamacooko O, Bagiire D, *et al.* Sustained virological response and drug resistance among female sex workers living with HIV on antiretroviral therapy in Kampala, Uganda: a cross-sectional study. *Sex Transm Infect* 2019; 95(6): 405-11. <http://dx.doi.org/10.1136/sextrans-2018-053854> PMID: 31266818
- [17] McCluskey SM, Siedner MJ, Marconi VC. Management of virologic failure and HIV drug resistance. *Infect Dis Clin North Am* 2019; 33(3): 707-42. <http://dx.doi.org/10.1016/j.idc.2019.05.004> PMID: 31255384
- [18] Armenia D, Di Carlo D, Maffongelli G, *et al.* Virological response and resistance profile in HIV-1-infected patients starting darunavir-containing regimens. *HIV Med* 2017; 18(1): 21-32. <http://dx.doi.org/10.1111/hiv.12388> PMID: 27353061
- [19] Adawaye C, Fokam J, Kamangu E, *et al.* Virological response, HIV-1 drug resistance mutations and genetic diversity among patients on first-line antiretroviral therapy in N'Djamena, Chad: findings from a cross-sectional study. *BMC Res Notes* 2017; 10(1): 589. <http://dx.doi.org/10.1186/s13104-017-2893-1> PMID: 29126456
- [20] Li Y, Gu L, Han Y, *et al.* HIV-1 subtype B/B' and baseline drug resistance mutation are associated with virologic failure: a multi-center cohort study in China. *J Acquir Immune Defic Syndr* 2015; 68(3): 289-97. <http://dx.doi.org/10.1097/QAI.0000000000000473> PMID: 25501612
- [21] Bertagnolio S, Perno CF, Vella S, Pillay D. The impact of HIV drug resistance on the selection of first- and second-line ART in resource-limited settings. *J Infect Dis* 2013; 207 (Suppl. 2): S45-8. <http://dx.doi.org/10.1093/infdis/jit121> PMID: 23687288
- [22] Hingankar NK, Thorat SR, Deshpande A, *et al.* Initial virologic response and HIV drug resistance among HIV-infected individuals initiating first-line antiretroviral therapy at 2 clinics in Chennai and Mumbai, India. *Clin Infect Dis* 2012; 54 (Suppl. 4): S348-54. <http://dx.doi.org/10.1093/cid/cis005> PMID: 22544202
- [23] Kiptoo M, Brooks J, Lihana RW, *et al.* HIV-1 drug resistance-associated mutations among HIV-1 infected drug-naïve antenatal clinic attendees in rural Kenya. *BMC Infect Dis* 2013; 13: 517. <http://dx.doi.org/10.1186/1471-2334-13-517> PMID: 24180455
- [24] Luo XL, Mo LD, Su GS, *et al.* Incidence and types of HIV-1 drug resistance mutation among patients failing first-line antiretroviral therapy. *J Pharmacol Sci* 2019; 139(4): 275-9. <http://dx.doi.org/10.1016/j.jpshs.2018.11.016> PMID: 30928089
- [25] Phanuphak P, Sirivichayukul S, Jiamsakul A, *et al.* Transmitted drug resistance and antiretroviral treatment outcomes in non-subtype B HIV-1-infected patients in South East Asia. *J Acquir Immune Defic Syndr* 2014; 66(1): 74-9. <http://dx.doi.org/10.1097/QAI.0000000000000108> PMID: 24413039
- [26] Vermund SH. Control of HIV epidemic: improve access to testing and ART. *Lancet HIV* 2017; 4(12): e533-4. [http://dx.doi.org/10.1016/S2352-3018\(17\)30166-2](http://dx.doi.org/10.1016/S2352-3018(17)30166-2) PMID: 28867268
- [27] Simonetti FR, Kearney MF. Review: Influence of ART on HIV genetics. *Curr Opin HIV AIDS* 2015; 10(1): 49-54. <http://dx.doi.org/10.1097/COH.0000000000000120> PMID: 25389802
- [28] Elinav H, Pops KO, Shasha D, *et al.* HIV/AIDS profile and realities at a regional antiretroviral therapy clinic in Jerusalem: 12 years analysis. *Scand J Infect Dis* 2012; 44(1): 65-9. <http://dx.doi.org/10.3109/00365548.2011.608713> PMID: 21923627
- [29] Zuo Z, Liang S, Sun X, *et al.* Drug resistance and virological failure among HIV-infected patients after a decade of antiretroviral treatment expansion in eight provinces of China. *PLoS One* 2016; 11(12): e0166661. <http://dx.doi.org/10.1371/journal.pone.0166661> PMID: 27997554
- [30] Bennett DE, Myatt M, Bertagnolio S, Sutherland D, Gilks CF. Recommendations for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment. *Antivir Ther* 2008; 13 (Suppl. 2): 25-36. PMID: 18575189
- [31] Lau KA, Wong JJ. Current trends of HIV recombination worldwide. *Infect Dis Rep* 2013; 5 (Suppl. 1): e4. <http://dx.doi.org/10.4081/idr.2013.s1.e4> PMID: 24470968
- [32] Wang Z, Zhang M, Zhang R, *et al.* Diversity of HIV-1 genotypes and high prevalence of pretreatment drug resistance in newly diagnosed HIV-infected patients in Shanghai, China. *BMC Infect Dis* 2019; 19(1): 313. <http://dx.doi.org/10.1186/s12879-019-3927-1> PMID: 30961560
- [33] Plantier JC, Leoz M, Dickerson JE, *et al.* A new human immunodeficiency virus derived from gorillas. *Nat Med* 2009; 15(8): 871-2. <http://dx.doi.org/10.1038/nm.2016> PMID: 19648927
- [34] Xiao P, Zhou Y, Lu J, *et al.* HIV-1 genotype diversity and distribution characteristics among heterosexually transmitted population in Jiangsu province, China. *Virol J* 2019; 16(1): 51. <http://dx.doi.org/10.1186/s12985-019-1162-4> PMID: 31023323
- [35] Vasylyeva TI, Liulchuk M, du Plessis L, *et al.* The Changing Epidemiological Profile of HIV-1 Subtype B Epidemic in Ukraine. *AIDS Res Hum Retroviruses* 2019; 35(2): 155-63. <http://dx.doi.org/10.1089/aid.2018.0167> PMID: 30430838
- [36] Bbosa N, Kaleebu P, Ssemwanga D. HIV subtype diversity worldwide. *Curr Opin HIV AIDS* 2019; 14(3): 153-60.



- <http://dx.doi.org/10.1097/COH.0000000000000534> PMID: 30882484
- [37] Daw MA, El-Bouzedi A, Ahmed MO, Dau AA. Molecular and epidemiological characterization of HIV-1 subtypes among Libyan patients. *BMC Res Notes* 2017; 10(1): 170. <http://dx.doi.org/10.1186/s13104-017-2491-2> PMID: 28454556
- [38] Lu X, Zhao C, Wang W, *et al.* HIV-1 genetic diversity and its distribution characteristics among newly diagnosed HIV-1 individuals in Hebei province, China. *AIDS Res Ther* 2016; 13: 3. <http://dx.doi.org/10.1186/s12981-015-0087-2> PMID: 26793263
- [39] Megens S, Laethem KV. HIV-1 genetic variation and drug resistance development. *Expert Rev Anti Infect Ther* 2013; 11(11): 1159-78. <http://dx.doi.org/10.1586/14787210.2013.844649> PMID: 24151833
- [40] He X, Xing H, Ruan Y, *et al.* A comprehensive mapping of HIV-1 genotypes in various risk groups and regions across China based on a nationwide molecular epidemiologic survey. *PLoS One* 2012; 7(10): e47289. <http://dx.doi.org/10.1371/journal.pone.0047289> PMID: 23056619
- [41] Yin YQ, Chen JS, Cheng H, *et al.* Transition and evolution of HIV-1 subtype among HIV-1 infections in Wuxi city, 2013-2016. *Zhonghua liu xing bing xue za zhi = Zhonghua liuxingbingxue zazhi* 2020; 41(2): 244-8.
- [42] Yuan D, Du Z, Zhou J, *et al.* HIV-1 subtype diversity, drug resistance, and genetic transmission networks in men who have sex with men with virologic failure in antiretroviral therapy in Sichuan, China, 2011 to 2017. *Medicine (Baltimore)* 2019; 98(43): e17585. <http://dx.doi.org/10.1097/MD.00000000000017585> PMID: 31651864
- [43] Chen H, Luo L, Pan SW, *et al.* HIV Epidemiology and Prevention in Southwestern China: Trends from 1996-2017. *Curr HIV Res* 2019; 17(2): 85-93. <http://dx.doi.org/10.2174/1570162X17666190703163838> PMID: 31269884
- [44] Xie H, Nie J, Chen Q, Huang W, Wang Y. Comparison of the genotypic and phenotypic properties of HIV-1 standard subtype B and subtype B/B' env molecular clones derived from infections in China. *Emerg Microbes Infect* 2018; 7(1): 90. <http://dx.doi.org/10.1038/s41426-018-0087-0> PMID: 29769530
- [45] Lin YL, Song B, Shao B, *et al.* Identification of a Novel HIV-1 Unique Recombinant Form Comprising CRF01\_AE, Subtype B', and CRF65\_cpx Among Men Who Have Sex with Men in Jilin, China. *AIDS Res Hum Retroviruses* 2018; 34(8): 714-8. <http://dx.doi.org/10.1089/aid.2018.0109> PMID: 29786452
- [46] Shin KH, Lee HJ, Lee JH, Chang CL, Kim HH. Comparison of the cobas human immunodeficiency virus 1 (HIV-1) test using the cobas 4800 system with COBAS ampliPrep/COBAS TaqMan HIV-1 test and abtort realTime HIV-1 assay and performance evaluation of cobas HIV-1. *Am J Clin Pathol* 2019; 152(5): 558-62. <http://dx.doi.org/10.1093/ajcp/aqz075> PMID: 31365738
- [47] Braun P, Delgado R, Drago M, *et al.* A European multicentre study on the comparison of HIV-1 viral loads between VERIS HIV-1 Assay and Roche COBAS® TAQMAN® HIV-1 test, Abbott RealTime HIV-1 Assay, and Siemens VERSANT HIV-1 Assay. *J Clin Virol* 2017; 92: 75-82.
- [48] Mouafo LC, Péré H, Ndjoyi-Mbiguino A, *et al.* LETTER TO THE EDITOR Performance of the ViroSeq® HIV-1 Genotyping System v2.0 in Central Africa. *Open AIDS J* 2015; 9: 9-13. <http://dx.doi.org/10.2174/1874613601509010009> PMID: 25767633
- [49] Thiam M, Diop-Ndiaye H, Kebe K, *et al.* Performance of the ViroSeq HIV-1 genotyping system v2.0 on HIV-1 strains circulating in Senegal. *J Virol Methods* 2013; 188(1-2): 97-103. <http://dx.doi.org/10.1016/j.jviromet.2012.11.044> PMID: 23266258
- [50] Zhao J, Chang L, Wang L. Nucleic acid testing and molecular characterization of HIV infections. *Eur J Clin Microbiol Infect Dis* 2019; 38(5): 829-42. <http://dx.doi.org/10.1007/s10096-019-03515-0> PMID: 30798399
- [51] Yang Z, Wei S, Liu J, *et al.* Characterization of HIV-1 subtypes and drug resistance mutations in Henan Province, China (2017-2019). *Arch Virol* 2020; 165(6): 1453-61. <http://dx.doi.org/10.1007/s00705-020-04606-6> PMID: 32279138
- [52] Li W, Zhu Z, Chu J, *et al.* Multiple HIV-1 genotypes circulating among college students in Nanjing, China. *AIDS Res Hum Retroviruses* 2020; 36(7): 616-24. <http://dx.doi.org/10.1089/aid.2019.0288> PMID: 32316742
- [53] Zhao YT, Han ZG, Wu H, *et al.* Characteristics and dynamics of HIV-1 subtype distribution among injected drug users in Guangzhou, 2008 - 2015. *Zhonghua liu xing bing xue za zhi = Zhonghua liuxingbingxue zazhi* 2019; 40(12): 1629-33.
- [54] Yin Y, Liu Y, Zhu J, *et al.* The prevalence, temporal trends, and geographical distribution of HIV-1 subtypes among men who have sex with men in China: A systematic review and meta-analysis. *Epidemiol Infect* 2019; 147: e83. <http://dx.doi.org/10.1017/S0950268818003400> PMID: 30869019
- [55] Sun L, Jia L, Liu Y, *et al.* Multiple HIV-1 subtypes were found circulating in shijingshan district of Beijing, China. *AIDS Res Hum Retroviruses* 2019; 35(5): 494-9. <http://dx.doi.org/10.1089/aid.2018.0263> PMID: 30681000
- [56] Han ZG, Zhang YL, Wu H. Characteristic and dynamic of HIV-1 subtype distribution in men who have sex with men in Guangzhou, 2008-2015. *Zhonghua liu xing bing xue za zhi = Zhonghua liuxingbingxue zazhi* 2018; 39(1): 67-71.
- [57] Li X, Zhu K, Xue Y, *et al.* Multiple introductions and onward transmission of HIV-1 subtype B strains in Shanghai, China. *J Infect* 2017; 75(2): 160-8. <http://dx.doi.org/10.1016/j.jinf.2017.05.009> PMID: 28551370
- [58] Yuan R, Cheng H, Chen LS, Zhang X, Wang B. Prevalence of different HIV-1 subtypes in sexual transmission in China: a systematic review and meta-analysis. *Epidemiol Infect* 2016; 144(10): 2144-53. <http://dx.doi.org/10.1017/S0950268816000212> PMID: 26892485
- [59] Liang B, Yang Y, Zhang F, *et al.* Characterization of a Novel HIV-1 Recombinant Form (CRF01\_AE/CRF07\_BC/CRF08\_BC) Identified from Guangxi, China. *AIDS Res Hum Retroviruses* 2020; 36(2): 143-52. <http://dx.doi.org/10.1089/aid.2019.0185> PMID: 31482724
- [60] Jiang J, Liang B, Li K, *et al.* Genomic characterization of a novel HIV type 1 strain originating from CRF07\_BC and CRF01\_AE by heterosexual transmission in the Lingshan prefecture of Guangxi province, China. *AIDS Res Hum Retroviruses* 2020; 36(2): 153-60. <http://dx.doi.org/10.1089/aid.2019.0182> PMID: 31547666
- [61] Zhang Y, Pei Z, Li H, *et al.* Characterization of a novel HIV-1 circulating recombinant form (CRF80\_0107) among men who have sex with men in China. *AIDS Res Hum Retroviruses* 2019; 35(4): 419-23. <http://dx.doi.org/10.1089/aid.2018.0226> PMID: 30259751
- [62] Zhang C, Feng Y, Gao L, *et al.* Genetic characterization and recombinant history of a novel HIV-1 circulating recombinant form (CRF101\_01B) identified in Yunnan, China. *Infect Gene Evol* 2019; 73: 109-12.
- [63] Miao J, Ran J, Song Y, *et al.* Characterization of a novel HIV-1 circulating recombinant form, CRF01\_AE/B'/C (CRF96\_cpx), in Yunnan, China. *AIDS Res Hum Retroviruses* 2018; 34(4): 393-7. <http://dx.doi.org/10.1089/aid.2017.0288> PMID: 29258320
- [64] Su L, Wei D, Yang H, *et al.* Identification of a novel HIV-1 circulating recombinant form (CRF85\_BC) in Sichuan, China. *AIDS Res Hum Retroviruses* 2016; 32(9): 895-9. <http://dx.doi.org/10.1089/aid.2016.0053> PMID: 27169981
- [65] Han X, Takebe Y, Zhang W, *et al.* A large-scale survey of CRF55\_01B from men-who-have-sex-with-men in China: Implying the evolutionary history and public health impact. *Sci Rep* 2015; 5: 18147. <http://dx.doi.org/10.1038/srep18147> PMID: 26667846
- [66] Zhang W, Han X, An M, *et al.* Identification and characterization of a novel HIV-1 circulating recombinant form (CRF59\_01B) identified among men-who-have-sex-with-men in China. *PLoS One* 2014; 9(6): e99693. <http://dx.doi.org/10.1371/journal.pone.0099693> PMID: 24978029
- [67] Guo W, Li H, Zhuang D, *et al.* Impact of Y181C and/or H221Y mutation patterns of HIV-1 reverse transcriptase on phenotypic re-

- sistance to available non-nucleoside and nucleoside inhibitors in China. *BMC Infect Dis* 2014; 14: 237.  
<http://dx.doi.org/10.1186/1471-2334-14-237> PMID: 24885612
- [68] Gatanaga H, Ode H, Hachiya A, Hayashida T, Sato H, Oka S. Combination of V106I and V179D polymorphic mutations in human immunodeficiency virus type 1 reverse transcriptase confers resistance to efavirenz and nevirapine but not etravirine. *Antimicrob Agents Chemother* 2010; 54(4): 1596-602.  
<http://dx.doi.org/10.1128/AAC.01480-09> PMID: 20124001
- [69] Megens S, De Wit S, Bernatchez J, *et al.* Characterization of amino acids Arg, Ser and Thr at position 70 within HIV-1 reverse transcriptase. *Acta Clin Belg* 2014; 69(5): 348-57.  
<http://dx.doi.org/10.1179/2295333714Y.0000000038> PMID: 25103592
- [70] Xu HT, Colby-Germinario SP, Oliveira M, *et al.* The connection domain mutation N348I in HIV-1 reverse transcriptase enhances resistance to etravirine and rilpivirine but restricts the emergence of the E138K resistance mutation by diminishing viral replication capacity. *J Virol* 2014; 88(3): 1536-47.  
<http://dx.doi.org/10.1128/JVI.02904-13> PMID: 24227862
- [71] Yap SH, Herman BD, Radzio J, Sluis-Cremer N, Tachedjian G. N348I in HIV-1 reverse transcriptase counteracts the synergy between zidovudine and nevirapine. *J Acquir Immune Defic Syndr* 2012; 61(2): 153-7.  
<http://dx.doi.org/10.1097/QAI.0b013e3182657990> PMID: 22743599
- [72] Li HP, Han Y, Zhu XP, *et al.* Studying on the prevalence and mutation pattern of N348I which related to the resistance of HIV-1. *Zhonghua liu xing bing xue za zhi = Zhonghua liuxingbingxue za zhi* 2011; 32(9): 908-12.
- [73] Sluis-Cremer N, Moore K, Radzio J, Sonza S, Tachedjian G. N348I in HIV-1 reverse transcriptase decreases susceptibility to tenofovir and etravirine in combination with other resistance mutations. *AIDS* 2010; 24(2): 317-9.  
<http://dx.doi.org/10.1097/QAD.0b013e3283315697> PMID: 20010074
- [74] Biondi MJ, Beilhartz GL, McCormick S, Götte M. N348I in HIV-1 reverse transcriptase can counteract the nevirapine-mediated bias toward RNase H cleavage during plus-strand initiation. *J Biol Chem* 2010; 285(35): 26966-75.  
<http://dx.doi.org/10.1074/jbc.M110.105775> PMID: 20530477
- [75] Ehteshami M, Beilhartz GL, Scarth BJ, *et al.* Connection domain mutations N348I and A360V in HIV-1 reverse transcriptase enhance resistance to 3'-azido-3'-deoxythymidine through both RNase H-dependent and -independent mechanisms. *J Biol Chem* 2008; 283(32): 22222-32.  
<http://dx.doi.org/10.1074/jbc.M803521200> PMID: 18547911
- [76] Yap SH, Sheen CW, Fahey J, *et al.* N348I in the connection domain of HIV-1 reverse transcriptase confers zidovudine and nevirapine resistance. *PLoS Med* 2007; 4(12): e335.  
<http://dx.doi.org/10.1371/journal.pmed.0040335> PMID: 18052601