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New Trends of Primary Drug Resistance Among HIV Type 1-Infected Men Who Have Sex with Men in Liaoning Province, China

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Abstract

To elucidate the recent changes in prevalence of HIV-1 primary resistance mutations in men who have sex with men (MSM) in Liaoning province, 217 samples from antiretroviral therapy-naive MSM were collected. For 201 samples, the entire protease gene and 256 amino acids of the reverse transcriptase gene were successfully amplified by reverse transcriptase polymerase chain reaction (RT-PCR) and nested PCR of viral RNA and were sequenced. Among the amplified *pol* sequences, HIV-1 CRF01_AE accounted for 87.6% (176/201), subtype B accounted for 8.0% (16/201), and subtype CRF07_BC accounted for 4.5% (9/201). The overall prevalence of mutations conferring resistance to any drug was 4.5%, representing 4.5% for protease inhibitor (PI)-related mutations, 0.5% for nucleoside/nucleotide reverse transcriptase inhibitor (NRTI)-related mutations, and 0.5% for nonnucleoside reverse transcriptase inhibitor (NNRTI)-related mutations. Included were V32I (0.5%), M46I (2.0%), L90M (2.0%), T215C (0.5%), and Y188L (0.5%). Only one case carried resistance mutations to all three drug classes (L90M, L10I, and A71T to PI; T215C to NRTI; and Y188L to NNRTI). L10I (4.5%), V118I/IV (17.4%), and K103R/KR (10.0%) were commonly observed mutations, but do not confer any drug resistance to PIs, NRTIs, and NNRTIs. CRF01_AE is becoming a major HIV-1 infection subtype among MSM of Liaoning province. Relatively high rates of HIV drug-resistant mutations to PIs in antiretroviral treatment-naive patients in the study represent a serious challenge for future HIV treatment programs in China.

Introduction

In recent years, the Chinese HIV-1 epidemic has been increasing rapidly, moving from high-risk groups to the general population.[1] Men who have sex with men (MSM) constitute a major group for HIV transmission because of many high-risk behaviors, including multiple sexual partners, a low rate of condom usage, increased opportunities for sexual relationships, and a low HIV detection rate.[2,3] In 2007, it was estimated that 12.2% of new Chinese HIV cases were infected through homosexual contacts,[4] a proportion that had increased to 32.5% by 2009.[5] The tradition of carrying on the ancestral line remains prevalent in China, and many MSM have married women to presumably avoid social pressure. Of Chinese MSM 41–97% have female sexual partners or are married to a female. This bisexual behavior facilitates the transmission of HIV and other sexually transmitted diseases (STDs) from high-risk populations to the general population.[6,7]

A study of 195 MSM (all infected with HIV subtype B between 1999 and 2003) from six cities in the United States found that 15.9% of participants showed primary HIV drug resistance.[8] Studies have reported numbers of MSM infected with subtype B strains in Beijing and in Liaoning province.[9,10] The same studies revealed that Chinese MSM had close relationships with foreign HIV-infected people through which they had likely become infected with drug-resistant strains. Free antiretroviral treatment (ART) has been available on a nationwide basis for 6 years in China. HIV strains with highly drug-resistant mutations were also reported in patients treated with ART.[11–13] It is thus possible that drug-resistant strains would increase in number among infected MSM.

In light of the recent rapid increase of HIV infection in Chinese MSM, the epidemiology of drug-resistant strains should be reassessed. The incidence of drug resistance among treatment-naive MSM can be partially attributed to new infections by drug-resistant strains of HIV. In 2009, our group initiated a

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comprehensive study of HIV-1 drug resistance among MSM within the past 5 years in Liaoning province, China.

Materials and Methods

Study subjects

A cohort of HIV-positive treatment-naive MSM was recruited through preexisting MSM nongovernmental organizations (NGO) from 2003 to 2009. All subjects self-reporting oral or anal sex at least once with another male in the past 6 months were eligible for entering the study. A total of 217 HIV-positive MSM participants met the entry criteria and attended the study. Informed consent was obtained from each participant before this study. Peripheral blood samples were collected and immediately mixed with 10 ml EDTA-3K to prevent coagulation. The study protocol was approved by the institutional review boards (IRB) of the China Medical University.

Viral RNA extraction and reverse transcriptase polymerase chain reaction amplification

Plasma was separated conventionally from anticoagulant blood samples within 6 h after collection and was subsequently cryopreserved at −80°C until detection for drug-resistant genotypes. Viral RNA was isolated from 140 μl of plasma using a QIAamp viral RNA kit (Qiagen Inc., Germany) according to the manufacturer's instructions. The entire protease and 256 codons of the reverse transcriptase sequence within the *pol* gene were reverse transcribed and amplified with the SuperScript Polymerase One-Step RT-PCR System (Invitrogen, USA) using primers MAW-26 and RT-21.[14]

Sequencing and purification

Nested PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and were sequenced in both directions.[14]

Data analysis

We used Binary Logistic regression to assess the relationships between antiretroviral drug resistance and demographic variables. Backward elimination with a stay criteria for covariates set at $p = 0.05$ was used for model selection. We performed all analyses using SPSS 17.0 software.

The *pol* sequences were then multiply aligned by the Vector NTI advance 10.0 software Contig Express component and were analyzed through the Stanford University HIV Drug Resistance Database HIVdb program, version 4.2.6 (http://hivdb.stanford.edu) for genotypic resistance interpretation. Drug resistance-associated mutations in PR and RT were defined according to the Stanford HIVdb, version 4.2.6. The HIV-1 subtypes of the *pol* sequences were identified by the neighbor-joining method in MEGA4.1.

Results

Demographic characteristics of HIV-positive MSM

The *pol* regions were successfully amplified for 201 individuals of 217 HIV-positive MSM participants enrolled in the study. The demographics of these 201 MSM HIV/AIDS patients are shown in Table 1. Their ages ranged between 18 and 71 years (mean, 36 years). Subjects all resided in Liaoning province, and 75% of subjects were natives of Shenyang city. Eighty-seven percent of subjects were of Han ethnicity. Over 60% of the HIV infections were identified in 2008 or 2009. Of the subjects 72% were unmarried, while 26% were married.

Prevalence of HIV-1 subtypes among MSM

Phylogenetic analysis of the 201 successfully amplified *pol* regions (1065 bp total, encoding the protease gene and part of the reverse transcriptase gene region) was performed (Fig. 1). Among the amplified pol sequences, HIV-1 CRF01_AE accounted for 88% (176/201), subtype B for 8% (16/201), and CRF07_BC for 4% (9/201).

Genotypic analysis of HIV-1 drug resistance

Amplified gene regions were assessed for drug-resistant mutations through the Stanford HIV Drug Resistance Database. Mutations were grouped according to whether they could potentially confer resistance to protease inhibitor (PI), nucleoside/nucleotide reverse transcriptase inhibitor (NRTI), or nonnucleoside reverse transcriptase inhibitor (NNRTI) drug classes. Overall mutation frequency for resistance to any drug was 4.5%, with 4.5% for PI mutations (mainly occurring in HIV/AIDS patients infected with the CRF01_AE subtype), 0.5% for NRTI mutations, and 0.5% for NNRTI mutations (all occurring in HIV/AIDS patients infected with the B subtype). The drug resistance rate was calculated only using these mutations at drug resistance loci because they are known to reduce drug susceptibility.

Among PI-related drug resistance mutations, the major mutation M46I, which decreases susceptibility to nelfinavir (NFV), was found in four patients (4 of 201). L90M, which decreases susceptibility to indinavir (IDV) and NFV, was also found in four patients (4 of 201). Other major mutations including V32I (1of 201) and I54FI (1of 201) do not affect susceptibility to PI. Various minor mutations were detected at the following positions in the PR gene: L10I (4.5%), T74S/ST (3.0%), A71T/V (1.5%), F53FS (0.5%), and A71T/V (22.0%) (Table 2).

Among RTI-related drug resistance mutations, the T215C, which confers low-level resistance to the NRTI drugs zidovudine (AZT) and stavudine (D4T), was detected in one specimen (0.5%). Y188L, which confers resistance to the NNRTI drugs including high-level resistance to efavirenz (EFV) and nevirapine (NVP), intermediate-level resistance to delavirdine (DLV), and low-level resistance to etravirine (ETR), was also detected in one specimen (0.5%). Several other mutations including V118I/IV (17.4%), K103KR/R (10.0%), V179D/DV/E/S (4.5%), V106I/IV (2.0%), L210M (1.5%), K101Q (1.0%), V75LV (0.5%), F77L (0.5%), Q151QR (0.5%), A62AV (0.5%), and Y181HY (0.5%) were detected (Table 2). However, these mutations do not affect susceptibility to NRTI.

All subjects except one showed mutations resistant to only one class of drug, the exception being a sample from one patient who developed resistance to all classes of antiretroviral drugs. M46I, T74S/ST, and K103KR/R were detected only in individuals infected with the CRF01_AE subtype virus, whereas V179E was detected only in individuals with the B subtype virus (Table 2).

Figure 2 shows the prevalence of transmitted drug resistance over time according to drug class. The prevalence of transmitted drug-resistant mutations was on the rise slightly, but no significant changes over time were observed (Table 1).

Table 1. Demographic Characteristics of Study Subjects

	All subjects (n = *201*) *number (%)*	*Resistant* (n = *9*) *number (%)*	*Nonresistant* (n = *192*) *number (%)*	*Odds ratio*	*95% Confidence interval*	p-*value*[a]
Age at enrollment (years)						
18–25	46 (23)	1 (11)	45 (23)	0.992	0.42–2.35	0.985
26–35	67 (33)	5 (56)	62 (32)			
36–45	44 (22)	2 (22)	42 (22)			
≥45	44 (22)	1 (11)	43 (22)			
Recruitment site						
Huanggu district of Shenyang	43 (21)	1 (11)	42 (22)	1.074	0.56–2.06	0.829
Heping district of Shenyang	42 (21)	2 (22)	40 (21)			
Other districts of Shenyang	66 (33)	5 (56)	61 (32)			
Other cities of Liaoning province	50 (25)	1 (11)	49 (26)			
Race/ethnicity						
Han	175 (87)	9 (100)	165 (86)	N/A	N/A	N/A
Other	22 (11)	0 (0)	22 (11)			
Unknown	4 (2)	0 (0)	4 (2)			
Marital status						
Married	82 (41)	5 (56)	77 (38)	1.079	0.20–5.92	0.930
Unmarried	115 (57)	4 (44)	111 (60)			
Unknown	4 (2)	0 (0)	4 (2)			
Sexual orientation						
Homosexual	76 (38)	2 (22)	74 (39)	0.457	0.066–3.160	0.427
Bisexual	125 (62)	7 (78)	118 (61)			
Year of seroconversion						
Before 2006	38 (19)	1 (11)	37 (19)	1.325	0.66–2.65	0.427
2007	39 (19)	2 (22)	37 (19)			
2008	81 (40)	2 (22)	79 (41)			
2009	43 (21)	4 (44)	39 (20)			
HIV subtype						
CRF01_AE	176 (88)	7 (78)	169 (88)	1.654	0.55–4.94	0.367
B	16 (8)	1 (11)	15 (8)			
CRF07_BC	9 (4)	1 (11)	8 (4)			

[a]Nominal *p*-values (resistant versus nonresistant) from binary logistic regression analysis; an observed nominal *p*-value of 0.05 or less would be considered significant.

Discussion

HIV-1 drug resistance is one of the main causes of highly active antiretroviral treatment (HAART) failure. In western countries including the United States, the level of HIV drug resistance has been reported at or above 70% among the patients receiving HAART, and HIV drug resistance to at least one drug was above 10% in new HIV infections.[15–17] Currently, HIV infection rates are increasing gradually in MSMs in China. Some MSMs have even progressed to AIDS and subsequently received HAART treatment. High rates of HIV drug-resistant mutations in MSM found in our study corroborate earlier results and represent a serious challenge for HIV prevention and treatment programs in China.[9]

HIV-1 molecular epidemiological surveillance carried out during 2001–2002 revealed that there were at least seven major HIV-1 subtypes circulating in China.[18] The most prevalent subtypes were the B/C recombinant (~50%; mainly found in injecting drug users), followed by Thailand B (29.1%; former plasma/blood donors), and CRF01_AE (15.5%; heterosexual transmission). Only 2.6% was identified as subtype B and was concentrated in MSM. In contrast to previous reports that the majority of HIV isolates from Beijing MSM were subtype B,[9,19] we observed that the majority (87.6%) of HIV-infected MSM in Liaoning province had subtype CRF01_AE. In addition, B (11.0%) and CRF07_BC (2.0%) were also identified, similar to our previous studies.[10] In Liaoning province, MSM concentrated in cities and had a balanced age distribution according to my studies. They have established their own NGO and also frequent specific bars, bath centers, nightclubs, and other venues. They have few chances for social and sexual contact with foreigners, but notably, about 62% of this cohort admitted to having bisexual contact. Therefore, it is possible they were primarily infected with drug-resistant subtype CRF01_AE viruses from sexual transmission with other Chinese. Infection with CRF01_AE as identified in this study has been confirmed through heterosexual transmission in China, and according to the recent reports MSM contact has become a major route of local CRF01_AE transmission in some cities of China and Singapore.[20–22] Our results suggest that MSM contact is becoming a major route of CRF01_AE transmission in China and that various forms of HIV transmission, including both homosexual and heterosexual behavior, likely exist among MSM of Liaoning province.

We observed a high prevalence of HIV drug-resistant mutations, which represents a challenging situation for HIV prevention and control in China. In 2005, the high prevalence of HIV drug-resistant mutations (20.8%) was reported in patients ($n = 124$) treated with ART in Henan province in central Chi-

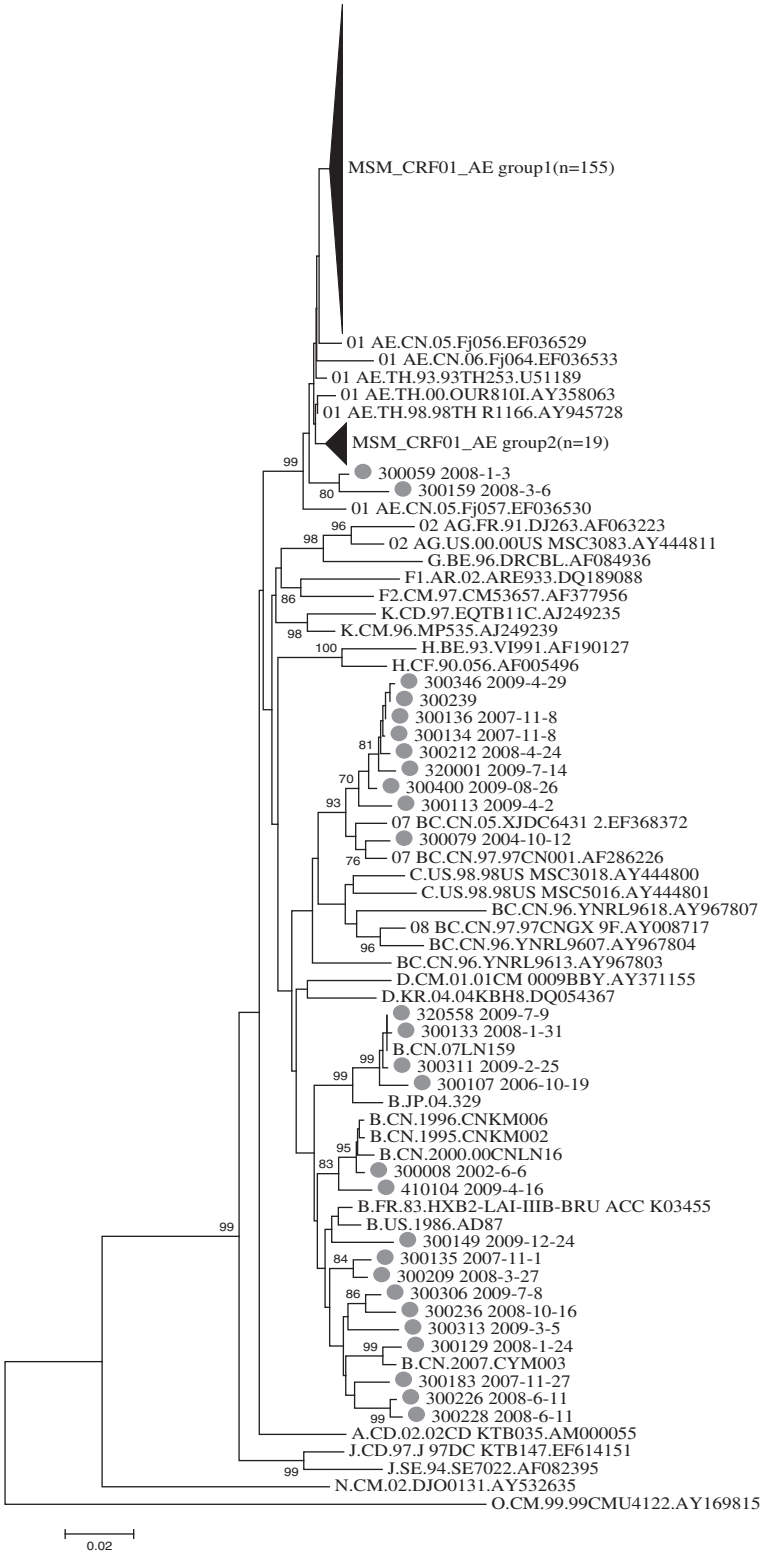

FIG. 1. A phylogenetic tree of *pol* gene regions (1065 bp, encoding the protease gene and part of the reverse transcriptase gene region) of HIV-1 strains was constructed using MEGA (version 4.1) based on the neighbor-joining method. Sample sequences (denoted with black circles) and reference HIV-1 subtypes obtained from the Los Alamos database were aligned using BioEdit 5.0.6 with minor manual adjustments. The statistical robustness of the neighbor-joining tree and reliability of the branching patterns were confirmed by bootstrapping with 1000 replicates. The trees were midpoint rooted. Values on the branches represent the percentage of 500 bootstrap replicates. Bootstrap values over 70% are marked in the tree.

na.[11] In addition, primary HIV drug resistance was reported as 0.6% to PIs, 5.8% to NRTIs, and 1.5% to NNRTIs in 2002 in a nationwide investigation,[12] and 4.4% in Liaoning province among ART-naive AIDS patients.[13] In this study, the rate of primary HIV drug resistance was 4.5% to any drug, 4.5% to PIs, 0.5% to NRTIs, and 0.5% to NNRTIs totally. The calculated drug resistance rate excluded potentially low degree or minor drug resistance loci and included only mutations demonstrated to have strong effects on resistance (denoted by the footnote to Table 2). Drug-resistant mutations to PI were much higher than previously reported and appear to be on a rise. Although PIs were not used in China, the majority of primary HIV drug resistance focused on PIs, which often happened in CRF01_AE-infected patients and the minority to NRTIs and NNRTIs

Table 2. Prevalence of Mutations Potentially Conferring Resistance to PIs, NRTIs, and NNRTIs

Mutations	Virus subtype (number of cases)			Total	Incidence (%)
	CRF01_AE	B	CRF07_BC		
PIs					
Major resistance mutations					
V32I[a]	0	0	1	1	0.5
M46I[a]	4	0	0	4	2.0
I54FI	1	0	0	1	0.5
L90M[a]	3	1	0	4	2.0
Minor resistance mutations					
L10I	7	2	0	9	4.5
F53FS	1	0	0	1	0.5
A71T	0	1	1	2	1.0
A7IV	0	1	0	1	0.5
T74S	4	0	0	4	2.0
T74ST	2	0	0	2	1.0
NRTIs					
V75LV	1	0	0	1	0.5
F77L	1	0	0	1	0.5
V118I	24	3	0	27	13.4
V118IV	7	0	1	8	4.0
Q151QR	1	0	0	1	0.5
L210M	2	1	0	3	1.5
T215C[a]	0	1	0	1	0.5
NNRTIs					
A62AV	1	0	0	1	0.5
K101Q	1	1	0	2	1.0
K103KR	4	0	0	4	2.0
K103R	16	0	0	16	8.0
V106I	0	3	0	3	1.5
V106IV	1	0	0	1	0.5
V179D	2	0	1	3	1.5
V179DV	1	0	0	1	0.5
V179E	0	4	0	4	2.0
V179S	1	0	0	1	0.5
Y181HY	1	0	0	1	0.5
Y188L[a]	0	1	0	1	0.5

[a]Mutations affecting susceptibility to drug.
PIs, protease inhibitors; NRTIs, nucleoside reverse transcriptase inhibitors; NNRTIs, nonnucleoside reverse transcriptase inhibitors.

among MSM of Liaoning province. Moreover, the relatively high rate of drug resistance to PIs suggests that primary drug-resistant strains may be derived from foreign countries with more diverse and prolonged ART experience. Resistance to NRTIs and NNRTIs happened in only one patient infected with subtype B. This suggests internal dissemination of drug-resistant strains of HIV of China.

In our study, we found the majority of primary HIV drug resistance focused on PIs including M46I and L90M. These two mutations were the most common PI major mutations. The results support this. In addition, we detected many minor mutations that were not always associated with a decrease in *in vitro* susceptibility and that can compensate for the reduced fitness of resistant mutants. Resistance mutations may occur with subtype specificity. For example, M46I, T74S/ST, and K103KR/R appeared only in individuals with strains of CRF01_AE, while V179E appeared only in individuals with B subtype strains. However, these mutations do not affect the susceptibility to ARV drugs. These amino acid substitutions may be a phenomenon of natural viral polymorphisms, which might influence the emergence of drug-resistant viruses, modifying drug susceptibility and/or virus replication capacity.[23] Some studies have reported that substitutions of A71T/V often appeared in B viruses, whereas L10I was often seen in CRF01_AE viruses.[24] Although V118I/IV, V179D/DV/E/S, and K103R often appeared in our subjects, they are frequent polymorphisms at drug resistance sites.[25] Some researchers have even questioned whether V118I/IV should be included from mutation lists because its effect on resistance may be overemphasized.[26] K103R combined with V179D has been shown to confer significant resistance against EFV and NVP.[27] However, K103R and V179D appeared only separately in our cohort. All subjects except one had mutations resistant to only one class of drug and had potential or low-level resistance only to ARV drugs. A single sample was resistant to all classes of ARV drugs and even had high-level resistance to NNRTIs. This patient may have been directly infected with a drug-resistant virus. In light of this, our data may represent only primary drug resistance in the MSM population of Liaoning province and therefore may not be generalizable to other districts. Nevertheless, these data warn us that HIV drug-resistant isolates may be prevalent among MSM, which will no doubt compromise the success of future ART in these patients and the women to whom they pass these drug-resistant viruses.

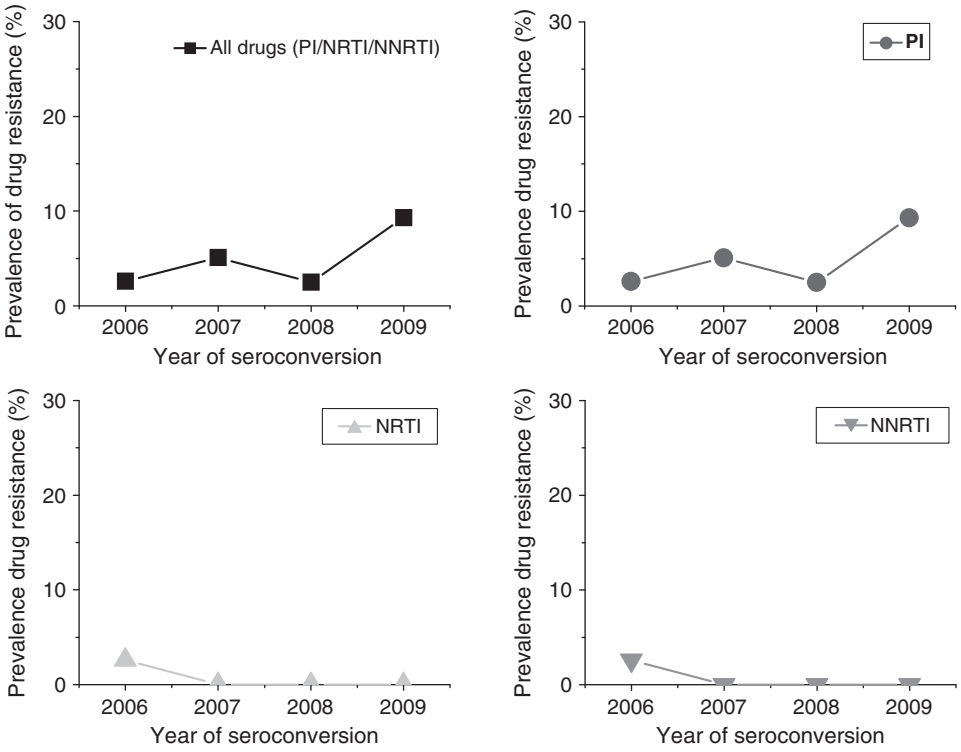

FIG. 2. Prevalence of transmitted drug resistance over time according to drug class. The prevalence of transmitted drug-resistant mutations was rising slightly, but no significant changes over time were observed.

In conclusion, CRF01_AE is becoming a major subtype among MSM of Liaoning province. This is the first report of polymorphisms and the emergence of primary drug-resistant mutations in a large cohort of MSM of Liaoning province. These findings provide useful information on HIV genetic variability of China. HIV drug-resistant mutations in ART-naive patients seen in this study represent a serious challenge for the future of HIV treatment programs in China.

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Author Disclosure Statement

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