

Human Immunodeficiency Virus Type 1 Subtypes Prevalence in Central China

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Purpose: To study the epidemic characteristics, transmission sources and routes of various subtypes of human immunodeficiency virus type 1 (HIV-1) and sequence variations in Henan, central China. To provide theoretical foundation for Acquired Immune Deficiency Syndrome (AIDS) prevention strategy in this region where the primary HIV transmission route was through former paid blood donation. **Materials and Methods:** HIV-1 gene env and gag were amplified by nested polymerase chain reaction (PCR) from uncultured peripheral blood mononuclear cells (PBMCs) obtained from 1,287 HIV-1 confirmed samples in Henan. **Results:** Among 1,287 samples, 5 HIV-1 strains were found including subtypes B' (95.9%), C (0.47%) and recombinant subtypes CRF 07_BC (1.09%), CRF 08_BC (1.79%) and CRF 01_AE (0.78%). Phylogenetic tree analysis found that 1,234 Henan subtype B' were closely related to those commonly found in Thailand, and were distantly related to other international subtypes. The dominant strain in former blood plasma donors (FPDs) was subtype B', and the dominant strains in sexual transmission were subtype B' and BC. Among HIV patients who were most likely infected through routes other than paid blood donation, the percentage of non-B' subtypes was much higher than those of FPD. **Conclusion:** These findings suggest that the prevailing strain of HIV-1 in Henan is subtype B', similar to the B' subtype found in Thailand. In addition, for the first time we found subtypes C and recombinant subtypes CRF07_BC, CRF08_BC and CRF01_AE in this region. Indicating that the subtype feature of HIV-1 became more complicated than before in central China.

Key Words : Human immunodeficiency virus type 1, subtype, sequence analysis

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INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) exhibits considerable genetic diversity resulting from the high mutation rate of reverse transcriptase, high viral turnover, viral genomic recombination, and immune and therapeutic selection pressures. This diversity is a challenge for viral load determination, drug resistance testing, and Acquired Immune Deficiency Syndrome (AIDS) vaccine development.^{1,2} Three phylogenetic groups of HIV-1, including main (M), outlier (O), non-M, non-O (N), have been recognized. Most HIV-1 infections are caused by group M viruses that comprise 9 clades (A-D, F-H, J, and K) and > 13 intersubtype recombinants known as circulating recombinant forms (CRFs).³ The genetic distance of each subtype in HIV-1 is 20-35%, whereas the genetic distance within the same subtype is only 7-20%.⁴ It is very important to consider the distribution of subtypes within a treatment population with the development and secondary transmission of drug resistant strains of HIV, as the data show that different subtypes have different sensitivity to non-nucleoside reverse transcriptase inhibitors (NNRTIs).^{5,6} A deeper understanding of HIV-1 subtypes can also inform ongoing vaccine research better.

That can more efficiently adapt to the localized outbreaks in regions such as Henan.

Henan province is located in central China where in the mid-1990s thousands of paid blood donors were infected with HIV through the use of unsanitary equipments and the practice of reinjecting communally centrifuged red blood cells back into donors. After the banning of such practices in 1998, the Chinese government carried out the first mass screening of the former blood plasma donors (FPDs) in 2004. By 2006, Henan was found to have 21,828 confirmed cases of AIDS, and 35,232 cases of HIV with 7,107 recorded deaths from AIDS.⁷⁻⁹ Henan province has the second highest concentration of HIV/AIDS in the country, and 53% of all AIDS patients undergoing ARV treatment live in Henan.¹⁰

Data collected from screenings in Henan have provided important information about the epidemiology of HIV in this community, but until now there has been little research into the subtypes distribution within this population. The following research and epidemiology analyses were carried out with 1287 HIV-1 samples found in Henan between year 2006 and 2007.

MATERIALS AND METHODS

Specimens

A total of 1,287 HIV-1 samples found in Henan between year 2006 and 2007, were confirmed as HIV positives. We collected 5-7 mL blood from peripheral vein of each subject. Anticoagulant ethylenediaminetetraacetic acid (EDTA) (1.5%) was used and centrifuged at 1,200 r.p.m. for 20 min within 4 hours after collection. Extracted Peripheral Blood Mononuclear Cells (PBMCs) of blood, preserving at -20°C.

Extract nucleic acid

We used DNA Blood Mini Kit (German QIAGEN Company, Hilden, Germany) to extract HIV-1 pre virus DNA from blood PBMCs according to the manual. Add 6 µg/mL the lysate into protease K solution (10 mmol/L tris-HCl, pH 8.4, 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 0.1% gelatin, 0.45% NP40, 0.45% Tween 228), making it 6 × 10⁶ cell density. Protease K was inactivated by heating at 95°C for 15 minutes, and the samples were stored at -20°C.

PCR amplification and cloning

According to the method of nested PCR, we designed several primers¹¹ (Table 1) and amplified HIV-1 gene env and gag. Primers were used for PCR sequence analysis of HIV-1 gene env and gag.

Thermal cycling conditions of gene env as follows: For the 1st PCR reaction with primer (Env-B1/Env-4), 94°C for 3 minutes, 52°C 30 seconds, 72°C 3 minutes, 1 cycle; 94°C 30 seconds, 52°C 30 seconds, 72°C 1 minute, 30 cycles; 72°C 10 minutes for extension. We take 1/10 matter of the 1st PCR reaction production, and process the 2nd PCR reaction with primer (Env-7/Env-8), the condition was at 94°C for 3 minutes, 55°C 30 seconds, 72°C 3 minutes, 1 cycle at 94°C for 30 seconds, 55°C 30 seconds, 72°C 1 minute, 30 cycles; 72°C for 10 minutes extension.

Thermal cycling conditions of gene gag was a follows: For the 1st PCR reaction with the primer (gagF2/gage2), at 94°C for 5 minutes, 52°C 1 minute, 72°C 150 seconds, 1 cycle; 94°C 30 seconds, 52°C 30 seconds, 72°C 90 seconds, 30 cycles; 72°C 10 minutes prolong. We take 1/10 matter of the 1st PCR reaction product, and processed the 2nd PCR reaction with primer (306/c--gag) as follows; at 94°C for 2 minutes, 50°C 50 seconds, 72°C 90 seconds, 1 cycle; 94°C 30s, 50°C 30s, 72°C 1 min, 35 cycles; 72°C for 10 minutes extension.

Table 1. Primers Used for PCR Sequence Analysis of HIV-1 Gene Env and Gag

Primer	Sequence	Direction	Location (HXB2)	Length
Env				
Env-B1	ATGGGATCAAAGCCTAAAGCCATGTG	Forward	6557 - 6582	1255 bp
Env-4	GCCCATAGTGCTTCCTGCTGCTCC	Reverse	7811 - 7792	
Env-7	TGTCAGCACAGTACAATGTACACATG	Forward	7002 - 7021	668 bp
Env-8	TTCACTTCTCCAATTGTCC	Reverse	7668 - 7648	
Env-D	CTGTAAATGGCAGTCTGGCAG			
Gag				
GagF2	ATG GGTGCG AGAGCGTCARTATTA	Forward	790 - 814	1243 bp
Gage2	TCCAACAGCCCTTTTCTTAGG	Reverse	2032 - 2011	
306	GGGAAA AAATTC GCT TAA GGCC	Forward	836 - 857	672 bp
c-gag	TAG TTC CTG CTA TRT CAC TTCC	Reverse	1507 - 1486	

PCR, polymerase chain reaction.

Purification of PCR product

PCR product after 1% gelose gelatin electrophoresis was checked and compared with standard markers, cutting peculiar amplify stripe (Qiaex of Qiaegen Company, Hilden, Germany) and HIV-1 DNA fragment was purified according to the manual. The circulatory DNA was dissolved as 10 mmol/L in Tris-HCl buffer (pH 8.7), after gelose gelatin electrophoresis and compared with standard molecular weight to estimate the amount of nucleic acid.

Nucleic acid sequence analyses

We used env-D and 306 as sequence analyses primer, and used purified PCR product as template, using ABI Company mark sequence analyses Kit. We performed the sequencing analysis by 9,600 PCR apparatus produced by PE Company. The dosage of template was about 1 µg and the dosage of primer was 6 pmol.

Sequence software analysis

The nucleotide sequence of 1,287 samples aligned with CLUSTAL W (European Bioinformatics Institute, Cambridge, UK) software,¹¹ and the results acquired were and used for phylogenetic tree analysis after artificial adjustment. Reference sequence, which was downloaded from Los Alamos HIV Sequence Database (Los Alamos National Laboratory, Los Alamos, NM, USA), was used to identify the subtype of obtained sequence; Genetic distance between sequences was assessed using the software MEGA 3.1 Kimura 2-parameter (Center for Evolutionary Functional Genomics The Biodesign Institute, Tempe, AZ, USA),¹² and phylogenetic tree was reestablished with neighbor-joining method.

Statistical analysis

The intraclass genetic diversity and interclass genetic divergence were calculated with Kimura 2 parameter model. Statistical analysis was done by Mann-Whitney U test¹³ and *p* value less than 0.05 was considered statistically significant.

RESULTS

Subtype analysis of HIV-1

There were subtypes B',C and recombinant subtypes CRF07_BC, CRF08_BC, CRF01_AE in 1,287 samples. And respective percentages were 95.9% (1,234/1,287), 0.47% (6/1,287), 1.09% (14/1,287), 1.79% (23/1,287), and 0.78% (10/1,287).

Transmission routes

As shown in Table 2, among the HIV patients whose transmission routes were not former paid blood donors, the percentage of no-B' subtype was much higher than that of former paid blood donors.

Area distributing characteristic of HIV-1 subtype

Among 1,287 HIV-1 samples, male constituted 680 samples and female 607 samples. Age was between 18-59 years. 1,144 as former paid blood donors (89.7%). 104 infected by sex transmission (8.02%), 21 infected by receiving blood (1.63%), 10 infected by mother-to-child (MTC) transmission of HIV (0.78%), and 8 infected by injection drug use (IDU) (0.62%). There were 388 individuals who took medicine; 212 individuals taking Stavudine (D4T) + Lamivudine (3TC) + Nevirapine (NVP). 147 individuals Zidovudine (AZT) + 3TC + NVP, and 29 individuals D4T + 3TC + Efavirenz (EFV).

The main transmission route in Henan was paid blood donation. In these populations, most of former paid blood donors were subtypes B'. Among them, 1,201 were Henan natives. And 86 came from other provinces. There were 53 samples that were no-B' subtype and, 45 samples from Henan native, including 11 samples that had outgoing working history. Eight no-B' subtype samples came from other provinces, including 1 sample from patient in Jiangmen Guangdong (subtype CRF07_BC), who had no regular job. Other 5 samples came from immigrants from ruili yunnan, and they were all female living in xiangxian xuch-

Table 2. The Transmit Routes of HIV-1 Subtype in Henan

	Sex transmission	MTC	Former paid blood donate	Receive blood	IDU	Total
C	3 (2.89%)	1 (10.0%)	2 (0.18%)	0	0	6
CRF07_BC	8 (7.71%)	2 (20.0%)	2 (0.174%)	0	2 (25.0%)	14
CRF08_BC	14 (13.49%)	0	8 (0.696%)	1 (4.76%)	0	23
CRF01_AE	4 (3.85%)	0	5 (0.44%)	1 (4.76%)	0	10
B'	75 (72.1%)	7 (70.0%)	1,127 (98.5%)	19 (90.5%)	6 (75.0%)	1,234
Total	104	10	1144	21	8	1,287

HIV-1, human immunodeficiency virus type 1; MTC, mother to child; IDU, injecting drug user.

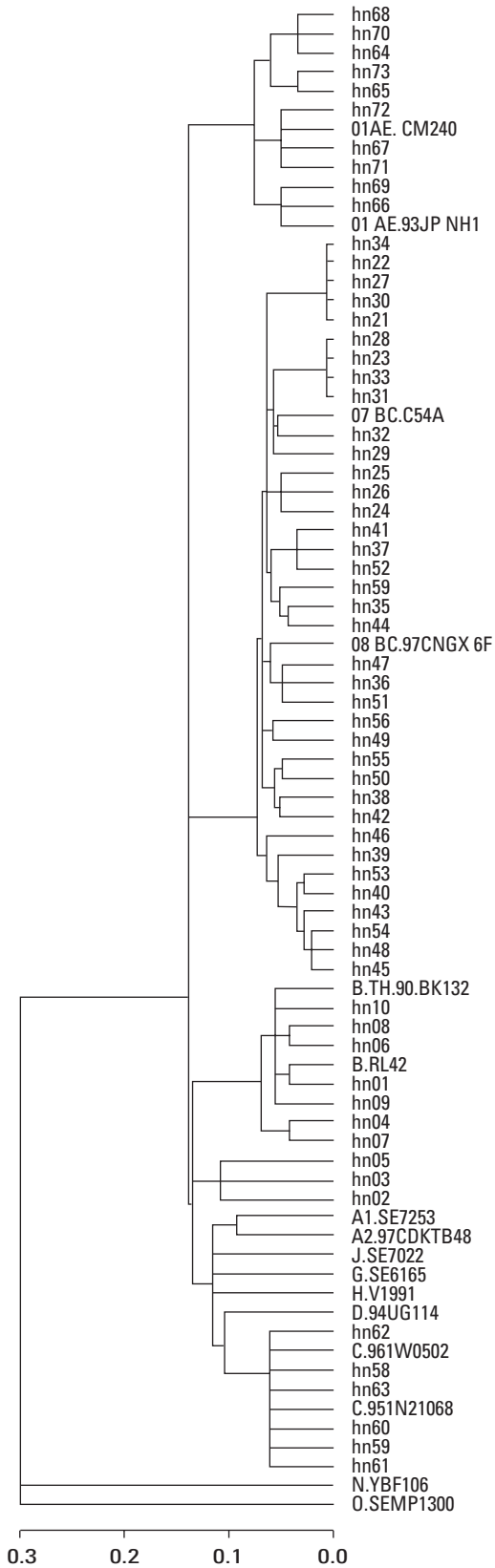


Fig. 1. Phylogenetic analysis of the HIV-1 env C2 - V3 coding region of samples from Henan. We get all 53 non-B' subtypes and choose 10 B' subtype.

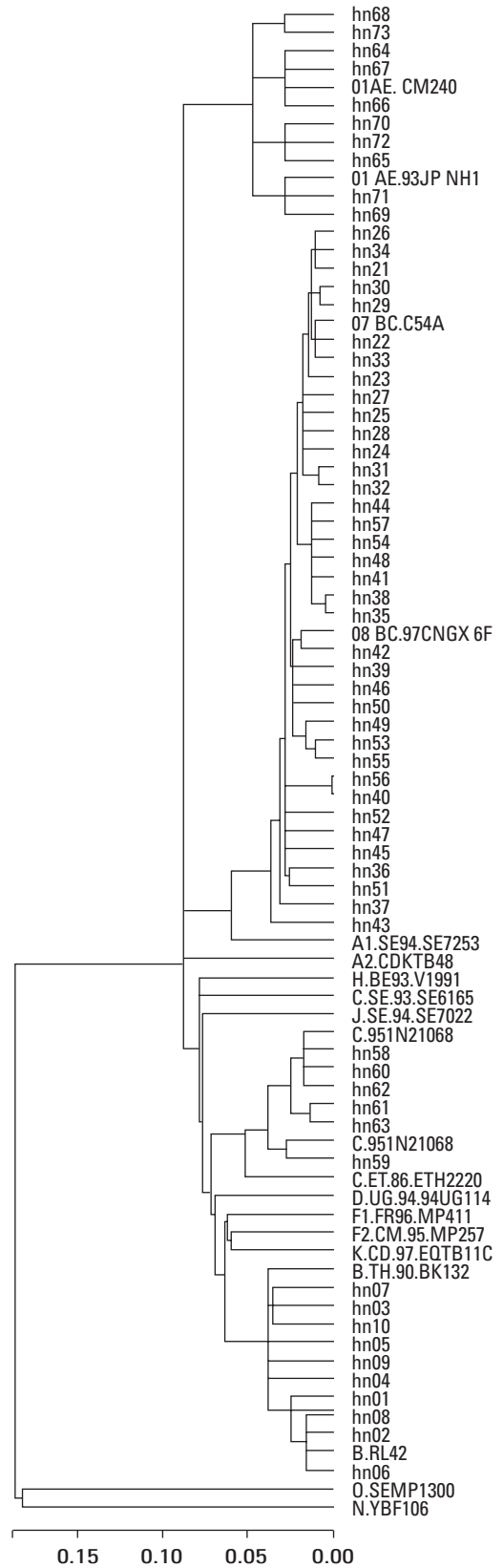


Fig. 2. Phylogenetic analysis of the HIV-1 gag P24 coding region of samples from Henan. We get all 53 non-B' subtypes and choose 10 B' subtype.

ang; 2 samples (all subtype CRF08_BC) had IDU history, 2 samples (subtype CRF01_AE and C) were infected by sex transmit, and 1 samples (subtype CRF01_AE) came from a former paid blood donor.

Phylogenetic tree analysis

Phylogenetic analysis of the HIV-1 env C2-V3 and gag P24 coding region of 1,278 samples from Henan by the neighbor-joining method. Genetic distances were calculated by the Kimura two-Parameter model. The scale indicates the relative phylogenetic distance. Bootstrap values were generated from bootstrapprep replicates, and values greater than 70% are labeled. Reference isolates from other types N, O, subtype A, B, C, D, G, H, J and recombinant from CRF01_AE, CRF07_BC and CRF08_BC were downloaded from the HIV-1 databases (<http://hiv-web.lanl.gov>). The bar represents 1% genetic distance.

From 2 figures, 10 AE sequence (hn 64-73) can be seen clustered together with subtype CRF01-AE.Th.Cm240. The inner group genetic distance of CRF01_AE were (5.676 ± 0.135)% (env), (4.329 ± 0.127)% (gag). In comparison with the sequence of respective international strains CRF01-AE.Th.Cm240, the genetic distance was (7.332 ± 0.158)% (env), (6.562 ± 0.208)% (gag).

10 B' sequence (hn1-10) clustered together with subtype B.CN.RL42. The inner group genetic distance of B' was (7.342 ± 0.265)% (env), (6.831 ± 0.211)% (gag). In comparison with the sequence of respective international strains B.CN.RL42, the genetic distances were (9.327 ± 0.245)% (env), and (8.562 ± 0.208)% (gag).

14 CRF07_BC (hn21-34) clustered together with subtype CRF07-BC.CN.97.CN54A.

The inner group genetic distance of CRF07_BC was (7.356 ± 0.265)% (env), (6.877 ± 0.201)% (gag). In comparison with the sequence of respective international strains B.CN.RL42, the genetic distances were (9.218 ± 0.167)% (env), and (8.551 ± 0.145)% (gag).

23 CRF08_BC (hn35-57) clustered together with subtype CRF08-BC.97CNGX6F.

The inner group genetic distances of CRF08_BC were (7.356 ± 0.265)% (env), and (6.877 ± 0.201)% (gag). In comparison with the sequence of respective international strains 08-BC.97CNGX.6F, the genetic distances were (6.278 ± 0.194)% (env), and (5.891 ± 0.121)% (gag).

6 C sequence (hn 58-63) clustered together with subtype C.95IN21068. The inner group genetic distances of C were (4.576 ± 0.126)% (env), and (4.655 ± 0.209)% (gag). In comparison with the sequence of respective international strains C.95in21068, the genetic distances were (5.214 ± 0.183)% (env), and (5.558 ± 0.156)% (gag).

DISCUSSION

Few countries to date have undertaken investigation of HIV molecular epidemiology in a multi-population setting in multiple locations. Although some have attempted to establish a database of comprehensive molecular epidemiology data, budgetary, logistical, and time constraints have impeded past efforts.¹⁴

Between 1996 and 1998, China launched a program known as the National HIV Molecular Epidemiology Investigation Research Plan in which a comprehensive survey of all HIV-1 subtypes in China was documented. HIV-1 subtypes, A, B, C, D, E, F, G, BC, were found in addition to HIV-2, and the study also investigated the distribution situation of different populations and geographical locations. Of the 19 confirmed samples of HIV-1 positive individuals from Henan, all were found to be Thailand subtype B'. From 2002 to 2004, the National Center for AIDS/STD Control and Prevention (NCAIDS) launched the National HIV Molecular Epidemiology Investigation Database Program, and in its first project 1048 HIV positives samples from 26 provinces and municipalities were collected.¹⁶ The results showed that 28 former paid blood donors from Henan were all of subtype B', confirming the results of the earlier survey study.¹⁵⁻¹⁸

As an inland province in central China, Henan province-along with Hubei, Shanxi, Hebei, Shandong, and Anhui-is relatively underdeveloped and its rural population was particularly vulnerable to the commercial blood/plasma collection that took place in the early to mid 1990s.¹⁹ As a result of unsanitary practices, many FPDs were infected with HIV, eventually accounting for 9.7% of all infections in China by 2003.^{20,21} In more developed areas of China such as Guangdong, Jiangsu, and Fujian, the most prevalent subtypes of HIV-1 are A, AE, BC and are primarily transmitted through heterosexual intercourse sex and needle-sharing practices among injecting drug users (IDU).^{22,23} In developing areas including Henan and Anhui, however, the prevailing subtype is B', with a low percentage of subtypes C and AE, and the main transmission route is through unsanitary blood donation practices.²⁴

The most recent round of research on HIV-1 strains in Henan is the most comprehensive and large scale HIV molecular epidemiology investigation to date, with a total of 1,287 samples from 18 cities. In addition, the populations sampled include not only FPD but also female sex workers (FSW), IDU, and men who have sex with men (MSM). Because this data were combined with information from earlier molecular epidemiology surveillance work in the early stages of the HIV epidemic in Henan, the

results in this paper were based on the most comprehensive dataset available on the topic.

Tracing the variation of HIV strains in an epidemic with regional diversity and multiple routes of transmission is important for developing better clinical treatment methods and to inform vaccine research. This study found that the HIV-1 subtypes found in Henan are more diverse than ever, signaling the need for corresponding preventive measure and further sophistication in the development of anti-retroviral treatment therapies .

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