Detection and analysis of hepatitis C virus in HIV-infected patients in the Guangxi province of China

SHU-ZHI WU*, JIN-LU WEI*, BIN XU, PENG-HAI WEI, YAN YANG, BIN QIN and ZHI-CHUN XIE

Department of Epidemiology, Public Health School, Guangxi Medical University, Nanning, Guangxi 530021, P.R. China

Received April 2, 2015; Accepted April 29, 2016

DOI: 10.3892/etm.2017.4067

Abstract. The aim of the present study was to investigate the prevalence rate of hepatitis C virus (HCV) infection in human immunodeficiency virus (HIV)-positive individuals and to study the infection status of HCV RNA in HIV-infected individuals who did not have anti-HCV antibodies in the Guangxi province of China, in order to provide basis for screening and clinical treatment of hepatitis C in future. Data were collected from patients recruited via a questionnaire. Between August 2008 and January 2009, 300 HIV-infected individuals were randomly selected from various HIV monitoring points in Liuzhou and Qinzhou (Guangxi, China). In addition, 41 patients with only hepatitis C were recruited from a hospital clinic (First Affiliated Hospital of Guangxi Medical University, Nanning, China). HCV antibodies in patient serum samples were detected by ELISA. HCV RNA expression was detected using nested polymerase chain reaction (PCR), HCV RNA levels in the serum were evaluated using quantitative fluorescence PCR, and HCV genotypes were confirmed using restriction fragment length polymorphism. The infection rate of HCV in the HIV-infected people was 48.67%. The anti-HCV positive rate differed between routes of disease transmission: Anti-HCV positive rate was 63.7% among drug users, 34.96% among sex-transmitted persons and 1.37% among other persons. In the anti-HCV-negative group, the HCV RNA-positive rate was 26.62%. In the anti-HCV-positive group, HCV RNA positive rate was 78.08%. HCV RNA level of HIV/HCV coinfected patients was higher than those infected with HCV alone, and there was no difference of anti-HCV-positive rate among different levels of HCV RNA. HCV genotypes of HIV/HCV coinfected persons showed diversity across Guangxi, and the predominant ones were the 1b and mixed subtypes. The predominant HCV genotypes were 6a, mixed subtypes and 3b amongst patients that contracted HCV via drug use-related routes of transmission. The patients with HCV transmission routes other than drug-related routes possessed 1b and 1a+1b genotypes. In conclusion, there was a large proportion of HIV infected persons with mixed HCV infection in the Guangxi province of China. The present results show that 26.62% of HCV-infected persons will be fail to be diagnosed with hepatitis C virus coinfection if we simply use ELISA to detect HCV antibody. The predominant HCV genotypes were 1b, mixed, 6a and 3b in HIV/HCV coinfected persons.

Correspondence to: Professor Zhi-Chun Xie, Department of Epidemiology, Public Health School, Guangxi Medical University, 22 Shuangyong Road, Nanning, Guangxi 530021, P.R. China
E-mail: 357154887@qq.com

*Contributed equally

Key words: hepatitis C virus, human immunodeficiency virus, coinfection

Introduction

Human immunodeficiency virus (HIV) infection is propagated primarily via blood transfusion (before 1985), intravenous drug use, professional exposure in medical institutes, sexual transmission and mother-to-child transmission (1,2).

Historically, hepatitis C virus (HCV) infection has developed in three waves: i) Medical care through needles and syringes reused without sterilization; ii) blood transfusion before 1991; iii) and through intravenous drug injection or the sharing of straws for cocaine inhalation (3).

HCV infection is often observed among HIV-infected persons, with one-third of HIV-infected Americans and 7 million worldwide being coinfected (4,5). HIV coinfection deteriorates HCV disease, increasing the likelihood of cirrhosis and HCV-related mortality (6,7).

There are 33.3 million people globally living with HIV infection (8). It is estimated that 20-30% of HIV patients are also infected with hepatitis C (HCV) (9). In China, small sample studies suggest that 8.25-56.9% of HIV-infected persons were simultaneously infected with HCV (10-14). Delayed testing for HIV and HCV is common among patients at methadone clinics in Guangdong, with numerous patients experiencing delays of ≥2 months (13,14). Early diagnosis and enrollment in care is particularly crucial in a highly populated country such as China; thus, expediting HIV and HCV testing is important (15).

Guangxi province is a high endemic area of HIV infection in China [the cumulated report number of HIV-infected persons is ranked second in the country (16)], which also has a high incidence of viral liver disease and hepatocellular carcinoma (17). Based on frequent HIV/HCV coinfection in other areas, there is a crucial requirement to investigate the HCV

infection status in HIV-infected people. Furthermore, there are likely to be numerous HCV-infected persons among the HIV-infected population for which the diagnosis of HCV has been missed due to the diagnosis being made only on the basis of HCV antibody detection.

Materials and methods

Ethics statement. This study was approved by the Health Bureau of Guangxi Province and the Ethics Review Committee at Guangxi Medical University (Nanning, China). Written informed ethical consent was provided by clinical directors who answered the questionnaire. All patients gave their written consent for their information to be used for research.

Study population. This study was performed between August 2008 and January 2011. The participants were recruited through acquired immune deficiency syndrome (AIDS) voluntary counselling and testing clinics, prisons, addiction treatment centers, hospitals and communities from Liuzhou and Qinzhou cities in Guangxi province. A total of 300 individuals with HIV-1 infection were recruited. All participants or their guardians voluntarily signed a consent form. Written informed ethical consent was provided by professionals who answered the questionnaire. A total of 41 patients who were only infected with hepatitis C were recruited from the First Affiliated Hospital of Guangxi Medical University to serve as the control group.

All participants were analyzed to characterize their serological status toward both HIV [screening and confirmation tests (18)] and HCV [screening, serological confirmation, search for HCV-RNA by polymerase chain reaction (PCR) and genotyping] as described below. Samples of serum and plasma (10 ml) were prepared from venous whole blood.

Reagents. Hepatitis C virus antibody diagnostic kits were purchased from Shanghai Kehua Bio-Engineering Co., Ltd., (Shanghai, China). QIAamp Viral RNA Mini kit was obtained from Qiagen (Hilden, Germany). A Revert Aid First Strand cDNA Synthesis kit was purchased from Fermentas, Inc., (Burlington, ON, Canada). Restriction endonucleases *Bsr*BI, *Hae*II, *Hinf*I, *Bst*UI, *Hae*III and *Apo*l were obtained from New England Biolabs (Ipswich, MA, USA).

Enzyme-linked immunosorbent assay (ELISA). Flavicheck-HCV, a commercial third generation, rapid, qualitative, two-site sandwich immunoassay test device was employed according to the manufacturer's instructions (Tulip Diagnostics (P), Ltd., Goa, India). The kit was used to detect total antibodies specific to HCV in serum or plasma by using a multiepitope recombinant peptide antigen that is broadly cross-reactive to all major HCV genotypes.

Nested-PCR and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from 140 μl plasma using a QIAamp Viral RNA Mini kit according to the manufacturer's instructions (Qiagen). RNA samples were DNase treated using an RQ1 RNase-Free DNase kit (Promega, Madison, WI, USA), according to the manufacturer's instructions. Nucleic acids were then aliquoted, and one aliquot was reverse-transcribed according to the SuperScript III First-Strand cDNA Synthesis SuperMix kit protocol using random hexamers (Invitrogen; ThermoFisher Scientific, Inc., Carlsbad, CA, USA). cDNA was synthesized from total RNA using reverse transcriptase using a First Strand cDNA synthesis kit (Fermentas, Inc.). The cDNA was amplified by nested PCR using a Tiangen 2X PCR mix kit (Tiangen Biotech Co., Ltd., Bejing, China) or by RT-qPCR using SYBR Green SuperMix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturers' instructions. The PCR cycling was conducted as follows: In the first round, the amplification mixture comprised 5.0 μl cDNA, 12.5 μl pre-mixed solution of Tiangen 2X PCR mix and 0.5 μl of each primer (P1 and P2). The enzyme was diluted with 6.5 μl water, and the total reaction volume was 25.0 μl. The reaction conditions were as follows: 94˚C for 5 min; 94˚C for 45 sec, 62˚C for 30 sec, and 72˚C for 20 sec for 35 cycles; followed by 72˚C for 5 min. In the second round, the amplification mixture comprised 3.0 μl product from the first round, 12.5 μl pre-mixed solution of Tiangen 2X PCR mix, 0.4 μl each primer (P3 and P4). The enzyme was diluted with 8.7 μl water, and the total reaction volume was 25.0 μl. The extension time in the second round was 15 sec, and all other times were as in the first round. For the RT-qPCR analysis, all experiments were performed in triplicate. The sample input was normalized against its own critical threshold (Cq) value of the housekeeping gene GAPDH. Primer sequences and PCR conditions used in this study are listed in Table I. The $2^{-\Delta\Delta Cq}$ method (19) was used to calculate the expression levels.

PCR product sequencing. The nested PCR products and sense primer were used for sequencing reaction. PCR products were recovered according to the manufacturer's instructions (Fermentas, Inc.). The sequencing was performed using the Sanger dideoxy method (20). This was followed by detection of the HCV sequence using GeneBank (https://www.ncbi.nlm.nih.gov/genbank/), BBLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), Clustalx (http://www.clustal.org/clustal2/) and BioEdit software (http://www.mbio.ncsu.edu/BioEdit/page2.html).

HCV genotype test. Restriction fragment length polymorphism (RFLP) analysis was conducted using the method described by Chinchai *et al* (21,22). In RFLP, the nested PCR products of RNA positive samples (20-30 μl) were digested using the restriction enzymes *Acc*1, *Mbo*l and *Bst*N1 and incubated at 37˚C for overnight in a specific endonuclease buffer (Shanghai Biological Engineering Co., Ltd., Shanghai, China). The digested product was loaded onto 3% nusieve agarose gel and the restriction pattern was analyzed using a Gel-Doc 2000 System (Bio-Rad Laboratories, Inc.).

Data analysis. Data were analyzed using SPSS software version 17.0 (SPSS, Inc., Chicago, IL, USA). Chi-square (χ^2) test was utilized to compare variables. $P<0.05$ was considered to indicate a statistically significant difference. Odds ratio (OR) and 95% confidence interval (CI) were used to measure the strength of association.

Results

Demographics and epidemiological characteristics. Demographic data were collected regarding the participant's

Table I. HCV primer sequences, annealing temperatures and product sizes for the nested PCR analyses.

Primer	Primer sequence	Annealing temperature (°C)	Product size (bp)
Nested outer primer	Forward: 5'-TGAGGAACTACTGTCTTCACG-3' Reverse: 5'-AGCACCCTATCAGGCAGTACC-3'	62	258
Nested inner primer	Forward: 5'-GGGAGAGCCATAGTGGTCTG-3' Reverse: 5'-CACTCGCAAGCACCCTATC-3'	62	186

HCV, hepatitis C virus; PCR, polymerase chain reaction.

Table II. Sociodemographic characteristics of HIV/HCV coinfected persons.

			HCV			
Parameter	Participants (n)	Participants (%)	Positive (n)	Positive rate	χ^2	P-value
Gender					13.37	<0.01
Male	195	65.0	110	59.46		
Female	105	35.0	36	34.29		
Age (years)					13.69	0.02
<20	6	2.0	2	33.33		
20-29	89	29.7	42	47.19		
30-39	105	35.0	63	60.0		
40-49	50	16.7	25	50		
50-59	22	7.3	6	27.27		
≥60	28	9.3	8	28.57		
Ethnicity					1.62	0.20
Han	201	67.0	103	51.24		
Minority	99	33.0	43	43.43		
Occupation					21.94	<0.01
Unemployed	164	54.7	100	60.98		
Employed	136	45.3	46	33.82		
Education					9.00	0.03
≤Primary school	51	17.0	30	58.82		
Junior high school	201	67.0	94	46.77		
High school/polytechnic	30	10.0	18	60.0		
≥College	18	6.0	4	22.22		
Marital status					18.85	<0.01
Married	95	31.67	33	34.74		
Unmarried	165	53.33	99	60.0		
Divorced/widowed	40	15.0	14	35.0		
Region					0.21	0.91
Liuzhou	141	47.0	68	48.23		
Qinzhou	159	53.0	78	49.06		

HIV, human immunodeficiency virus; HCV, hepatitis C virus.

gender, age, ethnicity, education, employment and marital status (Table II). Among the 300 HIV positive participants, 195 were male and 105 were female; the oldest patient was 88 years old and the youngest was 2 years old; the mean age was 37.39±13.73 years. Among all participants, 201 were of Han ethnicity and 99 were of minority ethnicity. Among the viral hepatitis C and HIV-infected participants, 103 were Han, while 43 patients were of the Zhuang ethnicity. The majority of the participants had no fixed occupation (54.7%); the majority of the participants were unmarried or single (53.33%); among the HIV positive patients in Liuzhou and Qinzhou, there were no significant differences in HCV infection rate (Table II).

Table III. Distribution of HIV/HCV coinfected patients in different transmission routes.

Transmission route	Participants (n)	HIV/HCV coinfection	Coinfection rate (%)	χ^2	P-value
Drug-related	101	93	92.08		
Sex-related	188	51	27.13		
Vertical transmission	5	1	20.0	127.4	<0.001
Blood transfusion	6	1	16.67		
Total	300	146	48.67		

HIV, human immunodeficiency virus; HCV, hepatitis C virus.

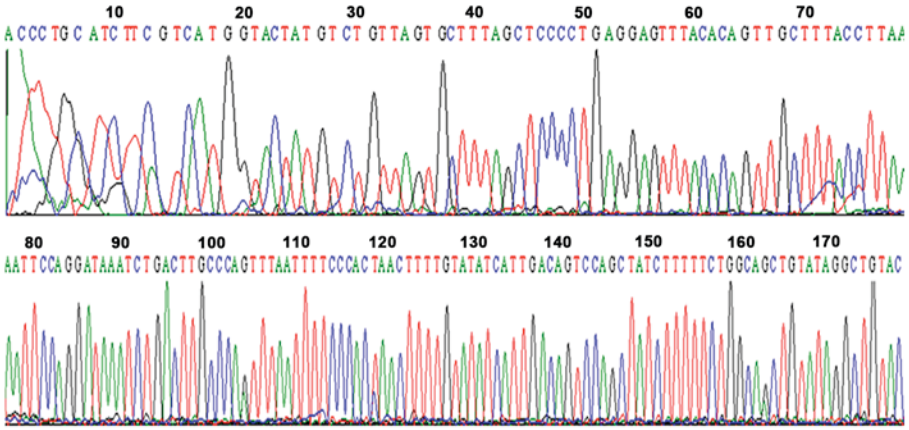

Figure 1. Sequence analysis of hepatitis C virus 5' untranslated regions of five positive serum samples.

Predominant route of transmission in HIV and *HCV coinfected participants*. The prevalence of HCV infection was 48.67% (146/300) among the HIV infected study population. Among the 101 individuals who were drug users, 93 (92.08%) exhibited HIV/HCV coinfection. One of the six subjects that had received a blood transfusion was infected with HCV (16.67%). One of the five subjects suffered HIV from vertical transmission was infected with HCV (20%). Among the 188 subjects that suffered from a history of sexually transmitted diseases, 51 persons were infected with HCV (27.13%). With respect to sexual behavior, ~69.18% of the subjects had more than one sexual partner. The rate of coinfection differed between transmission route according to the results of the χ^2 test (Table III).

Gene sequencing of HCV positive serum samples. Five cases of HCV RNA-positive serum samples were randomly selected, and nested PCR products were sequenced. The results showed that the sequences of the samples were the same as HCV 5' untranslated region in GeneBank by blasting HCV database (Fig. 1).

HCV prevalence at the RNA level. HCV RNA quantification was conducted using nested PCR. The results showed that among the 146 HCV antibody-positive serum samples, there were 114 HCV RNA positive samples. However, there were 41 HCV RNA positive samples among the HCV antibody-negative serum samples (Fig. 2).

HCV genotypes in the Guangxi province population. The digested results showed that among the 41 study participants

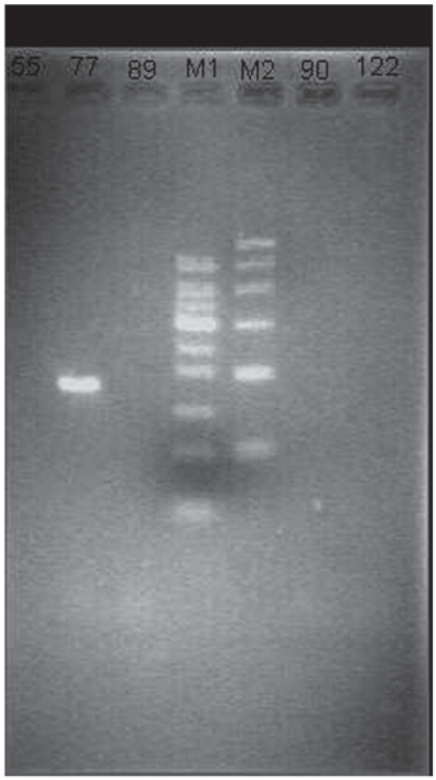

Figure 2. Electrophoretogram of hepatitis C virus RNA. M1 is Marker1 (bp): 50, 100, 150, 200, 250, 300, 350, 400 and 500. M2 is Marker2 (bp): 100, 200, 300, 500, 700 and 1,000. The length of electrophoretic bands of the nested PCR amplified product is 186 bp. The three lanes to the left and right of the markers are patient samples (nos. 55, 77, 89, 90 and 122), respectively.

Table IV. Comparison of HCV genotype distribution between two groups (n=155).

	Genotype									
HCV antibody	1a	1b	2a	2b	3a	3b	4a	6a	Mixed	Total
Negative	3	22	0	2	6	1	5	2	0	41
Positive	10	31	5	4	5	14	0	18	27	114
Total	13	53	5	6	11	15	5	20	27	155

The overall HCV RNA positive participants were 155, HCV genotypes were diverse among the 155 human immunodeficiency virus/HCV coinfection subjects, and the main type were 1b, mixed subtype, 6a, 3b and 3a. HCV, hepatitis C virus.

Table V. Comparison of positive HCV antibody and HCV RNA (n=300).

	HCV RNA		
HCV antibody	Positive	Negative	Total
Positive	114	32	146
Negative	41	113	154
Total	155	145	300

HCV, hepatitis C virus.

Table VI. Comparison of HCV RNA load between three age groups (n=41).

Age (years)	Cases	HCV RNA load (log)	F	P-value
<30	15	5.84±1.29		
30-45	12	6.40±0.76	0.83	0.44
>45	14	5.84±1.52		

HCV, hepatitis C virus.

Table VII. Comparison of HCV RNA load in different transmission routes (n=41).

Transmission	Cases	HCV RNA load (log)	t	P-value
Sex	33	5.94±1.24	0.31	0.74
Drugs	8	6.39±0.94		

HCV, hepatitis C virus.

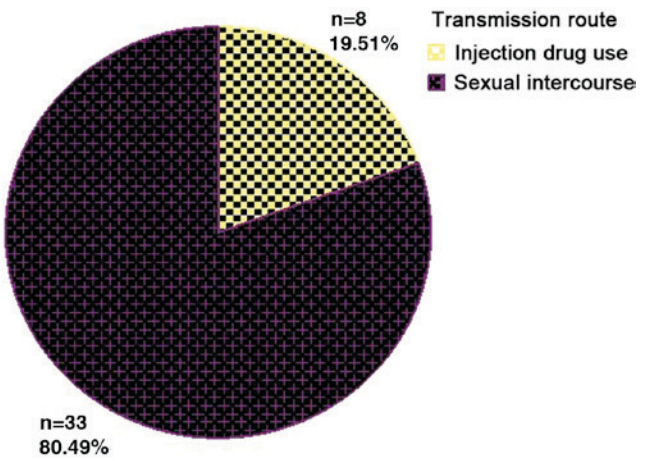

Figure 3. Distribution of transmission routs in 41 patients with hepatitis C virus RNA-positive. Sexual intercourse accounted for 80.49%; injection drug use accounted for 19.51%.

positive for HCV RNA but negative for HCV antibody, the prevalence rates of the 1b, 1a, 3a and 4a genotypes were 22 (53.7%), 3 (7.3%), 6 (14.6%) and 5 (12.2%), respectively. Among the 146 study participants that were positive for HCV antibody, the prevalence of the 1b, mixed, 6a, 3b, 1a, 3a, 2a and 2b genotypes were 31 (27.2%), 27 (23.7%), 18 (15.8%), 14 (12.3%), 10 (8.8%), 5 (4.4%), 5 (4.4%) and 4 (3.5%), respectively (Table IV).

Differences between positive HCV antibody and HCV RNA test results. Among the 300 study participants tested for anti-HCV antibodies and HCV RNA, 114 were positive for both, accounting for 38%. A total of 41 participants were anti-HCV negative and HCV RNA positive, accounting for 13.7%. A total of 32 participants were anti-HCV positive and HCV RNA negative, accounting for 10.67% (32/300) of all patients. Among the 300 individuals analyzed, 113 were negative for anti-HCV and HCV RNA, accounting for 37.67%. Among the anti-HCV negative participants, 41 were HCV RNA positive, accounting for 13.7% (41/300) of all patients (Table V).

Characteristics of the HCV antibody negative but HCV RNA positive participants. Among the 41 study participants positive for HCV RNA but negative for HCV antibody, there were 23 male and 18 female subjects, with an average age of 39.51±16.32 years, and the viral load differed, although not significantly, among age groups (Table VI). The prevalence of HCV RNA differed according to transmission route; most patients contracted HCV sexually and the second most prevalent route was drug-related transmission (Fig. 3); however, no significant differences in HCV RNA load were observed between the different transmission routes (Table VII).

Discussion

This study was aimed at determining the HCV infection prevalence and HCV genotype in HIV infected patients as well as obtaining demographic and epidemiological characteristics of HIV/HCV coinfection patients in the Guangxi province of China.

With respect to demographic characteristics, the majority of the HIV/HCV coinfection participants were males, and aged 20-40 years. The majority of the HIV/HCV coinfection participants had no fixed occupation, were unmarried or single, and 61.6% were drug users. The results showed that sexually active, young, single individuals with no fixed occupation were at the highest risk of HIV/HCV coinfection.

Hepatitis C virus (HCV) is an RNA virus which has been known to cause acute and chronic necroinflammatory disease of the liver (23,24). HCV is the leading cause of end-stage liver disease and hepatocellular carcinoma (25). HIV is known to have a negative impact on the natural disease outcome and immune response of HCV infection, whereas the reverse remains unclear (26). HIV infection is propagated primarily via blood transfusion (before 1985), intravenous drug use, professional exposure in medical personnel, sexual transmission and mother-to-child transmission (1,2). Furthermore, HCV contamination is caused by intravenous drug use and blood transfusion (before 1991), long term hemodialysis, organ transplantation and receipt of tattoos from unsanitary facilities (27). According to above statements, HIV and HCV share numerous transmission routes, and thus coinfection with HCV and HIV is quite common. In the present study, drug addiction, particularly intravenous drug use, was the primary transmission route in HIV/HCV coinfection subjects, which indicated that it is necessary to screen for HCV in HIV positive intravenous drug users. A number of studies have suggested that the presence of HIV infection accelerates the course of HCV-related liver disease in HCV/HIV coinfected patients (26,28,29). The frequency of HCV transmission to sexual partners is significantly higher when HIV virus is also transmitted (30). HIV is known to impair the T-helper type 1 immune response, which in turn changes the response of immune cells to HCV (31). This facilitates increased HCV replication and consequently easier infection. Epidemiologic studies have identified HIV as an independent factor in HCV transmission and acquisition since the earliest days of the HIV epidemic (32). HIV-infected persons are less likely to spontaneously clear HCV, and their HCV RNA set point tends to be higher, making them more infectious to their partners compared with HCV-monoinfected individuals (33). A previous study showed that coinfected men were more likely than HIV-uninfected men to produce HCV RNA in their semen (34). HIV-infected individuals may have compromised gastrointestinal mucosal barriers and be more likely to have chronic inflammation, facilitating HCV transmission (35). The present results indicate that intravenous drug use, sexual transmission and blood transfusion are the primary transmission routes of HIV/HCV coinfection in patients from Guangxi.

ELISA is the conventional method used to detect HCV antibody; however, the shortcoming of ELISA is the long window-period prior to antibodies becoming detectable and screening hepatitis C only by ELISA in HIV-infected persons is likely to miss the identification of some cases. Therefore, HCV antibody can not be used as an early diagnostic marker of HCV infection. However, serum HCV RNA detection could be a reliable indicator due to its shorter window-period and higher sensitivity. It has been reported that ~11% of HIV/HCV coinfection patients are HCV antibody negative (36). Liu *et al* showed that in HIV/HCV coinfection patients in Beijing, the nucleic acid positive rate was 19.5% in HCV antibody-negative group (37). In the present study, it was found that in HIV/HCV coinfection persons in Guangxi, the nucleic acid positive rate was 26.62% in HCV antibody-negative group, which was higher than reported previously.

Although the coinfection of HIV with HCV has been recognized worldwide, few studies have been conducted to investigate HIV/HCV coinfection prevalence in Guangxi and the association between prevalence and transmission route, particularly in patients infected via sexual transmission. The present study was conducted on different populations coinfected with HIV/HCV, providing important reference data for prevention and treatment. The data show that HIV/HCV coinfection prevalence was ~48.67%, which is higher reports from outside of China (30,38-41), and higher than the prevalence of HCV among domestic general populations (42), which indicates that HIV/HCV coinfection is a serious health concern. In addition, the results indicate that besides HCV, AIDS infected persons are also liable to coinfect with other hepatitis virus, tuberculosis and syphilis, which all indicate that it is important to improve approaches for the management of HIV coinfection.

In conclusion, the present data indicate that HIV/HCV coinfection is a serious health concern in the Guangxi province of China. Therefore, it may be necessary to manage and control HIV/HCV progression to prevent their potential outbreak or spread among the general population, on the basis of infection status, transmission routes and risk behaviors.

Acknowledgements

This study was financially supported by the fourth round of the Global Fund/Sino-British AIDS Project (grant no. 2008OR52).

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