High rate of seronegative HCV infection in HIV-positive patients

JUNIASTUTI[1,2], TAKAKO UTSUMI[2,5], NASRONUDIN[2,3,6], LINDAWATI ALIMSARDJONO[1], MOCHAMAD AMIN[2,3], MYRNA ADIANTI[3], YOSHIHIKO YANO[5], SOETJIPTO[2,4], YOSHITAKE HAYASHI[5], HAK HOTTA[5] and MARIA INGE LUSIDA[1,2,3]

[1]Department of Microbiology, School of Medicine, Airlangga University, Surabaya, East Java 60131; [2]Indonesia-Japan Collaborative Research Center for Emerging and Re-emerging Infectious Diseases; [3]Institute of Tropical Disease, Airlangga University, Surabaya, East Java 60115; [4]Department of Biochemistry, School of Medicine, Airlangga University, Surabaya, East Java 60131, Indonesia; [5]Center for Infectious Diseases, Kobe University Graduate School of Medicine, Kobe, Hyogo 6500017, Japan; [6]Department of Internal Medicine, Dr Soetomo General Hospital, Surabaya, East Java 60131, Indonesia

Received September 25, 2013; Accepted October 18, 2013

DOI: 10.3892/br.2013.188

Abstract. Co-infection with human immunodeficiency virus (HIV) and hepatitis C virus (HCV) is a significant global health problem. The two viruses are transmitted with high efficacy via blood-to-blood contact, mainly intravenous drug use (IVDU), whereas HCV is less easily transmitted sexually. Antibody testing is the main screening method for HCV infection, although it may not be the optimal option for HIV infection. The aim of this study was to investigate HCV infection in HIV-positive patients, with and without a detectable anti-HCV antibody response. A total of 187 plasma samples were obtained from HIV-positive patients in Surabaya, Indonesia and examined for anti-HCV [HCV enzyme immunoassay (EIA) 3.0], HCV genotype/subtype [reverse transcription-polymerase chain reaction (RT-PCR) using primers targeting a part of NS5B/5'UTR followed by sequencing] and HCV viral load (quantitative RT-PCR). A total of 119 patients (63.6%) were found to be anti-HCV-positive and, among these, HCV RNA was detected in 73 (61.3%), with HCV-1a as the predominant subtype (31.5%). Of the 68 anti-HCV-negative samples, HCV RNA was detected in 26/68 (38.2%) mostly as the HCV-3a subtype (50%). High HCV viral loads were more common among the HCV-seropositive patients. The HCV-seropositive samples with detected HCV RNA were mostly obtained from HIV-positive patients with parenteral transmission (IVDU) (76.7%); however, the HCV-seronegative samples with detected HCV RNA were mostly from patients who had acquired HCV through heterosexual transmission (61.5%). In conclusion, HIV-positive patients were at high risk of becoming co-infected with HCV and several remained HCV-seronegative. Furthermore, there may exist differences in HCV seropositivity and subtypes between HIV-positive patients who acquired HCV sexually and those who acquired HCV parenterally.

Correspondence to: Professor Maria Inge Lusida, Institute of Tropical Disease, Airlangga University, Campus C Jalan Mulyorejo, Surabaya, East Java 60115, Indonesia
E-mail: ingelusida@yahoo.com

Key words: hepatitis C virus, subtypes, anti-hepatitis C virus, human immunodeficiency virus co-infection

Introduction

The epidemic of human immunodeficiency virus (HIV) infection in Asia, including Indonesia, is rapidly expanding (1). The introduction of highly active antiretroviral therapy (HAART) has markedly reduced HIV-related morbidity and mortality. However, non-HIV-related conditions, particularly liver disease, currently constitute an increasingly high proportion of the causes of mortality among HIV-infected individuals (2). Hepatitis C virus (HCV) has emerged as an important cause of morbidity and mortality among HIV-positive individuals (3).

As the majority of individuals who acquire HCV are asymptomatic, it is difficult to determine some of the characteristics of acute infection (4). Early diagnosis is rare and the extent of this epidemic is unknown, since the majority of at-risk individuals are not tested for acute HCV infection (5). These and several other aspects of HCV infection may be further complicated by co-infection with HIV-1. In HIV-infected individuals, untreated acute HCV infection typically progresses to chronic HCV infection, a leading cause of non-AIDS-related morbidity and mortality among HIV-infected individuals in the HAART era (2).

HIV and HCV share common transmission pathways, which may explain the high rate of co-infection with the two viruses. Of the 33.4 million HIV-infected individuals worldwide in 2008, it is estimated that ≥5 million have concomitant HCV infection. Whereas the two viruses are transmitted with high efficacy via blood-to-blood contact [particularly in intra-

venous drug users (IVDUs)], HCV is less easily transmitted sexually and its risk remains controversial (6).

Antibody testing is the main screening method for HCV infection (7). However, serological screening in HIV-infected patients may not be the optimal screening method, possibly as a result of immunosuppression (8). Therefore, HCV RNA testing is recommended for the diagnosis of HCV infection (8,9).

The aim of this study was to investigate HCV infection in anti-HCV-positive and -negative HIV patients in Surabaya, Indonesia.

Materials and methods

Collection of field samples. Plasma samples were obtained from HIV-positive patients, who visited the Institute of Tropical Disease (ITD), Airlangga University, Surabaya, Indonesia, for an HIV viral load examination requested by a clinician. The majority of the patients (176/187, 94%) were on HAART with activity against AIDS (lamivudine+zidovudine+efavirenz or lamivudine+zidovudine+nevirapine) and exhibited no symptoms of acute hepatitis. The plasma samples were stored at -80˚C prior to examination. The study protocol was reviewed and approved by the Ethics Committees of Kobe University, Japan and Airlangga University, Indonesia and informed consent was obtained from all the patients. The HIV viral load data were retrieved from the patient database maintained at ITD, Airlangga University, Indonesia.

Anti-HCV tests. All the plasma samples were subjected to HCV enzyme immunoassay (EIA) 3.0 (Hepalisa Anti HCV; PT Indec Diagnostics, Jakarta, Indonesia) to detect anti-HCV, according to the manufacturer's instructions. The third-generation anti-HCV test which detects multiple antigenic determinants (core, NS3, NS4 and NS5) was used to increase sensitivity.

Viral RNA extraction, reverse transcription-polymerase chain reaction (RT-PCR) amplification and sequencing. HCV RNA was extracted from 140 μl plasma using a commercially available kit (QIAamp Viral RNA kit; Qiagen, Tokyo, Japan).

For the amplification of the NS5B region of the HCV genome, the extracted RNA was reverse-transcribed and amplified using SuperScript One-Step RT-PCR (Invitrogen, Tokyo, Japan) and a set of primers. The reaction was initially performed at 45˚C for 30 min for RT and at 94˚C for 2 min, followed by the first-round PCR over 40 cycles, with each cycle consisting of denaturation at 94˚C for 1 min, annealing at 45˚C for 1.5 min and extension at 72˚C for 2 min, using outer primers 166 (5'-TGGGGATCCCGTATGATACCCGCT GCTTTGA-3', nt 8230-8260, +) and 167R (5'-GGCGGA ATTCCTGGTCATAGCCTCCGTGAA-3', nt 8601-8630, -). All the PCRs were performed using Platinum Taq DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA). The second-round PCRs were performed under the same conditions to amplify the HCV genome, using different sets of primers as follows: i) HC23 (5'-TTTGACTCAACCGTCACT GA-3', nt 8256-8275, +), HC24 (5'-CTCAGGCTCGCC GCATCCTC-3', nt 8577-8596, -) and HC26 (5'-CTCAGG TTCCGCTCGTCCTC-3', nt 8577-8596, -); ii) HC15 (5'-ACT GTCACTGAACAGGACAT-3', nt 8265-8284, +) and HC16 (5'-GCTCTATCCTCATCGACGCC-3', nt 8568-8587, -); iii) HC23 and HC28 (5'-CACGAGCATGGTGCAGTC CCGGAGC-3', nt 8507-8531, -); iv) HC23 and HC32 (5'-AGGTAGCACGTCAGCGTGTTTCC-3', nt 8454-8476, -); and v) HC23 and HC34 (5'-TAGCACGTCATGGTGTTT CCCAT-3', nt 8451-8473, -) (10). If the result of amplification of the NS5B region was negative, the extracted RNA was reverse transcribed and amplified using the same condition but using a different set of primers as follows: i) UTR1 (5'-CCG GGAGAGCCATAGTGGTC-3', +) and UTR2 (5'-AGTACC ACAAGGCCTTTCGC-3', -) (first-round PCR); ii) UTR3 (5'-TGGTCTGCGGAACCGGTGAG-3', +) and UTR4 (5'-ACCCAACACTACTCGGCTAG-3', -) (second-round PCR) (11).

The PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide and were visualized under UV illumination. Amplified cDNA fragments were sequenced by a direct sequencing method with the BigDye Terminator v1.1 Cycle Sequencing kit and an ABI Prism 310 sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence analysis. Each obtained sequence was compared to those of the reported subtypes and on the basis of the percent homologies on the NS5B region, each isolate was assigned a subtype (10,12). When a subtype assignment was not possible due to the lack of NS5B amplification, the nucleotide sequences of the 5'UTR region were determined and compared with the consensus sequence motifs for each of the major genotypes previously reported (13).

Quantification of plasma HCV RNA titers. The quantification of plasma HCV RNA titers was performed with TaqMan® Gene Expression Master Mix (Applied Biosystems) using Applied Biosystems 7300 Real-Time PCR system.

Results

Samples. A total of 187 plasma samples were obtained from 187 HIV-positive patients (153 men and 34 women; mean age, 29.4 years; age range, 3 months-58 years). The risk factors for infection were IVDU (62.6%), heterosexual and homosexual intercourse (35.8 and 1.1%, respectively) and vertical transmission (0.5%) (Table I).

A total of 119 patients (63.6%) were anti-HCV-positive and the remaining 68 (36.4%) were anti-HCV-negative. The group of HCV-seropositive patients was most likely (73.1%) to have acquired HCV through parenteral transmission (IVDU), followed by heterosexual transmission (26.9%) (Table I).

HCV RNA detection. HCV RNA was detected in 73 of the 119 anti-HCV-positive samples (61.3%); among these, HCV-1a (31.5%) was the predominant subtype, followed by 3a (23.3%), 1 (16.4%), 1c (10.9%), 1b (9.6%), 3k (4.1%) and 2, 2a and 4a (1.4% each). Of the 68 anti-HCV-negative samples, HCV RNA was detected in 26 (38.2%), with HCV-3a being the most prevalent subtype (50%), followed by 3k (23.1%), 1c (15.4%) and 1b (11.5%) (Table II). An HCV viral load of >100,000 IU/ml was more commonly observed among the group of HCV-seropositive and HCV RNA-positive patients (33/73,

Table I. Modes of transmission according to the anti-HCV status.

	Modes of transmission (%)				
Anti-HCV	IVDU	Heterosexual	Homosexual	Vertical	Total (%)
Positive	87 (73.1)	32 (26.9)	-	-	119 (63.6)
Negative	30 (44.1)	35 (51.5)	2 (2.9)	1 (1.5)	68 (36.4)
Total	117 (62.6)	67 (35.8)	2 (1.1)	1 (0.5)	187 (100)

HCV, hepatitis C virus; IVDU, intravenous drug use.

Table II. Distribution of HCV genotypes/subtypes among samples with detected HCV RNA according to the anti-HCV status.

	HCV genotypes/subtypes (%)								
Anti-HCV	1	1a	1b	1c	2	2a	3a	3k	4a
Positive (n=73)	12 (16.4)	23 (31.5)	7 (9.6)	8 (10.9)	1 (1.4)	1 (1.4)	17 (23.3)	3 (4.1)	1 (1.4)
Negative (n=26)	-	-	3 (11.5)	4 (15.4)	-	-	13 (50)	6 (23.1)	-

HCV, hepatitis C virus.

Table III. Modes of transmission among samples with detected HCV RNA according to the anti-HCV status.

	Modes of HCV transmission (%)		
Anti-HCV	IVDU	Heterosexual	Homosexual
Positive (n=73)	56 (76.7)	17 (23.3)	-
Negative (n=26)	9 (34.6)	16 (61.5)	1 (3.9)

HCV, hepatitis C virus; IVDU, intravenous drug use.

Table IV. HIV viral load among anti-HCV-positive and HCV RNA-positive patients vs. anti-HCV-negative and HCV RNA-positive patients.

	Anti-HCV (%)	
HIV viral load (copies/ml)	Negative	Positive
≥100,000	14 (53.9)	41 (56.2)
<100,000	7 (26.9)	27 (37.0)
Undetected	5 (19.2)	5 (6.8)
Total	26	73

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

45.2%) compared to the group of HCV-seronegative and HCV RNA-positive patients (1/26, 3.9%).

The majority of HCV-seropositive samples with detected HCV RNA were obtained from HIV-positive patients who had acquired HCV through parenteral transmission (IVDU) (56/73, 76.7%), followed by those with a history of heterosexual transmission (17/73, 23.3%). However, HCV-seronegative samples with detected HCV RNA were mostly obtained from HIV-positive patients with a history of heterosexual transmission (16/26, 61.5%), followed by those with parenteral (IVDU) (9/26, 34.6%) and homosexual transmission (1/26, 3.9%) (Table III).

HIV viral load. High HIV viral loads (≥100,000 copies/ml) were mostly detected among the group of HCV-seropositive and HCV RNA-positive patients (41/73, 56.2%) and the group of HCV-seronegative and HCV RNA-positive patients (14/26, 53.9%) (Table IV).

Discussion

HIV/HCV co-infection affects over one-third of HIV-infected individuals worldwide (14). Following the introduction of HAART, HCV infection has been considered as the principal cause of morbidity and mortality among HIV-infected individuals (15). Co-infected patients exhibit a higher mortality rate compared to singly HIV-infected and HCV infection is considered a predictor of mortality (16). Despite these emerging trends, screening for HCV in HIV-infected patients is not routinely performed, since HCV is perceived as a slowly progressive disease, which would be unlikely to affect the natural history of HIV and associated opportunistic infections (4). In Indonesia, the rapidly increasing number of new HIV infections makes the epidemic one of the fastest growing in Asia (1). However, the incidence rate of HCV infection among HIV-positive patients has not yet been determined in Indonesia. We examined repositoried blood specimens obtained from HIV-positive patients in Surabaya to identify and describe cases of newly acquired HCV infection.

Antibody testing is the main screening method for HCV infection in HIV-infected individuals (7). Tests available for the diagnosis of HCV infection in the acute phase vary in sensitivity and the third-generation antibody ELISA tests were developed to detect multiple antigenic determinants (core, NS3, NS4 and NS5) to increase the sensitivity. In this study, the result of anti-HCV tests using EIA 3.0 demonstrated that, of the 187 plasma samples obtained from HIV-positive patients, 119 (63.6%) were anti-HCV-positive and the remaining 68 (36.4%) were anti-HCV-negative (Table I). However, serological screening in HIV-infected patients may not be the optimal screening method, possibly as a result of immunosuppression (8).

In our study, the group of HCV-seropositive patients was more likely (73.1%) to have acquired HCV through parenteral transmission (IVDUs), followed by heterosexual transmission (26.9%) (Table I). Individuals infected with HIV are frequently co-infected with HCV due to the shared modes of transmission (9). The risk of HCV transmission is significantly higher for patients who acquire HIV infection parenterally compared to those who acquire it sexually. The role of homosexual and heterosexual transmission of HCV remains controversial; it is considered to occur, although with a low efficiency. The sexual route is a common mode of HIV transmission, although it is not as effective for HCV (17). A previous study reported that ≤90% of HIV-positive IVDUs tested positive for HCV antibodies (18), although only 4-8% of the individuals who acquire HIV through sexual contact have detectable HCV antibodies (19,20). In our study, the heterosexual transmission history in HCV-seropositive patients (26.9%) was significantly higher compared to previously reported findings, which may be due to certain mechanisms involved in HCV transmission between sexual partners, particularly those who engage in habits that are associated with a high risk of virus transmission. Transmission may also result from exposure to unreported parenteral risk factors or from sharing certain personal items, such as toothbrushes or razors, which may result in accidental exposure to the partner's blood (21).

In HIV/HCV co-infection, abnormal antibody and cellular immune responses to HCV have been described (22-24). Immunosuppression by HIV infection may impair antibody formation and false-negative HCV antibody tests have been reported in individuals co-infected with HIV (22,25). Loss of HCV antibodies is observed in rare cases with advanced immune deficiency in HIV/HCV co-infection and does not necessarily indicate viral clearance (23). Identifying HCV seroconversion in serial samples is suboptimal, since the antibody development may be delayed in HIV-infected individuals to >1 year after the initial infection (26), compared to HIV-negative patients, who generally produce antibodies to HCV within 6 weeks of infection (27). This delay in the formation of antibodies may result in a significant delay in the diagnosis of HCV in HIV-positive individuals and the patients may miss the opportunity to receive effective treatment for their infection (59 vs. 40% success rate) (28,29). The European AIDS Clinical Society co-infection guidelines endorse an approach that should be considered, with serological testing for HCV for all patients upon entry into HIV care and annually thereafter for HCV-uninfected individuals, with HCV RNA testing for all HCV antibody-negative patients exhibiting an unexplained increase in alanine transaminase levels and at high risk for HCV infection (IVDU, mucosal trauma during intercourse) (30). Matthews and Dore (31) suggested that consideration should be given to HCV RNA testing, despite a negative HCV antibody status, in cases of unexplained transaminase elevation in patients with CD4 cell counts <200/mm^3 when acute hepatitis C is suspected. Frequent serum HCV RNA testing is thus a possible screening strategy (32). Of note, the HCV antibody status may be negative, despite active HCV viremia, in 10-15% of immunosuppressed patients (31).

Our study demonstrated that HCV RNA was detected in 73 (61.3%) of the 119 anti-HCV-positive samples; of the 68 anti-HCV-negative samples, HCV RNA was detected in 26 (38.2%) (Table II). In the HCV-seropositive and HCV RNA-positive group of patients, a high HCV viral load (>100,000 IU/ml) was more frequently detected (33/73, 45.2%) compared to the HCV-seronegative and HCV RNA-positive group of patients (1/26, 3.9%). Higher HCV viral loads may also be responsible for the increased transmissibility, as has been noted in HIV-co-infected patients (17); in this study, they were mostly found among anti-HCV-positive patients. A previous study demonstrated that HCV RNA was detected more frequently among the 31 HCV-seropositive patients (84.4% of tests) compared to the 20 HCV viremic HCV-seronegative patients (51.5% of tests) (9). Another study reported that HCV RNA was detected in the blood of >80% of HIV-positive individuals who were positive for HCV antibodies (33). Those findings are consistent with those of our study, according to which HCV RNA was highly detected among HIV-positive and HCV-seropositive patients, but was also relatively highly detected among HIV-positive and HCV-seronegative patients. There was a predominantly high HIV viral load (≥100,000 copies/ml) among the HCV-seropositive and HCV RNA-positive patients (41/73, 56.2%) and also among HCV-seronegative and HCV RNA-positive patients (14/26, 53.9%) (Table IV), which may have contributed to these results. As previously reported, patients with higher HIV RNA titers tend to have lower CD4 cell counts. The impaired T-helper type 1 immune response in turn may alter the response of immune cells to HCV, permitting greater HCV replication (34,35).

IVDU continues to be the primary risk factor for acquisition of HCV/HIV co-infection, with HCV usually acquired within the first year of IVDU (36); however, patients with a sexual transmission history generally acquired HIV prior to HCV (3,37). It was previously suggested that the presence of HIV may increase the heterosexual transmission of HCV (20). HCV infection appears to occur more frequently among HIV-infected HCV-seronegative individuals than appreciated, particularly if HIV acquisition was through sexual as opposed to parenteral risk factors (9). Those findings confirmed the results of our study, demonstrating that the majority of the HCV-seronegative samples with detected HCV RNA were obtained from HIV-positive patients with heterosexual transmission history (61.5%), differed from HCV-seropositive samples with detected HCV RNA, which were mostly obtained from HIV-positive patients with parenteral transmission (IVDU) history (76.7%) (Table III). However, men engaging in sexual intercourse with other men did not appear to exhibit an overall increased risk for co-infection (38-40), although

epidemics of acute HCV were previously described among HIV-infected men engaging in sexual intercourse with other men exhibiting high-risk behaviors (41). The two HIV-infected homosexual males included in this study were anti-HCV-negative, although HCV RNA was detected in one of them (data not shown). HCV/HIV co-infection among homosexual men requires further investigation.

The distribution of HCV genotypes/subtypes in HIV-positive patients reflects the route of transmission. HCV RNA was detected in 73 (61.3%) of the 119 anti-HCV-positive samples and, among these, HCV-1a (31.5%) was the predominant subtype, followed by 3a (23.3%), 1 (16.4%), 1c (10.9%), 1b (9.6%), 3k (4.1%) and 2, 2a and 4a (1.4% each). Of the 68 anti-HCV-negative samples, HCV RNA was detected in 26 (38.2%), with HCV-3a being the most prevalent subtype (50%), followed by 3k (23.1%), 1c (15.4%) and 1b (11.6%) (Table II). HCV subtypes 1a and 3a were more commonly reported among IVDUs (42). The high incidence of HCV-3a in the group with a history of heterosexual transmission may be attributed to the sexual partners, who were IVDUs; however, this hypothesis requires further investigation. Considering the differences in the predominant transmission mode between HCV-seropositive and -seronegative individuals, it is likely that the mode of HCV transmission is associated with the HCV subtypes.

In conclusion, HIV-positive patients are at a high risk of becoming co-infected with HCV, several of whom may remain HCV-seronegative, partly due to their immunodeficiency status. These data may also suggest that there may exist differences in HCV seropositivity and subtypes between HIV-positive patients who acquired HCV sexually and those who acquired HCV parenterally. It seems prudent to consider HCV infection in HIV-positive patients who test negative for HCV antibodies, as delayed HCV seroconversion in HIV-positive patients may result in delayed diagnosis and treatment.

Acknowledgements

The authors are grateful to all the participants in this study. This study was supported by a grant from the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

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