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Rapidly Spreading of hepatitis C virus among injecting drug users in the Philippines - Implications for HIV epidemics

Shortened title: Rapid HCV spreading in the Philippines

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ABSTRACT

From the trends of human immunodeficiency virus (HIV) epidemics in South and Southeast Asia, it was postulated that an HIV epidemic would start as a blood-borne infection among injecting drug users in the Philippines. In 2002, 560 individuals were recruited in Metro Cebu, Philippines and tested for HIV, hepatitis C virus (HCV) and hepatitis B virus (HBV) infections. Seroprevalence of anti-HCV among injecting drug users (70.1%, 61/87) was significantly higher than those among inhalation drug users (16.3%, 7/43; $P=0.00$; OR=12), sex workers (0%, 0/130; $P=0.00$; OR= ∞), antenatal clinic attendees (0%, 0/100; $P=0.00$; OR= ∞), and students/health care workers (2.0%, 4/200; $P=0.00$; OR=115). Seroprevalence of HBsAg among injecting drug users (10.3%, 9/87) was significantly higher than those among sex workers (2.3%, 3/130; $P=0.01$; OR=4.9), and antenatal clinic attendees (3%, 3/100; $P=0.04$; OR=3.7), but was not statistically different from those among inhalation drug users (9.3%, 4/43; $P=0.9$) and students/health care workers (4.5%, 9/200; $P=0.06$). None of the study population was reactive to anti-HIV antibody. The HCV strains obtained from the injecting drug users belonged to either genotype 1a or 2b and the strains in each genotype clustered closely to each other. There was no dual infection with genotype 1a and 2b. These suggest that the HCV infection in injecting drug users may be rapidly emanating from limited source individuals in Metro Cebu, Philippines.

INTRODUCTION

The Philippines is one of the low prevalence countries for human immunodeficiency virus (HIV). Based on the AIDS registry of the Department of Health in the Philippines, the total number of HIV cases has increased but remained at low level at a cumulative total of 2,107 as of June 2004. Main mode of HIV transmission has been reported to be heterosexual contact since 1984. Although HIV-positive cases have appeared sporadically among sexually active populations such as sex workers, no outbreak has occurred among them in this country. However, wide-range HIV strains have already been introduced in the country, *i.e.*; five HIV-1 subtypes (A, B, C, D, F), a circulating recombinant form (CRF01_AE) [Espantaleon et al., 2003; Paladin et al., 1998; Santiago et al., 1998], a recombinant strain (*gag-A/env-B*) [Espantaleon et al., 2003]. Even HIV-2 [Leano et al., 2003] have been identified. Among these, HIV-1 subtype B was the most predominant, followed by CRF01_AE [Paladin et al., 1998; Santiago et al., 1998; Espantaleon et al., 2003]. The low prevalence and the variety of HIV strains in the Philippines indicate that HIV has mainly been imported from abroad and the gateway of HIV into the Philippines has been quite open. Therefore, the migration sites and the subsequent circulation pathways of HIV have become one of the most important concerns for the prevention of an AIDS outbreak in the Philippines.

The past trend of HIV/AIDS outbreak in South and Southeast Asia reported by World Health Organization (WHO; HIV/AIDS in Asia and the Pacific Region 2003) and others [Ruxrungtham et al., 2004] have implied that the Asian AIDS epidemic may start among injecting drug users with secondary new infections being evident among sex workers. This is reasonable when considering the fact that the probability of HIV infection is 10-fold higher for the transmission through

contaminated needle sharing than that through sexual contact [Royce et al., 1997]. Therefore, it could be postulated that an HIV outbreak would start as a blood-borne infection among injecting drug users in the low HIV-prevalence countries including the Philippines, and that the HIV outbreak could be preceded by other blood-borne infections, such as hepatitis C virus (HCV) and hepatitis B virus (HBV) infections.

HIV, HCV and HBV are the major blood-borne pathogens spreading among injecting drug users via shared syringes and other injection devices [Lauer and Walker, 2001]. Seroprevalence of HCV antibody (anti-HCV) has been globally reported to be 65-90% among injecting drug users [Chamot et al., 1992; Crofts et al., 1993; Lauer and Walker, 2001; Soriano et al., 2002; Van Ameijden et al., 1993; van den Hoek et al., 1990] and 82.9-100% among HIV-infected injecting drug users [van Asten et al., 2004]. However, the reports on the prevalence and the characteristics of HCV and HBV have been limited in the Philippines. According to the available data, the positive rate for anti-HCV was 2.2% (9/392 tested) and the same rate was also noted for HBsAg among blood donors in 1990 [Arguillas et al., 1991], and anti-HCV was reported to be 4.6% (23/502 tested) among inmates [Katayama et al., 1996].

In this study, an HCV-epidemic site was identified in the Philippines and the genetic links of the HCV strains infecting injecting drug users were analyzed to determine their migration site, circulation pathways and the spreading speed.

MATERIALS AND METHODS

Subjects

From June to August 2002, 560 individuals were recruited in Metro Cebu of the Philippines. Study population was categorized into five groups; injecting drug users (n=87), inhalation drug users (n=43), sex workers (n=130), antenatal clinic attendees (n=100), and students and health care workers (n=200). Characteristics of the study population are shown in Table I. Injecting drug users were from two areas; an urban area where there was easy access to the prohibited drugs and the drug rehabilitation centers where they were trying to be accustomed to be free from the drugs. Injecting drug users were identified through the pre-tested interview questionnaire conducted by a trained study staff. All of the 560 participants agreed to be part of the study after the researchers explained the objectives and the conduct of the study, and signified their intent to join the study by signing an informed consent form.

Serological testing

A total of 5 ml whole blood was collected from each participant. Plasma was separated and subjected to each test.

Determine HIV-1/2 (ABBOTT JAPAN, Tokyo, Japan) and Determine HBsAg (ABBOTT JAPAN) were used for the detection of anti-HIV antibody and Hepatitis B surface antigen, respectively. HCV PHA "ABBOTT" (ABBOTT HCV 2nd Generation) was kindly provided by ABBOTT JAPAN for research purpose and was used for the detection of anti-HCV in this study. All the systems were cautiously used according to the manufacturer's instructions.

RNA Extraction, Reverse Transcription and Polymerase Chain Reaction (PCR)

HCV-RNA was extracted from 100 μ l of plasma using SMITEST EX-R&D (Genome Science Laboratories, Fukushima, Japan), and reverse-transcribed according to First-Strand cDNA Synthesis protocol (Invitrogen, Carlsbad, CA) with antisense gene-specific primers, hep32 (5'-GCDGARTACCTGGTCATAGC-3') for NS5B regions of HCV genome. A part of NS5B region of HCV gene was amplified by nested PCR with primers, hep31b (5'-TGGGTTCTCDTATGAYACC-3')/hep32 in the first round, and hep33b (5'-AYACCCGMTGYTTTGACTC-3')/hep34b (5'-CCTCCGTGAAKRCTCKCAG-3') in the second round. Nested PCR was performed with 20 μ l reaction mixture containing 2.5mM MgCl₂, 200 μ M each dNTP, 0.5 μ M primers and one unit of Amplitaq Gold[®] (Applied Biosystems, Foster City, CA). First-round PCR was done with one cycle of 94°C for 10 min, and 35 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec with a final extension of 72°C for 10 min. Second-round PCR was done in the same condition except for the annealing temperature at 60°C. PCR amplification was confirmed by visualization with ethidium bromide staining of the gel [White et al., 2000].

Genotyping

PCR product was subjected to nucleotide sequence determination directly with the primers of hep33b and hep34b for NS5B region. Some of the PCR-products were cloned with TOPO TA cloning kit (Invitrogen) and sequenced as previously described [Thompson et al., 1994]. At least 11 clones per sample were analyzed to investigate the possible co-existence of different HCV genotypes.

The sample sequences were aligned with HCV sequences from the database in STD AIDS Cooperative Central Laboratory (Manila, the Philippines) and HCV sequence database (http://gluttony.lanl.gov/content/hcv-db/combined_search/search) by ClustalW with subsequent inspection and manual modification [Thompson et al., 1994]. The frequency of nucleotide substitution in each base of

the sequences was estimated by the Kimura two-parameter method. A phylogenetic tree was constructed by the neighbor-joining method, and its reliability was estimated by 1000 bootstrap replications. The profile of the tree was visualized with the program of Njplot [Perriere and Gouy, 1996].

Statistical analysis

Prevalence data of HCV and HBV infection was analyzed by χ^2 test and p value less than 0.05 was considered to be significant.

RESULTS

Prevalence of HCV, HBV and HIV infections

Of the 87 injecting drug users, 61 (70.1%) were positive for anti-HCV. Twenty-eight of the injecting drug users were recruited from an area at the downtown of Metro Cebu, and all of them (100%, 28/28) had anti-HCV. Of the 43 inhalation drug users, only seven (16.3%) had anti-HCV. No one was positive for anti-HCV in the 130 sex workers and the 100 antenatal clinic attendees. Among the students/health care workers (n=200), only 4 (2.0%) were positive for anti-HCV (Table II). Thus, the prevalence of anti-HCV was significantly higher among injecting drug users than inhalation drug users ($P=0.00$; Odds ratio (OR)=12, 95% Confidence interval (CI): 5-31), sex workers ($P=0.00$; OR= ∞), antenatal clinic attendees ($P=0.00$; OR= ∞), and students/health care workers ($P=0.00$; OR=115, 95% CI: 38-346), indicating that injecting drug use is significantly associated with the HCV infection.

Seroprevalence of HBsAg among injecting drug users (10.3%, 9/87) was significantly higher than that among sex workers (2.3%, 3/130; $P=0.01$; OR=5, 95% CI: 1-19) and antenatal clinic attendees (3.0%, 3/100; $P=0.04$; OR=4, 95% CI: 1-14), but not than that among inhalation drug users (9.3%, 4/43; $P=0.9$) and students/health care workers (4.5%, 9/200; $P=0.06$) (Table II).

HIV antibody was not detected in any of these groups (Table II).

Seven (8.0%) of the 87 injecting drug users were dually positive for HBsAg and anti-HCV. Among other population groups, there was no dual positive case.

HCV Genotypes

Of the 61 injecting drug users positive for anti-HCV (Table II), 52 samples were available for further analysis and 38 samples were positive for PCR with NS5B

primers. Twenty-three of the PCR-positive samples were randomly selected and were subjected to nucleotide sequencing. The PCR products were directly sequenced and analyzed phylogenetically. A phylogenetic tree (Fig.1.) based on NS5B sequences (nucleotides, 7975 to 8196 [Choo et al., 1991]) showed two HCV genotypes, 1a and 2b. Of the 23 HCV strains analyzed, 15 clustered significantly with genotype 1a reference sequences (with bootstrap value 97%), and most of them sub-clustered together while two strains (02dx02 and 02du98) did not. The remaining 8 clustered significantly with genotype 2b reference sequences and formed a significant sub-cluster (with bootstrap value 96%), suggesting that the source of HCV 2b circulation among the injecting drug users in Metro Cebu is limited and 02du49 could be a founder strain (Fig.1.).

Heterogeneity of HCV strains in an injecting drug user

To investigate the possible co-existence of different HCV genotypes in an injecting drug users, the PCR products of randomly selected 9 strains (5 genotype 1a strains: 02dz02, 02ccdm6, 02ccmk2, 02du98, 02qq01; and 4 genotype 2b strains: 02ww8, 02ccdq4, 02ccdq5, 02du49) were cloned. At least 11 clones per sample were sequenced in the regions of NS5B and analyzed phylogenetically. Phylogenetic trees based on NS5B sequences showed that nucleotide sequences of all the clones in each individual were homogeneous, and co-existence of genotype 1a and 2b were not observed.

DISCUSSION

In the current study, it was found that an HCV infection was epidemic in Metro Cebu of the Philippines, where 70% of injecting drug users were positive for anti-HCV. The prevalence of anti-HCV among injecting drug users has been reported to be 65-90% globally [Chamot et al., 1992; Crofts et al., 1993; Van Ameijden et al., 1993; van den Hoek et al., 1990], and that of Metro Cebu in our study was consistent with the previous reports. Despite the high prevalence of anti-HCV positive cases among the tested injecting drug users, HIV infection was not observed at all.

Like most of RNA viruses, HCV exhibits genetic heterogeneity [Bukh et al., 1995; Zuckerman and Zuckerman, 1995], which has been reported even within the same individual [Chen et al., 1992; Higashi et al., 1993; Houghton et al., 1991; Martell et al., 1992; Okamoto et al., 1991]. In our study, two HCV genotypes, 1a and 2b, were circulating among injecting drug users in Metro Cebu, and each injecting drug user had homogeneous HCV population regardless of the genotypes. These results suggest that these HCV strains have been introduced recently into injecting drug users in Metro Cebu and spreading rapidly among them from a few origins. However, the origins have not been specified yet and further investigation is required.

The rate of HBsAg was found to be from 2% to 10% among the different population groups in Metro Cebu. However, there was no significant difference in the seroprevalence of HBsAg between injecting drug users and inhalation drug users ($P= 0.85$). This may be because newly acquired HBV results in acute infection, needle sharing among injecting drug users may not contribute to the increase in the HBV chronic infection, and HBV antigen carrier state may mainly

be induced by vertical infections. For the further discussion, the detection of anti-HBs antibody will be required.

The Philippines and Indonesia are both island countries and have similar distances from Thailand and Cambodia where HIV infection is most prevalent in Asia. By the year 1999, Indonesia had been considered to be one of the low and slow HIV prevalence countries like the Philippines. However, in the late 2000, sharp increase in HIV prevalence among injecting drug users (up to over 35% in Jakarta) was noted (HIV/AIDS in Asia and the Pacific Region 2001, WHO). This increasing trend of HIV prevalence was also noted among blood donors thereafter, suggesting that the behavior of contaminated needle sharing (causing HCV infection) triggered an AIDS outbreak before the increase in the number of HIV-positives through sexual transmission. As seen in Indonesia, HIV spreads first among injecting drug users, followed by sex workers in other Asian countries especially if drug users are the clients of sex workers [Ruxrungtham et al., 2004]. However, it seems that HIV has not yet deeply migrated through the blood-borne pathway in the Philippines. As shown in this study, HIV infection was very rare even among HCV-positive injecting drug users. However, convincing evidence will be given through the further analyses with increasing the number of subjects and in geographically different places in the Philippines. Although HIV is of low prevalence, the rapid spread of HCV infection indicates that the injecting drug users can be at highest risk in causing an AIDS epidemic in this country.

In this study, it was demonstrated that the HCV infection clustered among injecting drug users in Metro Cebu of the Philippines. HCV infection seemed to be rapidly spreading among injecting drug users from limited sources. Further studies must be conducted to specify the migration site(s) and the subsequent circulation mode of HCV infection more precisely, which can serve as a model for

probable migration sites of HIV infections at early phase of a possible AIDS epidemic in the Philippines.

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TABLE I. Characteristics of Injecting and Inhalation Drug Users and Others

Population	Tested (Male/Female)		Mean Age (Range)
Injecting drug users	87	(80/7)	30 (13-46)
Inhalation drug users	43	(42/1)	29 (11-53)
Sex workers	130	(2/128)	25 (18-46)
Antenatal clinic attendees	100	(0/100)	26 (17-42)
Students/Health care workers	200	(65/135)	31 (6-61)

TABLE II. Seroprevalence of Hepatitis B Virus, Hepatitis C Virus and HIV Infections among Selected Population in Metro Cebu

Population	Tested	Positive cases (%) for:		
		HBsAg	Anti-HCV	Anti-HIV
Injecting drug users	87	9 (10%)	61 (70)	0
Downtown of Metro Cebu ^a	28	3 (11)	28 (100)	0
Drug rehabilitation centers	59	6 (10)	33 (56)	0
Inhalation drug users ^b	43	4 (9.3)	7 (16)	0
Sex workers	130	3 (2.3)	0	0
Antenatal clinic attendees	100	3 (3.0)	0	0
Students/Health care workers	200	9 (4.5)	4 (2.0)	0

^aClients from the downtown of Metro Cebu (n= 28) were all injecting drug users.

^bAll the inhalation drug users were from drug rehabilitation centers.

Figure legends

Fig.1.

Phylogenetic trees of 23 HCV strains (highlighted in the boxes) from injecting drug users in Metro Cebu and 31 HCV strains from other area of the Philippines, performed on 227 nt within the NS5B region by the neighbor-joining method with GBV-B (accession no. NC 001655) as an outgroup. Analyzed samples were indicated with two digits of the collecting year at the head of the ID (e.g. 02ES). If the collecting year is unknown, IDs are shown with the symbol of “?” (e.g.?JF). Accession numbers were used for the IDs of the genotype-known reference strains with two digits indicating genotypes at the end of the number (e.g. L23471-5a). Bootstrap values are given on the branches as percentage from 1,000 replicates.

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GBV-B

