VirusDis. (October–December 2019) 30(4):490–497
https://doi.org/10.1007/s13337-019-00553-2

ORIGINAL ARTICLE

Diversity of hepatitis C virus infection among HIV-infected people who inject drugs in India

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Received: 19 June 2019 / Accepted: 11 November 2019 / Published online: 7 December 2019

Abstract The availability of generic direct acting antivirals (DAAs) for hepatitis C virus (HCV) treatment has prompted many low-and-middle-income countries to launch HCV elimination programs. Because the efficacy of some of these generic DAAs varies by HCV viral subtype, information on subtype distribution can contribute important information to these elimination programs. We conducted a cross-sectional serosurvey to characterize HCV subtype diversity among HIV positive people who inject drugs (PWID) across 14 cities in India. Of 801 HIV positive PWID sampled, 639 tested HCV antibody positive (78.9%). Among 105 samples sequenced, genotype 3 (58.1%) was the most commonly observed followed by genotype 1 (36.2%) and genotype 6 (5.7%). Of the genotype 3 infections, 65% were subtype 3a and 35% were subtype 3b. Of the genotype 1 infections, 94% were subtype 1a and 6% were subtype 1b. All genotype 6 samples were subtype 6n. There was some variability in genotype diversity depending on geographic region and PWID epidemic stage with greater diversity observed in older PWID epidemics. One sequence, HY018, did not cluster with any known reference sequences in phylogenetic analysis. Nearly 80% of HIV infected PWID across India are co-infected with HCV, and subtype prevalence and genetic diversity varied by region and PWID epidemic stage. HCV elimination programs in India will need to consider HCV subtype.

Keywords Hepatitis C virus · People who inject drugs · India · Low-and-middle-income country · Genotype

Introduction

Approximately 71 million persons globally are chronically infected with hepatitis C virus (HCV, family *Flaviviridae*, genus *Hepacivirus*, species *Hepacivirus C*) [1]. People who inject drugs (PWID) bear a significantly higher burden of HCV infection with estimates of prevalence ranging from 50 to 90% [2, 3]. The burden of HCV is highest among HIV-infected PWID [2, 3]. Since the advent of antiretroviral therapy, HIV-related mortality has declined in nearly all high-income settings [4], and HCV-related mortality has subsequently overtaken HIV-related mortality [5]. However, there have been dramatic advances in HCV therapy. Treatment for HCV previously comprised of weekly interferon injections and daily ribavirin for up to 48 weeks with sub-optimal viral clearance rates and high-levels of toxicity. However, current treatment consists of 8–12 weeks of all-oral direct acting antivirals (DAAs) with minimal side effects and clearance rates greater than 95% [6–8]. However, access challenges remain across nearly all settings.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13337-019-00553-2) contains supplementary material, which is available to authorized users.

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It has been estimated that the general population HCV prevalence in India is between 1 and 2.5% [9]; this translates to nearly 20 million persons living with HCV infection in India. Access to HCV therapy in India has been facilitated by preferential pricing agreements and the production of generic DAAs including sofosbuvir, ledipasvir, daclatasvir and velpatasvir. Consequently, some states, including Punjab, have launched HCV elimination programs that include the provision of free treatment. However, the generic DAA regimens currently available in India have differential efficacy by subtype among persons with cirrhosis [10] making knowledge of HCV subtype distribution important for treatment and elimination program planning.

Prior reports have suggested that genotype 3 HCV infection predominates in most parts of Asia including India, followed by genotype 1. This is in contrast to western settings where genotype 1 infection predominates and may impact the relative effectiveness of currently available HCV DAAs in the region [9, 11]. Moreover, some studies have suggested that there is regional variability in HCV subtype distribution across India [12–14]. Even less data is available on the distribution of subtypes among people who inject drugs (PWID) in India. Yet, India is home to the largest number of opioid users globally, with top-end estimates of PWID as high as 1.1 million [15]. A recent large multi-city survey of PWID across India demonstrated a high burden of HCV infection and near negligible uptake of treatment [16]. However, the study did not collect information on HCV subtype. Accordingly, in this study, we characterize HCV prevalence and subtype distribution among HIV-infected PWID in 14 states across India.

Materials and methods

Study population

From October 2009–January 2011, 801 persons with a history of injection drug use were sampled across 14 cities in India to examine the viral genetic diversity of HIV and HCV among PWID in India. In each city, we partnered with a single existing non-governmental organization (NGO) that worked with PWID. Each NGO had a roster of HIV-positive individuals. To be included in the study, individuals had to be $\geq$ 18 years old, HIV-infected and ART naïve (by self-report). They also had to report injection drug use in the prior 2 years and provide informed consent. In order to examine the viral diversity in the absence of antiretroviral pressure on the viral genome, we selected a random sample of antiretroviral-naïve individuals from these rosters (every third person in the roster was selected until the desired sample size of $\sim$ 60 per site was reached). In each location, individuals were sampled at multiple times per day over the course of 2 weeks to 1 month to prevent oversampling from a single social/injecting network.

A total of 234 participants were recruited from 4 cities in the Northeastern states (Guwahati, Assam [AS]; Imphal, Manipur [MP]; Aizawl, Mizoram [MZ]; and Dimapur, Nagaland [NG]); 294 participants from 5 cities in the Northern states (Amritsar, Punjab [PB]; Faridabad, Haryana [HY]; Ghaziabad, Uttar Pradesh [UP]; Jaipur, Rajasthan [RJ]; New Delhi [ND]); 91 participants from 2 cities in the Western states (Panaji, Goa [GA]; Mumbai, Maharashtra [MH]); 60 participants from one city in the East (Kolkatta, West Bengal [WB]); and 122 participants from 2 cites in the Southern states (Chennai, Tamil Nadu [TN]; Ananthapur, Andhra Pradesh [AP]). These locations were also selected to represent drug use epidemics of varying stages: established epidemics (AS, MP, MZ, NG), emerging epidemics (PB, HY, UP, RJ, GA, AP) and large cities (ND, MH, WB, TN).

Study procedures

Following oral consent, participants responded to a survey that collected information about demographics, lifetime and recent drug-related risk behavior (including alcohol use), perceived need for services and perceived causes of mortality among PWID in the region. Following the survey, dried blood spot (DBS) samples were collected from all participants. EDTA-anticoagulated whole blood (50 µl) was spotted onto each of five circles of filter paper (Whatman No. 903; Whatman, Springfield Mill, UK) and air-dried. DBS samples were stored according to WHO guidelines in air-impermeable bags with silica desiccant from 2 to 12 months prior to testing. The study was approved by the Johns Hopkins School of Public Health (JHSPH) and the YR Gaitonde Centre for AIDS Research and Education (YRGCARE) Institutional Review Boards and all participants provided consent.

Laboratory methods

DBS samples from all study sites were shipped to the central laboratory in Chennai, India. First, all samples were tested for antibodies to HCV infection (Abbott Murex anti-HCV, Version 4.0, Republic of South Africa) as per manufacturer's instructions. Samples in 11 of the 15 cities representing geographical variability were selected for further analysis. In specimens from eight of these cities (ND, PB, UP, RJ, MP, MZ, WB, and TN), we used the

following approach. First, all samples within a site that tested positive for HCV antibodies were randomly ordered. Samples were subsequently tested in batches of 10 and 5 persons. If any detected HCV genotype in the first batch of 10 was represented by < 2 sequences, additional samples were tested in batches of five until each site included at least two sequences of each genotype detected. In three additional sites, HY, AS, and MH only 3, 2, and 3 samples, respectively were sequenced because these were the only specimens viable for sequencing.

For each specimen, two 50 µL dried spots were punched and eluted with 1.7 mL of Abbott lysis buffer at 56 °C with constant shaking for 2 h. The tubes were then centrifuged for 5 min at 15,000×g, and supernatants were processed manually using the CE marked Abbott Sample Preparation System (Abbott Laboratories, Abbott Park, IL), as per the manufacturer's instructions (1 mL protocol). The extracted RNA was subjected to one step RT PCR using HCV Core/E1 gene specific primers followed by nested PCR as previously described [17]. The amplified product was sequenced unidirectionally using the ABI prism 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The ab1 files containing HCV Core/E1 FASTA sequences were blasted in the NCBI tool (https://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi) to determine HCV subtypes. Sequences have been deposited in GenBank under accession numbers MN153064-MN153168 (Online Resource 1).

Phylogenetic and clustering analysis

Sequence alignments were performed using ClustalW in the MEGA version 6 package [18]. Parameter values for the best-fit model of nucleotide substitution were determined to be TPM2uf+G (three parameter model with unequal frequencies; gamma distribution shape parameter [G = 0.67]) using the corrected Akaike information criteria (AICc) as implemented in jModelTest [19, 20]. A phylogenetic tree was inferred by maximum-likelihood reconstruction with 1000 bootstrap replicates based on the nucleotide alignment of the core/E1 sequences as implemented in the MEGA version 6 package [18]. The tree was displayed with the Fig Tree version 1.4.2 program (http://tree.bio.ed.ac.uk/software/figtree/). Clusters were determined using a range of genetic distance thresholds from 1.5% through 5% in increments of 0.5% until the number did not increase using MicrobeTRACE version 1.9 (https://github.com/CDCgov/MicrobeTRACE).

Results and discussion

Characteristics of study participants

The median age of the 801 participants was 33 years (interquartile range [IQR], 28–38); 88.1% were male. The majority had no (27.2%) or only primary school education (31.6%). The majority were either unemployed (31.0%) or daily wage laborers (48.9%). The median age at first injection was 20 years (IQR, 18–25). Types of drugs injected varied by city; overall, the drug that was most commonly reported was heroin injection (68.5%) followed by buprenorphine (39.3%) and other pharmaceutical drugs (45.1%). A large proportion (39.9%) reported drinking three or more days per week with a median of three drinks per drinking episode (IQR, 3–4). The majority (74.4%) reported that they typically injected with at least one other person with 35.3% reporting that they typically injected with multiple people. The median lifetime number of injection partners was 9 (IQR, 4–20), and 91.4% reported that they had ever shared needles with a median of five (IQR, 3–10) sharing partners. There was variability by site with respect to most sociodemographic and risk behaviors (Table 1).

Hepatitis C prevalence and subtypes

Overall, 639 of 801 PWID were infected with HCV for an overall HCV prevalence of 78.9%; prevalence ranged from 2 to 98% across sites but was greater than 70% in in all but two sites (Fig. 1a). Among 211 available samples where sequencing was attempted, 106 could not be amplified. Among the remaining 105 samples that were sequenced, genotype 3 (58.1%) was most commonly detected, followed by genotype 1 (36.2%) and genotype 6 (5.7%). Of those that were genotype 3, 65% were subtype 3a and 35% were subtype 3b. Of those that were genotype 1, 94% were subtype 1a and 6% were subtype 1b. All genotype 6 samples were subtype 6n. There was some variability in subtype diversity depending on geographic region and stage of the PWID epidemic (Fig. 1b). In the cities with historical PWID epidemics, subtypes 1b, 3a, 3b and 6n were detected with 3b and 6n predominating. In sites with emerging epidemics, subtypes 1a, 3a and 3b were detected with a predominance of 3a. In the large cities, subtypes 1a, 1b, and 3a were detected.

Genetic diversity

All HCV sequences clustered in a monophyletic manner with their respective genotype reference sequence(s) with greater than 80% bootstrap support, except for sequence

Table 1 Characteristics of participants by study site[a] (n = 801)

	South		West		North					Northeast				East
	AP	TN	GA	MH	HY	PB	RJ	UP	ND	AS	MP	MZ	NG	WB
Number of participants	61	61	37	54	53	70	60	51	60	48	63	60	63	60
Median age (years)	32	40	35	30	28	29	32	34	36	38	35	30	31	34
IQR	27,38	34,43	28,40	24,35	25, 32	25,35	26,40	29,41	30,41	34,41	32,38	26,35	26,36	30,38
Gender (%)														
Male	47.5	98.4	54.1	94.4	100	100	96.7	100	100	100	98.4	78.3	65.1	93.3
Female	42.6	1.6	43.2	5.6	0	0	3.3	0	0	0	1.6	21.7	34.9	6.7
Hijrah	9.9	0	2.7	0	0	0	0	0	0	0	0	0	0	0
Married (%)	63.9	45.9	43.2	25.9	37.7	25.7	20.0	56.9	20.0	56.3	58.7	31.7	60.3	70.0
Education (%)														
None	24.6	29.5	0	33.3	24.5	52.9	43.3	47.1	48.3	4.2	1.6	1.7	28.6	26.7
Primary school	36.1	49.2	18.9	51.9	24.5	31.4	46.7	27.5	31.7	16.7	14.3	6.7	23.8	56.7
Secondary school	13.1	6.6	32.4	7.4	32.1	12.9	1.7	11.8	13.3	50.0	28.6	21.7	27.0	15.0
High school or more	26.2	14.8	48.7	7.4	18.9	2.9	8.3	13.7	6.7	29.2	55.6	70.0	20.6	1.7
Employment (%)														
Unemployed	16.4	13.1	24.3	25.9	9.4	40.0	40.0	27.5	3.4	43.8	55.6	53.3	44.4	30.0
Daily wages	63.9	77.1	24.3	70.4	60.4	45.7	46.7	54.9	81.4	35.4	15.9	23.3	38.1	41.7
Weekly wages	4.9	1.6	2.7	1.9	17.0	7.1	3.3	3.9	5.1	4.2	0	0	0	18.3
Monthly wages	14.8	8.2	48.7	1.9	13.2	7.1	10	13.7	10.2	16.7	28.6	23.3	17.5	10.0
Alcohol use[b] (%)														
Never	18.0	9.8	24.3	51.0	15.1	7.1	41.7	15.7	26.7	0	47.6	8.3	1.6	26.7
Light use	31.2	16.4	16.2	47.1	32.1	61.4	45.0	66.7	66.7	77.1	33.3	45.0	57.1	56.7
Heavy use	50.8	73.8	59.5	2.0	52.8	31.4	13.3	17.7	6.7	22.9	19.1	46.7	41.3	16.7
Median number of drinks	3	3	3	3	4	3	3	3	3	2	4	3	3	3
IQR	3,6	3,6	2,3	3,3	3,4	3,4	3,5	3,5	3,4	2,3	2,6	2,5	3,4	2,5
Median age at 1st injection (years)	24	18	22	17	21	20	21	27	23	21	19	18	19	18
IQR	22,32	17,20	19,25	14,22	20,26	16,25	18,27	30,32	19,29	19,25	17,21	17,22	17,20	17,20
Lifetime injection drug use (%)														
Heroin	65.6	100	89.2	98.2	1.9	39.0	56.7	58.8	55.0	97.9	100	68.3	54.0	95.0
Buprenorphine	0	4.9	35.1	9.3	75.5	42.9	83.3	76.5	95.0	18.8	14.3	0	1.6	98.3
Other pharmaceutical drugs	62.3	62.3	27.0	64.8	84.9	57.1	83.3	88.2	80.0	37.5	50.8	73.3	98.4	91.7
Cocaine	13.1	1.6	75.7	7.4	28.3	15.7	1.7	2.0	0	2.1	0	3.3	0	0
Methamphetamine	11.5	1.6	27.0	3.7	1.9	5.7	0	0	0	0	0	0	0	3.3
Pattern of drug injection (%)														
Alone	18	6.6	2.7	20.4	52.8	18.6	45	37.3	28.3	33.3	34.9	18.3	1.6	40
With one sexual partner	11.5	0	35.1	11.1	0	5.7	3.3	0	5	0	0	0	19.1	0
With one good friend	52.5	9.8	24.3	46.3	17	48.6	30	43.1	61.7	6.3	23.8	21.7	20.6	50
With multiple friends	18	82	32.4	22.2	28.3	24.3	21.7	13.7	5	60.4	41.3	56.7	58.7	6.7
With unknown persons	0	1.6	5.4	0	1.9	2.9	0	5.9	0	0	0	3.3	0	3.3
Medan lifetime injection partners	6	9	6	9.5	4	4	6	20	15	65	40	14	5	9
IQR	5,15	5,10	4.5,12	5,15	3,6	3,6	3,25	7,70	6.5,27.5	30,100	20,100	5.5,40	3,10	6,20
Ever shared needles (%)	49.2	98.4	97.3	100	98.1	88.6	93.3	86.3	93.3	97.9	100	93.3	93.7	95

Table 1 continued

	South		West		North					Northeast				East
	AP	TN	GA	MH	HY	PB	RJ	UP	ND	AS	MP	MZ	NG	WB
Lifetime median number of needle sharing partners	7	4	6	5	3	2.5	6	7	8	18	20	10	4	5
IQR	3,10	3,6	4,7	3,8	2,3	2,3	2,12	3,20	4,15	7,35	5,40	5,30	2,6	4,6

[a]Study sites: South: Ananthapur, Andhra Pradesh (AP); Chennai, Tamil Nadu (TN). West: Panaji, Goa (GA); Mumbai, Maharashtra (MH). North: Faridabad, Haryana (HY); Amritsar, Punjab (PB); Ghaziabad, Uttar Pradesh (UP); Jaipur, Rajasthan (RJ); New Delhi (ND). Northeast: Guwhati, Assam (AS); Imphal, Manipur (MP); Aizawl, Mizoram (MZ); and Dimapur, Nagaland (NG). East: Kolkatta, West Bengal (WB); interquartile range (IQR)

[b]Light alcohol use $\leq$ 3 days per week; heavy alcohol use $\geq$ 3 days per week

HY018 which clustered with genotype 3, but did not cluster in a monophyletic manner with the other known subtypes (Fig. 2). Within each genotype, sequences from the large cities of Chennai and Kolkatta tended to cluster almost exclusively with sequences from their respective city, while sequences from the Northern and Northeastern cities did not exclusively cluster by city, but tended to cluster within both city and region. Of the large cities, sequences from Kolkatta showed very little within city diversity, while sequences from Chennai and New Delhi had comparatively greater within genotype diversity. Sequences from the Northeastern cities, where the most established epidemics are, showed greater within site and regional diversity compared to sequences from the large cities and cities with emerging PWID epidemics.

Nine clusters of sequences, ranging in size from two to fourteen sequences, were found using a genetic distance threshold of $< 4.0\%$ (Online Resources 2 and 3). Three of the clusters were limited to sequences from a single city, while six of the clusters contained sequences from multiple cities. A single cluster contained sequences from multiple regions. The largest cluster contained thirteen genotype 1a sequences from Kolkatta, West Bengal.

Implications of study findings

With the newly available all-oral therapies for HCV infection, there has been much discussion about the best way to deliver these treatments across settings and populations, particularly those that will be most difficult to reach. These data from this resource-limited setting reinforce the high prevalence of HCV among HIV-infected PWID, and the diversity in risk behaviors and subtype distribution have important implications for treatment delivery as well as HCV transmission.

A predominance of genotype 3 HCV infection was observed in this sample of HIV positive PWID across India, followed by genotype 1 and then 6. In nearly all cities, more than one subtype was present with the highest levels of diversity observed in cities with the oldest injection drug use epidemics (Northeast). These findings are consistent with some prior reports, which have demonstrated a predominance of genotype 3 in Southern India [21]. By contrast, reports from Northeastern India have suggested that HCV genotype 4 is most predominant followed by genotype 6 with a relatively low prevalence of genotype 3 infection [22]. However, these studies were conducted in tribal populations who might be underrepresented in our study. Studies in Burma have suggested the most common subtypes to be 6, 3b and 1b [23], which is consistent with the results of our study. Genotype 3 HCV has also been reported as the most common genotype in other parts of south and southeast Asia [24].

The phylogenetic analysis provides insight into the HCV epidemics in both individual sites and regionally. The greatest diversity of HCV genotypes was observed in the Northeastern cities, where drug use and likely HCV transmission have been endemic for decades [25]. Moreover, the sequences collected from these cities showed the greatest within genotype genetic diversity consistent with what would be expected from older more established epidemics. By contrast in Kolkatta, West Bengal, only subtype 1a was found and very little genotype genetic diversity (maximum distance = 0.037; data not shown) was observed between all 18 samples collected from this site. This could indicate a new emerging epidemic in this location with limited introduction of viruses from outside the city. In comparison, the subtype 3a and 1a sequences from Chennai, Tamil Nadu demonstrated about double the genetic diversity seen in the 1a samples from Kolkatta with maximum diversity within genotype with genetic distances of 0.070, and 0.078 (data not shown) respectively.

Interestingly, one sequence, HY018, could not be assigned to a known subtype in the phylogenetic analysis. HY018, from Faridabad, Haryana, was one of three samples sequenced from the site, which all clustered within genotype 3. The other two sequences were subtype 3a, and while HY018 more closely resembled the genotype 3b

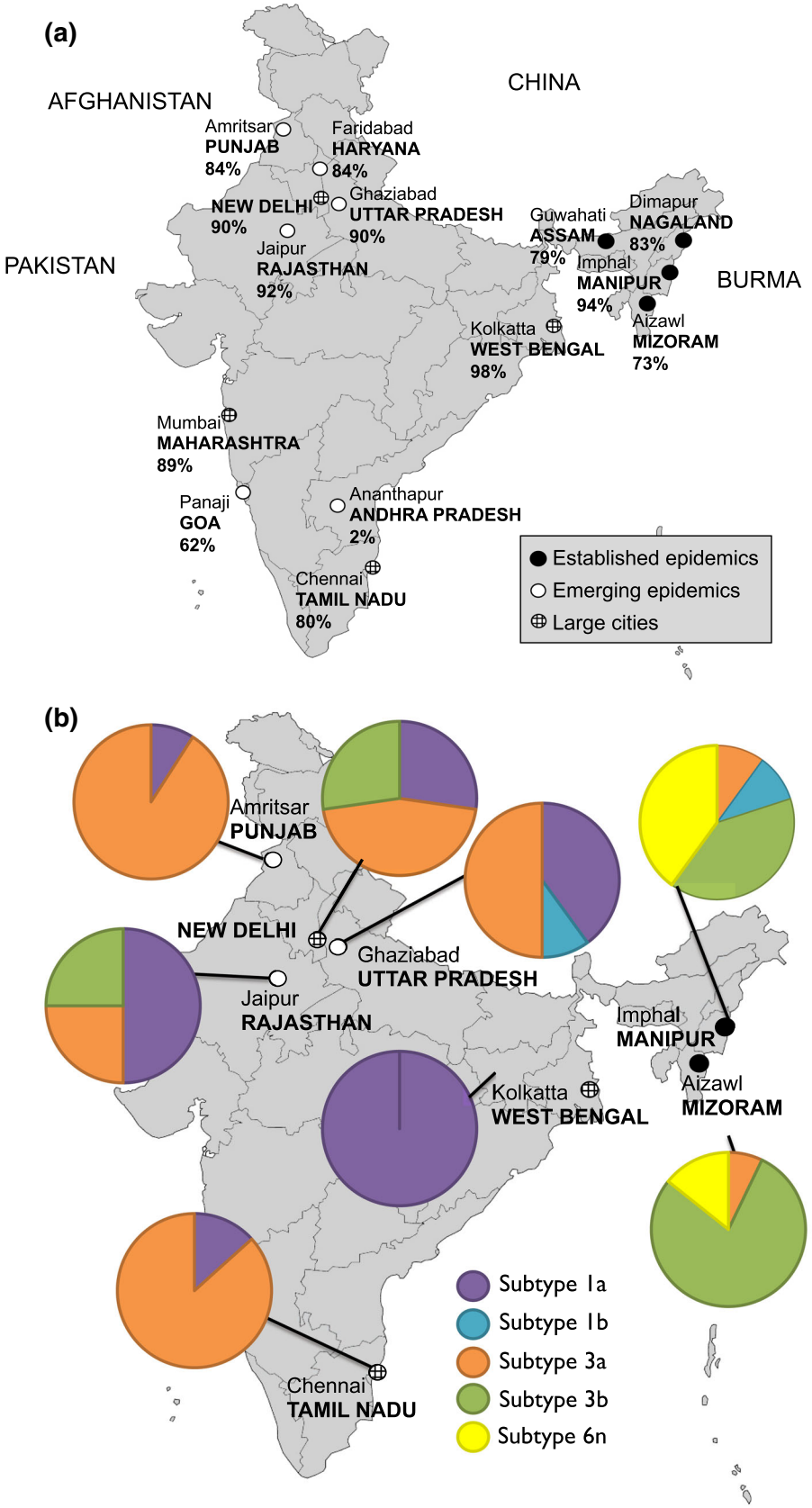

Fig. 1 **a** HCV antibody prevalence by study site (n = 14 sites, 801 participants), **b** distribution of HCV subtype by study site (n = 8 study sites, 105 participants)

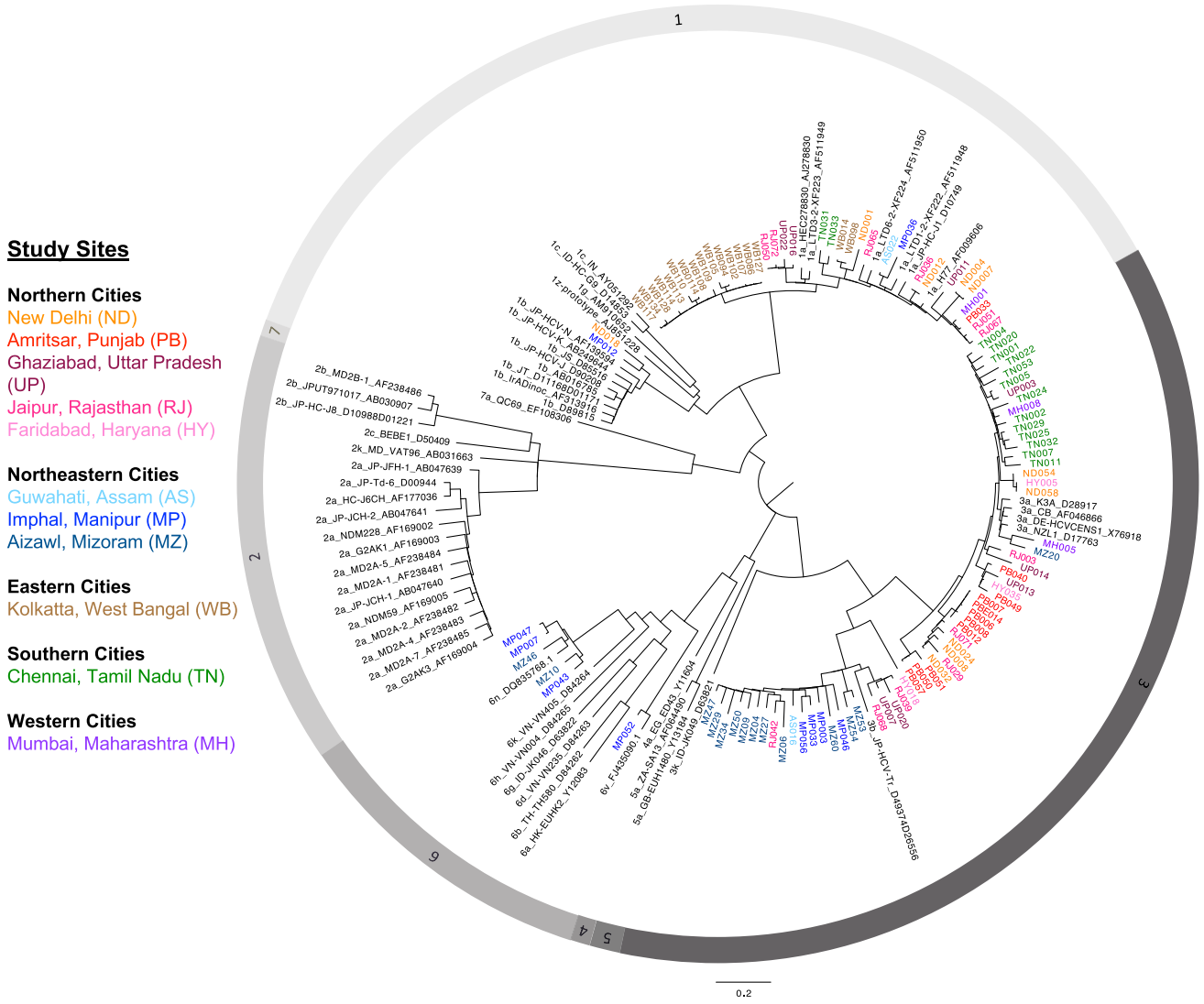

Fig. 2 Phylogenetic tree of HCV sequences from 105 participants in 8 study sites

sequences, it did not cluster significantly with the 3b reference sequence nor with the other 3b sequences from this study. HY018 may be a new HCV subtype not previously reported. Additional sampling and sequencing will be helpful in determining whether this is a new subtype and further whether this subtype is common in the population.

Study limitations

This study was limited by the relatively small sample size from each city that was available for sequence analysis. Moreover, the samples were collected from several years ago, and we did not have available samples from some cities to assess subtype distribution and genetic diversity. However, the aforementioned findings are consistent with what has been demonstrated in the general population within India [9].

Taken together, this study demonstrates the large burden of HCV among HIV-infected PWID across India, many of whom are still actively engaged in high risk behaviors. While HCV subtype prevalence and genetic diversity within each genotype varied by region and PWID epidemic stage, HCV prevalence remained high in HIV-infected PWID in all sites. These findings collectively reinforce the importance of considering HCV subtype when implementing HCV treatment efforts across India.

Acknowledgements This work was supported by grants from the National Institutes of Health (DA12568, DA26727, T32DA007292). We thank the site staff and study participants, without whom this work would not be possible.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest.

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