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Letter

HCV 6a was expanding and became the predominant subtype among blood donors between 2004 and 2019 in Guangdong, China

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Dear Editor,

There were an estimated 9.4 million patients with chronic hepatitis C virus (HCV) in China, putting a considerable burden on public health (Polaris Observatory HCV Collaborators, 2022). What is more, the incidence of HCV infection increased 14.5% every year based on data from China's National Notifiable Diseases Reporting System (Zhang et al., 2019). Approximately 75% of individuals infected with HCV develops chronic hepatitis, while long-term chronic HCV infection is the most important cause of liver fibrosis, cirrhosis, and hepatocellular carcinoma. HCV is commonly transmitted through unsafe transfusion practice, injection drug users (IDU), and high-risk sexual behaviors. The Chinese government has launched a series of activities to guarantee the safety of blood transfusion, and the blood banks in China constitute the largest network system that screened HCV infection in the general population across the nation. But the problem of residual transmission from blood donors still exists today (Wang et al., 2013; Du et al., 2019). Previous study showed that the overall HCV prevalence ranges from 166.56 per 100,000 in first-time donors, and 15.21 per 100,000 in repeated donors in China (Fu et al., 2019). Hence, it is of paramount importance to explore the current patterns of HCV transmission among blood donors in China.

Based on the diversity of nucleic acid sequence, HCV can be classified into eight genotypes (from 1 to 8) with sequences differed by 31%–33% between each other, and at least 90 subtypes (a, b, c, etc.) with the sequences differed by 20%–25% (Simmonds et al., 2005). The distribution of HCV subtypes varies in geography and population in China. Specifically, HCV 1b was widely distributed throughout the whole country, HCV 2a was prevalent in Northwest and Northeast China, while HCV 3a and 6a were more common in South China (Ye et al., 2013). Multiple researchers have demonstrated that HCV 1b was primarily transmitted by blood donors (Zhang et al., 2017, 2020), and that the spread of HCV 6a was principally attributed to IDU (Zhao et al., 2013; Zhang et al., 2017). Since certain HCV subtypes manifest unique features of geographic distribution, viral biology, transmission routes, and even medical management, exploring the distribution of HCV subtypes would help us to acknowledge its high-risk transmission route and provide clues for public-prevention policy. Besides, as a forefront of reform and openness in South China, Guangdong Province is at risk of importing HCV directly from abroad. HCV 6a was introduced into Guangdong Province in the late 1970s from Southeast Asia, and then became the second major local strain (Lu et al., 2005; Fu et al., 2011). It has been documented that the distribution of HCV subtypes may change over time. Thus, it is noteworthy to explore the recent status of HCV distribution and its time trends in blood donors during recent years in Guangdong.

In the present study, we recruited 800 blood donors with HCV infection from Guangzhou Blood Center between 2012 and 2019. The blood samples were centrifuged at 1800 $\times g$ for at least 10 minutes and the plasmas were stored at −80 °C until use. HCV RNA was extracted from 250 μL of plasma for each sample using the MagNA pure LC total nucleic acid isolation kit (large volume) (Roche Diagnostics, Indianapolis, IN, USA), and then reverse-transcribed to DNA fragments using the PrimeScriptII1st strand cDNA synthesis kit (Takara Bio INC, Shiga, Japan). The HCV subtypes were determined using *E1* and *NS5B* genes. The cDNA was amplified using a nested PCR with E1-and NS5B-specific primers (strain H77, E1:nt 717–1315; NS5B:nt 8254–8636) (Rong et al., 2014). Then the amplified products were sent to BGI Tech Co., Ltd. (Guangzhou, China) for purification and sequencing. All sequences were then aligned to HCV reference sequences (http://hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html) using BioEdit software (http://www.mbio.ncsu.edu/bioedit/). Further, we executed phylogenetic analysis with the maximum-likelihood method in MEGA 7.0 [using the general time-reversible model and gamma distributed with invariant site (G + I) rate], and bootstrap sampling was performed in 500 replicates. The samples were classified into different subtypes based on the

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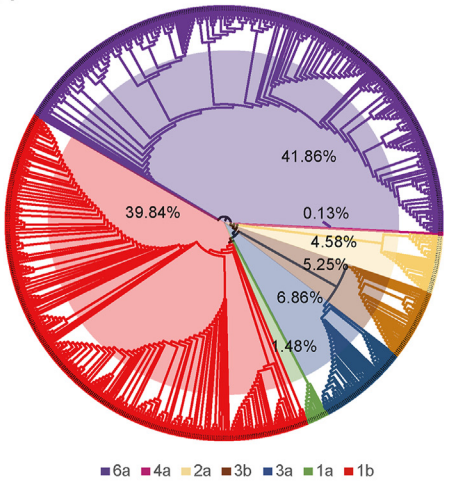
clustering patterns (bootstrap >70%) for the sample sequences with the reference sequences of the subtypes. The visualization of the phylogenetic tree was performed using iTOL (https://itol.embl.de/).

The HCV subtypes were determined successfully in 769 samples, accounting for 96.13% (769/800) of all HCV-positive cases. Of these 769 samples, *E1* region was successfully amplified and sequenced in 743 (96.62%) samples and *NS5B* region was obtained in 659 (85.70%) samples, both regions were obtained in 633 (82.31%) samples. Phylogenetic tree based on *E1* gene is shown in Fig. 1A. Seven subtypes, including 1a, 1b, 2a, 3a, 3b, 4a, and 6a, were identified based on the sequences of both *E1* and *NS5B*. The genotyping of samples determined by *E1* gene was consistent with that with *NS5B* gene region, and no co-infection or recombination was observed for any sample. HCV 6a was the predominant subtype, accounting for 41.35%, followed by 1b (39.92%), 3a (7.15%), 3b (5.72%), 2a (4.16%), 1a (1.56%), and 4a (0.13%) (Table 1). These results are similar to previous studies conducted in Guangzhou (Rong et al., 2014; Yuan et al., 2017), which reported that HCV 1b, 6a, 3a, and 3b were the main subtypes.

A



B

The characteristics of the HCV infected blood donors in Guangzhou from 2004 to 2019

		2004-2007 N (%)	2008-2011 N (%)	2012-2015 N (%)	2016-2019 N (%)	*P* value
Gender	Male	179 (75.32)	338 (80.48)	349 (85.54)	303 (83.93)	**<0.05**[a]
	Female	57 (24.68)	82 (19.52)	59 (14.46)	58 (16.07)	
Age	<40	196 (83.05)	312 (74.29)	280 (68.63)	187 (50.69)	**<0.05**[a]
	≥40	35 (14.83)	108 (25.71)	128 (31.37)	178 (49.79)	
Region origin	Guangdong	145(61.44)	258(61.43)	266(65.20)	241(66.76)	>0.05[a]
	Non-Guangdong	91(38.56)	162(38.57)	142(34.80)	120(33.24)	
Genotype	1a	3 (1.27)	2 (0.47)	7 (1.72)	5 (1.39)	>0.05[b]
	1b	97 (41.10)	184 (43.81)	172 (42.16)	135 (37.40)	>0.05[a]
	2a	18 (7.63)	32 (7.62)	17 (4.17)	15 (4.16)	**<0.05**[a]
	3a	15 (6.36)	35 (8.33)	36 (8.82)	19 (5.26)	>0.05[a]
	3b	17 (7.20)	21 (5.00)	18 (4.41)	26 (7.20)	>0.05[a]
	4a	0	0	1 (0.25)	0	>0.05[b]
	6a	82 (34.75)	144 (34.29)	157 (38.48)	161 (44.60)	**<0.05**[a]
	6e	2(0.85)	1 (0.24)	0	0	>0.05[b]
	6n	2(0.85)	1 (0.24)	0	0	>0.05[b]
Total		236	420	408	361	

[a] *P* values were estimated using Chi-square trend test; [b] *P* values were estimated using fisher's exact test.
P values less than 0.05 were in bold and considered significant.

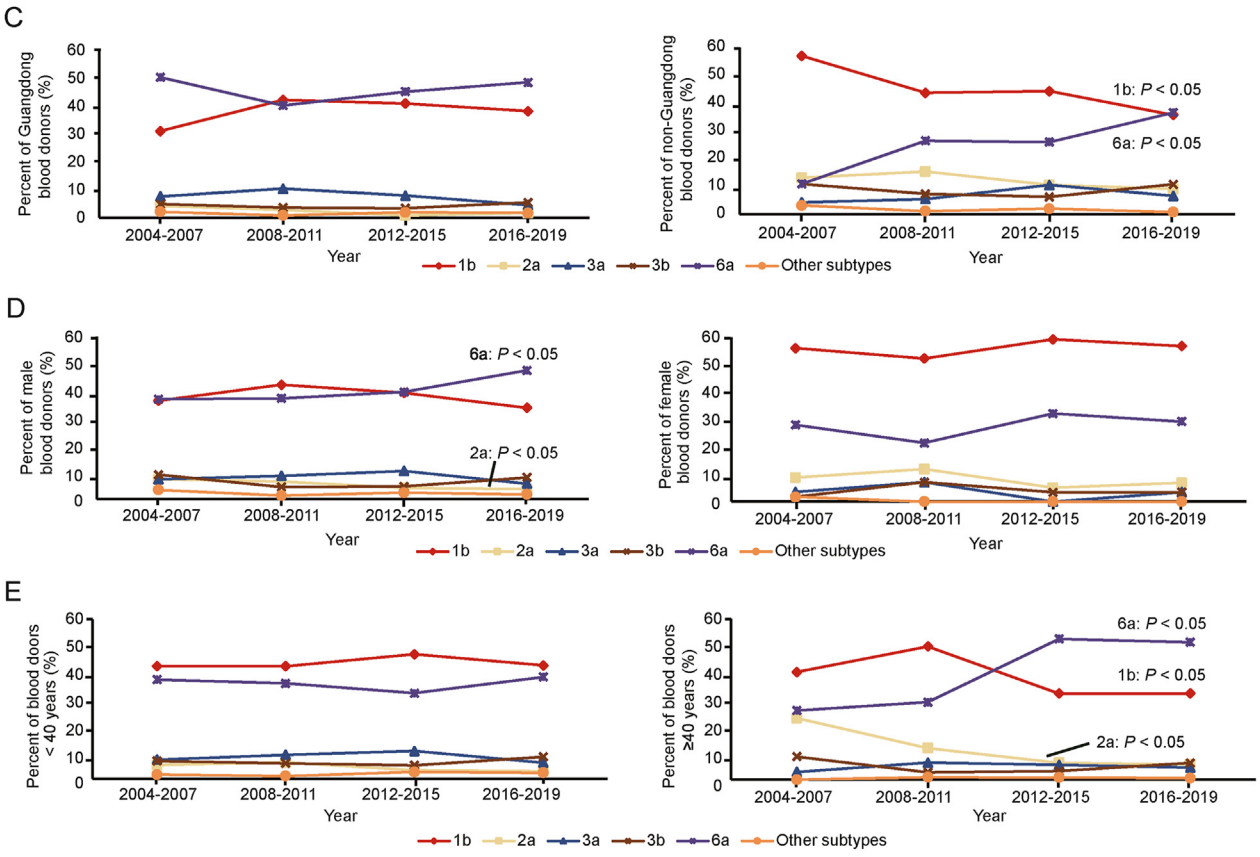


Fig. 1. Distribution and time trends of different HCV subtypes among blood donors between 2004 and 2019 in Guangdong, China. **A** Circular form of the phylogenetic tree based on all 743 *E1* sequences. Different subtypes are shown in different colors, as indicated on the tree. The pie chart inside the tree indicate the percentages occupied by different HCV subtypes. **B** Characteristics of HCV infected blood donors in Guangzhou from 2004 to 2019. **C** Temporal distribution of HCV genotypes in individuals of Guangdong and non-Guangdong origin over the four study periods (2004–2019). Each study period consisted of four years, and Chi-square trend test (linear-by-linear association) was used to calculate the *P*-value for 1b, 6a, and others (including 1a, 4a, 6e, and 6n), respectively. **D** Temporal distribution of HCV genotypes in male and female blood donors. **E** Temporal distribution of HCV genotypes in the younger age group (<40 years) and older age group (≥40 years).

Table 1
The association between HCV subtypes and demographic characteristics in 2012–2019 cohort.

	Total N (%)	1a N (%)	1b N (%)	2a N (%)	3a N (%)	3b N (%)	4a N (%)	6a N (%)	*P* value[a]
Gender									**<0.01**
Male	652 (84.79)	12 (1.84)	239 (36.67)	25 (3.83)	53 (8.13)	40 (6.14)	1 (0.15)	282 (43.25)	
Female	117 (15.21)	0	68 (58.12)	7 (5.98)	2 (1.71)	4 (3.42)	0	36 (30.77)	
Age (years)									**<0.01**
<40	463 (60.21)	10 (2.16)	209 (45.14)	14 (3.02)	40 (8.64)	29 (6.26)	1 (0.22)	160 (34.56)	
≥40	306 (39.79)	2 (0.65)	98 (32.03)	18 (5.88)	15 (4.90)	15 (4.90)	0	158 (51.63)	
Region origin									**<0.01**
Guangdong	507 (65.93)	9 (1.78)	201 (39.64)	6 (1.18)	32 (6.31)	22 (4.34)	0	237 (46.75)	
Non-Guangdong	262 (34.07)	3 (1.15)	106 (40.46)	26 (9.92)	23 (8.79)	22 (8.40)	1 (0.38)	81 (30.40)	
Total		12 (1.56)	307 (39.92)	32 (4.16)	55 (7.15)	44 (5.72)	1 (0.13)	318 (41.35)	

[a] The statistical analysis was performed by fisher's exact test. *P* values less than 0.05 were considered significant and shown in bold.

We observed that the distributions of HCV subtypes were significantly different between gender ($P < 0.01$) and age groups ($P < 0.01$) (Table 1). In different gender groups, HCV 6a was the predominant subtype in male donors, accounting for 43.25% (N = 282), followed by 1b (N = 239, 36.66%), 3a (N = 53, 8.13%) and 3b (N = 40, 6.14%). While in female donors, HCV 1b comprised the majority of HCV-positive samples (N = 68, 58.12%), and 6a contributed 30.77% (N = 36). The proportions of subtypes 2a, 3b, and 3a were 5.98%, 3.42%, and 1.71%, respectively. In age groups, HCV 1b was detected in nearly half of the HCV-positive samples from donors younger than 40 years of age (N = 209, 45.14%); about one third of the samples were infected with subtype 6a (N = 160, 34.56%). In contrast, the distribution of these two subtypes in the older blood donors (≥40 years) were the opposite, with proportions of 32.03% (N = 98) and 51.63% (N = 158) for 1b and 6a, respectively. The percentages for other subtypes in these two age groups were all less than 10%. Our results were consistent with the surveys conducted in Central and North China (Niu et al., 2016; Zhang et al., 2017), which also reported disparities of HCV subtype distribution between age and sex groups.

Notably, we found significant differences in genotypic distribution between Guangdong origin (GDO) and non-Guangdong origin (NGDO) ($P < 0.01$) (Table 1). In GDOs, HCV 6a was detected in nearly half (N = 237, 46.75%) of the 507 positive samples, with the second most prevalent subtype being 1b (N = 201, 39.64%). The percentages for other subtypes were 6.31% (3a), 4.34% (3b), 1.77% (1a), and 1.18% (2a), respectively; subtype 4a was not detected in these samples. For NGDOs, the most prevalent subtype was 1b (N = 106, 40.46%), while the second most prevalent was 6a (N = 81, 30.92%). Subtypes 2a, 3a, and 3b comprised 9.92%, 8.78%, and 8.40% of the samples, respectively. The subtype 1a and 4a were detected in only three (1.15%) and one (0.38%) sample, respectively (Table 1).

We further retrieved data of HCV positive donors from the Guangzhou Blood Center between 2004 and 2011 (Fu et al., 2011; Rong et al., 2014) to explore the potential time trends of HCV subtype distribution (Fig. 1B). We observed that 6a and 1b were the dominant subtypes during the study period, but the two subtypes showed different time-variation trends. From 2004 to 2019, the proportion of HCV 6a in blood donors was gradually increased from 34.75% to 44.60% ($P < 0.05$), while the proportions of 1b decreased from 41.10% to 37.40%. In general, HCV 6a increased obviously and replaced HCV 1b as the most prevalent subtype in the period 2016–2019. HCV 2a comprised 7%–8% of the HCV-positive donors during 2004–2011, but its proportion dropped to 4%–5% in 2012–2019 ($P < 0.05$). We did not observe statistical differences in the distributions of other subtypes during these periods (Fig. 1B). It was reported that in recent years HCV 6a increased in Guangdong, Jiangxi, Chongqing, and other places as well (Zhang et al., 2017, 2020). However, our study was the first-ever observation of HCV 6a replacing 1b as the predominant subtype among blood donors in the mainland of China.

Lu et al. reported that HCV 6a developed into a local epidemic strain in Guangdong in 2005 (Lu et al., 2005), and our previous studies indicated that it may spread to other provinces of China (Fu et al., 2011, 2012). In this study, we plot the temporal distribution of HCV subtypes in GDO and NGDO blood donors to discriminate their time trends. As shown in Fig. 1C, we observed a remarkable upward trend for 6a and a significant downward trend for 1b during 2004–2019 in NGDOs ($P < 0.05$). In contrast, the proportions for these HCV subtypes were relatively stable during this same period in GDOs ($P > 0.05$). These results showed that 6a had maintained a high prevalence in GDOs from 2004 to 2019, while it had a rapid expansion and rise in NGDOs at the same time. Some reasons may explain the increase of subtype 6a in NGDOs: 1) It was found that the patients infected with HCV 6a had higher viral loads, indicating higher potential to be transmitted through blood or other relevant routes (Rong et al., 2012; Zhang et al., 2017). 2) The increase in the number of drug users resulted in transmission from high risk population to the general population. Guangdong plays an important role in China's drug trafficking network, and the registered drug users in Guangdong accounted for about one-sixth of the total drug users in China in 2015. HCV 6a is mainly transmitted through drug user, since it exhibited a very high prevalence of 63.05% in HCV positive drug users (Yan et al., 2019). The increasing of the proportion of 6a in blood donors may indicate its spread from high risk population such as intravenous drug users to the general population which may be partly represented by blood donors. 3) Influx of migrant populations to Guangdong, and the frequent exchanges of culture and business between Guangdong and other district facilitate the spread of 6a (Fu et al., 2012; Zhang et al., 2019).

Considering the distribution of HCV subtypes may be associated with sex and age, the proportions of HCV subtype distributions were plotted by sex and age groups separately. A significantly upward trend for 6a and a downward trend for 2a were observed in males and older donors, respectively (Fig. 1D & E). The significantly downward trend for 1b proportions, however, was only exhibited in the older blood donors (Fig. 1E). Since the increase and decrease of proportion of certain HCV subtype may reflect the variation of its transmission ability, we speculated that some behavior factors associated with sex or age may influence the transmission of HCV 6a and 1b. In addition, the decline of proportions of subtype 1b and 2a may be because of the implementation of HCV screening for donated blood, well-planned strategy in safe blood donations/transfusions and the high sustained virological response rate for the particular subtype (Yan et al., 2012; Zhang et al., 2017). Furthermore, we present data about the age and sex groups for HCV negative donors from Guangzhou Blood Center during 2004–2019. The results showed that sex ratio was stable in this period. Although the proportions of older group in HCV negative donors increased from 8.56% to 17.19% in 2004–2019 (Supplementary Table S1), the discrepancy was far less than that in HCV positive donors (increased from 14.83% to 49.79%, Fig. 1B). So, the changes observed in HCV infected donors may not be induced by the changed demographics of total blood donors.

This study had some limitations. Firstly, most blood donors didn't know how they were infected, so the lack of information about potential

behaviors and risk factors in blood donors hindered further analysis of the association between HCV subtypes and transmission routes. Besides, volunteer blood donors cannot fully represent the general population because people younger than 18 years or older than 55 years were excluded. However, the blood donors in current study may better represent the healthy general population and source of natural HCV transmission, comparing to studies conducted in the IDU, or in patients with chronic liver disease and noticeable symptoms.

In summary, we revealed the time trends of HCV subtypes over a long duration (2004–2019) in blood donors. HCV 6a replaced 1b as the most prevalent subtype in the period of 2016–2019, not only in all donors but also in the subgroups of males, older individuals, and those of Guangdong origin. The significantly increased proportion of HCV 6a in non-Guangdong origin indicated an intensive expansion of 6a from Guangdong to other districts. Our results highlight the importance of continuous surveillance of subtype distributions and the necessity to implement prevention and treatment strategies against HCV 6a.

Footnotes

This study was approved by the Ethical Review Committee of the Guangzhou Blood Center. Additional informed consent was obtained from all patients for which identifying information is included in this article. The author(s) declare that there are no conflicts of interest. This work was supported by grants from National Natural Science Foundation of China (32000666 and 81772208); Guangdong Basic and Applied Research Foundation (2020A1515010118); Medical Science and Technology Research Foundation of Guangdong Province 2020 (B2020090).

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