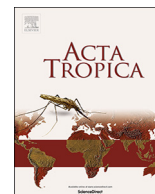




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High prevalence and genetic diversity of hepatitis B viruses in insectivorous bats from China



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ABSTRACT

Bats have been identified as the hosts of hepatitis B virus (HBV) in recent years and bats HBV can infect human hepatocyte. We investigated the prevalence and genetic diversity of HBV in bats in China. In this study, a total of 197 insectivorous bats belonging to 10 bat species were captured from karst caves in Mengyin County, Shandong Province and Xianning City, Hubei Province, China. PCR amplification indicated that in total 6.6% (13/197) bats were positive to HBVs. The HBV positive rate in bats was 7.1% (9/127) and 5.7% (4/70) in Shandong Province and Hubei Province, respectively. Phylogenetic analysis indicated that HBV from the two places were in the same cluster with 90.5%–99.5% homology, but distinct from bat HBVs from other places in China and other countries. We concluded that HBV was prevalent and genetic diversified in bats, supporting the hypothesis that bats may be the origin of primate hepadnaviruses.

1. Introduction

The family Hepadnaviridae include 2 genera: the genus *Avihepadnavirus* and the genus *Orthohepadnavirus*. The genus *Avihepadnavirus* includes hepatitis B viruses (HBV) that infect birds and the genus *Orthohepadnavirus* contains HBVs that infect mammals, including humans, woodchucks, ground squirrels, Arctic squirrels, and woolly monkeys (Bonvicino et al., 2014; Seeger and Mason, 2015; Valaydon and Locarnini, 2017; Winer and Ploss, 2015). Hepadnaviruses are partially double stranded DNA viruses that replicate their DNA by reverse transcription of an RNA intermediate (Bonvicino et al., 2014). The human HBV is a cosmopolitan infectious agent and causes a serious public health problem with over 350 million people chronically infected with HBV worldwide and one to two million deaths per year (Hepatitis, 2019).

With over 1100 species of bats in the world, which represent about 25% of all known species of mammals on Earth. Multiple factors, such as longevity, migratory activity, large and dense roosting communities, and close social interaction, predispose bats as reservoirs for a wide variety of viruses, including Ebola viruses, lyssaviruses, severe acute respiratory syndrome (SARS)-related coronaviruses, filoviruses, henipaviruses, and other paramyxoviruses (Han et al., 2015; Schountz,

2014). In recent years, HBVs have been identified in many places in the world and Tent-making bat HBV can infect human hepatocyte in vitro, suggesting that bat HBVs may infect humans (Bonvicino et al., 2014; Drexler et al., 2013; Littlejohn et al., 2016). Phylogenetic studies of HBVs detected in different bat species put forward a theory that bat hepadnaviruses may be the origin of primate hepadnaviruses (Drexler et al., 2013; Littlejohn et al., 2016; Locarnini et al., 2013). In Asia, bat HBVs have been detected in Vietnam, Myanmar, Malaysia and China (He et al., 2013; He et al., 2015; Nie et al., 2018; Van Nguyen et al., 2018; Wang et al., 2017; Yang et al., 2018). In China, bat HBVs were studied in bats collected mainly in the South China, which are close to Vietnam and Myanmar including Yunnan and Guangxi provinces (He et al., 2013, 2015; Nie et al., 2018; Yang et al., 2018). The prevalence and diversity of HBVs in bats from other places in China is not well studied. The aim of this study is to determine the prevalence and diversity of bat HBV in the East and Central China.

2. Materials and methods

2.1. Bat collection

Bats from Shandong Province were captured and identified in 2015

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Table 1
PCR primers for amplification of Hepadnaviridae.

Primers	Primer sequences	Primary/Nested PCR	Amplicon size	Reference
HBV_266os	GTGGTGGAYTTCTCWCARTT	Primary		
HBV_763oa	CCCCAAWACCANRTCATCCATA			
HBV_386is	GATGTRTCTGCGGCGTYYTATC	Nested	299 bp	Van Nguyen et al. (2018)
HBV_687ia	CTAGTAAAYTGAGCCARGAGAAA			
HBV-F1	CTGCGGCGTTTTATCATATACC	Primary		
HBV-R1	CCAGGAGAAACGGGCTGAGG			
HBV-F2	CTGCTGCTATGTGCCATCTTC	Nested	220 bp	This study
HBV-R2	CCAGGAGAAACGGGCTGAGG			
HBV211-F1	TTWCAGGTGGGKTKTTTC	Primary		
HBV1424-R1	AGSATCCARTTSGCARYRCAGCC			
HBV211-F1	AGSATCCARTTSGCARYRCAGCC	Nested	551 bp	This study
HBV762-R2	ACCACATCATCCATATAASTAA			
HBV225-F1	TTTCTGTGGACAAAATCCCTC	Primary		
HBV1424-R1	AGSATCCARTTSGCARYRCAGCC			
HBV397-F2	CTGCGGCGTTTTATCATCATA	Nested	452 bp	This study
HBV822-R2	AAAAGAGMRCAMACAGAGG			
HBV397-F2	CTGCGGCGTTTTATCATCATA	Nested	838 bp	
HBV1235-R3	ATGATTAACAGGCCCARCC			
HBV331-F3	TCCMAACYTSCARKCACTYACC	Nested	331 bp	
HBV762-R4	ACCACATCATCCATATAASTAA			

in a previous study (Han et al., 2017). Bats from Hubei Province were captured in a karst cave in Xianning City in May 18, 2018 in this study. All bats were identified morphologically and molecularly by PCR amplification and DNA sequencing of the mitochondrial cytochrome b (*cytB*) gene as previously described (Ishii et al., 2014).

2.2. PCR amplification of HBV from bats

DNA was extracted from bat liver tissues with the AllPrep DNA/RNA Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. Bat DNA was used as template for nested PCR amplification of the polymerase gene of viruses in the family Hepadnaviridae (Table 1).

PCR was performed under the following conditions: 1 denaturing cycle of 95 °C 5 min, 16 cycles of 95 °C 40 s, 60 °C (−1 °C for each cycle, end at 45 °C) 50 s, and 72 °C 1 min, followed by 20 cycles of 95 °C 40 s, 45 °C 50 s, 72 °C 1 min, and a final cycle of 72 °C 5 min.

PCR products were analyzed using 1.2% agarose gel electrophoresis and detected with ethidium bromide staining under UV light. PCR products with expected size were excised from gels and extracted with Gel Extraction Kit (Omega, Norcross, Georgia). The purified PCR products were cloned into PMD 19-T vectors (TaKaRa, Shiga, Japan) and the recombinant plasmids were sequenced bidirectionally.

2.3. Phylogenetic analysis

Sequence chromatograms and sequence analysis were examined with Chromas and BLAST program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), respectively. Sequences were aligned and trimmed with MAGA 7 (www.megasoftware.net). Phylogenetic tree was constructed with MEGA 7 using the Maximum Likelihood method with nucleotide sequence and bootstrap values were calculated with 1000 replicates.

3. Results

3.1. Bat HBV detection

Among 197 bat samples collected, 127 bats were from Mengyin County of Shandong Province and 70 bats were from Xianning City, Hubei Province. The bat species were confirmed by PCR amplification of *cytB* gene and the bat *cytB* gene sequences were deposited in GenBank (Accession numbers: MH888177–MH888183). The 197 bats comprised 10 species of 3 genera of the family Vespertilionidae

Table 2

Prevalence of HBV in bat species in Shandong and Hubei provinces of China.

Bat species	No. of samples	PCR positive of HBV	Rates (%)
<i>Eptesicus andersoni</i>	13	0	0
<i>Eptesicus serotinus</i>	14	0	0
<i>Miniopterus schreibersii</i>	9	1	11.1
<i>Myotis adversus</i>	15	0	0
<i>Myotis altarium</i>	2	0	0
<i>Myotis davidii</i>	42	3	7.1
<i>Myotis fimbriatus</i>	32	3	9.4
<i>Myotis laniger</i>	2	0	0
<i>Myotis pequinus</i>	58	5	8.6
<i>Myotis ricketti</i>	10	1	10
Total	197	13	6.6

(Table 2).

PCR amplification indicated that in total 6.6% (13/197) bats were positive to HBV. The positive rate for HBV was 7.1% (9/127) for bats from Shandong Province and 5.7% (4/70) for bats from Hubei Province. Five species of bats including *Miniopterus schreibersii*, *Myotis davidii*, *Myotis fimbriatus*, *Myotis pequinus*, *Myotis ricketti* were positive to HBV and the prevalence of HBV was 11.1%, 7.1%, 9.4%, 8.6%, and 10%, respectively in these bat species (Table 2).

3.2. Bat HBV genomic analysis

Initially we obtained 220 bp DNA fragments in 13 bats by nested PCR with primary primer pairs (HBV-F1 and HBV-R) and nested primer pair (HBV-F2 and HBV-R) as previous described (Van Nguyen et al., 2018). Then we tried to extend the sequences of the initial PCR amplified region on both directions with degenerated primers. Degenerated primers were designed on the unknown sequences outside the initial PCR targeted region using bat HBV sequences from GenBank as template and paired with primers derived from the known sequence of the initial PCR product. By trial and error, only HBV sequences from four positive bats (2 from Shandong and 2 from Hubei) were successfully extended up to 883 bp. The identity between HBV sequences from our study was 93.7%–97.2%. BLAST search indicated that the sequence homology between the bat HBV from this study and bat HBV sequences from GenBank were 75.7%–91.6%. The bat HBV from our study was most closely related to a bat HBV sequence from Myanmar (JX941466.1) with 91.6% identity.

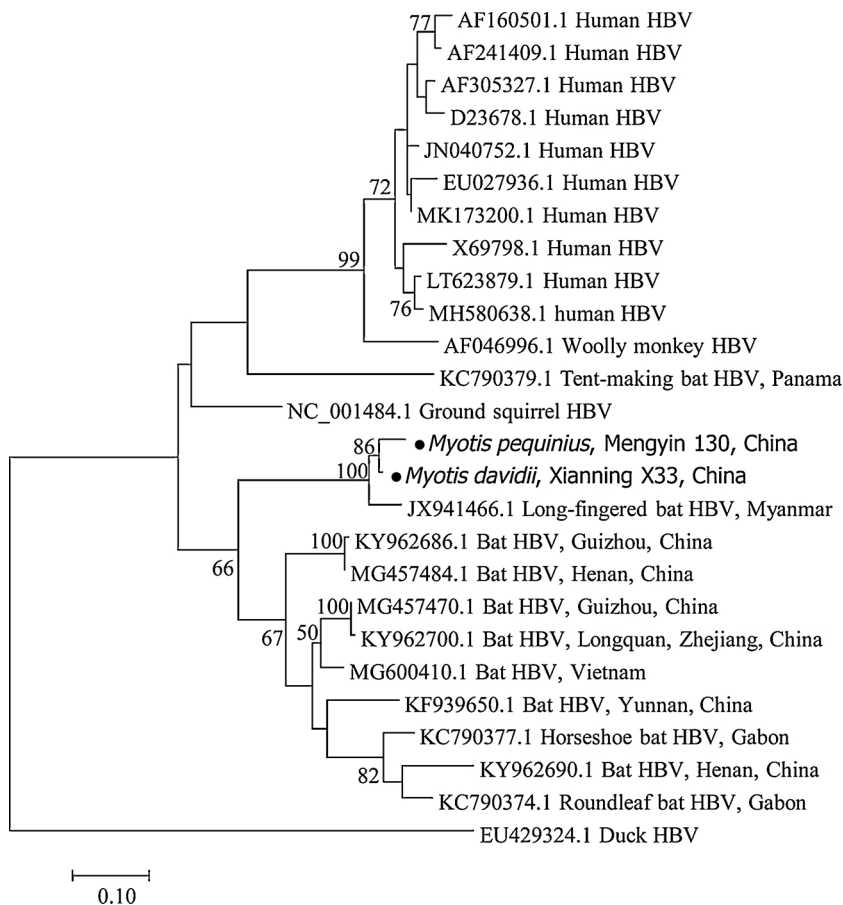


Fig. 1. Phylogenetic tree of bat HBVs. The two fragments performed phylogenetic tree were two long positive samples from Xianning (491 bp) and Mengyin (892 bp). The tree was constructed with MEGA 7.0 by using the Maximum Likelihood method with the Kimura 2-parameter model. Only bootstrap values > 50% were shown. HBVs detected in bats in this study were shown with dots.

3.3. Phylogenetic analysis

Phylogenetic analysis indicated that the sequences from bats in this study and a bat HBV sequence from Myanmar (JX941466.1) formed a monophyletic group, which is in the same clade with bat HBVs from Vietnam and other places in China including Henan, Guizhou, and Zhejiang provinces (Fig. 1). All HBVs forms two major clades including one clade that consists of only bat HBVs, which were from Asia and Africa and another clade that contains both bat HBV from America, Woolly monkey HBVs and human HBVs (Fig. 1). The bat HBV sequences identified in this study were deposited in GenBank with accession numbers: MH099251–MH099263

4. Discussion

We have identified HBVs in 5 bat species collected from two geographic locations in China, which were more than a thousand kilometers apart with Shandong Province in the East and Hubei Province in the Central China. The HBV prevalence was very high in bats from both locations. The positive bat species included *Miniopterus schreibersii*, *Myotis davidii*, *Myotis fimbriatus*, *Myotis pequinius*, and *Myotis ricketti*. This is the first time to report HBV in *Myotis fimbriatus*, *Myotis pequinius*, and *Myotis ricketti* bats. *Miniopterus schreibersii* and *Myotis davidii* had been found positive to HBV previously (He et al., 2013; He et al., 2015; Nie et al., 2018; Van Nguyen et al., 2018; Wang et al., 2017; Yang et al., 2018). Bat species that were negative to HBV in this study might be caused by small sample size and whether they carry HBV need to be further investigated by increasing sample size.

HBV classification is based on partial viral S gene or whole genome sequences. When whole genomes are used, the established nucleotide divergence must be of at least 7.5% for defining a genotype, while a classification exclusively based on the S gene requires at least a 4%

divergence (Bonvicino et al., 2014; Littlejohn et al., 2016; Velkov et al., 2018). Because full-length nucleotide sequence was not successfully determined in this study, a partial nucleotide sequence of the S gene was used for phylogeny. The bat HBV sequences found in this study were less than 4% divergence among the bat species investigated, but they were more than 4% divergence from bat HBVs from other places in China and other countries. Therefore, our results suggest that the bat HBV found in Hubei Province and Shandong Province is a new genotype.

Phylogenetic analysis indicated the bat HBV strains from China and other Asian countries are highly diversified. Bat HBV from different provinces in China distributed in several clusters. Asian bat HBVs is distinct from the human HBVs. One bat HBV from Panama were in the same clade as the human and monkey HBVs, but other bat HBVs from Gabon were in the same clade with Asian bat HBVs. Our study and previous studies indicated that bat HBV in different places were highly divergent and some bat HBV strains are closer to primate HBVs. These results suggesting that HBV originated in bats a long time ago, possibly during the early phases of bat evolution, and the different species of bat HBVs have since co-evolved with their host species. Combined with previous studies (Drexler et al., 2013; He et al., 2013, 2015; Nie et al., 2018; Van Nguyen et al., 2018; Velkov et al., 2018; Wang et al., 2017; Yang et al., 2018), these data indicate that bats should be an important natural reservoir of *orthohepadnaviruses*.

The origin of HBV is not clear and a recently detected HBV from a New World bat, the tent-making bat, is antigenically related to primate HBV and can infect human hepatocytes (Bonvicino et al., 2014; Drexler et al., 2013; Littlejohn et al., 2016; Locarnini et al., 2013; Nie et al., 2018; Seeger and Mason, 2015; Winer and Ploss, 2015). Phylogenetic analysis indicated that the New World bat viruses formed a sister clade to all primate hepadnaviruses and primate and New World bat viruses together were in sister relationship to all Old World bat viruses. Our

study further indicated that bat HBV in China are highly genetically divergent, supporting the hypothesis that bats might be the origin of ancestral hepadnaviruses.

We have tried to amplify the whole genome of HBV from different bats with degenerate primers derived from different conserved regions of published bat HBV sequences. However, none of the primer pairs worked. The most likely possibility is that the genome sequences of bat HBVs are highly divergent making it difficult to be amplified with degenerated primers. Another possibility is that the genome copy number of HBVs in bat liver tissue is extremely low making it hardly to be detected by PCR. To decipher the genome sequences of different HBV strains in bat in Central China, the bat HBV strains need to be isolated by cell culture in future.

5. Conclusion

We identified a novel genotype of HBV in bats from Shandong and Hubei provinces of China and our results indicated that bat HBVs were highly prevalent and diversified in different locations in China. The diversity of bat HBVs support the hypothesis that HBV might originated in bats.

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Consent for publication

Not applicable.

Authors' contributions

SCL designed the study and performed the experiments; SCL, HJH, JWL, XX, XQG, XRQ, MZ and LJW participated in bat sampling; SCL, HJH and XJY wrote the manuscript.

Ethics approval and consent to participate

The collection of bats was approved by the Ethics Committee of Prevention Medicine of Wuhan University (No. 2018010).

Declaration of Competing Interest

The authors declared to have no competing interests.

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