Virology 533 (2019) 93–98

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Natural recombination of equine hepacivirus subtype 1 within the NS5A and NS5B genes

Gang Lu[a,b,c,1], Jiajun Ou[a,b,c,1], Yankuo Sun[a,1], Liyan Wu[a,b,c], Haibin Xu[a,b,c], Guihong Zhang[a,*], Shoujun Li[a,b,c,**]

[a] College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong Province, People's Republic of China
[b] Guangdong Provincial Key Laboratory of Prevention and Control for Severe Clinical Animal Diseases, Guangzhou, Guangdong Province, People's Republic of China
[c] Guangdong Technological Engineering Research Center for Pet, Guangzhou, Guangdong Province, People's Republic of China

ARTICLE INFO

Keywords:
Equine hepacivirus
Subtype
Recombination event
Intra-subtype
China

ABSTRACT

Equine hepacivirus (EqHV) was first reported in 2012 and is the closest known homolog of hepatitis C virus (HCV). A number of studies have reported HCV recombination events. The aim of this study was to determine whether recombination events occur in EqHV strains. Considering that no information on the Chinese EqHV genome sequence is available, we first sequenced the near-complete genomes of three field EqHV strains. Through systemic analysis, we obtained strong evidence supporting a recombination event within the NS5A and NS5B genes in the American EqHV strains, but not in the strains from China or other countries. Finally, using cut-off values for determination of HCV genotypes and subtypes, we classified the EqHV strains from around the world into one unique genotype and three subtypes. The recombination event occurred in subtype 1 EqHV strains. This study provides critical insights into the genetic variability and evolution of EqHV.

1. Introduction

In 1989, the nearly complete genome of a new bloodborne flavi-like virus, which is now known as hepatitis C virus (HCV), was determined in humans (Choo et al., 1989). Recently, HCV-related viruses have been discovered in several animal hosts, including dogs, equines, rodents, bats, monkeys, cattle, sharks, and shrews (Guo et al., 2019; Pybus and Theze, 2016). According to the International Committee on Taxonomy of Viruses (ICTV), these newly identified viruses are proposed to be in the *Hepacivirus* genus of the *Flaviviridae* family.

HCV is a major cause of liver disease in humans and can be transmitted via blood. It has been estimated that HCV infection is present in approximately 185 million people worldwide, conferring a substantial threat to human health (Kohli et al., 2014). The pathology and transmission routes of animal-derived HCV-related viruses have rarely been investigated, except with regard to Norway rat hepacivirus, bovine hepacivirus, and equine hepacivirus (EqHV). All three viruses are hepatotropic and can cause both acute and persistent infections in their hosts (Billerbeck et al., 2017; Pfaender et al., 2015; Ramsay et al., 2015; Trivedi et al., 2018). Norway rat hepacivirus and EqHV can be transmitted via the blood and induce liver damage, but subclinical signs of hepatitis have also been observed in some horses with experimental EqHV infection (Pfaender et al., 2015).

EqHV is the single type in the *Hepacivirus A* species and is the closest known homolog of HCV. EqHV was first identified by Peter D. Burbelo et al. in America in 2012 (Burbelo et al., 2012). Through the use of a luciferase-based immunoprecipitation system, EqHV was determined to be circulating in the American equine population with a high rate of detection of 33.96%. Further investigation demonstrated that EqHV was distributed worldwide, with no apparent geographical restrictions (Lu et al., 2016; Pronost et al., 2017; Badenhorst et al., 2018).

Like the HCV genome, the EqHV genome contains a single long ORF flanked by a 5′ UTR and a 3′ UTR that encodes a polyprotein predicted to be cleaved into 3 structural proteins (Core, E1, and E2) and 7 non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B)(Burbelo et al., 2012). HCV shows very high levels of genetic diversity based on genetic distance and is classified into 8 genotypes and 67 subtypes (Smith et al., 2014). For another HCV-like homolog found in cattle, bovine hepacivirus, one genotype and five subtypes have been determined (da Silva et al., 2018; Lu et al., 2018). In contrast, EqHV is highly conserved and has been classified into two subtypes based on genetic analysis of the partial genome (5' UTR, NS3, and NS5B)(Pronost et al., 2017).

* Corresponding author.
** Corresponding author. College of Veterinary Medicine, South China Agricultural University, Guangzhou, Guangdong Province, People's Republic of China.
E-mail addresses: guihongzh@scau.edu.cn (G. Zhang), shoujunli@scau.edu.cn (S. Li).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.virol.2019.05.006
Received 31 March 2019; Received in revised form 15 May 2019; Accepted 16 May 2019
Available online 17 May 2019
0042-6822/

Genetic recombination can create novel genetic variations that combine genomic fragments from two or more virus strains. As a consequence, recombination plays a significant role in the genetic diversity of RNA viruses. Recombination also plays an essential role in the evolution of RNA viruses. A number of HCV recombination events have been reported worldwide (Kalinina et al., 2002), and recombination is considered to be a feature of HCV genetic diversity (Simmonds, 2004). Recombination can be classified as inter-genotype (Bhattacharya et al., 2011; Demetriou et al., 2009), intra-genotype (Moreno et al., 2009; Shi et al., 2012), or intra-subtype recombination (Moreno et al., 2006; Sentandreu et al., 2008). Until now, no recombination events within EqHV strains have been reported.

The aim of this study was to determine whether recombination events occur in EqHV strains circulating in the equine population worldwide. We first reported EqHV in China in 2016 (Lu et al., 2016). However, only the partial genome (5' UTR, NS3B, and NS5B) of EqHV has been sequenced (< 500 bp). In the present study, to provide more genetic information on Chinese strains for global recombination analysis, we sequenced the near-complete genomes of three field EqHV strains in China. Through further genetic analysis, we found the first evidence of recombination within American EqHV strains; similar evidence was not present in EqHV strains from China or other countries.

2. Results

2.1. Genome sequencing of EqHV

To sequence the genomes of EqHVs from China, 13 serum samples were collected from racehorses at an equestrian club and further tested for EqHV RNA (Table 1). After nested PCR targeting the NS3 gene, sequencing, and subsequent BLAST analysis were performed, 6 serum samples were confirmed to be EqHV RNA-positive, and the rate of detection of EqHV in the studied animals was 46.2% (6/13). A high nucleotide similarity of > 98% was observed among the NS3 genes of the six field EqHV strains. However, it was noted that three of the six serum samples (from equines S3, S8, and S13) tested negative for EqHV RNA after gel electrophoresis following the first round of nested PCR, indicating a low viral RNA content in the three samples.

The genomes of three EqHV strains (Guangdong18, Guangdong21, and Guangdong22) were sequenced in this study from equines S2, S5, and S6, respectively. Using long-range PCR and a gap-filling PCR strategy (Table S1), five fragments covering nearly the complete genomes of Guangdong18, Guangdong21, and Guangdong22 were obtained, sequenced and assembled. The near-complete genomes of Guangdong18, Guangdong21, and Guangdong22 were 9033 nt long, 9023 nt long, and 9023 nt long, respectively, with partial 5' UTRs of 192 nt, 191 nt, and 191 nt and complete polyprotein genes of 8841 nt, 8832 nt, and 8832 nt, respectively. The G + C content of the polyprotein genes of these three strains varied between 50.23% and 50.46%, which was comparable to that of the polyprotein genes of EqHV strains from other countries (49.72%–50.69%). Interestingly, a unique base insert of six nucleotides, ^{1179}ACCTCAGAA^{1181}, was found in Guangdong18 but was not observed in Guangdong21, Guangdong22 or the EqHV strains from other countries.

Table 1
Information on the equines tested for EqHV in this study.

Equine ID	Sex (F/G)[a]	Age (years)	Equine hepacivirus[b]
S1	G	12	-
S2	G	13	+
S3	G	11	+
S4	G	8	-
S5	G	8	+
S6	G	8	+
S7	G	11	-
S8	G	9	+
S9	F	8	-
S10	G	9	-
S11	G	12	-
S12	G	9	-
S13	G	10	+

[a] F, female; G, gelding.
[b] "+", EqHV RNA-positive; "-", EqHV RNA-negative.

Thus far, the complete 5′ UTRs and 3′ UTRs of most of the published EqHV strains have not been sequenced. In addition, the 3′ terminal regions of the complete polyprotein genes of some EqHV strains have not been provided. The nearly complete polyprotein gene sequences (lacking the 22 nt sequence at the 3' terminal) of 20 EqHV strains from other countries were obtained and processed, together with the sequences of Guangdong18, Guangdong21, and Guangdong22 obtained in this study, for subsequent sequence recombination and similarity analysis.

2.2. Recombination event detection

To screen for any possible recombination events in the EqHV strains, standard similarity plots for the polyprotein genes of the EqHV strains were generated using SimPlot software (Fig. 1). The cut-off value was set as 94%, and a value of > 94% was considered to indicate high genetic similarity. When the American EqHV strain WSU-2013 was used as the inquiry strain, the sequence of WSU-2013 had a high genetic similarity with that of the American EqHV strain NPHV-H3-011; however, the partial 3′ terminal NS5A gene and the partial 5' terminal NS5B gene of WSU-2013 had high genetic similarities with the corresponding genes of the American EqHV strain NPHV-F8-068. In contrast, WSU-2013 did not have high genetic similarity with the reference EqHV strain R09-250 in the entire polyprotein gene.

In the analysis of the three EqHV strains (WSU-2013, NPHV-H3-011, and NPHV-F8-068) based on seven methods using RDP software, all *p* values were < 0.001 (Table S2). Similar to the SimPlot analyses, bootstrap analysis also demonstrated that the major parent-like strain of WSU-2013 was NPHV-H3-011 and that the minor parent-like strain of WSU-2013 was NPHV-F8-068 (Fig. 2). The break points were determined to be at positions 6737 and 8281. This evidence indicated that WSU-2013 is a recombinant EqHV strain.

When other EqHV strains (including Guangdong18, Guangdong21, and Guangdong22) were analyzed using the above strategy, no recombination events were detected.

2.3. EqHV sequence similarity and genotype analyses

Since EqHV was first reported, a systemic genotyping study on EqHV has not been published. Considering this, EqHV sequence similarity and genotype analyses were performed in this study to determine the subtype of EqHV in which the recombination event occurred.

The nucleotide and amino acid similarities of the EqHV strains were calculated, and those among Guangdong18, Guangdong21, and Guangdong22 ranged from 95.2% to 99.11% and from 98.2% to 99.3%, respectively. These three field strains had nucleotide and amino acid similarities of 83.8%–95.5% and 94.6%–98.0%, respectively, with other EqHV strains.

It has been reported that different HCV genotypes differ by a *P*-distance of > 0.23 at the amino acid level and that different HCV subtypes differ by a *P*-distance of > 0.15 at the nucleotide level (Smith et al., 2016). Upon using these two cut-off values as references to investigate the published EqHV strains (with the exception of the recombinant EqHV strain WSU-2013) and the field EqHV strains in China (Table S3), it was observed that all the EqHV strains differed from each other with a low *P*-distance ranging from 0.001 to 0.066 at the amino acid level, indicating that EqHV could be classified into a single genotype; this genotype was provisionally designated as genotype 1. In contrast, it was found that EqHV could be classified into three subtypes,

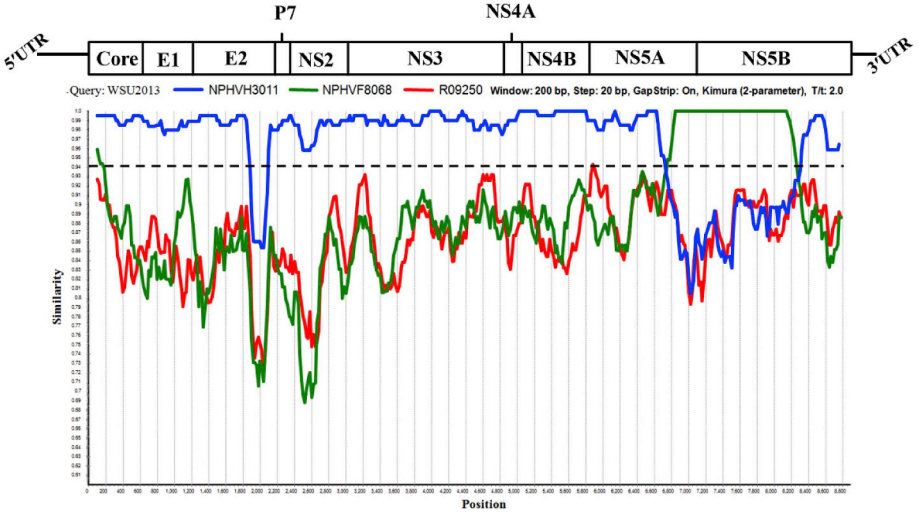

Fig. 1. SimPlot analysis of the EqHV strains based on the polyprotein gene. WSU-2013 and R09-250 were used as the inquiry and reference strains, respectively. The cut-off value was set as 94% and is indicated by a dotted line.

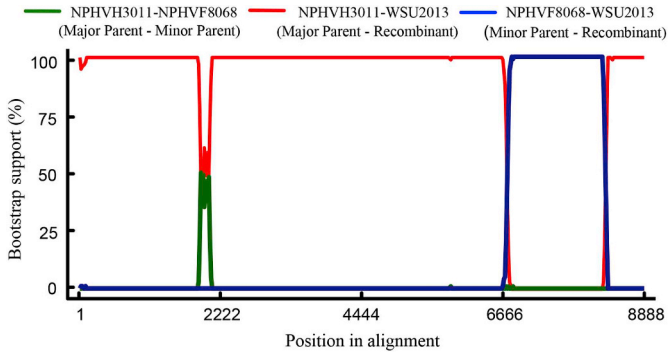

Fig. 2. Bootstrap analysis of the EqHV strains based on the polyprotein gene.

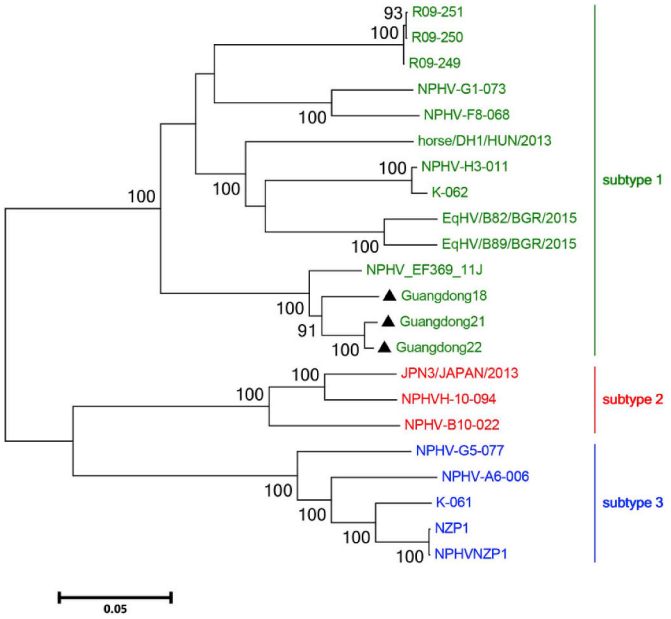

Fig. 3. Phylogenetic analysis of the EqHV strains based on the polyprotein gene. The strains of subtypes 1, 2, and 3 are indicated in green, red, and blue, respectively. The three Chinese strains sequenced in this study are indicated by a triangle.

provisionally designated as subtype 1, subtype 2, and subtype 3, which included 14, 3, and 5 strains, respectively. The different subtypes differed by a *P*-distance of 0.172–0.207 at the nucleotide level. All three field strains in China, Guangdong18, Guangdong21, and Guangdong22, belonged to subtype 1.

Phylogenetic analysis based on the entire polyprotein genes also demonstrated that the EqHV strains could be divided into three groups (Fig. 3): subtype 1, subtype 2, and subtype 3. Guangdong18, Guangdong21, and Guangdong22 clustered with each other and had the closest relationship with the UK EqHV strain NPHV_EF369_11J among all the analyzed strains.

2.4. Recombination events occurred within EqHV subtype 1

As suggested in Figs. 1–3, both the major parent-like (NPHV–H3-011) and minor parent-like (NPHV–F8-068) strains of the recombinant EqHV strain WSU-2013 were grouped in subtype 1, indicating that a recombination event occurred in this subtype.

In addition, a nucleotide sequence *P*-distance analysis was performed on WSU-2013 based on the recombinant portion of its polyprotein gene (6738–8280 nt) and the portion free of recombination (1–6737 nt + 8281–8804 nt) (Table S4). The results clearly demonstrated that both portions of WSU-2013 had higher nucleotide homology with EqHV subtype 1 strains than with subtype 2 or subtype 3 strains.

Two phylogenetic trees were constructed using either the 1–6737 nt + 8281–8804 nt or the 6738–8280 nt region (Fig. 4). When the 1–6737 nt + 8281–8804 nt region was analyzed, WSU-2013 and NPHV-H3-011 had the closest relationship with K-062, and NPHV-F8-068 had the closest relationship with NPHV-G1-073. When the 6738–8280 nt region was analyzed, NPHV-H3-011 still had the closest relationship with K-062, but WSU-2013 had the closest relationship with NPHV-F8-068 and NPHV-G1-073. In addition, when both regions (1–6737 nt + 8281–8804 nt and 6738–8280 nt) were analyzed, some strains always clustered with each other (Guangdong18, Guangdong21, Guangdong22, and NPHV_EF369_11J; R09–249, R09-250, and R09-251; EqHV/B82/BGR/2015 and EqHV/B89/BGR/2015; JPN3/JAPAN/2013, NPHVH-10-094, and NPHV-B10-022; and NPHV-G5-077, NPHV-A6-006, K-061, NZP1, and NPHVNZP1). The phylogenetic analysis also indicated that a recombination event occurred, which was determined to have occurred in the EqHV subtype 1 strain WSU-2013.

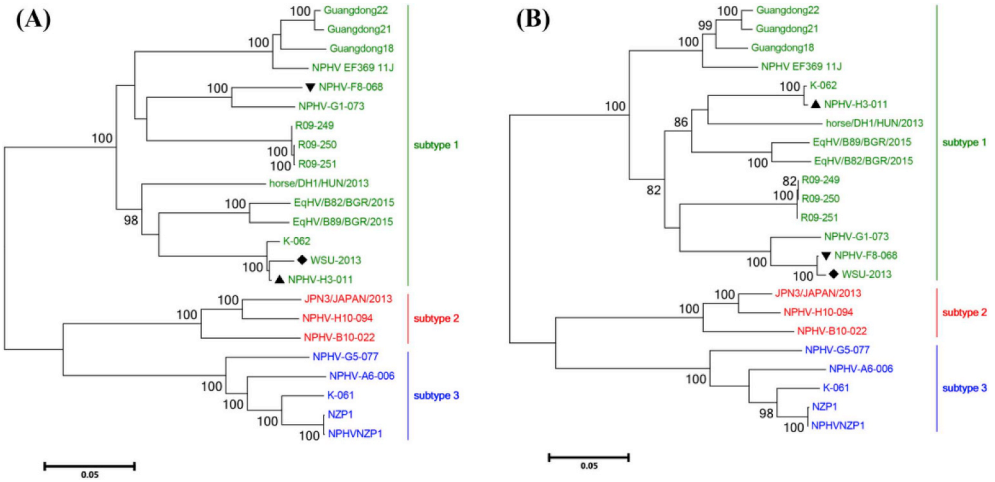

Fig. 4. Phylogenetic analysis of the EqHV strains based on the 1–6737 nt + 8281–8804 nt region (A) and the 6738–8280 nt (B) region. The strains of subtypes 1, 2, and 3 are indicated by green, red, and blue, respectively. NPHV-H3-011, NPHV-F8-068, and WSU-2013 are indicated by an upright triangle, an upside-down triangle, and a diamond, respectively.

3. Discussion

Since its first discovery in 2012, EqHV infection has been found in horses on all continents except Australia (Badenhorst et al., 2018). However, all the tested racehorses in this study were imported from Australia or New Zealand, and it is unclear whether the animals were infected with EqHV before or after coming to China. Whether EqHV is circulating in the equine population on the Australian continent still needs further investigation.

Previous studies have detected EqHV RNA in equines with a rate of detection of 0.9%–34.1% (Badenhorst et al., 2018; Lu et al., 2016). In 2016, we first reported 6 serum samples determined to be EqHV RNA-positive by nested PCR from 177 tested equines in China, with a rate of detection of 3.4% (Lu et al., 2016). In the present study, EqHV was found to be circulating in racehorses at an equestrian club with a rate of detection of 46.2% (6/13), which was higher than the rate reported in our previous study in China and higher than the rates in studies in other countries. However, only a small number of racehorses at the equestrian club were tested for EqHV in this study. Large-scale surveillance is necessary to assess the rate of detection of EqHV in equines in China. Thus far, no systematic studies on EqHV transmission routes have been reported. However, it has been demonstrated that equines can be infected with EqHV via inoculation with plasma containing this virus (Ramsay et al., 2015). According to the equine owners, the equines in this study were not treated with any equine plasma products; in addition, the syringes and needles used were disposable and were used only one time. The cause for the high rate of detection of EqHV and the possible transmission route of EqHV at this equestrian club require further investigation.

Using cut-off values for classification of HCV genotypes and subtypes (Smith et al., 2016), EqHV strains from around the world were classified into one genotype and three subtypes based on their nearly complete polyprotein gene sequences. Compared with a previously reported classification of EqHV strains based on a 1455 bp partial genome (Pronost et al., 2017), the classification based on the nearly complete polyprotein gene used in our study can provide more genetic information for genotype and subtype classification. Previously, only a < 500 bp partial genome of the Chinese EqHV strains had been sequenced. In the present study, the nearly complete genomes of three field EqHV strains were obtained. All of the field EqHV strains in China were of subtype 1. An epidemiological survey of EqHV in wider geographical regions and sequencing of more EqHV genomes may help us fully understand the EqHV subtypes in China.

In this study, the first recombination event was detected in the subtype 1 EqHV strains, a finding that was strongly supported by the results of SimPlot analysis, bootstrap analysis, and phylogenetic analysis. The recombination event occurred in the American strains but not in other strains. The nearly complete genome sequences of only 23 EqHV strains (including the three strains in this study) were used for analysis. More EqHV genome sequences from around the world are needed to estimate the frequency of recombination and to determine the genome regions in which recombination has occurred. Some recombination events in RNA viruses have been reported to influence viral replication and pathogenicity (Stedman, 2018; Xiao et al., 2016). The recombination determined in this study occurred within the NS5A and NS5B genes. It has been demonstrated that HCV NS5B possesses RNA-dependent RNA polymerase (RdRP) activity and that HCV NS5A can modulate RdRP activity by binding NS5B(Shirota et al., 2002). Whether recombination within the NS5A and NS5B genes influences EqHV replication and pathogenicity needs to be determined.

Until now, most of the recombination events in HCV strains have been determined to be inter-genotypic or intra-genotypic (Raghwani et al., 2012). There are only two reports on intra-subtype recombination between HCV strains. In 2006, an intra-subtype recombination event in NS5A was identified in a quasispecies from one of six HCV-infected patients undergoing antiviral therapy (Moreno et al., 2006). In 2008, intra-subtype recombination events within NS5A and/or E1-E2 regions were detected in treated and treatment-naïve HCV- or HCV/HIV-infected patients (Sentandreu et al., 2008). The biological relevance and clinical implications of intra-subtype HCV or EqHV recombination remain unclear.

Previous animal experiments indicated that equines previously infected with EqHV could be protected against rechallenge with homologous or distinct strains (Pfaender et al., 2017). However, a lack of sterilizing immunity was observed in the animals, as viral RNA of the challenge strain was detected. This lack of sterilizing immunity may provide an opportunity for co-infection of EqHV strains. The recombination event detected in EqHV in the present study indicated that the equine host was simultaneously co-infected with multiple virus strains. Mixed infection with different strains has been reported in several studies on HCV patients (Laskus et al., 2001; Pham et al., 2010; Schroter et al., 2003). Mixed infection could generate recombinants efficiently, as determined in chimpanzees experimentally infected with HCV of different genotypes (Gao et al., 2007). Although they have been reported in a number of studies, recombination events for HCV are considered to be rare. However, we successfully determined the existence of one intra-subtype recombination event among only 23 EqHV strains. It is possible that recombination is an occasional event; alternatively, these results may suggest that EqHV is more readily able to produce recombinants than HCV.

In conclusion, in this study, we sequenced the nearly complete genomes of three EqHV strains in China for the first time. We also classified EqHV strains from around the world into one genotype and three subtypes, and we identified the first recombination event in EqHV

subtype 1 strains, which occurred within the NS5A and NS5B genes. The results of this study will enable a better understanding of the genetic variability and evolution of EqHV.

4. Materials and methods

4.1. Serum collection and detection

In 2018, a total of 13 equine serum samples were collected from racehorses in the province of Guangdong in southern China (Table 1). These animals were imported from Australia or New Zealand, with ages ranging from 8 to 13 years. The presence of EqHV RNA was detected using a previously described method. Briefly, total RNA was extracted from 200 μL of equine serum sample, diluted in 20 μL of RNase-free water, and then reverse transcribed into cDNA using a HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) with random primers. Next, nested PCR targeting the EqHV NS3 partial gene was performed as described in our previous study (Lu et al., 2016). The PCR products with the expected bands after 1% gel electrophoresis were sent for sequencing from both ends (BGI, Guangzhou, China).

4.2. Genome sequencing

To sequence the genome of the field EqHV strains in China, the published EqHV genome sequence was obtained from the online GenBank database (Table S5). After alignment with BioEdit 5.0.7.0, two primer pairs targeting a 3506 bp and a 3908 bp EqHV partial genome were designed using Oligo 7.0 (Table S1). After PCR, the DNA fragments were purified using a universal DNA purification kit (Tiangen, Beijing, China) and then cloned into a pCloneEZ-blunt plasmid vector (CloneSmarter, Houston, TX, USA). After sequencing, three other primer pairs were designed to obtain the genome of the field EqHV strains, which were finally edited and assembled using BioEdit 5.0.7.0 and SeqMan 7.1.0. The nucleotide and amino acid similarity of the polyprotein gene between the EqHV strains in this study and those from other countries were calculated in MegAlign 7.1.0.

4.3. Phylogenetic analysis

To determine the evolutionary relationships between the Chinese EqHV strains and the EqHV strains from other countries, the genome sequences were aligned using BioEdit 5.0.7.0., and then the nucleotide sequences were used to conduct a phylogenetic analysis. After genetic distance was estimated with the “Find Best DNA Models” program, a maximum likelihood (ML) phylogenetic tree of polyprotein genes was ultimately established using MEGA 5.05 under the Hasegawa-Kishino-Yano model + Gamma distribution + invariant sites method based on 1000 bootstrap replicates (Fig. 3). In addition, two ML phylogenetic trees for the putative recombination region (Kimura 2-parameter model + Gamma distribution method) and the nonrecombination region (general time-reversible model + Gamma distribution method) were constructed based on 1000 bootstrap replicates (Fig. 4).

4.4. Recombinant analysis

To detect potential recombination events, the genome similarity between EqHV strains circulating during the same period was displayed using SimPlot 3.5.1 software with a 1000-nucleotide sliding window and a 100-nucleotide step. A schematic diagram of the EqHV genome was generated to show the site of each viral protein (Fig. 1). Recombination Detection Program (RDP) 4.27 was used to further identify the putative recombinant EqHV strains and the parent EqHV strains. A total of seven methods (RDP, GENECONV, Chimaera, MaxChi, BootScan, SiSican and 3Seq) were implemented to detect recombination events, with the p value set to 0.001 (Table S2). A recombination score of > 0.6 was considered to indicate a potential recombination event. A boot-scanning analysis between EqHV strains was performed using RDP 4.27 (Fig. 2). The break points of the recombination events were determined.

Conflicts of interest

The authors declare that they have no competing interests.

Funding

This work was supported by funds from Guangdong Natural Science Foundation (2017A030310367), Science and Technology Planning Project of Guangdong Province (2017A030303068), and Guangdong Provincial Key Laboratory of Prevention and Control for Severe Clinical Animal Diseases (2017B030314142).

Author contributions

G.L., G.H.Z., and S.J.L.designed the research and supervised the experiments; G.L., J.J.O., Y.K.S., L.Y.W., and H.B.X. collected the samples and performed the experiments; G.L. analyzed the data; G.L., G.H.Z., and S.J. L. wrote the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.05.006.

References

Badenhorst, M., Tegtmeyer, B., Todt, D., Guthrie, A., Feige, K., Campe, A., Steinmann, E., Cavalleri, J.M.V., 2018. First detection and frequent occurrence of Equine Hepacivirus in horses on the African continent. Vet. Microbiol. 223, 51–58.

Bhattacharya, D., Accola, M.A., Ansari, I.H., Striker, R., Rehrauer, W.M., 2011. Naturally occurring genotype 2b/1a hepatitis C virus in the United States. Virol. J. 8, 458.

Billerbeck, E., Wolfisberg, R., Fahnoe, U., Xiao, J.W., Quirk, C., Luna, J.M., Cullen, J.M., Hartlage, A.S., Chiriboga, L., Ghoshal, K., Lipkin, W.I., Bukh, J., Scheel, T.K.H., Kapoor, A., Rice, C.M., 2017. Mouse models of acute and chronic hepacivirus infection. Science 357, 204–208.

Burbelo, P.D., Dubovi, E.J., Simmonds, P., Medina, J.L., Henriquez, J.A., Mishra, N., Wagner, J., Tokarz, R., Cullen, J.M., Iadarola, M.J., Rice, C.M., Lipkin, W.I., Kapoor, A., 2012. Serology-enabled discovery of genetically diverse hepaciviruses in a new host. J. Virol. 86, 6171–6178.

Choo, Q.L., Kuo, G., Weiner, A.J., Overby, L.R., Bradley, D.W., Houghton, M., 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 244, 359–362.

da Silva, M.S., Junqueira, D.M., Baumbach, L.F., Cibulski, S.P., Mosena, A.C.S., Weber, M.N., Silveira, S., de Moraes, G.M., Maia, R.D., Coimbra, V.C.S., Canal, C.W., 2018. Comprehensive evolutionary and phylogenetic analysis of Hepacivirus N (HNV). J. Gen. Virol. 99, 890–896.

Demetriou, V.L., van de Vijver, D.A., Cyprus, H.C.V.N., Kostrikis, L.G., 2009. Molecular epidemiology of hepatitis C infection in Cyprus: evidence of polyphyletic infection. J. Med. Virol. 81, 238–248.

Gao, F., Nainan, O.V., Khudyakov, Y., Li, J., Hong, Y., Gonzales, A.C., Spelbring, J., Margolis, H.S., 2007. Recombinant hepatitis C virus in experimentally infected chimpanzees. J. Gen. Virol. 88, 143–147.

Guo, H., Cai, C., Wang, B., Zhuo, F., Jiang, R., Wang, N., Li, B., Zhang, W., Zhu, Y., Fan, Y., Chen, W., Chen, W., Yang, X., Shi, Z., 2019. Novel hepacivirus in Asian house shrew. China. Sci. China Life Sci. 62, 701–704.

Kalinina, O., Norder, H., Mukomolov, S., Magnius, L.O., 2002. A natural intergenotypic recombinant of hepatitis C virus identified in St. Petersburg. J. Virol. 76, 4034–4043.

Kohli, A., Shaffer, A., Sherman, A., Kottilil, S., 2014. Treatment of hepatitis C: a systematic review. JAMA 312, 631–640.

Laskus, T., Wang, L.F., Radkowski, M., Vargas, H., Nowicki, M., Wilkinson, J., Rakela, J., 2001. Exposure of hepatitis C virus (HCV) RNA-positive recipients to HCV RNA-positive blood donors results in rapid predominance of a single donor strain and exclusion and/or suppression of the recipient strain. J. Virol. 75, 2059–2066.

Lu, G., Jia, K., Ping, X., Huang, J., Luo, A., Wu, P., Li, S., 2018. Novel bovine hepacivirus in dairy cattle, China. Emerg. Microb. Infect. 7, 54.

Lu, G., Sun, L., Xu, T., He, D., Wang, Z., Ou, S., Jia, K., Yuan, L., Li, S., 2016. First description of hepacivirus and pegivirus infection in domestic horses in China: a study in Guangdong province, heilongjiang province and Hong Kong district. PLoS One 11, e0155662.

Moreno, M.P., Casane, D., Lopez, L., Cristina, J., 2006. Evidence of recombination in quasispecies populations of a Hepatitis C Virus patient undergoing anti-viral therapy. Virol. J. 3, 87.

Moreno, P., Alvarez, M., Lopez, L., Moratorio, G., Casane, D., Castells, M., Castro, S., Cristina, J., Colina, R., 2009. Evidence of recombination in Hepatitis C Virus populations infecting a hemophiliac patient. Virol. J. 6, 203.
Pfaender, S., Cavalleri, J.M., Walter, S., Doerrbecker, J., Campana, B., Brown, R.J., Burbelo, P.D., Postel, A., Hahn, K., Anggakusuma, Riebesehl, N., Baumgartner, W., Becher, P., Heim, M.H., Pietschmann, T., Feige, K., Steinmann, E., 2015. Clinical course of infection and viral tissue tropism of hepatitis C virus-like nonprimate hepaciviruses in horses. Hepatology 61, 447–459.
Pfaender, S., Walter, S., Grabski, E., Todt, D., Bruening, J., Romero-Brey, I., Gather, T., Brown, R.J., Hahn, K., Puff, C., Pfankuche, V.M., Hansmann, F., Postel, A., Becher, P., Thiel, V., Kalinke, U., Wagner, B., Bartenschlager, R., Baumgartner, W., Feige, K., Pietschmann, T., Cavalleri, J.M., Steinmann, E., 2017. Immune protection against reinfection with nonprimate hepacivirus. Proc. Natl. Acad. Sci. U. S. A 114, E2430–E2439.
Pham, S.T., Bull, R.A., Bennett, J.M., Rawlinson, W.D., Dore, G.J., Lloyd, A.R., White, P.A., 2010. Frequent multiple hepatitis C virus infections among injection drug users in a prison setting. Hepatology 52, 1564–1572.
Pronost, S., Hue, E., Fortier, C., Foursin, M., Fortier, G., Desbrosse, F., Rey, F.A., Pitel, P.H., Richard, E., Saunier, B., 2017. Prevalence of equine hepacivirus infections in France and evidence for two viral subtypes circulating worldwide. *Transbound. Emerg. Dis.* 64, 1884–1897.
Pybus, O.G., Theze, J., 2016. Hepacivirus cross-species transmission and the origins of the hepatitis C virus. Curr. Opin. Virol. 16, 1–7.
Raghwani, J., Thomas, X.V., Koekkoek, S.M., Schinkel, J., Molenkamp, R., van de Laar, T.J., Takebe, Y., Tanaka, Y., Mizokami, M., Rambaut, A., Pybus, O.G., 2012. Origin and evolution of the unique hepatitis C virus circulating recombinant form 2k/1b. J. Virol. 86, 2212–2220.
Ramsay, J.D., Evanoff, R., Wilkinson Jr., T.E., Divers, T.J., Knowles, D.P., Mealey, R.H., 2015. Experimental transmission of equine hepacivirus in horses as a model for hepatitis C virus. Hepatology 61, 1533–1546.
Schroter, M., Feucht, H.H., Zollner, B., Schafer, P., Laufs, R., 2003. Multiple infections with different HCV genotypes: prevalence and clinical impact. J. Clin. Virol. 27, 200–204.
Sentandreu, V., Jimenez-Hernandez, N., Torres-Puente, M., Bracho, M.A., Valero, A., Gosalbes, M.J., Ortega, E., Moya, A., Gonzalez-Candelas, F., 2008. Evidence of recombination in intrapatient populations of hepatitis C virus. PLoS One 3, e3239.
Shi, W., Freitas, I.T., Zhu, C., Zheng, W., Hall, W.W., Higgins, D.G., 2012. Recombination in hepatitis C virus: identification of four novel naturally occurring inter-subtype recombinants. PLoS One 7, e41997.
Shirota, Y., Luo, H., Qin, W., Kaneko, S., Yamashita, T., Kobayashi, K., Murakami, S., 2002. Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. J. Biol. Chem. 277, 11149–11155.
Simmonds, P., 2004. Genetic diversity and evolution of hepatitis C virus–15 years on. J. Gen. Virol. 85, 3173–3188.
Smith, D.B., Becher, P., Bukh, J., Gould, E.A., Meyers, G., Monath, T., Muerhoff, A.S., Pletnev, A., Rico-Hesse, R., Stapleton, J.T., Simmonds, P., 2016. Proposed update to the taxonomy of the genera Hepacivirus and Pegivirus within the Flaviviridae family. J. Gen. Virol. 97, 2894–2907.
Smith, D.B., Bukh, J., Kuiken, C., Muerhoff, A.S., Rice, C.M., Stapleton, J.T., Simmonds, P., 2014. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. Hepatology 59, 318–327.
Stedman, K.M., 2018. Viral recombination: ecology, evolution, and pathogenesis. Viruses 10, 358.
Trivedi, S., Murthy, S., Sharma, H., Hartlage, A.S., Kumar, A., Gadi, S.V., Simmonds, P., Chauhan, L.V., Scheel, T.K.H., Billerbeck, E., Burbelo, P.D., Rice, C.M., Lipkin, W.I., Vandegrift, K., Cullen, J.M., Kapoor, A., 2018. Viral persistence, liver disease, and host response in a hepatitis C-like virus rat model. Hepatology 68, 435–448.
Xiao, Y., Rouzine, I.M., Bianco, S., Acevedo, A., Goldstein, E.F., Farkov, M., Brodsky, L., Andino, R., 2016. RNA recombination enhances adaptability and is required for virus spread and virulence. Cell Host Microbe 19, 493–503.