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Short communication

First identification and phylogenetic analysis of equine hepacivirus in Korea

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ABSTRACT

Non-primate hepacivirus (NPHV) corresponds a group of isolates recently characterized in horses and dogs that present similar genomic organization and are closely related to hepatitis C virus. Since canine hapacivirus, NPHV identified in dogs, was first discovered in dogs in the United States, equine hepacivirus (EqHV, NPHV identified in horses) has been identified in horses in several countries. However, no epidemiological studies have investigated EqHV in horses in Korea. In this study, a total of 74 ($n = 74$) serum samples collected from horses in four regions of Korea were tested for EqHV RNA using nested RT-PCR. Overall, 14 samples were identified as positive (18.9%) and further analyzed according to gender, age, breed, and region. There were high positive rates in males, young horses, and Thoroughbreds; however, these rates differed regionally. Sequencing of the partial NS3 region of 12 samples and the polyprotein encoding regions of two samples positive for EqHV RNA revealed that the Korean EqHV isolates shared approximately 85.3–99.6% and 97.7–100% homology at the nucleotide and deduced amino acid level, respectively. Phylogenetic analysis revealed that the partial NS3 genes clustered with sequences previously reported as NPHV. Notably, sequences of EqHV detected in horses in the same region showed sequence divergence. The sequences of the polyprotein encoding region of two representative EqHVs shared 83.9% and 95.7% homology with each other at the nucleotide and deduced amino acid level, respectively. Comparison of the sequences of polyprotein encoding regions of Korean EqHV isolates and hepaciviruses from different hosts revealed that the NS3 and NS5B regions were most conserved among hepaciviruses. The results of the present study demonstrate that there is a high positive rate of EqHV in Korea and provide significant information regarding the geographical distribution and genetic variability of Korean EqHV isolates that will help improve global epidemiology of EqHV.

1. Introduction

Hepatitis C virus (HCV) is a positive sense single-stranded RNA virus that belongs to the genus *Hepacivirus*, a member of the family *Flaviridae* (Stapleton et al. 2011). This organism, which can only infect humans and chimpanzees, causes cirrhosis, liver failure and hepatocellular carcinoma in humans (Shimotohno 1995). It is estimated that >170 million people are infected with HCV worldwide (Simmonds 2013). Since non-primate hepacivirus (NPHV) was first isolated from the respiratory samples of a domestic dog in the United States in 2011 (Kapoor et al. 2011), many homologs of HCV have been detected in various animals, including horses (Burbelo et al. 2012; Tanaka et al. 2014; Gemaque et al., 2014), dogs (El-Attar et al. 2015), cows (Corman et al. 2015), bats (Quan et al. 2013), rodents (Kapoor et al. 2013; Drexler et al. 2013) and non-human primates (Lauck et al. 2013).

Phylogenetic analyses using hepaciviruses identified from diverse hosts have revealed that equine hepacivirus (EqHV, NPHV identified in horses) and canine hepacivirus (NPHV identified in dogs) are most closely related to HCV (Pfaender et al. 2014; Thézé et al. 2015; Pybus and Thézé 2016). Genetic analysis of the NPHV genome has revealed that its structure is similar to that of HCV, and that its one open reading frame (ORF) encodes approximately 2940–2950 amino acid polyproteins, which yields the structural proteins core, E1, and E2, as well as the nonstructural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Pfaender et al. 2014; Thézé et al. 2015). Accordingly, there is now significant interest in EqHV because the origin and natural reservoirs of HCV are not clear and only the chimpanzee experimental model is available for research and development of drugs and vaccines for HCV (Pybus and Thézé 2016; Pfaender et al. 2014; Bukh, 2012). Although some studies did not support the evidence of an association between active EqHV infection and hepatopathy (Gemaque et al., 2014; Lyons et al. 2014), one study showed that the occurrence of acute and chronic hepatic disease in horses was closely related to the presence of EqHV RNA, mainly within the liver (Pfaender et al. 2015). Experimental intrahepatic inoculation of RNA synthesized from functional cDNA clones constructed using EqHV and experimental transmission of EqHV in horses demonstrated that the hepatotropic characteristics,

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Table 1
Detailed information describing geographical location, number, breed, purpose, and age of equine samples tested for equine hepacivirus (EqHV) in Korea.

Region (province)	Number (sample ID)	Breed				Gender		Purpose			EqHV positive	Age (years)		
		Pony	Thorough-bred	Warm-blood	Appaloosa	Female	Male	Riding	Racing	Breeding		1–2	3–10	>10
Chuncheon (Gangwon)	12 (K-001–012)	0/5[a]	2/6	0/1	0/0	1/8	1/4	2/12	0/0	0/0	2/12 (16.7%)	0/0	2/8	0/4
Gwacheon (Gyeonggi)	12 (K013–024)	0/11	0/0	0/0	0/1	0/10	0/2	0/12	0/0	0/0	0/12 (0%)	0/0	0/6	0/6
Icheon (Gyeonggi)	37 (K025–061)	0/0	8/37	0/0	0/0	2/19	6/18	0/0	8/37	0/0	8/37 (21.6%)	4/8	4/29	0/0
JeJu island (Jeju)	13 (K062–074)	0/0	4/13	0/0	0/0	4/13	0/0	0/0	0/3	4/10	4/13 (30.8%)	0/2	4/8	0/3
Total	74	0/16	14/56	0/1	0/1	7/50	7/24	2/24	8/40	4/10	14/74 (18.9%)	4/10	10/51	0/13

[a] Number of positive samples/total number tested.

infection kinetics and liver pathology of EqHV were similar to those of HCV infection in humans, suggesting the possibility of using the horse as a surrogate model for HCV research (Scheel et al. 2015; Ramsay et al. 2015).

Since EqHV was first detected in the United States in 2012 (Burbelo et al. 2012), it has been found in several other countries including the United Kingdom (Lyons et al. 2012; Reuter et al. 2014; Tanaka et al. 2014; Gemaque et al., 2014; Pfaender et al. 2015; Lu et al. 2016). Therefore, it is important to investigate the prevalence of EqHV infection in Korea and genetically characterize EqHV to assess the global epidemiology of this emerging virus. Accordingly, the objectives of the present study were (1) to evaluate the molecular prevalence of EqHV infection in horses from four regions of Korea and (2) to conduct sequencing and phylogenetic analysis of isolates.

2. Materials and methods

A total of 74 serum samples were collected from horses across three provinces (Gangwon, Gyeonggi, Jeju) in Korea between August and December 2015. Animals belong to the different properties and transmission among them was not confirmed. Twelve samples were collected from Chuncheon, Gangwon province, Northern Korea, while 49 were from Gwacheon ($n = 12$) and Icheon ($n = 37$), Gyeonggi province, Central Korea, and 13 were from Jeju Island, Jeju province, Southern Korea. Detailed information regarding serum samples is shown in Table 1. All serum samples were collected from clinically normal horses and stored at −70 °C until use.

Viral RNA in serum samples was extracted using a NucleoSpin® RNA Plus kit (Macherey-Nagel, Germany). Nested RT-PCR to detect EqHV RNA was performed to amplify a partial sequence of the NS3 region. RT-PCR was performed using a QIAGEN® OneStep RT-PCR kit (Qiagen, UK) with previously published primers (Lyons et al. 2012). Nested PCR was performed using a Maxime PCR Premix kit (IntRON, Korea) with the following primers that had been modified based on recently published sequence data for EqHV: 5′-ACG GGG CAG ART CYA AAG GYG TW-3′ (forward inner sense, 0.4 μM) and 5′-TCC AAR CCC CGA TAG TAR GTG AC-3′ (reverse inner sense, 0.4 μM). The expected product sizes of RT-PCR and nested PCR were 320 bp and 265 bp, respectively. The near complete genomic sequences, including the polyprotein encoding region (reference sequences: KP325401, position 385–9213, 2942 aa; and JQ434008, position 388–9222, 2944 aa) of the two Korean EqHV K-061 and K-062 isolates were determined by the primer walking method. The viral genome of two Korean EqHV isolates was segmentally amplified by RT-PCR using specific primers (Supplementary Table 1). Detailed information regarding the RT-PCR and PCR conditions used are available on request.

PCR products of the expected size were purified, cloned, and sequenced. The nucleotide and deduced amino acid sequence data were compiled and analyzed using the Lasergene version 12 software (DNASTAR). The sequences were compared to each other and those of published hepaciviruses identified in other species including human (HCV) using the MegAlign Clustal W or V multiple sequence alignment algorithm. Phylogenetic trees based on sequences of the partial NS3 region and the polyprotein encoding region of the Korean EqHV isolates were generated by the neighbor-joining (NJ) method as implemented in the Lasergene software with 1000 bootstrap replicates and confirmed by the NJ method in the MEGA 6 software with 1000 bootstrap replicates.

The sequences of the polyprotein encoding regions of NPHVs were inspected for recombination using the Genetic Algorithm Recombination Detection (GARD) (Kosakovsky Pond et al. 2006) program and employed to calculate the ratios of nonsynonymous to synonymous evolutionary changes (dN/dS) in both structural and nonstructural proteins by the PARtitioning approach for Robust Inference of Selection (PARRIS) in the DataMonkey web server (Scheffler et al. 2006; Delport et al. 2010). To calculate the amino acid pairwise distances among hepaciviruses, amino acid sequences of polyproteins were aligned using the MUSCLE program as implemented in the SSE package (Simmonds 2012). Sequence divergence scans and summary values for different genome regions were analyzed by the Sequence Distance program in the SSE package (Simmonds 2012).

The nucleotide sequence data of the 12 partial NS3 regions and the near complete genomic sequence data of the two Korean EqHV isolates reported in this study were deposited in GenBank under accession numbers KX056104 to KX056117.

3. Results and discussion

To investigate the prevalence of EqHV in horses in Korea, a total of 74 horse serum samples from four geographic regions of three provinces in Korea were collected and screened for the presence of EqHV RNA. EqHV RNAs were detected in 14 out of 74 horse serum samples (18.9%) by NS3 gene-based nested RT-PCR assay (Table 1). Based on the detection of EqHV RNA, the positive rate of EqHV in Korea was relatively higher than in most other countries, including the United States (7.8%), while it was lower than in one of two investigations in Japan (13.68% and 35.5%) (Burbelo et al. 2012; Lyons et al. 2012; Drexler et al. 2013; Tanaka et al. 2014; Gemaque et al., 2014; Lyons et al. 2014; Matsuu et al. 2015; Figueiredo et al., 2015; Pfaender et al. 2015). The proportions of positive rates for EqHV RNA were investigated based on horse gender, age, breed and region (Table 1). There were higher positive rates in male horses (29.2%) than female horses (14.0%). There were also higher positive rates for NPHV RNA in horses 1–2 years old (40%) than in those 3–10 years old (19.6%). However, horses 11 or more years of age were all negative for EqHV RNA ($n = 13$). These results were similar to those obtained for Japan (Matsuu et al. 2015). However, the reason for the high positive rate of EqHV infection and exact route of infection in young horses is not clear. Thoroughbred horses had higher positive rates of EqHV RNA (14/56, 25.0%) than other breeds. Moreover, other breeds including pony ($n = 16$), Warmblood ($n = 1$), and Appaloosa ($n = 1$) were all negative for EqHV RNA. The high prevalence of EqHV infections in Korea seemed to be due to the high proportion of Thoroughbreds