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Short communication

Serological evidence of hepatitis E virus infection in zoo animals and identification of a rodent-borne strain in a Syrian brown bear

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ABSTRACT

Hepatitis E virus (HEV) is the causative agent of hepatitis E, an emerging infectious disease of humans. HEV infections have also been described in various animal species. Whereas domestic pigs and wild boars are well-known animal reservoirs for HEV, the knowledge on natural HEV infection in zoo animals is scarce so far. Here, we analysed 244 sera from 66 mammal species derived from three zoos in Germany using a commercial double antigen sandwich ELISA. HEV-specific antibodies were detected in 16 animal species, with the highest detection rates in suids (33.3%) and carnivores (27.0%). However, RNA of the human pathogenic HEV genotypes 1–4 was not detected in the serum samples from suids or carnivores. Using a broad spectrum RT-PCR, a ratHEV-related sequence was identified in a sample of a female Syrian brown bear (*Ursus arctos syriacus*). Subsequent serum samples within a period of five years confirmed a HEV seroconversion in this animal. No symptoms of hepatitis were recorded. In a follow-up investigation at the same location, closely related ratHEV sequences were identified in free-living Norway rats (*Rattus norvegicus*), whereas feeder rats (*Rattus norvegicus forma domestica*) were negative for HEV-specific antibodies and RNA. Therefore, a spillover infection of ratHEV from free-living Norway rats is most likely. The results indicate that a wide range of zoo animals can be naturally infected with HEV or HEV-related viruses. Their distinct role as possible reservoir animals for HEV and sources of HEV infection for humans and other animals remains to be investigated.

1. Introduction

Hepatitis E virus (HEV) infections represent the most common cause of acute hepatitis in humans worldwide (Rein et al., 2012). In several European countries, the number of recorded human hepatitis E cases steadily increased during the past ten years (Adlhoch et al., 2016). The disease is mostly characterized by mild to moderate acute hepatitis; subclinical infections appear to be frequent. However, pregnant women in endemic regions with HEV-1 and persons with underlying liver disease portray a risk group for severe acute hepatitis including lethal outcomes. In addition, chronic infections, which can develop to liver cirrhosis, have been identified in immunosuppressed transplant patients (Kamar et al., 2012).

HEV belongs to the family *Hepeviridae* and possesses an RNA genome containing three open reading frames (ORFs). ORF1 encodes a non-structural polyprotein, ORF2 the capsid protein and ORF3 a small phosphoprotein. The human-pathogenic genotypes (GT) HEV-1 to HEV-4 are classified together with additional GT from wild boars and camels into the species *Orthohepevirus A* (Smith et al., 2014). The species *Orthohepevirus B* contains avian HEV strains, whereas mainly strains from rats and ferrets are found in *Orthohepevirus C* and batHEV strains in *Orthohepevirus D* (Smith et al., 2014).

The sources of infection with human-pathogenic HEV are GT-dependent (Johne et al., 2014). HEV-1 and HEV-2 are restricted to humans and mainly transmitted by fecally contaminated water. In contrast, HEV-3 and HEV-4 are zoonotic viruses, with pigs and wild boars representing the main animal reservoirs. These animals do not show any clinical symptoms due to HEV infection. Direct contact between humans and animals and ingestion of virus-containing food are the main transmission routes of these genotypes.

RNA of HEV-3 or HEV-4 as well as HEV-specific antibodies have also been detected in a considerable variety of other wildlife, farmed and pet

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animal species (Spahr et al., 2017b; Doceul et al., 2016; Pavio et al., 2010). To gain knowledge about the distribution of HEV infections in different animal species, zoo-like locations with a large diversity of mammal species represent interesting sites. However, only a few studies analysing zoo animals have been published yet (Spahr et al., 2017a; Li et al., 2015; Zhang et al., 2008).

To analyse HEV infections in zoo animals, a serological survey on HEV-specific antibodies was performed with animals from three zoos in Germany. Animals of taxa showing comparably high seroprevalences were additionally analysed by RT-PCR for the presence of HEV RNA. Follow-up investigations in free-living and feeder Norway rats should identify the source of HEV infections in zoo animals. The results of the investigation should contribute to further clarify the role of zoo animals as susceptible hosts of HEV.

2. Materials and methods

2.1. Sampling

In total, 244 individual sera from 66 mammal species were collected in three zoos (A–C) in Germany (Suppl. Table 1), though most sera were obtained from zoo A. The sera were obtained between 2006 and 2016 during animal immobilizations for different purposes, e.g. routine health checks, and stored at −20 °C. Additionally, liver samples from 12 animals were taken during routine dissections of died zoo animals between 2015 and 2016 and stored at −20 °C. No animal was sampled for the sole profit of this study. All animals in the zoos were routinely checked by their keepers for physical health, which was documented daily. 73 free-living Norway rats were collected between 2009 and 2016 from two zoos (A and D) and stored at −20 °C (Suppl. Table 2). These rats were collected routinely for use in the network "Rodent-borne pathogens" and standard protocols of the network were used for preparation of liver samples and extraction of transudates from the thoracic cavity (Ulrich et al., 2008). Additionally, 20 randomly selected feeder rats from zoo A were killed for internal stock control, using CO_2 inhalation in accordance with animal welfare regulations. All liver and transudate samples were stored at −20 °C until further investigation.

2.2. Serological analysis

The serum samples were analysed for HEV-specific antibodies using the Axiom® HEV-Ab EIA (Axiom Diagnostik, Bürstadt, Germany) and the results were evaluated according to the recommendations of the manufacturer. This assay is based on HEV-1 capsid protein antigens and uses the test principle of a double antigen sandwich ELISA. By this, it is species-independent and can detect all immunoglobulin classes.

2.3. RNA isolation

RNA was extracted from serum samples using the NucleoMag®VET kit (Macherey-Nagel, Düren, Germany) in a King Fisher 96 Flex Workstation (Thermo Fisher Scientific GmbH, Schwerte, Germany), following the manufacturer's instructions. Liver samples were homogenized using a TissueLyser (Qiagen GmbH, Hilden, Germany) and QIAzol®Lysis Reagent (Qiagen GmbH), and RNA was extracted by a modified QIAzol protocol method as described before (Schmidt et al., 2016). The RNA pellets were resolved in 100 µl DEPC-treated water and stored at −80 °C until further use.

2.4. Real-time RT-PCR (RT-qPCR)

RNA samples were tested for the presence of HEV-1 to HEV-4 using a previously described RT-qPCR protocol (Jothikumar et al., 2006). The QuantiTect® Probe RT-PCR Kit (Qiagen GmbH) was used in 20 µl reactions with conditions as previously described (Schielke et al., 2011). The limit of detection of this RT-qPCR as determined by dilution series of in vitro transcribed RNA was seven genome equivalents per PCR reaction (Schielke et al., 2011).

2.5. Nested broad-spectrum RT-PCR (NBS-RT-PCR)

The NBS-RT-PCR was performed according to Johne et al. (2010). This assay amplifies a conserved region within the RNA-dependent RNA polymerase (RdRp)-encoding region of OFR1 and has been demonstrated to be capable of detection of HEV strains from the species *Orthohepevirus A*, *B* and *C* (Johne et al., 2010). The RT-PCR was performed using the One-Step RT-PCR kit (Qiagen GmbH) and the nested PCR using the TaKaRa ExTaq kit (TaKaRa Bio, Japan) as described before (Johne et al., 2010). The nested PCR products were separated by agarose gel electrophoresis and bands according to a length of 331–334 nucleotides (nt) were excised and purified using the QIAquick Gel Extraction Kit® (Qiagen GmbH).

2.6. SW-RT-PCR

The SW-RT-PCR targets a similar genomic region of the HEV genome like the NBS-RT-PCR, but is designed as one-step RT-PCR (Wolf et al., 2013). It has been shown to efficiently detect ratHEV, but should also be able to detect strains of the species *Orthohepevirus A* based on the primer sequences. This RT-PCR was performed using the SuperScriptIII with PlatinumTaq Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) in a 25 µl reaction (Wolf et al., 2013). RT-PCR products with a length of 282 bp were purified using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel).

2.7. Sequence analyses

Purified amplification products were either sequenced by a commercial company (Eurofins GmbH, Hamburg, Germany) or sequenced in-house using the BigDye® Terminator version 1.1 Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) in an HITACHI 3130 Genetic Analyser (Applied Biosystems, Darmstadt, Germany). For sequence comparisons and phylogenetic analyses, a sequence fragment of the RdRp-encoding region with a length of 279 nt (nt 4108–4387; numbering according to ratHEV reference strain R63, acc. no. GU345042), derived from the products of the NBS-RT-PCR and/or the SW-RT-PCR, was used. The newly generated HEV sequences were deposited at GenBank (sequence from the Syrian brown bear: acc. no. MF480313, sequences from rats: acc. nos. MF480314–480320). Sequence alignments were performed using BioEdit 7.2.0 (Hall, 1999) and MEGA 7 (Kumar et al., 2016). The GTR + G model was used as it was identified as the best suited substitution model by MEGA 7. The phylogenetic analyses were performed by Bayesian algorithms via the CIPRES online portal (Ronquist et al., 2012) with 8 million generations and by Maximum likelihood algorithm performed via MEGA7 (Kumar et al., 2016) with 1.000 bootstrap replicates and a consensus tree was generated. Reference sequences for phylogenetic reconstructions were taken from Smith et al. (2014).

3. Results

3.1. HEV-specific antibodies are mainly detected in zoo animals of the family Suidae and the order Carnivora

A total of 244 serum samples from mammalian zoo animals, belonging to 66 species, were tested for the presence of HEV-specific antibodies (Table 1 and Suppl. Table 1). In total 28/244 (11.5%) turned out to be anti-HEV antibody-positive. Animals from 16 species in three orders (Artiodactyla, Carnivora, Perissodactyla) were tested positive. The highest seroprevalence was found in animals from the family Suidae with 9/27 (33.3%) positive samples originating from three different species. A high seroprevalence was also recorded for animals of