Hepatitis E virus in bottlenose dolphins

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ABSTRACT: Hepatitis E virus (HEV) infects several animal species that act as zoonotic reservoirs for viral transmission. Solid and liquid residues from infected animals could lead to HEV contamination of food and surface waters. Evidence of human HEV infection through ingestion of seafood (shellfish, mussels) has been reported. Dolphins generally feed on fish and squid but are able to adapt to an environment and consume whatever prey is available. Clinical signs of infected dolphins include lethargy, inappetence, behavioral aberrations and increased serum alanine aminotransferase (ALT). The dolphins examined in this study were maintained at the National Aquarium, Havana, Cuba. A total of 31 dolphins were evaluated for HEV markers. Sera were collected and screened for total immunoglobin (Ig) anti-HEV. Sera and liver homogenate were tested for HEV RNA by nested RT-PCR using primers targeting the open reading frame 1. Phylogenetic analysis was performed using partial nucleotide sequences at the amplified RNA-dependent RNA polymerase gene. Total anti-HEV Ig was detected in 32.2% (10 of 31), and 16.1% (5 of 31) of these dolphins were positive by both serology and HEV RNA testing. Nucleotide sequence analyses revealed that HEV strains identified in dolphins were genotype 3. This virus may represent an environmental contamination of food or wastewater as a source of HEV exposure and infection. Our findings provide evidence that HEV is associated with liver disorders in cetaceans and that it is advisable to screen for exposure of this virus in captive dolphins, particularly animals with elevated serum ALT or compromised liver function test results of undetermined cause.

KEY WORDS: Viral diseases · Hepatitis E virus · Bottlenose dolphins · Zoonotic · Genotype

INTRODUCTION

Hepatitis E virus (HEV) is widely distributed around the world. It is estimated that 2 billion people live in endemic areas, where the fecal–oral route is the primary means of HEV transmission (Pérez-Gracia et al. 2014). Seroprevalence of HEV is high in countries with suboptimal sanitation; however, HEV infection also occurs in people in developed and industrialized areas, with no travel history to endemic regions. Therefore, some researchers explain these epidemiological characteristics by the existence of a zoonotic reservoir for HEV (Meng et al. 1997).

HEV is a positive-strand RNA virus that is a member of the family Hepeviridae. Recent taxonomic consensus grouped all mammalian and avian HEV isolates to the genus Orthohepevirus. Species Orthohepevirus A is subdivided into 7 genotypes designated as HEV1 to HEV7. HEV1 and HEV2 are restricted to
humans, and HEV3 and HEV4 are found in humans as well as animal species (pig, deer, mongoose and rabbit) and are considered zoonotic. Genotypes HEV5 to HEV7 have been isolated from wild boars (HEV5, HEV6) and dromedaries (HEV7) (Smith et al. 2014).

In Cuba, 2 HEV genotypes have been detected. HEV1 has been reported in people, and HEV3 has been reported in swine and people working on pig farms. This finding constituted the first evidence for zoonotic transmission of HEV3 in the Caribbean region (Montalvo Villalba et al. 2008, 2013).

Several environmental sources have been associated with HEV infection in animals including consumption of untreated water or contaminated animal byproducts. In cetaceans, hepatitis and abnormal levels of hepatic enzymes have been detected and are primarily attributed to metabolic or autoimmune disorders as well as parasites or viral infections. Bossart et al. (1990) showed that hepatitis B-like virus may be responsible for liver inflammation in cetaceans, and Dotzauer et al. (1994) demonstrated the susceptibility of dolphin cell lines to hepatitis A virus infection.

At the National Aquarium in Cuba, bottle-nose dolphins Tursiops truncatus are maintained in exhibit tanks (A–D) with open water circulation systems (not recycled), and fresh seawater is constantly provided. Seawater is supplied to each tank by pumping and then cleaned by a filtering sand system. Each swimming pool has an independent filtered system. Fresh fish, shellfish and squid are the main food for dolphins. Usually, their average weight ranged between 200 and 250 kg, and animals were fed 8 to 15 kg daily with fresh as well as frozen fish (Trachurus murphyi). These fishes are acquired in Cuba and also are imported from Chile; dolphin diets also include vitamin supplements. In December 2007 and June 2014, veterinarians observed that some dolphins were showing abnormal behavior including depression, inappetence, decreased physical contact and synchronous movements, vomiting and lethargy. These animals also had increased serum ALT levels (≥60 U l⁻¹; mean of 187 U l⁻¹, range from 75 to 980 U l⁻¹), and acute hepatitis was suspected. All sera from clinically affected animals had been previously tested for hepatitis B virus marker (UMELISA HBsAg PLUS, Center for Immunossay), and no positive cases were detected.

In the 2007 episode, in 12 of 17 dolphins, paired sera were recovered 8 mo before the onset of acute illness. In this group of animals, 1 dolphin died, and a liver sample was collected for attempted virus isolation. Additionally, in this period (2007), serum samples from humans (n = 32) who worked in close contact with dolphins (keepers, trainers and veterinarians) were collected. At the time of the 2007 study, no humans showed evidence of clinical disease, including acute hepatitis (Institutional Ethics Committee Code CIE-IPK-LVH1/2007).

**MATERIALS AND METHODS**

**Sample collection**

The National Aquarium is located at coordinates 23.1156° N, 82.4366° W in Havana, Cuba. The facility includes 5 dolphin pools with a volume of 120 000 gallons (~454 250 l) of water per pool. Dolphin distribution is usually 4 to 6 animals per pool, and these individuals change periodically to different locations in Cuba. The dolphins in captivity in 2007 were not the same animals as in 2014. In 2014, no dolphins were transferred between facilities during the outbreak. New arrival dolphins are quarantined in separate pools to avoid infections that could transmit to resident dolphins at the aquarium. Water for filling the tanks is drawn from seawater and subsequently filtered and treated with chloride.

In December 2007 and June 2014, during regular observations of display dolphins Tursiops truncatus at the National Aquarium in Havana, Cuban veterinarians identified 31 (17 animals in 2007 and 14 animals in 2014) dolphins with abnormal behaviors. Dolphins showed low interactive communication and a variety of signs including depression, inappetence, decreased physical contact and synchronous movements, vomiting and lethargy. These animals also had increased serum ALT levels (≥60 U l⁻¹; mean of 187 U l⁻¹, range from 75 to 980 U l⁻¹), and acute hepatitis was suspected. All sera from clinically affected animals had been previously tested for hepatitis B virus marker (UMELISA HBsAg PLUS, Center for Immunossay), and no positive cases were detected.

**ELISA for detection of serum antibodies to HEV**

An ELISA purchased from Genelabs Diagnostics was used to detect total immunoglobulin (Ig) anti-HEV in sera collected from dolphins and humans working in close contact with dolphins. This immunoassay is based on the double-antigen HEV
Nested RT-PCR, nucleotide sequence accession numbers and phylogenetic analysis

All serum samples obtained from dolphins with clinical signs and elevated ALT enzymes (n = 31) and workers (n = 32) were tested for HEV RNA by nested RT-PCR (nRT-PCR). In addition, a liver suspension obtained from a dead dolphin in 2007 was also tested. RNA was extracted from 200 µl of serum and liver homogenate using QIAamp Viral RNA Mini Kit (QIAGEN). An nRT-PCR previously described (Zhai et al. 2006) was performed using specific primers that amplified a fragment of the open reading frame 1 (ORF1), RNA-dependent RNA polymerase (RdRp) region. PCR products were purified, and DNA fragments at a concentration of >50 ng µl⁻¹ (GenomeLab DTCS_Quick Start protocol) were selected for sequencing. Partial nucleotide sequences of RdRp of HEV were submitted to GenBank, and Accession Nos. KM065571 and KP096349 were assigned. These 2 sequences were compared with 25 HEV nucleotide sequences (RdRp) deposited in the GenBank database (www.ncbi.nlm.nih.gov/nuccore). Phylogenetic analysis was performed using MEGA 6 software (Tamura et al. 2013). For each nRT-PCR assay run, negative (no template control) and positive controls (full-length HEV pSK-HEV) were included (Emerson et al. 2001). The sensitivity of the nRT-PCR was 10² copies ml⁻¹. This assay was evaluated as an in-house technique using an in vitro transcript of a full-length clone of genotype 1 pSK-HEV diluted from 10⁹ to 10⁰ copies ml⁻¹ and used for quantification.

Ethical considerations

All procedures were carried out in accordance with international ethical guidelines for human and animal research. For collecting animal samples, care was taken to avoid or minimize discomfort, distress and pain. All blood sampling was done by experienced veterinarians or medical personnel. Blood sampling of humans required prior informed consent before starting this investigation. The present protocol was approved by the Institutional Ethics Committee under Code CIE-IPK-LVH1/2007.

RESULTS AND DISCUSSION

Detection of HEV serological and genetic markers (HEV ELISA and HEV RNA)

Antibodies were detected in 10 of 31 (32.2%) dolphin sera tested with the ELISA kit. The prevalence of antibodies against HEV among dolphins with clinical signs and elevated levels of ALT by year was 41.1% (7 of 17) in 2007 and 21.4% (3 of 14) in 2014. Both male and female bottlenose dolphins were infected with HEV (Table 1). The OD values of positive samples ranged from 0.744 to 3.528, while those of negative samples ranged from 0.023 to 0.133.

HEV RNA was detected in 5 of 31 (16.1%) dolphin serum samples. Five of 10 dolphin sera (50%) with ELISA antibodies were positive in the nRT-PCR. The RdRp (ORF1) amplicon was also detected in liver sus-
pension from the dead animal, Romina (Rm). Dolphins positive for both anti-HEV antibodies and HEV RNA had elevated serum ALT values that ranged between 96 and 237 U l\(^{-1}\); the highest value was detected in dolphin Rm. Evidences of anti-HEV seroconversion were identified in 5 animals (Table 1). Based on clinical chemistry and signs, dolphins with HEV antibodies to HEV and/or HEV RNA were detected between 24 and 96 h after the index case of acute hepatitis. HEV-positive dolphins shared the same swimming pools; however, no additional cases were detected 5 d after the onset of clinical signs with increased ALT in 2007 or 2014 (Table 1).

Antibodies against HEV have been detected across a wide variety of terrestrial animal species, with the highest prevalence reported in swine samples (Dong et al. 2011). In aquatic environments, HEV infections have been reported in shellfish (Crossan et al. 2012, Gao et al. 2016), while evidence of HEV exposure in marine mammals has not previously been documented.

Dolphins in captivity are generally fed fish and squid but are able to consume other fish or invertebrate species. In this present study, ingestion of ecologically contaminated food could have been the source of HEV. However, only a few (10%) of the exposed dolphins that exhibited clinical signs like hepatitis were anti-HEV positive.

At the Cuban National Aquarium, water quality is monitored at the pools and other indoor areas by weekly physico-chemical water analyses and coliform counts. The results from these counts have always been lower than acceptable limits because the water is usually chlorinated. However, food and water screening does not include surveillance for viral agents.

Antibodies to HEV with OD values that ranged from 0.926 to 3.622 were detected by ELISA in 4 of 32 (12.5%) humans exposed to the dolphins with no history of acute viral hepatitis or illness, whereas values in the seronegative samples ranged from 0.019 to 0.098. HEV RNA (nRT-PCR) was not detected in serum from any of the workers. These findings are consistent with previous observations of subclinical hepatitis E infections in Cuba (Montalvo Villalba et al. 2010). The prevalence of HEV antibodies in aquarium staff exposed to the dolphins is similar to levels reported in the general population in Havana (10%). However, the prevalence of HEV antibodies found in animal handlers was lower than levels reported in Cuban swine farmers (12.5 vs. 35.8%). HEV1 and HEV3 genotypes are currently present in Cuba, and sporadic cases and outbreaks have been reported (Rodriguez Lay et al. 2008, Montalvo Villalba et al. 2008, 2010, 2013).

As no evidence of seroconversion or HEV RNA was detected in any of the trainers or veterinarians, it is unlikely that the captive dolphins in the present study were reservoirs and potential sources of HEV infection. However, given the intimate contact between dolphins and humans in aquarium conditions, the potential risk of zoonotic transmission of HEV from these animals to animal handlers should not be ignored.

**Table 1. General characteristics and clinical and epidemiological relevant data among bottlenose dolphins (Tursiops truncatus) with positivity for anti-hepatitis E virus (HEV) and HEV RNA markers.** ALT: alanine aminotransferase (reference concentration = 41–60 U l\(^{-1}\)); OS: day of onset of clinical signs; CS: day of the collection of the sample. Optical density (OD):cutoff (CO) ratios ≥1 were considered to be reactive; first number (1\(^{\text{st}}\)) indicates OD:CO ratio anti-HEV in sera 8 mo before clinical signs appeared; second number (2\(^{\text{nd}}\)) indicates OD:CO ratio anti-HEV in sera after clinical signs appeared and ALT increased. Water tank ID indicates animals with positivity for anti-HEV and HEV RNA markers that shared the water tank at that time. nRT-PCR: nested RT-PCR; NA: not available

<table>
<thead>
<tr>
<th>Dolphin ID (abbreviation)</th>
<th>Sex</th>
<th>ALT (U l(^{-1}))</th>
<th>OS/CS (d/d.mo.yr)</th>
<th>OD:CO ratio 1(^{\text{st}})</th>
<th>HEV RNA nRT-PCR in sera</th>
<th>Water tank ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cristal (Cri)</td>
<td>Female</td>
<td>102</td>
<td>04/12 Nov 2007</td>
<td>NA</td>
<td>5.1</td>
<td>Negative B</td>
</tr>
<tr>
<td>Javi (Jav)</td>
<td>Male</td>
<td>83</td>
<td>04/12 Nov 2007</td>
<td>0.7</td>
<td>8.0</td>
<td>Negative B</td>
</tr>
<tr>
<td>Lía (Li)</td>
<td>Female</td>
<td>96</td>
<td>05/12 Nov 2007</td>
<td>0.2</td>
<td>3.0</td>
<td>Positive C</td>
</tr>
<tr>
<td>Marina (Mar)</td>
<td>Female</td>
<td>144</td>
<td>10/13 Jun 2014</td>
<td>NA</td>
<td>3.1</td>
<td>Positive A</td>
</tr>
<tr>
<td>Nina (Nin)</td>
<td>Female</td>
<td>323</td>
<td>12/14 Jun 2014</td>
<td>NA</td>
<td>3.3</td>
<td>Positive C</td>
</tr>
<tr>
<td>Rambo (Rbo)</td>
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<td>123</td>
<td>04/12 Nov 2007</td>
<td>0.2</td>
<td>3.2</td>
<td>Negative C</td>
</tr>
<tr>
<td>Raquel (Rq)</td>
<td>Female</td>
<td>153</td>
<td>10/14 Jun 2014</td>
<td>NA</td>
<td>14.1</td>
<td>Negative A</td>
</tr>
<tr>
<td>Romel (Rmel)</td>
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<td>237</td>
<td>02/12 Nov 2007</td>
<td>0.1</td>
<td>13.3</td>
<td>Positive A</td>
</tr>
<tr>
<td>Romina (Rm)(^{a})</td>
<td>Female</td>
<td>980</td>
<td>02/04 Nov 2007</td>
<td>0.2</td>
<td>14.0</td>
<td>Positive A</td>
</tr>
<tr>
<td>Rufo (Rf)</td>
<td>Male</td>
<td>557</td>
<td>01/04 Dec 2007</td>
<td>NA</td>
<td>14.5</td>
<td>Negative D</td>
</tr>
</tbody>
</table>

\(^{a}\)Dolphin that died
Nucleotide sequencing and phylogenetic analysis

Two of the 5 PCR amplicons obtained from sera were sufficiently concentrated to sequence. PCR amplicons obtained from homogenized liver were also sequenced. The nucleotide sequences obtained from the serum and liver samples from the dead dolphin (Rm) were 100% identical, and the sequence corresponds to the liver sample deposited in the GenBank database (Accession No. KM065571). This partial sequence was translated into a 99 amino acid protein. The second sequence obtained from the serum of dolphin Marina was also deposited in GenBank (KP096349), and its deduced amino acid sequence was 99% identical to the Romina RdRp sequence.

Basic local alignment search tool (BLAST) analysis of these nucleotide and amino acid sequences showed that they were 100% identical to homologous sequences of HEV genotype 3 (Fig. 1) from humans in Germany (ADY18330) and the Netherlands (A6E 94813). Similar viruses have previously been identified in Cuba (Montalvo Villalba et al. 2013).

Virus propagation in cell culture

Two of 5 serum samples positive for HEV RNA by nRT-PCR had viral loads \( \geq 10^4 \) copies ml\(^{-1} \) by qRT-PCR. Sera collected from the dead dolphin (Rm) contained \( 4.51 \times 10^4 \) copies ml\(^{-1} \), whereas dolphin Rmel had \( 4.41 \times 10^4 \) copies ml\(^{-1} \). HEV RNA was quantified in supernatants from cell cultures infected with the serum of dolphin Rm, and viral titers increased to \( \sim 2.23 \times 10^7 \) on Days 4 and 7 and then decreased to \( 1.30 \times 10^4 \) on Day 24. In contrast, viral titers decreased to \( 2.43 \times 10^2 \) in culture supernatant of cells infected with the Rmel sample at 4 dpi, and no HEV virus was detected at 7 and 24 dpi. Cytopathic effect was not detected in any flask (Rm and Rmel samples) even after several passages.

The highest viral titer was found at 4 and 7 dpi for the Rm sample, similar to previous results using sera, stool suspensions and homogenate liver samples (Takahashi et al. 2012). These authors found that viral load reached \( 10^5 \) copies ml\(^{-1} \) at 4 to 6 dpi. Non-specific chronic reactive hepatitis and increased activities of serum aminotransferases are not uncommon in dolphins (Venn-Watson et al. 2008). Some causes included Sarcocystis spp. (Resendes et al. 2002), biliary trematodes (Dailey & Stroud 1978), hepatitis B-like virus infection (Bossart et al. 1990), acquired immunodeficiency (Bossart 1984) and metabolic disorders (Venn-Watson et al. 2008).

Although the amount of genomic sequence data generated from 2 HEV-positive dolphin samples is limited, based on the clinical presentation and serol-
ogy results, these dolphins may have been infected through hitherto unidentified source(s) of contaminants containing human or swine feces harboring HEV genotype 3 strains. The identical or almost identical amino acid sequences of the dolphin HEV RdRp to the many reported worldwide in humans and swine substantiate this hypothesis.

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LITERATURE CITED


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