Virus Research 251 (2018) 17–21

Contents lists available at ScienceDirect

Virus Research

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

Serological and molecular investigation for hepatitis E virus (HEV) in captive non-human primates, Italy

Irene Melegari[a], Federica Di Profio[a], Fulvio Marsilio[a], Vittorio Sarchese[a], Andrea Palombieri[a], Klaus Gunther Friedrich[b], Federico Coccia[b], Barbara Di Martino[a,*]

[a] Faculty of Veterinary Medicine, Università degli Studi di Teramo, Italy
[b] Fondazione Bioparco, Viale del Giardino Zoologico, 00197, Rome, Italy

ARTICLE INFO

Keywords:
Hepatitis E virus
Zoonoses
IgG antibodies
Non-human primate

ABSTRACT

Hepatitis E virus (HEV) is the leading cause of human enterically-transmitted viral hepatitis occurring around the world both as outbreaks and as sporadic cases. Non-human primates (NHPs) have been experimentally infected with HEV, but few studies have been reported about natural infection in wild-living and zoo monkeys. In order to provide a more complete picture on the epidemiology of HEV in NHPs living in controlled environment, we investigated the presence of HEV by screening serologically and molecularly a historical collection of 86 sera from seven different species of primates housed at the Zoological Garden (Bioparco) of Rome, Italy. By using an enzyme-linked immunosorbent assay based on the recombinant capsid protein of a Gt3 HEV strain, IgG antibodies were detected in three macaques (4.8%; 3/62) and in a white-crowned mangabey (16.6%; 1/6), with an overall prevalence of 4.6% (4/86). This positivity was confirmed when assessed the sera by western blotting. Rescreening the sera for IgM and viral RNA, all the samples resulted negative. Also, HEV RNA was not found when 17 stool samples were analyzed by RT-PCR. Although these results suggest that none of the monkeys housed at the Bioparco of Rome in the 17-year time frame spanning 2001 to 2017 developed acute or at least sub-acute HEV disease, the detection of IgG antibodies demonstrated that animals living in this setting were exposed to HEV or to antigenically related viruses.

1. Introduction

Hepatitis E virus (HEV) infection is highly prevalent in humans. Every year, there are an estimated 20 million HEV infections worldwide, with 3.3 million symptomatic cases of hepatitis E (Rein et al., 2012). HEV is a small non-enveloped RNA virus classified in the genus *Orthohepevirus*, family *Hepeviridae* (Smith et al., 2014). Based on the full-length genome analysis (Smith et al., 2014), HEV strains are classified into 7 genotypes (Gt1-Gt7). Four major Gt (1-4), all representing a single serotype (Nan et al., 2017), have been implicated in human disease. More recently, chronic infection due to a camelid HEV strain (Gt7) was reported in a liver transplant recipient (Lee et al., 2016). Gt1 and Gt2 are endemic in developing countries and restricted to humans, where they are predominantly transmitted through the faecal–oral route, either indirectly through contaminated drinking water or food. Gt3 and Gt4 infect humans and animals and are responsible for sporadic cases of autochthonous human hepatitis E in industrialized countries (Kamar et al., 2012). The accumulating literature indicates that human infections by Gt3 and Gt4 HEVs are due to consumption of raw or undercooked pork or game meat, thus raising public health concerns about the zoonotic transmission of HEV (Meng et al., 1998). Pigs, wild boars and deer are recognized as the main reservoirs for Gt3 and Gt4 infections, although molecular evidence indicates that several additional animal species may act as HEV hosts, including rabbits, yaks, sheep, goats and cattle (Cossaboom et al., 2011; Xu et al., 2014; Wu et al., 2015; Huang et al., 2016a; Di Martino et al., 2016). Studies to investigate animal models for human HEV suggest that also non-human primates (NHPs) are susceptible to HEV infection, developing virologic, biochemical, immunologic, and histopathologic changes similar to those observed in acute infection in humans (Aggarwal et al., 2001; Huang et al., 2016b). Direct evidence for natural HEV infection in NHPs has been reported only occasionally (Yamamoto et al., 2012; Zhou et al., 2014; Spahr et al., 2018b). An outbreak of hepatitis E has been described in a monkey breeding facility in Japan during 2004–2006. Molecular analysis of the complete genome of the monkey HEV Inuyama strain revealed the high genetic correlation (92.0%-93.0% nucleotide identity) with Gt3 HEVs identified in pigs in the same geographical area (Yamamoto et al., 2012). More recently, by screening a

* Corresponding author.
E-mail address: bdimartino@unite.it (B. Di Martino).

https://doi.org/10.1016/j.virusres.2018.04.017
Received 16 March 2018; Received in revised form 25 April 2018; Accepted 27 April 2018
Available online 02 May 2018
0168-1702/

collection of stool samples from 37 chimpanzees living in two different zoos in China (Zhou et al., 2014) HEV-like strains have been found with an overall prevalence rate of 18.9%. Serologic studies documenting the prevalence of HEV in NHPs have been performed since the early 1994 (Arankalle et al., 1994; Huang et al., 2011; Spahr et al., 2018a) revealing rates ranging from 3.9% to 78.5%. Although the source of infections for NHPs housing in zoos is still unclear, it was hypothesized that widespread of HEV in a monkey facility may be subsequent to the direct or indirect contact with other possible reservoirs such as wild mice and rats. In this study, we investigated the presence of HEV in NHPs housed at the Zoological Garden (Bioparco) of Rome, Italy, assessing serologically and molecularly a historical collection of sera obtained from seven different species of monkeys. Also, NHP stool samples were tested for the presence of HEV RNA.

2. Materials and methods

2.1. Sampling

A total of 86 serum samples of NHPs were collected in the 17-year time frame spanning 2001 to 2017. The sera were obtained from 62 Japanese macaques (*Macaca fuscata*), 10 mandrills (*Mandrillus sphinx*), 6 white-crowned mangabeys (*Cercocebus atys lunulatus*), 3 Bornean orangutans (*Pongo pygmaeus*), 2 gorillas (*Gorilla gorilla*), 2 chimpanzees (*Pan troglodytes*) and 1 hamadryas baboon (*Papio hamadryas*). While the majority of the animals (72) consisted of adults (6–20 years old), the sample set contained also 7 sera from juveniles (< 3 years old) and 7 from monkeys older than 20 years of age. None of the animals was specifically bled for this study, but only for the regular health checks, performed mostly at the time of their introduction in the Bioparco.

A total of 17 faecal specimens were collected during December 2017 from 3 Japanese macaques, 4 mandrills, 4 chimpanzees, 3 Bornean orangutans and 3 white-crowned mangabeys. All of the animals were clinically healthy at the time of sampling. Faecal stools were placed in isothermal boxes using ice bags and transferred in the lab. Samples were kept frozen at −80 °C until tested.

2.2. Expression of HEV capsid protein by baculovirus system

The complete ORF2 gene of a Gt3 HEV strain, the WB/P6-15/ITA subtype c (GenBank accession number KT245136) (Di Profio et al., 2016), was synthesized and cloned into the *Bam*HI site of the vector pUC57 (GeneScript) and used as template to amplify by PCR the 112-*N*-terminal-amino acids (aa)-truncated ORF2, using the primer set HEV/111d/VP1 start (5′ - AGG ATC CAT GGC CAC ATC ACC TGC CCC T - 3′) and HEV/111d/VP1 stop (5′ - AGA GGA TCC TTA AAA CTC CC - 3′) both including recognition sites for *Bam*H1, as previously described (Li et al., 2005). The amplified gene was cloned into the *Bam*HI site of the baculovirus transfer vector pAcYM1 (Matsuura et al., 1987) under the control of the polyhedrin promoter. The correct orientation of the insert was evaluated by PCR and sequence analysis. Recombinant vector pAcYM1 with 112-*N*-terminal-aa-truncated ORF2 gene was purified and co-transfected into *Spodoptera frugiperda* (*Sf21*) cells with linearized baculovirus DNA (BacPAK6, Bsu36 I digest, Clontech), using Cellfectin II Reagent (Invitrogen Ltd, Milan, Italy). The recombinant baculovirus was plaque purified on *Sf21* cells and selected using X-Gal blue/white screening. For baculovirus amplification, each plaque was resuspended in 500 µl TC-100 medium (Invitrogen Ltd, Milan, Italy) and subjected to 3 rounds of amplification in fresh *Sf*9 insect cells. For large-scale production of the capsid protein, 100 ml *Sf9* cells (1×10^6 cell/ml) suspension culture, were infected with the recombinant baculovirus at a multiplicity of infection of 3 plaque forming units/cell. The culture medium after separation from the cell debris at 72 h post-infection (PI), was concentrated by ultracentrifugation through a 17% sucrose cushion in TEN-buffer (100 mM NaCl; 50 mM Tris-HCl, pH 7.5; 1 mM EDTA). The resulting pellet was mixed with CsCl to obtain a solution with a density of 1.34 *g*/ml by adding 5.11 *g* CsCl to 10 ml suspension. Gradients were formed by centrifugation at 35,000 rpm (150,000 × *g*) for 27 h in a SW 41Ti rotor using a Beckman Coulter Optima L-90 K ultracentrifuge. The gradients were fractionated by bottom puncture, and aliquots of each fraction were analysed by SDS-12% polyacrylamide gel electrophoresis (PAGE) and western blotting (WB) assays. The concentration of the HEV capsid protein was determined by measuring the optical density at 280 nm (OD_{280}) and by running aliquots of purified recombinant protein on SDS-12% PAGE containing bovine serum albumin (BSA) standards.

2.3. SDS-12% polyacrylamide gel electrophoresis (PAGE) and western blotting

HEV 112-*N*-aa- truncated capsid protein, as well as mock infected *Sf9* cells and wild-type baculovirus-infected Sf9 insect cells, were separated on SDS-12% PAGE and stained with Coomassie blue. WB analysis was performed by transferring the separated proteins from the SDS-PAGE gel to Immobilon-P Transfer membranes (Millipore Corporation, Billerica, USA). In order, to assess the immunogenicity of the recombinant capsid protein, swine sera resulted positive either in RT-PCR for HEV or when tested with a commercial double-antigen sandwich ELISA kit (Bijing Wantai Bio-Pharmaceutical-Co., Ltd.) were used. Briefly, membranes were incubated with each swine serum at final dilution of 1:100 for 1 h at room temperature. After washing three times with 0.1% Tween-Phosphate-Buffered Saline (PBS-T), membranes were incubated with horseradish peroxidase-conjugated goat anti-swine IgG (Invitrogen, Ltd, Milan, Italy) at 1:3,000 dilution for 30 min at 37 °C. Antibody binding was detected using 3,3-Diaminobenzidine tetrahydrochloride (DAB) substrate. WB analysis was also used to screen NHP sera either for the presence of IgM or IgG against HEV. Each serum was assessed at dilution of 1:100, while horseradish peroxidase-conjugated goat anti-human IgG or IgM (Invitrogen, Ltd, Milan, Italy) were used both at dilution of 1:3,000.

2.4. Antibody detection enzyme-linked imunnosorbent assay (ELISA)

For the development of the ELISA, mock infected *Sf9* insect cells lysate and the HEV antigen, both at final concentrations of 1 µg/ml, were coated onto 96 well EIA plates (Costar, Italy) at 100 µl per well in carbonate-bicarbonate buffer (0.05 M, pH 9.6). The plates were incubated at 4 °C overnight. The wells were washed five times with 0.1% PBS-T and then blocked with 200 µl of PBS containing 1% BSA at room temperature for two hours.

To determine the working dilutions of the NHP sera, human sera resulted positive for the presence of IgG or IgM anti-HEV by using commercial antibodies detection ELISA kits (Wantai, Biologic Pharmacy Enterprise, Beijing, China), were 2-fold diluted starting at initial dilution of 1:25 until 1:400, using an antigen concentration of 1 µg/ml. Therefore, each NHPs serum sample (100 µl), used at the optimal dilution established as 1:100 in 1% dried milk (Blotto, Santa Cruz Biotechnology, Inc., Heidelberg, Germany) in PBS, was added either to the HEV antigen or mock infected *Sf9* cells coated wells, and the plates were incubated at 37 °C for 1 h. Also, human sera resulted positive or negative for IgG or IgM anti-HEV were employed as controls at final dilution of 1:100. Plates were washed 5 times with 0.1% PBS-T and then incubated with horseradish peroxidase-conjugated goat anti-human IgG (Invitrogen Ltd, Milan, Italy) or with horseradish peroxidase-conjugated goat anti-human IgM (Invitrogen Ltd, Milan, Italy), both at 1:5000 dilution for 30 min at 37 °C. The reaction was developed after the addition of 100 µl per well of 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) substrate for 15 min and stopped after addition of an equal volume of 1 M/l phosphoric acid. Absorbance was measured at 405 nm using a multiskan automatic plate reader (ThermoLabsystems, Finland).

The cut-off point of the ELISA ($OD_{405} \geq 0.45$ for IgG and $OD_{405} \geq 0.15$ for IgM) was established as the mean of the OD_{405}