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Human bocavirus 1 infection of CACO-2 cell line cultures

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ABSTRACT

Human bocavirus 1 (HBoV1) is a parvovirus associated with pneumonia in infants. It has been detected in different tissues, including colorectal tumors. In this study, we investigated whether Caco-2 cell line, derived from human colon cancer, can be utilized as a model for HBoV1 replication. We demonstrate HBoV1 replication in Caco-2 cultures supplemented with DEAE-dextran after inoculation with respiratory material from infected patients presenting with acute respiratory infection. A viral cycle of rapid development is displayed. However, in spite of HBoV1 DNA 4-fold increment in the supernatants and monolayers by day 1, evidencing that the system allows the virus genome replication after the entry occurred, infectious progeny particles were not produced. These results are consistent with an infection that is limited to a single growth cycle, which can be associated to mutations in the NS1 and VP1/VP2 regions of HBoV1 genome. Further research will contribute to fully elucidate these observations.

1. Introduction

Human bocavirus 1 (HBoV1) belongs to the species *Primate bocaparvovirus 1* in the genus *Bocaparvovirus*, subfamily *Parvovirinae*, family *Parvoviridae* (Qiu et al., 2017). It causes lower acute respiratory tract infections (ARTI) especially in infants less than 2 years old (Ghietto et al., 2015; Martin et al., 2009; Meriluoto et al., 2012), and the frequency of detection varies from 1% to 33% (Bicer et al., 2013; Garcia-Garcia et al., 2008; Ghietto et al., 2012b; Martin et al., 2009). Severe cases are associated with high viral load, anti-HBoV1 IgM antibody detection or an increase in the levels of IgG antibodies, comorbidity and age (Christensen et al., 2010; Ghietto et al., 2015; Nascimento-Carvalho et al., 2012; Wang et al., 2010). A longitudinal study of children from infancy to puberty documented a clear association of primary HBoV1 infection with respiratory symptoms (Meriluoto et al., 2012). All of that strongly supports the etiological role of HBoV1 in ARTI.

In vitro, HBoV1 viral particles from nasopharyngeal clinical specimens infect polarized primary human airway epithelium cultures developed at an air-liquid interface (HAE-ALI) (Dijkman et al., 2009). Other authors obtained HBoV1 virions from HEK293 cells transfected with an infectious plasmid and demonstrated that this reverse genetic system generated HBoV1 virions that productively infect HAE-ALI at a high multiplicity of infection (MOI) -using 750 viral genome copies per cell- and cause cytopathic effect (Deng et al., 2014, 2013; Huang et al., 2012; Khalfaoui et al., 2016). On the other hand, HBoV1 DNA was detected in tissue from tonsils, adenoids (Gunel et al., 2015; Norja et al., 2012), lung and colorectal tumors (Abdel-Moneim et al., 2016; Schildgen et al., 2013), providing evidence of the capacity of the virus to infect different cell types.

In this study, we investigated whether epithelial cells derived from human colorectal adenocarcinoma (Caco-2) can be utilized as a model for HBoV1 replication. We demonstrate HBoV1 replication in Caco-2 cell culture supplemented with diethylaminoethyl-dextran (DEAE-dextran) after inoculation with respiratory material from HBoV1-infected patients with ARTI.

2. Materials and method

2.1. Inoculum preparation

The inoculum was prepared from HBoV1-positive respiratory secretions obtained by nasopharyngeal aspirate from two children. Samples 2526 and 307 had different virus concentrations 1.34×10^9 DNA copies/μl and 1.14×10^3 DNA copies/μl, respectively. These two samples were used for high- and low-MOI assays. The patients from whom the clinical specimens were obtained were 9 and 10 years old and were hospitalized due to lower respiratory tract illness. They were hemoculture negative and also negative by molecular method (real time RT-PCR) for other common respiratory viruses, such as influenza A and B, parainfluenza 1, -2 and -3, adenovirus, respiratory syncytial virus and metapneumovirus (Ghietto et al., 2015). HBoV1 was

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confirmed by sequencing [Gen' database accession numbers JX034732 and JN632491 (Ghietto et al., 2012b, 2015)]. Fifty-µl respiratory secretion was diluted in 500 µl Eagle's Minimum Essential Medium (EMEM) (GIBCO) with penicillin-streptomycin, centrifuged 3 min at 1000*g* and filtered. This preparation was re-tested by PCR and qPCR, aliquoted and kept at − 70 °C until used in the infection assays.

2.2. Cell cultures

Human colon adenocarcinoma Caco-2 cells (ATCC® HTB-37), and Vero cells (ATCC ® CCL-81) were grown in EMEM (GIBCO) supplemented with penicillin-streptomycin or gentamicin, glutamine and 10% fetal bovine serum (FBS); this is referred to as complete growth medium. Cell monolayers were trypsinized and reseeded every 6 or 7 days, using 0.25% trypsin and 0.02% EDTA, and maintained at 37 °C in a 5% CO_2 incubator. To obtain differentiated Caco-2 cultures, the cells were placed in 6-well plates with 3 ml complete growth medium. After the cells reached 100% confluence, the cultures were maintained during 4 weeks with changes of the supernatant medium every 5–7 days until use. In these cultures, after reaching confluence the cells differentiate spontaneously under normal culture conditions into enterocyte-like cells, developing tight junctions and expressing transporters found in the small intestine. These cells are homogeneously polarized and differentiated after 30 days (Buhrke et al., 2011; Lnenickova et al., 2016).

2.3. Experimental conditions and HBoV1 infection

Control (mock–infected) and infected Caco-2 and Vero cultures were maintained in EMEM culture medium, standard or supplemented with 1 or 10 µg/ml of DEAE-dextran. DEAE-dextran is a polycationic derivative of the carbohydrate polymer dextran. It was one of the first chemical reagents used to transfer nucleic acids into cultured mammalian cells (Vaheri and Pagano, 1965) and has been used to enhance the growth of other viruses such as coronaviruses, rubella, and picornaviruses. Postinoculation temperatures of incubation were 33 °C or 37 °C. Previous studies described that respiratory viruses replicated slightly better at 33 °C than at 37 °C, since the lower temperature seems to replicate the conditions in the upper respiratory tract (Gangl et al., 2015; Papadopoulos et al., 1999). Three independent repetitions of infection assays were performed in 60 mm-Petri dishes. During the optimization of DEAE-dextran concentration to be used in the culture, we observed cytotoxicity and decreased cell survival in the control and infected cultures supplemented with 10 µg/ml DEAE-dextran. Consequently, the assays with DEAE-dextran were performed at 1 µg/ml.

Cultures were washed with phosphate-buffered saline (PBS) and 50-µl virus suspension prepared as indicated before was directly inoculated on the cells. At the moment of inoculation, regular cultures were 70–80% confluent monolayers and differentiated cultures were multilayers of 30 days. The virus was allowed to adsorb for 1 h at 37 °C or 33 °C. Next, the inoculum was removed and fresh culture medium was added (standard or supplemented with DEAE-dextran). Samples from the supernatant medium and the cells attached to the substratum were collected at 0 h postinfection (hpi) to 6 days postinfection (dpi). The samples at time point “0 hpi” corresponded to medium or cells collected immediately after inoculation (that is, following the addition of 50 µl-inoculum, removing it and adding fresh culture medium). Sterile EMEM or DEAE-dextran EMEM were used in as controls of reagents. Additionally, a negative nasopharyngeal aspirate (for HBoV1 and all the other above mentioned common respiratory viruses), obtained from a 1 year old patient hospitalized with lower ARTI, was processed exactly as the HBoV1+ specimen and inoculated into cultures to serve as negative control of infection.

Blind passage assays (8 successive passages after the starting culture inoculated with the diluted and filtered clinical specimens) were performed with inoculums from both samples 307 and 2526. For the blind passage assays, a 50-µl aliquot of supernatant or monolayers taken at 48 hpi from these cultures was used to infect fresh cultures. This procedure was repeated six more times. To release the intracellular virions, monolayers were lysed by 3 freeze-thaw cycles. Control cultures as explained above were also included in the series. Samples from the supernatant medium and the cell monolayer were preserved for virus detection in every passage.

Infected monolayers of Caco-2 cells were subcultured using the standard technique for cell passage, in order to determine if a persistent infection could be established. Sequences of 5 passages were performed, with a 1:5 dilution rate of the cell suspension at each passage. In addition, Caco-2 cell cultures were infected at 70–80% confluence and maintained during 28 dpi with changes of supernatant media every 5–7 days.

2.4. Nucleic acid extraction and HBoV1 detection

Nucleic acids were extracted from 200 µl of supernatant and 20 cm^2 monolayers using Axyprep Body Fluid Viral DNA/RNA Miniprep Kit (Axygen Bioscience). Extracts were stored at − 20 °C for subsequent HBoV1 detection. HBoV1 was detected by PCR as described previously (Ghietto et al., 2012a). PCR products were visualized in 8.5% polyacrylamide gels stained with silver solution (0.11 M $AgNO_3$).

2.5. Determination of cytopathic effect in cell culture

Cultures were observed daily under a reversed phase light microscopy to determinate cytopathic effect (CPE). To monitor the morphological changes in the monolayers photographs were taken at intervals of 24 h until 6 dpi. Sample monolayers were fixed with cold methanol and stained with hematoxylin-eosin.

2.6. HBoV1 quantification

HBoV1 DNA load in the supernatant and monolayers 1–6 dpi from Caco-2 and Vero cultures maintained in the different conditions described was determined by absolute quantification in Applied Biosystems 7500 Real-Time PCR System equipment, using the NP1 gene as the target (Allander et al., 2007). Samples of supernatant medium and cells attached to the surface form Caco-2 cultures at 3, 6 and 12 hpi were also quantified. The viral load value of the samples at 0 hpi were interpreted as remnants of the inoculum. The PCR protocol was essentially as described elsewhere (Ghietto et al., 2015), with 25 µl of amplification reaction final volume containing 2.5 µl of DNA sample, 5 U/µl of Platinum Taq DNA polymerase (Invitrogen), 0.04 µM each primer and 0.1 µl of a 1/100 SYBR Green (Invitrogen S-7563) dilution in DMSO. The viral load in each sample was calculated from a standard curve performed with a synthetic oligonucleotide (Marcrogen Korea) of known concentration.

2.7. Immunofluorescence assay

In order to confirm the infection of Caco-2 cells, an immunofluorescence assay (IFA) was optimized using as primary antibody a pool of sera obtained from children in whom HBoV1 respiratory infection had been confirmed. During the procedure, different concentrations of first antibody, conjugate antibody, and blocking solution as well as incubation time and washings were tested. The definite IFA applied was as follows: the cultures were fixed at 24 and 96 hpi, using freshly prepared 4% paraformaldehyde, for 20 min at room temperature. Cells were then permeabilized with 1% NP40, 5 min at room temperature and blocked with 5% bovine serum albumin, 1 h at 37 °C. The primary antibody was added at a 1/1000 dilution in 5% bovine serum albumin, incubating overnight at 4 °C. Subsequently, the conjugate antibody (FITC-anti-human IgG) was added at a 1/400 dilution and incubated