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Isolation of an equine coronavirus from adult horses with pyrogenic and enteric disease and its antigenic and genomic characterization in comparison with the NC99 strain

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ABSTRACT

A new equine coronavirus was isolated from the feces of adult horses with pyrogenic and enteric disease. The disease outbreak was mainly observed among 2- to 4-year-old horses living in stables of a draft-horse racetrack in Japan. On comparing the isolated virus (isolate Tokachi09) with the equine coronavirus NC99 strain, no significant differences were observed in several biological properties such as hemagglutinating activity, antigenicity (in indirect immunofluorescence and neutralization tests), and one-step growth (in cell culture). The sequences of the nucleocapsid and spike genes of isolate Tokachi09 showed identical size (1341 and 4092 nucleotides, 446 and 1363 amino acids, respectively) and high similarity (98.0% and 99.0% at the nucleotides, 97.3% and 99.0% at the amino acids, respectively) to those of strain NC99. However, the isolate had a 185-nucleotide deletion from four bases after the 3′-terminal end of the spike gene, resulting in the absence of the open reading frame predicted to encode a 4.7-kDa nonstructural protein in strain NC99. These results suggest that the 4.7-kDa nonstructural protein is not essential for viral replication, at least in cell culture, and that the Japanese strain probably originated from a different lineage to the North American strain. This is the first equine coronavirus to be isolated from adult horses with pyrogenic and enteric disease.

1. Introduction

Coronaviruses are enveloped, positive-stranded RNA viruses that cause respiratory and enteric disease in a variety of avian and mammalian species, including humans, cattle, pigs, chickens, turkeys, dogs, cats, mice, rats, rabbits, and bats (Lai et al., 2006). On the basis of their serological cross-reactivity and genomic organization, these viruses are divided into three categories: group 1, group 2, and group 3 (Brian and Baric, 2005; Lai et al., 2006). Recently, the Coronavirus Study Group of the International Committee for Taxonomy of Viruses (ICTV) has proposed three genera—*Alphacoronavirus*, *Betacoronavirus*, and *Gammacoronavirus*—to replace the group 1, group 2, and group 3 nomenclature, respectively (Woo et al., 2009). Equine coronavirus (ECoV) belongs to the genus

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Betacoronavirus, the species *Betacoronavirus*-1 with human coronaviruses OC43 (HCoV-OC43), human enteric coronavirus, bovine coronavirus (BCoV), porcine hemagglutinating encephalomyelitis virus (PHEV), canine respiratory coronavirus (CRCoV) (Erles et al., 2007), and bubaline coronavirus (BuCoV) (Decaro et al., 2010).

Coronavirus-like agents have been previously identified by electron microscopy in the feces of foals and adult horses having enteric disease with fever (Bass and Sharpee, 1975; Huang et al., 1983). A coronavirus antigen detected by antigen-capture enzyme-linked immunosorbent assay and immunohistochemistry in the feces and intestine of a foal suffering from neonatal enterocolitis have also been reported (Davis et al., 2000); however, isolation and characterization of the causative agent was not successfully performed. An ECoV was isolated from the feces of a diarrheic foal in 1999 (ECoV-NC99) in North Carolina, USA (Guy et al., 2000); its complete genomic sequence is composed of 11 open reading frames (ORFs) encoding two replicase polyproteins, five structural proteins (hemagglutinin esterase [HE], spike [S], envelope [E], membrane [M], and nucleocapsid [N]), and four accessory proteins (ns2, p4.7, p12.7, and I) (Zhang et al., 2007). Antigenic and genomic characterization studies revealed that this virus is a new member of species *Betacoronavirus*-1. Identification of new coronaviruses in horses and foals will help in understanding the etiologic role and epidemiology of this virus in equine species worldwide.

In this study, we isolated an ECoV from the feces of adult horses with pyrogenic and enteric disease and performed antigenic and genomic characterization of the isolate in comparison with strain NC99 and BCoV, which has a close antigenic relationship with ECoV.

2. Materials and methods

2.1. Clinical history

In mid-June 2009, an outbreak of a disease with symptoms of pyrexia occurred in mainly 2- to 4-year-old horses living in stables of a draft-horse racetrack in Tokachi, Hokkaido, Japan. Some diseased horses had diarrhea, and most animals recovered in two to four days. The outbreak subsided by the end of August, and 132 of about 600 horses finally became diseased in this period. During the outbreak period, various animal health measures were implemented at the racetrack, including a race ban, movement restriction, and disinfection, resulting in economic losses for the involved parties. Feces and nasal discharge were collected and examined to determine the causative agent.

2.2. Diagnostic test for identifying the causative agent

Reverse transcriptase (RT)-PCR was performed to detect the N gene of the ECoV. The oligonucleotide primers used for RT-PCR were ECoV-midf, 5′-gtgatgaggctattccgacta-3′ (nt 29862–29882, sense primer), and ECoV-Nr, 5′-ccaggtgccgacataaggttcat-3′ (nt 30708–30730, antisense primer), which were modifications of the primers used by Guy et al. (2000) to remove the *Eco*RI site. Viral RNA was extracted from a 10% suspension of feces in Dulbecco's modified Eagle's medium (DMEM) and nasal discharge of three diseased horses by using ISOGEN-LS (NIPPON GENE, Tokyo, Japan). RT-PCR was performed with a QIAGEN OneStep RT-PCR kit (QIAGEN, Tokyo, Japan). To investigate the role of enteric bacterial pathogens, several cultures were obtained by isolation from the fecal samples.

2.3. Virus isolation

Feces obtained from the diarrheic horses were prepared as a 10% suspension in DMEM containing 50 μg/ml gentamicin and 0.25 μg/ml TPCK trypsin (Thermo Fisher Scientific, Kanagawa, Japan) and clarified by low-speed centrifugation at 3000 × *g* for 10 min. The supernatant was inoculated onto confluent monolayers of human rectal adenocarcinoma (HRT-18G) cells in 12-well plates and incubated for 60 min at 37 °C for adsorption. After washing twice with phosphate-buffered saline (PBS), fresh medium of the same composition except 0.125 μg/ml TPCK trypsin was added. The monolayers were examined daily for a cytopathic effect (CPE) and passaged at 4- to 5-day intervals to fresh monolayers of HRT-18G cells until a distinct CPE appeared. The isolate (Tokachi09) was further passaged in HRT-18G cells, and the fifth passage of the cell cultures was used as the working stock for the following studies except the one-step growth, hemagglutinating activity (HA) and receptor-destroying enzyme (RDE) activity studies, in which the eight passage of the cell cultures was used, as obtained for strain NC99.

2.4. Electron-microscopic examination

The supernatant of the HRT-18G cell cultures infected with isolate Tokachi09 was clarified by centrifugation at 5000 × *g* for 20 min to remove cell debris and filtered through a 0.45 μm pore size filter. The filtered supernatant was concentrated by centrifugation at 148,600 × *g* for 2 h through a 35% (w/v) sucrose cushion, and the resultant pellet was resuspended in PBS at pH 7.2. A drop of the supernatant was examined by using the phosphotungstic acid negative-staining technique.

2.5. Assays for HA and RDE activity

HA was tested by using the microtiter method. Cell cultures with isolate Tokachi09 and NC99 were tested for HA by using mouse, chicken, horse, and guinea pig erythrocytes. Suspensions (1%) of mouse, horse, and guinea pig erythrocytes and those (0.5%) of chicken erythrocytes were incubated with the isolate for 1 h at 4 °C or 37 °C, and the HA titers were expressed as the reciprocal of the highest viral dilution showing HA. The plates incubated at 4 °C for 1 h were then incubated at 37 °C for 2 h in order to determine the RDE activity, and the RDE titers were expressed as the reciprocal of the highest viral dilution causing complete disappearance of the HA patterns.

2.6. Indirect immunofluorescence test

HRT-18G cell monolayers grown on a chamber slide were fixed in acetone 3 days after the isolate inoculation.