



# 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.

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## 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 1) (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'-ccaggtgccgacataaggttcac-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  $3000 \times g$  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  $5000 \times g$  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  $148,600 \times g$  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.

Hyperimmune rabbit sera against NC99 and the BCoV Kakegawa strain (Akashi et al., 1980; Kanno et al., 2007) were incubated with these infected monolayer cells. For detection, fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (MP Biomedicals, Inc., Solon, OH, USA) was used.

## 2.7. Virus neutralization

For characterizing the antigenicity of isolate Tokachi09, hyperimmune serum was produced by immunizing rabbits. The neutralization test was performed by using HRT-18G cell cultures grown in 96-well microplates to elucidate the antigenic relationship between the isolate and NC99 as well as that between the isolate and the BCoV Kakegawa (Akashi et al., 1980) and Kumamoto/1/07 strains (Kanno et al., 2009), which have a very close antigenic relationship with ECoVs (Davis et al., 2000; Guy et al., 2000). In brief, twofold serial dilutions of serum were mixed with an equal volume of viral suspensions containing two hundred 50% tissue culture infective dose (TCID<sub>50</sub>) per 0.1 ml and incubated for 60 min at 37 °C. The neutralizing antibody titers were expressed as the reciprocal of the highest serum dilution showing complete inhibition of the CPE. To further our understanding of the antigenic correlation between ECoV and BCoV, six-paired sera of horses in the same stable block were examined by neutralization tests against isolate Tokachi09 and BCoV Kakegawa.

## 2.8. One-step growth

One-step growth curves of the isolate and NC99 were examined in HRT-18G cell cultures. The culture supernatant was harvested at various time points after inoculation. To investigate the viral titer in the cells at the harvest times, the cells were washed twice with PBS, and then, an equal volume of PBS as the culture supernatant was added. After freeze–thawing thrice, low-speed centrifugation at 3000 × *g* for 10 min was conducted. The viral titer was determined by TCID<sub>50</sub> in 96-well microplates.

## 2.9. N and S gene sequencing

The oligonucleotide primers used for RT-PCR were designed from the nucleotide sequence data of NC99 (GenBank accession no. EF446615). The following primers were used: ECoV-Nf, 5'-tcaggcatggacaccgcattgtt-3' (nt 29315–29337, sense primer), and ECoV-Nr, 5'-ccaggtgcc-gacataaggttcat-3' (nt 30708–30730, antisense primer), for N gene amplification; ECoV-Sf, 5'-attttttttatgtgtgataatg-3' (nt 23684–23706, sense primer), and ECoV-Sr, 5'-aaaacagacatcttctaattctg-3' (nt 28134–28156, antisense primer), for S gene amplification. Viral RNA was extracted from the viral culture by using the High Pure viral RNA kit (Roche Diagnostics, Tokyo, Japan), followed by reverse transcription with PrimeScript reverse transcriptase (TaKaRa Bio, Shiga, Japan) by using a random primer (6 mers) according to the manufacturer's instructions. PCR amplification was performed by using PrimeSTAR GXL DNA polymerase (TaKaRa Bio). In brief, a 50 µl reaction

mixture was prepared with 10 µl of PrimeSTAR GXL buffer (5×), 4 µl of dNTP mix (2.5 mM each), 1 µl of each primer (20 µM), 2 µl of the cDNA template, 1 µl of PrimeSTAR DNA polymerase (1.25 U), and nuclease-free water. The reaction mixture was incubated at 98 °C for 1 min, followed by 30 cycles of amplification at 98 °C for 10 s, 55 °C for 15 s, and 68 °C for 1.5 min or 4.5 min (N and S gene amplification, respectively), with a final incubation at 68 °C for 7 min. The PCR products were purified by using a QIAquick gel extraction kit (QIAGEN). The sequencing reaction was performed by using a BigDye Terminator v.3.1 cycle sequencing kit (Life Technologies, Tokyo, Japan), and sequencing and data analysis were carried out on an ABI 3130 genetic analyzer (Life Technologies). The sequencing primers were designed on the basis of the nucleotide sequence data of the NC99 strain, and both sense and antisense strands were sequenced.

The amino acid sequences of the N and S protein regions were aligned by using CLUSTAL W (Thompson et al., 1994). Phylogenetic trees were generated by using the neighbor-joining method with CLUSTAL W and constructed by using the TreeView program (Page, 1996).

## 2.10. Nucleotide sequence accession numbers

The nucleotide sequences of the N and S genes have been deposited in GenBank/EMBL/DBJ under the accession numbers AB555559 and AB555560, respectively.

# 3. Results

## 3.1. Diagnostic test for identifying the causative agent

In the RT-PCR for diagnosing the causative agent, all three fecal samples showed 869-bp products, but no PCR products were obtained from the nasal discharge samples. Therefore, the fecal samples were used for virus isolation. The bacterial cultures of all the fecal samples were negative for enteric pathogens including *Salmonella* sp.

## 3.2. Virus isolation and electron-microscopic examination

The HRT-18G monolayer cells in 12-well plates showed a distinct CPE at the fourth passage in the case of one of the three fecal samples. Electron-microscopic examination of the supernatants of the HRT-18G cell cultures infected with isolate Tokachi09 revealed the presence of numerous particles of 80–130 nm diameter, with a layer of large club-shaped peplomers, which is the typical morphology of coronaviruses (data not shown).

## 3.3. Indirect immunofluorescence test

The hyperimmune rabbit sera against the ECoV NC99 and BCoV Kakegawa strains reacted with the isolates in the indirect immunofluorescence test (data not shown).

## 3.4. HA and RDE activity

Mouse erythrocytes were agglutinated by the supernatants of the HRT-18G cell cultures infected with both

**Table 1**

Hemagglutinating (HA) and receptor-destroying enzyme (RDE) activities of isolate Tokachi09 and ECoV NC99.

Virus	Viral titer <sup>a</sup>	HA titer <sup>b</sup>				RDE titer <sup>c</sup>	
		4 °C		37 °C			
		Mouse	Chicken	Mouse	Chicken	Mouse	Chicken
Tokachi09	4.8	64	<2	8	<2	8	<2
ECoV NC99	5.7	256	8	128	4	4	<2

<sup>a</sup> Log<sub>10</sub> TCID<sub>50</sub>/0.1 ml.<sup>b</sup> Reciprocal of the highest viral dilution showing complete hemagglutination.<sup>c</sup> Reciprocal of the highest viral dilution causing complete disappearance of the HA patterns.**Table 2**

Cross virus-neutralization test among isolate Tokachi09, ECoV NC99, and BCoVs.

Virus	Anti-serum			
	Anti-Tokachi09	Anti-NC99	Anti-BCoV Kakegawa	Anti-BCoV Kumamoto1
Tokachi09	2048 <sup>a</sup>	1448	2048	1024
ECoV NC99	724	4096	1448	362
BCoV Kakegawa	16	23	65536	8192
BCoV Kumamoto1	23	32	32768	32768

<sup>a</sup> Expressed as the reciprocal of the highest serum dilution that inhibited CPE.**Table 3**

Antibody titers of sera of the horses in the racetrack against isolate Tokachi09 and BCoV Kakegawa.

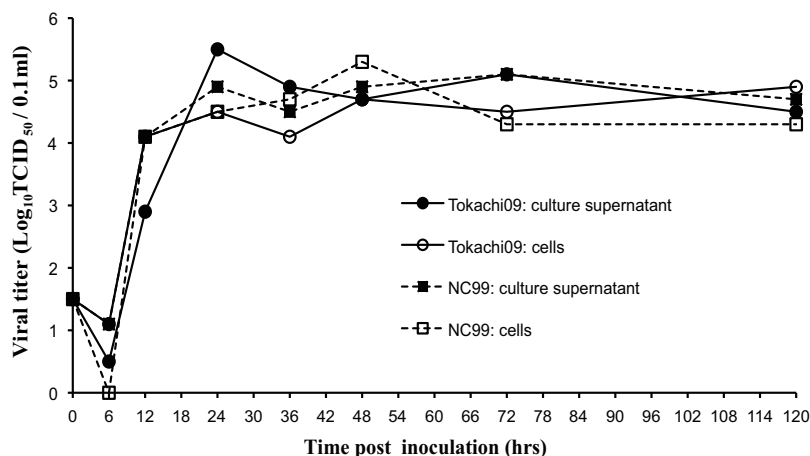
Horse no.	Tokachi09		BCoV Kakegawa	
	Pre	Post	Pre	Post
1	90.5 <sup>a</sup>	256	1	2.8
2	2	181	1	1
3	22.6	512	8	362
4	4	181	1	1
5	181	362	1	1
6	64	512	1	1
GM <sup>b</sup>	24	304.4	1.4	3.2

<sup>a</sup> Expressed as the reciprocal of the highest serum dilution that inhibited CPE.<sup>b</sup> Geometric mean.

viruses. The HA titers of isolate Tokachi09 and NC99 were 64 and 256 at 4 °C and 8 and 128 at 37 °C, respectively (Table 1). The RDE activities of isolate Tokachi09 and NC99 for receptors on mouse erythrocytes were detected at titers of 8 and 4, respectively. Chicken erythrocytes were agglutinated by NC99 but not by isolate Tokachi09. The HA titer of NC99 was 8 at 4 °C and 4 at 37 °C, whereas the RDE activity was undetectable. Both viruses did not agglutinate the horse and guinea pig erythrocytes.

### 3.5. Cross-neutralization test

Isolate Tokachi09, NC99, and BCoV Kakegawa were compared for antigenicity by cross-neutralization studies using antisera to these viruses (Table 2). No significant difference was observed between the isolate and NC99, whereas a certain antigenic difference was seen between



**Fig. 1.** One-step growth curves of the isolate Tokachi09 and NC99. Samples of culture supernatant and cells were harvested at various time points after inoculation. The viral titer was determined by TCID<sub>50</sub> in 96-well microplates.

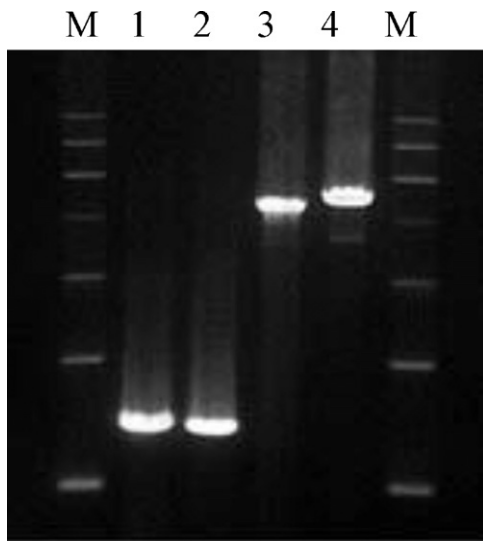


Fig. 2. RT-PCR products obtained by using the primers, which predicted to amplify the N (lanes 1 and 2) and S (lanes 3 and 4) genes were run on 0.6% agarose gel. Lane M, 1-kb DNA ladder marker; lanes 1 and 3, isolate Tokachi09; lanes 2 and 4, ECoV NC99.

ECoV and BCoV, although they have cross-reactivity. The six-paired horse sera showed seroconversion between before and after using isolate Tokachi09 (Table 3). However, only one horse serum showed positive seroconversion when BCoV Kakegawa was used as the antigen.

### 3.6. One-step growth

Both isolate Tokachi09 and NC99 showed replication at 12-h post-inoculation and a peak at 24-h post-inoculation (Fig. 1). Thereafter, the viral titers were maintained until 120-h post-inoculation in both the culture supernatant and the cells. The slope of the growth curve and the viral titers of Tokachi09 were similar to those of NC99.

### 3.7. RT-PCR and sequencing

RT-PCR was performed to amplify the N and S genes of isolate Tokachi09 by using the primers designed on the basis of the nucleotide sequence data of strain NC99. The PCR product obtained by using the primers predicted to amplify the N gene was about 1.4 kb and identical in size to the product obtained from NC99 as the template (Fig. 2). However, the PCR product obtained by using the primers predicted to amplify the S gene was about 4.3 kb, which was smaller than the product from NC99 (about 4.5 kb). From the sequencing data of these PCR products, the N gene of isolate Tokachi09 contains an ORF of 1341 nucleotides encoding a predicted protein of 446 amino acid residues. This size is identical to that of NC99; the sequence identity with NC99 is 98.0% at the nucleotides and 97.3% at the amino acids. The S gene of isolate Tokachi09 contains an ORF of 4092 nucleotides encoding a predicted protein of 1363 amino acid residues. This size is also identical to that of NC99, and the sequence identity with NC99 is 99.0% at the nucleotides and 99.0% at the

amino acids. Interestingly, isolate Tokachi09 has a 185-nucleotide deletion from four bases after the 3'-terminal end of the S gene, resulting in the absence of an ORF potentially encoding a nonstructural protein of 4.7 kDa (Fig. 3). This deletion was also observed in RT-PCR products derived from the original fecal sample (data not shown).

Phylogenetic analyses of isolate Tokachi09 and other *Betacoronavirus*-1 were performed on the basis of the amino acid sequences of the N and S proteins (Fig. 4). Both trees showed that isolate Tokachi09 is closely related to ECoV NC99 than to other *Betacoronavirus*-1.

## 4. Discussion

Coronavirus-like particles have been identified in the feces of diarrheic foals and horses (Bass and Sharpee, 1975; Durham et al., 1979; Huang et al., 1983; Mair et al., 1990); however, isolation and characterization of these agents were not reported until an ECoV (NC99 strain) was isolated from the feces of a diarrheic foal in North Carolina (Guy et al., 2000). Its complete genomic sequence and phylogenetic studies suggest that ECoV NC99 belongs to the cluster of *Betacoronavirus*-1 (formerly group 2a coronavirus) and is the most closely related to BCoV, HCoV-OC43, and PHEV (Zhang et al., 2007). However, little is still known about ECoV, especially with regard to the genetic diversity of field strains and their clinical significance. Therefore, it is necessary to isolate other ECoVs and compare their antigenic, pathogenic, and genomic characteristics in detail to understand the etiologic role and epidemiology of this virus in horses worldwide. Here, we have reported a new ECoV isolate, Tokachi09, which is the first isolated strain from adult horses with pyrogenic and enteric disease.

No significant differences were observed between NC99 and isolate Tokachi09 in several biological properties such as HA, antigenicity, and one-step growth, except that isolate Tokachi09 did not agglutinate chicken erythrocytes. Differences in the HA patterns of virus strains have also been demonstrated among in BCoVs (Storz et al., 1992). However, the reason for such differences is unknown. ECoV has been reported to have a close antigenic relationship with BCoV (Davis et al., 2000; Guy et al., 2000). However, only one of six horses showed antibody positivity against BCoV in the neutralization test whereas all sera showed seroconversion against isolate Tokachi09 (Table 2). Furthermore, when we performed the neutralization test using cattle sera seropositive against BCoV, all sera showed seropositivity against isolate Tokachi09 (data not shown). This inconsistency was also seen in the cross-neutralization test (Table 2). The neutralization titers of anti-ECoV serum to BCoV were significantly low when compared with those of anti-BCoV serum to ECoV. Although the reason for this difference is unknown, the result suggests that BCoV should not be used as an antigen in neutralization tests for diagnosing equine cases.

The N and S gene sequences of isolate Tokachi09 showed high similarity to those of NC99. However, isolate Tokachi09 has a 185-nucleotide deletion from four bases after the 3'-terminal end of the S gene, resulting in the

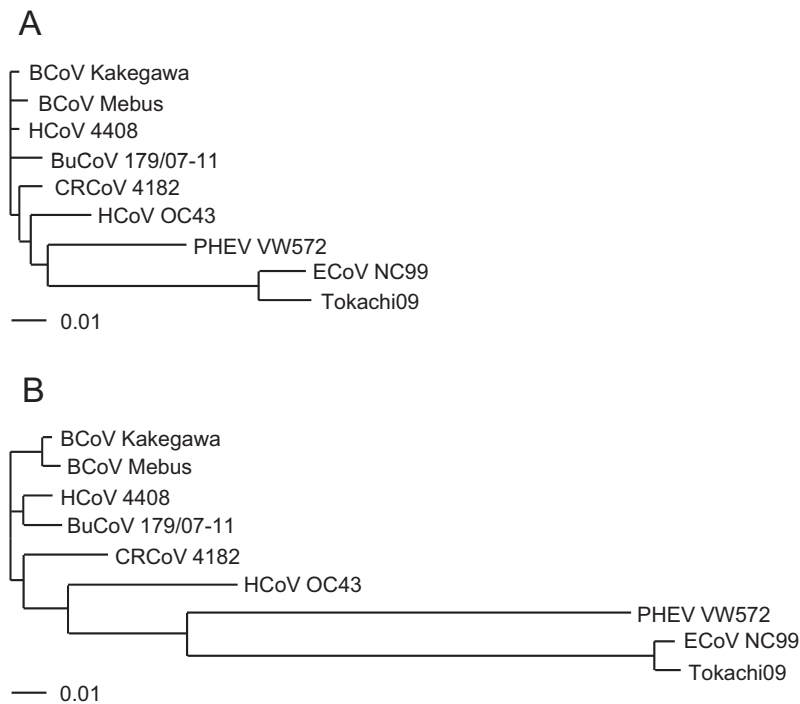


	nt27825	4.7 kDa protein	
ECoV NC99	atgacgattaatttcgtcattttaactatttttatatgacctgcctttgtgggttaaactt	→	60
Tokachi09	*****-----		14
	nt27885		
ECoV NC99	gctacaaccactcatgggtgtactaatattaatactagtagctctacattggcttatt	→	120
Tokachi09	-----		14
	nt27945		
ECoV NC99	taggcattttttatatgtgttaacaccaccaccatcgggttaaaccgggtttatggtgctag	→	180
Tokachi09	-----		14
	nt28005		
ECoV NC99	tgccaaattatatattttgtttatactttataactttaagcagttgctaaagttcttaaggcc		240
Tokachi09	-----*****t*****		55
	nt28065	12.7 kDa protein	
ECoV NC99	aacccttattaatggacatctggagacctgagaggaaatttctccgttttactaacggat	→	300
Tokachi09	*****		115
	nt28125		
ECoV NC99	ttaacgtcccagaattagaagatgtctgttttaaatttaattaccaatttcctaaagtag	→	360
Tokachi09	****t*****		175
	nt28185		
ECoV NC99	gatattgtagagtccctaattatgcttggtgtcgtaatcaaggtagcttttgtgctacat	→	420
Tokachi09	*****		235
	nt28245		
ECoV NC99	tcaccctttacggcaaatccaaacattatgataaatattttagaataataactggtttca	→	480
Tokachi09	*t*****		295
	nt28305		
ECoV NC99	cagcgttcgccaatactatagaggaggctgttaacaaactggtttttagaggctgttgatt	→	540
Tokachi09	*****t*****		355
	nt28365		
ECoV NC99	ttattatctggcgtagccagaatttaaatgcttatggctga	→	581
Tokachi09	*****		396

Fig. 3. Nucleotide sequence comparison of ns 4.7 and 12.7 genes of ECoV NC99 (nt 27825–28405) and the isolate Tokachi09. (–) Position of missing nucleotide; (\*) conserved nucleotide.

absence of the ORF predicted to encode a 4.7-kDa nonstructural protein (p4.7), which corresponds to the p4.9-coding gene in BCoV. There are ORFs predicted to encode nonstructural proteins between the S and the E genes in *Betacoronavirus*-1. In this region, BCoV has p4.9-, p4.8-, and p12.7-coding genes, although HCoV-OC43 and

PHEV have a deletion leading to the absence of p4.9- (in PHEV, it might be truncated) and p4.8-coding genes (Mounir et al., 1993; Vieler et al., 1995). The functions of these nonstructural proteins between the S and the E genes in coronaviruses are unknown. However, it can be concluded that the two ORFs coding p4.9 and p4.8 are not



**Fig. 4.** Phylogenetic relationship of isolate Tokachi09 and other *Betacoronavirus-1* based on a comparison of N protein (A) and S protein (B) sequences. Amino acid sequence alignments were carried out by using CLUSTAL W, and phylogenetic trees were constructed by using the neighbor-joining method. BCoV Kakegawa, bovine coronavirus Kakegawa (GenBank accession number AB354579); BCoV Mebus, bovine coronavirus Mebus (BCU00735); HCoV 4408, human enteric coronavirus 4408 (FJ415324); BuCoV 179/07-11, bovine coronavirus Bubalus 179/07-11 (EU019216); CRCoV 4182, canine respiratory coronavirus 4182 (DQ682406); HCoV OC43, human coronavirus OC43 (AY391777); PHEV VW572, porcine hemagglutinating encephalomyelitis virus VW572 (DQ011855); ECoV NC99, equine coronavirus NC99 (EF446615).

essential for the replication of ECoVs as well as in HCoV-OC43 and PHEV in cell culture. Interestingly, an antisense mutation resulting in the truncation of p4.9 has been identified only in respiratory bovine coronaviruses (Gelinas et al., 2001). From this findings and HCoV-OC43 and PHEV, this protein could be involved in preferential respiratory tropism (Gelinas et al., 2001; Sasseville et al., 2002). Indeed, the main clinical symptom of the affected Japanese horses was pyrexia, whereas the horse infected with NC99 had diarrhea and pyrexia (Guy et al., 2000). These symptoms might be related to the different pathogenic properties of NC99 and isolate Tokachi09. Evidence shows that non-structural proteins in coronaviruses may play a significant role *in vivo* rather than *in vitro*. Although the ns2 protein encoded by ORF2 of MHV is not required for viral replication in tissue culture (Schwarz et al., 1990), deletion of this protein or a single amino acid substitution leads to a significant *in vivo* attenuation of the virus (de Haan et al., 2002; Sperry et al., 2005). Whether p4.7 of ECoV plays a role in viral pathogenicity should be elucidated by animal experiments using NC99 and isolate Tokachi09.

Furthermore, the genetic diversity of coronaviruses caused by this kind of long deletion could provide clues to their evolutionary history. The occurrence of a long deletion, corresponding to the absence of BCoV p4.9 and p4.8, in HCoV-OC43 supports the speculation regarding a bovine-to-human direction of transmission (Vijgen et al., 2005). To elucidate the evolutionary relationship of *Betacoronavirus-1*, complete genomic sequence data of

isolate Tokachi09 would be required. Moreover, the finding that ECoV in Japan has a 185-nucleotide deletion resulting in the absence of p4.7 suggests that the Japanese strain probably originated from a different lineage to the North American strain. Virus isolation in other areas, especially Europe and Australia, is required to conduct epidemiological studies of this virus in horses.

In this racetrack, an outbreak of pyrogenic disease similar to this case occurred in 2004. Although isolation of the causative agent was unsuccessful, the results of an RT-PCR study using fecal samples suggested that the causative agent has high similarity of the genomic sequence of the N gene (97.8%) to that of NC99 (data not shown). Therefore, it was suspected that an ECoV caused this disease outbreak. However, the agent of 2004 does not have an identical genomic sequence of the N gene (98.4%) to that of isolate Tokachi09 and does not have a 185-nucleotide deletion between the S and the E genes (data not shown), suggesting that the disease in 2009 was not caused by the same virus as that causing the 2004 cases. The reason for disease recurrence is considered to be reduction in herd immunity of horses against ECoV during the 5 years since 2004. This is suggested by the fact that the pre-sera of the horses had low antibody titers against Tokachi09 (Table 3). For preventing such disease occurrence, and bovine enteritis by BCoV, herd immunity is crucial. Furthermore, considering the economic losses, vaccine development is necessary for biosecurity measures and to curtail this disease especially in the horse-racing field.

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