Epidemic of Equine Coronavirus at Obihiro Racecourse, Hokkaido, Japan in 2012

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ABSTRACT. Equine coronavirus (ECoV) outbreaks have occurred three times at Obihiro Racecourse in Hokkaido, Japan. The third ECoV outbreak occurred between late February and early April 2012. The main clinical signs of affected horses were anorexia, pyrexia and leucopenia; gastrointestinal disease was observed in about 10% of affected horses. Two ECoV strains were isolated from diarrheal samples. All paired sera (9/9) collected from febrile horses showed seroconversion by neutralization test. Sequence and phylogenetic analysis of the ECoV isolated showed that putative amino acid sequences in S and N genes were highly conserved among ECoV strains. In contrast, sequences of the region coding 4.7kDa non-structural protein (p4.7) differed among the strains. Because of the diversity of the p4.7 region, this region should be useful for epidemiological investigation of ECoV.
KEY WORDS: equine, equine coronavirus, Obihiro12-1, Obihiro12-2, outbreak.

Coronaviruses are large, enveloped, positive-stranded RNA viruses that can cause respiratory and gastrointestinal diseases in many avian and mammalian species [11]. On the basis of serological and genetic differences, coronaviruses are categorized into four different genera, *Alphacoronavirus*, *Betacoronavirus*, *Gammacoronavirus* and *Deltacoronavirus* [1, 21]. Equine coronavirus (ECoV) belongs to the species *Betacoronavirus 1* in the genus *Betacoronavirus* [24], which includes bovine coronavirus (BCoV) [20], a bovine-like coronavirus isolated from ruminants [2, 9], human coronavirus (HCoV) 4408 [25], HCoV OC43 [19], porcine hemagglutinating encephalomyelitis virus (PHEV) [19], canine respiratory coronavirus (CRCoV) [6] and bubaline coronavirus (BuCoV) [5].

ECoV-like agents have been detected by electron microscopy [3, 10] and antigen-capture ELISA in fecal samples and by immunohistochemistry in an intestinal tissue collected from a diarrheal horse [4]. ECoV strains were isolated from diarrheal samples in 1999 (NC99 strain) in the U.S.A. [8] and in 2009 in Japan (Tokachi09 strain) [16]. Outbreaks associated with ECoV occurred in 2011 and 2012 in the U.S.A. [17]. In 2004 and 2009, ECoV outbreaks occurred at Obihiro Racecourse in Hokkaido, Japan [14, 16]; they were followed by a third outbreak in adult horses at the same location between February and April 2012. Here, we report the clinical and epidemiological features and molecular characterization of the ECoV isolated in this third Japanese outbreak.

We collected fecal samples, nasal swabs and paired sera from 9 pyretic horses and a fecal sample and a nasal swab from 1 pyretic horse. Paired sera were collected at the onset of pyrexia and 19 to 22 days later. EDTA bloods were collected from another 9 pyretic horses. White blood cell counts were performed commercially on the EDTA bloods at Dai-ichi Kishimoto Laboratories Inc. (Obihiro, Japan).

Fecal samples were diluted 1: 10 in Dulbecco's modified Eagle's medium. Nasal swabs were suspended in 2 m*l* saline. Fecal suspensions were clarified by centrifugation at 2,000 $\times g$ for 10 min and used for virus isolation and reverse transcription-polymerase chain reaction (RT-PCR). Viral RNA was extracted from fecal samples and nasal swabs by using a High Pure Viral RNA Kit (Roche Diagnostics GmbH, Mannheim, Germany) in accordance with the manufacturer's instructions. RT-PCR was performed with a primer pair targeting the N gene of coronavirus using an OneStep RT-PCR kit (Qiagen GmbH, Hilden, Germany) [16]. PCR was also performed to detect the specific genes of equid herpesvirus (EHV) 1 and 4 in nasal swabs [12]. Rapid antigen detection kits were used to detect equine influenza virus in nasal swabs (Espline Influenza A&B-N, Fujirebio Inc., Tokyo, Japan) [22] and equine rotavirus in fecal samples (Dipstick "Eiken" Rota, Eiken Chemical Co., Ltd., Shimotsuga-gun, Japan) [15]. MLCB agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) were used to isolate *Salmonella* species from samples selectively enriched with Hajna tetrathionate broth (Eiken Chemical Co., Ltd.).

ECoV was isolated by using HRT-18G cells. Electron microscopy was performed on the supernatant of the HRT-18G cell cultures to observe the morphology of virus particles, as described previously [16]. The neutralization test for ECoV was performed with HRT-18G cells, as described previously [16]. Virus-neutralizing antibody titers were expressed as

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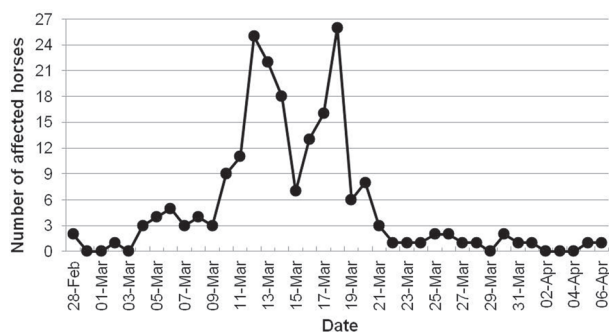
Fig. 1.   Numbers of horses newly affected by equine coronavirus at Obihiro Racecourse between 28 February and 6 April 2012.

Table 1.   Antibody titers of paired sera of febrile horses against Obihiro12-1 and Tokachi09 strains

Horse no.	Age	Obihiro12-1		Tokachi09	
		Pre	Post	Pre	Post
1	4	<2[b]	32	2	128
2	3	<2	64	2	128
3	4	<2	1,024	2	256
4	7	8	256	32	512
5	4	<2	2,048	16	4,096<[c]
6	2	8	1,024	256	1,024
7	3	<2	2,048	4	512
8	8	<2	1,024	64	1,024
9	2	<2	32	16	256
GMT[a]		1.6	348.4	11.8	512

a) Geometric mean titer, b) The titer was calculated as 1, c) The titer was calculated as 8,192.

the reciprocal of the highest serum dilution that inhibited cytopathic effects (CPE). Seroconversion was defined as a 4-fold or greater increase in antibody titers between paired sera. Type-specific ELISA to detect EHV-1 and -4 antibodies was performed as described previously [23].

The genes encoding S and N structural proteins and also those of non-structural proteins from 4.7 kDa (p4.7) to 12.7 kDa (p12.7) were sequenced commercially at the Dragon Genomics Center (Takara Bio Inc., Otsu, Japan). A preliminary analysis was performed via sequencing alignment using the web-based BLAST and CLUSTALW programs and Vector NTI Advance 11 software (Invitrogen, Carlsbad, CA, U.S.A.). Phylogenetic analysis of amino acid sequences was conducted with MEGA software Version 5.0 [18]. Phylogenetic trees based on S and N amino acid sequences were constructed by using the neighbor-joining method. Statistical analysis of the tree was performed with the bootstrap test (1,000 replicates) for multiple alignments. The nucleotide sequences of the S and N genes have been deposited in Gen-Bank/EMBL/DDBJ under the accession numbers AB775894 and AB775893, respectively.

From late February to early April 2012, an outbreak of disease with signs of pyrexia and anorexia occurred in draft horses at the Obihiro Racecourse in Hokkaido, Japan (Fig. 1). In this outbreak, 204 of about 650 horses became sick; the diseased horses ranged in age from 2 to 11 years (median 3 years). The rectal temperatures of these horses ranged from 37.5 to 41.5°C (median 39.6°C), and 196 horses were febrile (≥38.5°C). Among the diseased horses, 19 had diarrhea, 5 had colic and 3 had both. The white blood cell counts of 9 febrile horses ranged from 1,300 to 6,500 (median 3,300) cells/μl, and leucopenia (<4,500 cells/μl) was observed in 7 horses. Most horses recovered in 2 to 4 days with fluid therapy to prevent dehydration and antibiotics to prevent secondary infection. However, horses with watery diarrhea required 5 to 10 days to recover, and 2 horses with severe watery diarrhea died during the outbreak.

Out of 10 fecal samples tested, 5 were positive for ECoV by RT-PCR. All fecal samples were negative for equine rotavirus and *Salmonella* species. One horse with fever and nasal discharge was positive for EHV-4 in a nasal swab and for ECoV in a fecal sample. All nasal swabs were negative

for EHV-1 and equine influenza virus, as well as for ECoV. After the second passage, CPE appeared in HRT-18G cells inoculated with 2 ECoV-positive samples collected from horses with watery diarrhea. These isolates were named Obihiro12-1 and Obihiro12-2. Electron microscopy of the culture supernatants revealed the typical morphology of coronaviruses—round or petal-shaped particles 80 to 180 nm in diameter (data not shown). No other virus-like particles were observed in the culture supernatants. Upon neutralization testing, all paired sera showed seroconversion (>4-fold increase) to Obihiro12-1 strain (Table 1). One horse, the nasal swab of which was positive for EHV-4 by PCR, showed seroconversion to ECoV and EHV-4 (data not shown).

Sequence analysis was performed for the S (4,092 nucleotides and 1,363 amino acids) and N (1,341 nucleotides and 446 amino acids) genes of Obihiro12-1. The amino acid sequence identities between Obihiro12-1 and 2 other ECoVs—the NC99 and Tokachi09 strains—were 98.6% and 98.7%, respectively, in S protein and 97.5% and 98.9% in N protein. Phylogenetic analyses were performed for the amino acid sequences of S and N proteins (Fig. 2). Phylogenetic analyses using the *Betacoronavirus 1* strains showed that Obihiro12-1 was clustered with the other ECoVs. Analysis of the region from the p4.7 to p12.7 genes in Obihiro12-1, NC99 and Tokachi09 revealed 541, 581 and 396 base pairs, respectively (Fig. 3). Compared with NC99, Obihiro12-1 had deletions of a total of 40 nucleotides within p4.7 and the non-coding region following the p4.7 gene. These deletions were confirmed by sequence analysis using the RT-PCR product amplified from the original fecal sample (data not shown). The amino acid sequence identities of p12.7 protein were 99.1% and 99.1% between Obihiro12-1 and the other two ECoVs (strains NC99 and Tokachi09), respectively. The p4.7 to p12.7 sequence of Obihiro12-2 was the same as that of Obihiro12-1.

Serological tests (Table 1) showed that ECoV had caused the outbreak. Most affected horses developed anorexia, pyrexia and leucopenia. Gastrointestinal disease was observed
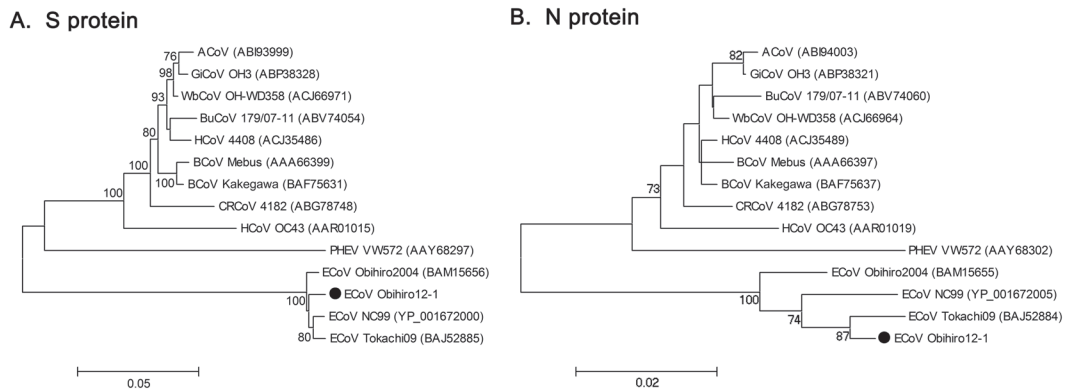
A. S protein

B. N protein

Fig. 2. Phylogenetic analysis of the amino acid sequences of the (A) S protein and (B) N protein of *Betacoronavirus 1* strains. ECoV Obihiro2004 was the ECoV associated with the 2004 outbreak at Obihiro racecourse [14]. Black dots show Obihiro12-1. Percentages of bootstrap support are indicated by the values at each node; values of<70 have been omitted. ACoV, alpaca coronavirus; BCoV, bovine coronavirus; BuCoV, bubaline coronavirus; CRCoV, canine respiratory coronavirus; ECoV, equine coronavirus; GiCoV, giraffe coronavirus; HCoV, human coronavirus; PHEV, porcine hemagglutinating encephalomyelitis virus; WbCoV, waterbuck coronavirus.

in 13.2% of diseased horses, regardless of age. ECoV infection can reliably be characterized by these clinical signs, because these features were similar to those in other reported outbreaks [14, 16, 17]. The affected horses generally recovered well with treatment for dehydration and secondary infection. However, two died during the outbreak; these horses showed stomatitis and severe watery diarrhea. One of the horses that died was positive for ECoV by neutralizing test using paired sera; the status of the other was unknown, because no samples were tested. ECoV likely caused the clinical signs in the ECoV-positive horse that died. Antibody titers against ECoV Obihiro12-1 strain were low in the pre-sera (Table 1); these low antibody titers against ECoV also occurred in pre-sera in the 2009 outbreak. Antibody titers of two horses (Nos. 4 and 8), which experienced the 2009 ECoV outbreak, against Tokachi09 strain were higher than those against Obihiro12-1 strain in the pre-sera. This result suggests that antigenic characteristics were slightly different between Obihiro12-1 and Tokachi09 strains. Although the invasion route is unclear, resurgence of ECoV may be caused by a reduction in the herd immunity of horses against ECoV [16] and by antigenic shift of ECoV.

Two previous ECoV outbreaks had been reported at this racecourse. The first occurred from December 2004 to March 2005 [14] and the second from June to August 2009 [16]. This third outbreak occurred from February to April 2012 (Fig. 1). The numbers of horses affected in the first, second and third outbreaks were 191, 132 and 204, respectively. About 20% to 30% of horses were affected in each outbreak. In 4 states of the U.S.A., such outbreaks have occurred in November 2011 and February and April 2012, and 20% to 57% of horses have been affected each time. These epidemiological data indicate that such outbreaks become epidemics regardless of the season and have high morbidity rates.

We sequenced only S, N and the region coding p4.7 to

p12.7 genes, and therefore other genes of the isolates should be sequenced and analyzed for further understanding of ECoV. Sequence and phylogenetic analysis showed that structural proteins (S and N) were highly conserved among ECoV strains (Fig. 2). The p4.7 to p12.7 genes in ECoV, which corresponds to p4.9, p4.8 and p12.7 genes in BCoV [7], are between S and envelope genes in *Betacoronavirus 1*. In this region, CRCoVs have 3 (p4.9, p2.7 and p12.8) genes or 2 (p8.8 and p12.8) genes depending on virus strains [6, 13]. The functions of these non-structural proteins are unclear in *Betacoronairus 1*. The p4.7 gene differed, while p12.8 gene highly conserved among the 3 ECoV strains (Fig. 3). Deletions in the p4.7 gene were observed in Obihiro12-1 and Tokachi09. These deletions showed that the p4.7 gene was not important for propagation of ECoV [16], while the conserved p12.7 gene may be important for propagation. The region between S and p12.7 or p12.8 genes widely varies in *Betacoronavirus 1* including ECoV. This region may relate to be specificity of species susceptible to each *Betacoronavirus 1*. In addition, the polymorphism of the p4.7 region suggests that this region should be useful for epidemiological investigation of origin of ECoVs. The differences in the p4.7 region in Obihiro12-1 and Tokachi09 showed that this outbreak was caused by a strain different from the one that caused the 2009 outbreak.

p4.7 gene
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Obihiro12-1   1:ATGACGATTAATTTCGTCATTT-AACTATTTTATATGA──────────────────────── 37
Tokachi09     1:ATGACGATTAATTT─────────────────────────────────────────────── 14
NC99          1:ATGACGATTAATTTCGTCATTTTAACTATTTTATATGACCTGCCTTTGTGGGTTAAACTT 60
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              ─────────────────────────────────────────────────────────────────►
Obihiro12-1  38:────CAACCACTCATGGGTGTAC───TATTAATACTAGTACAGCTCTACATTGGC───TT 87
Tokachi09    14:─────────────────────────────────────────────────────────── 14
NC99         61:GCTACAACCACTCATGGGTGTACTAATATTAATACTAGTACAGCTCTACATTGGCTTATT 120
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              ──►
Obihiro12-1  88:TAGGCATTTT───────GTTAACACAGCCACCATCGGGTTAAACCGGTTTATGGTGCTAG 140
Tokachi09    14:─────────────────────────────────────────────────────────── 14
NC99        121:TAGGCATTTTTATATGTGTTAACACCACCACCATCGGGTTAAACCGGTTTATGGTGCTAG 180
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Obihiro12-1 141:TGCTAAATTATATTTTGTTATACTTTATAACTTTAAGCATTTGCTAAAGTTTTTAAGGCC 200
Tokachi09    15:────────────────────ATACTTTATAACTTTAAGCATTTGCTAAAGTTCTTAAGGCC 55
NC99        181:TGCCAAATTATATTTTGTTATACTTTATAACTTTAAGCAGTTGCTAAAGTTCTTAAGGCC 240
                                    ******************** *********** ********
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p12.7 gene
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              ─────────────────────────────────────────────────────────────────►
Obihiro12-1 201:AACCCTTATTAATGGACATCTGGAGACCTGAGAGGAAATTTCTCCGTTTTACTAACGGAT 260
Tokachi09    56:AACCCTTATTAATGGACATCTGGAGACCTGAGAGGAAATTTCTCCGTTTTACTAACGGAT 115
NC99        241:AACCCTTATTAATGGACATCTGGAGACCTGAGAGGAAATTTCTCCGTTTTACTAACGGAT 300
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Obihiro12-1 261:TTAACGTCCCAGAATTAGAAGATGTCTGTTTTAAATTTAATTACCAATTGCCTAAAGTAG 320
Tokachi09   116:TTAATGTCCCAGAATTAGAAGATGTCTGTTTTAAATTTAATTACCAATTTCCTAAAGTAG 175
NC99        301:TTAACGTCCCAGAATTAGAAGATGTCTGTTTTAAATTTAATTACCAATTTCCTAAAGTAG 360
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              ─────────────────────────────────────────────────────────────────►
Obihiro12-1 321:GATATTGTAGAGTTCCTAATTATGCTTGGTGTCGTAATCAAGGTAGCTTTTGTGCTACAT 380
Tokachi09   176:GATATTGTAGAGTTCCTAATTATGCTTGGTGTCGTAATCAAGGTAGCTTTTGTGCTACAT 235
NC99        361:GATATTGTAGAGTTCCTAATTATGCTTGGTGTCGTAATCAAGGTAGCTTTTGTGCTACAT 420
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Obihiro12-1 381:TTACCCTTTACGGCAAATCCAAACATTATGATAAATATTTTAGAATAATAACTGGTTTCA 440
Tokachi09   236:TTACCCTTTACGGCAAATCCAAACATTATGATAAATATTTTAGAATAATAACTGGTTTCA 295
NC99        421:TCACCCTTTACGGCAAATCCAAACATTATGATAAATATTTTAGAATAATAACTGGTTTCA 480
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Obihiro12-1 441:CAGCGTTCGCCAATACTATAGAGGAGGCTGTTAATAAACTGGTTTTAGAGGCTGTTGATT 500
Tokachi09   296:CAGCGTTCGCTAATACTATAGAGGAGGCTGTTAACAAACTGGTTTTAGAGGCTGTTGATT 355
NC99        481:CAGCGTTCGCCAATACTATAGAGGAGGCTGTTAATAAACTGGTTTTAGAGGCTGTTGATT 540
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              ──────────────────────────────────────►
Obihiro12-1 501:TTATTATCTGGCGTAGCCAGAATTTAAATGCTTATGGCTGA                     541
Tokachi09   356:TTATTATCTGGCGTAGCCAGAATTTAAATGCTTATGGCTGA                     396
NC99        541:TTATTATCTGGCGTAGCCAGAATTTAAATGCTTATGGCTGA                     581
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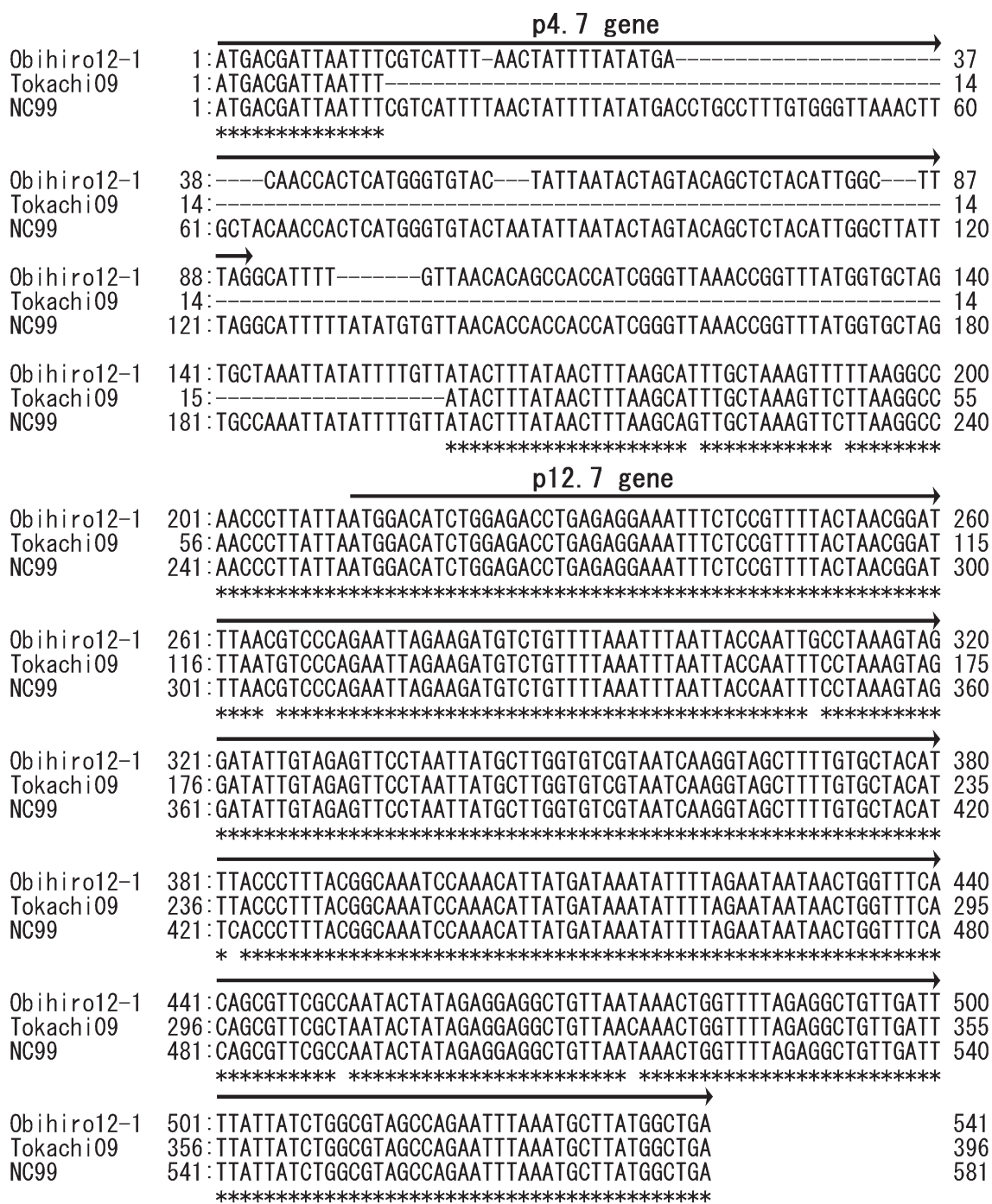
Fig. 3.   Alignment of the nucleotide sequences of the region from the p4.7 to p12.7 genes of the ECoV NC99, Tokachi09 and Obihiro12-1 strains. Minus signs (−) show missing nucleotides and asterisks (*) show conserved nucleotides.

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