Studies on the Epidemiology and Clinicopathology of Equine Coronavirus Infection

ウマコロナウイルス感染症の疫学及び臨床病理学的性状に関する研究

Joint Graduate School of Veterinary Medicine

Yamaguchi University

Yoshinori Kambayashi

March 2024

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GENERAL INTRODUCTION

Coronaviruses (CoVs) are members of the subfamily *Orthocoronavirinae* in the family *Coronaviridae*. They are enveloped viruses with a positive-sense and single-stranded RNA genome of approximately 26–32 kilobases in size, which is the largest genome of all known RNA viruses [58]. Based on phylogenetic relationships and genome structures, this subfamily is divided into four genera — *Alphacoronaviruses* (α-CoVs), *Betacoronaviruses* (β-CoVs), *Gammacoronaviruses* (γ-CoVs), and *Deltacoronaviruses* (Δ-CoVs). α-CoVs and β-CoVs originate from bats and predominantly infect mammals, while γ-CoVs and Δ-CoVs originate from birds and infect primarily birds and marine mammals [61].

CoVs commonly cause respiratory disease in mammals and birds. So far, seven different human coronavirus (HCoV) species consisting of HCoV-NL63, HCoV-229E, HCoV-OC43, HCoV-HKU1, severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome coronavirus (MERS-CoV), and SARS-CoV-2 has been reported [20, 53]. SARS-CoV-2 is the recently emerged CoVs species that resulted in a worldwide pandemic. The phylogenetic tree based on the full genome of CoVs shows that HCoV-NL63 and 229E belong to α-CoVs, and the rest of HCoVs belong to β-CoVs. Among the β-CoVs species, HCoV-OC43 and HKU1, SARS-CoV and SARS-CoV-2, and MERS-CoV belongs to subgenera *Embecovirus*, *Sarbecovirus*, and *Merbecovirus*, respectively [53]. HCoV-NL63, 229E, OC43, and HKU1 species are circulating around the world and generally cause mild upper respiratory tract illnesses characterized by cough, nasal discharge, sneezing and a sore throat, especially during the cold season [22]. In contrast, SARS-CoV and MERS-CoV can cause more severe symptoms. Patients initially show fever, myalgia, headache, and malaise. In severe cases,

patients suffer from dyspnea and respiratory distress leading to pneumonia [63, 65]. Approximately 30–40% of the patients also show gastrointestinal symptoms such as diarrhea and vomiting. At the end of 2019, a novel CoV, SARS-CoV-2, emerged in the city of Wuhan, China and is still spreading and threatening human life around the world [64]. SARS-CoV-2 causes coronavirus disease 2019 (COVID-19) represented mainly by respiratory symptoms as well as headache, diarrhea, vomiting, and olfactory and taste disorders [20]. The World Health Organization has reported that, by January 2024, approximately seven million people have died due to a SARS-CoV-2 infection [https://covid19.who.int/].

Infection with CoVs has also been reported in a variety of animal species including domestic, companion, and wild animals [17, 57, 60]. Transmissible gastroenteritis virus (TGEV), porcine epidemic diarrhea virus, porcine respiratory coronavirus, and feline infectious peritonitis virus belong to α-CoVs. In β-CoVs, β-CoV 1 species in the subgenus *Embecovirus* includes porcine hemagglutinating encephalomyelitis virus (PHEV), canine respiratory coronavirus, and bovine coronavirus (BCoV). *Sarbecovirus*, *Merbecovirus*, *Hibecovirus*, and *Nobecovirus* include some bat derived CoVs. γ-CoVs include avian coronaviruses such as infectious bronchitis virus (IBV) and a few virus species originating from marine mammals. Δ-CoVs includes several avian and swine coronaviruses.

The association of CoV infection in horses was first reported in 1975 [2]. CoV-like particles were detected by electron microscopy (EM) in the feces of three diarrheic foals that had died or were euthanized due to severe enterocolitis. CoV-like particles were also detected in the feces collected from diarrheic foals in New Zealand [10] and adult horses presenting with fever and diarrhea in the USA [21]. These reports suggest that CoVs are

associated with diarrhea in horses, as are rotaviruses, also known to cause enteric disease [11, 29]. A comprehensive survey to estimate the prevalence of enteric pathogens showed that CoVs particles were also detected in healthy horses [4]. Davis *et al.* performed a serological test with paired sera using BCoV as an antigen, fecal antigen-capture enzyme-linked immunosorbent assay (ELISA), and intestinal immunohistochemistry (IHC) using anti-BCoV antibodies to demonstrate CoV involvement in severe diarrhea in a neonatal foal [9]. Since there were no isolated equine CoV (ECoV) strains at the time, these tests were performed using BCoV as the antigen or antibodies against BCoV. The increase of serum antibody titers and confirmed CoV antigen captured in the feces and intestine demonstrated the association of this case with a CoV infection. Furthermore, these results suggested that ECoV belongs to the same genus β-CoVs as BCoV, due to ECoV and BCoV showing antigenic cross-reactivity.

The isolation of ECoV from fecal samples of an Arabian foal with diarrhea was first reported in North Carolina, USA in 2000 [16]. The isolated CoV strain was designated as NC99. In the virus neutralization (VN) test, NC99 showed high cross-reactivity with BCoV but not with PHEV and TGEV. In addition, the amino acid sequence analysis revealed that the nucleocapsid (N) protein sequence of NC99 had a higher identity with that of BCoV and HCoV-OC43 than those of α-CoVs (TGEV and HCoV-229E) and γ-CoVs (IBV and turkey CoV). Phylogenetic analysis further supported these results. This study strongly suggested that ECoV is a member of β-CoVs and is most closely related to BCoV.

The first complete genome sequence of ECoV strain NC99 was determined in 2007 by Zhang *et al.* [62]. It was shown that the NC99 genome consists of 30,992 nucleotides (nt) and contains 11 open reading frames (ORFs): 1a, 1b, 2–8, 9a, and 9b. The 5'-proximal

two-thirds of the genome consist of ORFs 1a and 1b, which encode the replicase polyprotein (pp) 1a and pp1ab. They are processed into 16 non-structural proteins (nsp 1–16) that regulate viral replication and transcription. ORF2 encodes the NS2 protein, but its function is not well known. ORF3, 4, 7, 8, and 9a encode the structural proteins of hemagglutinin esterase (HE), spike (S), envelope (E), membrane (M), and nucleocapsid (N), respectively. ORF9b encodes the hypothetical protein (I), and this region is included in ORF9a which encodes the N protein. ORF5 encodes a hypothetical protein with an estimated molecular weight of 4.7 kDa which is designated as the p4.7 protein. ORF6 encodes a protein corresponding to the BCoV 12.7 kDa non-structural protein (p12.7). Phylogenetic analysis based on amino acid sequences of pp1a, pp1ab, S, E, M, and N proteins showed that NC99 is most closely related to BCoV, HCoV-OC43, and PHEV. Therefore, ECoV was proven to belong to the β-CoV1 species in the subgenus *Embecovirus* in the genus β-CoVs.

ECoV commonly causes fever, lethargy, and anorexia [46]. Some affected horses show gastrointestinal signs represented by diarrhea as shown in the previous studies, but less than 30% of affected horses show these signs [40, 46]. ECoV infection is usually self-limiting, and most affected horses recover within a few days without intensive treatment [15]. However, severely affected horses occasionally present with acute progression of necrotizing enterocolitis or neurological disorders such as depression, ataxia, and recumbency [12, 46]. Neurological signs are associated with encephalopathy, which is predicted to be caused by hyperammonemia [12, 13]. Hyperammonemia is likely to be caused by an increased production and absorption of ammonia within the gastrointestinal tract. ECoV is known to be primarily transmitted via the fecal-oral route, and infected horses shed a large amount of the virus in their feces [36, 50]. Historically,

detection of ECoV has relied on EM or antigen-capture ELISA [10, 21], but these methods lack sensitivity unless sufficient numbers of viral particles are present in clinical samples. Nowadays, real-time RT-PCR (qPCR) has replaced these conventional assays for detecting viral RNA because of its high throughput capability, short turnaround time, and high sensitivity and specificity [6, 46].

As there is no antiviral agent for ECoV, infected horses are usually treated conservatively with fluid therapy to prevent dehydration and/or antibiotics to prevent secondary bacterial infection. So far, no vaccine has been developed for ECoV. Inoculation of horses with a modified-live BCoV vaccine was proven to be safe but did not induce a significant increase in serum titers against ECoV [43]. In contrast, another study shows that inoculation with an inactivated BCoV vaccine increased the VN titer against ECoV at 14 days post inoculation (dpi), but the titer against ECoV was much lower than that against BCoV [31]. Thus, it is still unclear whether the BCoV vaccine prevents ECoV infection.

Since 2010, several outbreaks of ECoV infection have been reported in the USA, France, Switzerland, and Japan [19, 27, 40, 46]. Morbidity rates varied among reports, ranging from 17–57% at each premise [15, 40, 46]. Mortality rates also varied among these outbreaks, ranging from 0–27% [12, 15, 40, 46]. In Japan, there were three outbreaks of ECoV infection among adult draft horses in 2005, 2009, and 2012 at the same premise, Obihiro racetrack in Hokkaido [28, 40, 41]. However, there was no ECoV outbreak involving other horse breeds including Thoroughbreds in Japan. ECoV genes have also been detected sporadically in the feces collected from diarrheic horses in France, the United Kingdom, Ireland, and Oman [5, 18, 27, 30]. One study showed that similar proportions of healthy and diarrheic foals shed ECoV in their feces [52]. In Japan, RT-

loop-mediated isothermal amplification (RT-LAMP) and qPCR were performed on rectal swabs from 307 diarrheic and 120 healthy Thoroughbred foals that were reared in Hokkaido, the largest breeding area in Japan [32, 34]. ECoV RNA was detected in only three healthy foals, and no diarrheic foal tested positive, suggesting that the virus is not associated with diarrhea in foals in this district. However, the prevalence of ECoV in the yearling or racehorse populations has never been investigated in Japan.

The duration of viral shedding in the feces varies among studies. Two experimental studies showed that horses began to shed the virus in their feces at 2–3 dpi and the shedding continued for up to 14 days [36, 50]. In some outbreaks, the duration of viral shedding in naturally infected horses ranged from 2 to 11 days [12, 46]. In an outbreak in the American miniature horse population, three horses shed the virus for relatively long periods of at least 18, 22, and 25 days [15]. It was reported that ECoV infected horses that died had significantly higher viral loads than the surviving horses [12]. In contrast, other studies showed that there was no correlation between the viral cycle threshold (Ct) values or the number of ECoV RNA present in fecal samples and the presence of clinical signs [15, 46]. Therefore, it remains controversial whether there is a correlation between the viral load and the presence or severity of clinical signs.

The characteristic clinical sign of ECoV infection is diarrhea, and ECoV RNA is detected in the feces and small intestinal contents [12, 46]. Histological evaluation in fatal cases has shown a variety of findings consistent with severe diffuse necrotizing enteritis. IHC staining using monoclonal antibodies against BCoV has identified the presence of coronaviral antigen in the superficial necrotic layer, cytoplasm of crypt enterocytes, undefined cells within the lamina propria, and rarely in the submucosa [9, 13]. These studies suggest that ECoV possesses a strong tropism toward infecting

enterocytes and other cells in the intestine. On the other hand, ECoV RNA has been detected in nasal swabs from horses that were naturally and experimentally infected with ECoV [36, 44, 50]. Furthermore, respiratory specimen collected from a foal with respiratory signs tested positive for ECoV, although fecal specimen was not tested [27]. BCoV, which is most closely related to ECoV phylogenetically, has been associated with the development of bovine respiratory disease complex and causes mild respiratory disease or pneumonia in growing calves [47, 48]. However, unlike BCoV, the etiological role of ECoV in respiratory tissues has not been thoroughly determined, and in some studies, the detection of viral RNA in nasal swabs was interpreted as environmental contamination from excreted feces containing ECoV [36, 44]. Thus, whether ECoV can infect the respiratory tissues is still unclear.

Although CoV has been known to be associated with equine diarrhea since the 1970s, it was only around 20 years ago that ECoV was first isolated [16]. Moreover, there are few reports describing the epidemiology and pathology of ECoV infection, and the information on this disease is insufficient. Here, the author has experienced an outbreak of ECoV infection at the Japan Racing Association (JRA) facility and had the opportunity to conduct an epidemiological survey and an experimental infection study. In this thesis, three studies are reported to elucidate the epidemiological and clinicopathological characteristics of ECoV infection and to evaluate the potential risk of this disease to the horse industry in Japan.

In CHAPTER 1, the author describes an outbreak of the ECoV infection that occurred among 41 riding horses on the same premise. So far, this is the first outbreak report involving equine breeds other than draft horses in Japan. The epidemiologic and clinical features of this outbreak as well as a genomic analysis of the ECoV responsible are

described. In CHAPTER 2, a serological surveillance was performed in the population of Thoroughbred yearlings and racehorses in Japan. The epidemiological characteristics of ECoV infection in each population and the impact of this disease on the horse industry in Japan were evaluated. In CHAPTER 3, the intestinal and respiratory tract tissues collected from horses experimentally infected with ECoV were analyzed by qPCR and *in situ* hybridization (ISH). This study investigated the tissue tropism and distribution of the virus to elucidate the etiological and pathological aspects of ECoV infection.

CHAPTER 1

Outbreak of equine coronavirus infection among riding horses in Tokyo, Japan

Summary

Before 2020, all confirmed outbreaks of ECoV infection in Japan had occurred in a herd of draft horses. In 2020, an outbreak of ECoV infection occurred among 41 horses at a riding stable in Tokyo, Japan. This stable housed 16 Thoroughbreds and 25 horses of other breeds, including Andalusians, ponies, and miniature horses. During the outbreak, 15 horses (37%) were affected, and fever was the most common clinical sign, followed by lethargy, anorexia, and diarrhea. All affected horses recovered within three days of disease onset. The VN test using paired sera confirmed that all 41 horses were infected with ECoV, suggesting that ECoV is highly contagious. Of the infected horses, 26 horses (63%) did not show any clinical signs, and longer viral shedding period was recorded in asymptomatic horses. These results suggest that subclinical horses could play an important role as spreaders. The viral shedding period in non-Thoroughbreds was significantly longer than that observed in Thoroughbreds. This result suggests that there is a difference in the persistence of ECoV among horse breeds and that some breeds can contribute to the spread of the virus. A genome sequence analysis revealed that the nucleotide lengths from genes *p4.7* to *p12.7* or *NS2* of the virus causing this outbreak differed from those of ECoVs detected in the previous outbreaks, suggesting that a different virus was responsible for the current outbreak.

Introduction

ECoV causes fever, anorexia, lethargy, and gastrointestinal signs such as diarrhea and colic in foals and adult horses [45]. Most affected horses show mild clinical signs and recover within a few days without the need for intensive care [15]. ECoV infection is occasionally fatal when the affected horses present with severe watery diarrhea or neurological disorders due to hyperammonemia [12]. Several outbreaks of ECoV have occurred in the USA, France, and Switzerland [15, 19, 27, 46], in which 17–57% of horses were affected at each premise. In Japan, there have been three outbreaks of ECoV infection among approximately 600 draft horses at the Obihiro racetrack in 2004, 2009, and 2012 [28, 40, 41]. Three ECoV strains—Tokachi09 in 2009 [41] and Obihiro12-1 and Obihiro12-2 in 2012 [40]—were isolated from adult horses with fever or diarrhea during these outbreaks, and the complete genome sequences were determined [33]. Although neurological disease was not observed in any of the outbreaks, two horses died due to severe enterocolitis in the most recent outbreak in 2012. However, these three outbreaks occurred in a population of draft horses, and there have been no reported ECoV outbreaks involving horse breeds other than draft horses in Japan. In 2020, the fourth outbreak of ECoV infection has occurred in a herd of 41 riding horses composed of various breeds, including Thoroughbreds and eight other breeds. This study describes the epidemiology and clinical features of this outbreak and a genomic analysis of the ECoV detected.

Materials and Methods

Horse population

An ECoV outbreak occurred in the riding stable at Tokyo racecourse, which is managed by the JRA. The stable housed 41 horses: 16 Thoroughbreds, 6 Andalusians, 5

ponies, 4 miniature horses, 3 Japanese-bred riding horses, 2 Japanese sport horses, 2 Friesians, 2 Lusitanos and 1 mixed breed. These horses were reared for a variety of uses such as horse shows, jumping, pets, and race leading. Detailed information is shown in Table 1. The age of the horses ranged from 1 to 19 years old (mean, 10.8 years). Almost all horses were housed in individual stalls, while four miniature horses shared the stalls according to their sex (two males and two females). Rectal temperature, clinical signs, and changes in fecal characteristics were monitored daily by the horse grooms for all horses throughout the duration of this outbreak. When abnormalities were observed, a veterinarian assessed their condition and treated them as needed.

Molecular detection of viral DNA and RNA

qPCR was performed on the fecal samples to detect ECoV RNA. Fresh feces were collected from each stall once a week after the onset of the outbreak. Although qPCR was performed on all horses in the barn regardless of whether they showed clinical signs or not, the first day of testing was different for each horse. Feces were temporarily stored at −20 °C at the veterinary clinic in the Tokyo racecourse and transported to the JRA Equine Research Institute (Tochigi Prefecture). Upon arrival, they were stored at −80 °C until testing.

Samples were diluted 1:10 in phosphate-buffered saline (PBS), and the suspensions were mixed vigorously for 1 min and centrifuged at 3000 × *g* for 10 min at 4 °C. Nucleic acid was extracted from 100 µL of supernatant with an automated nucleic acid extractor (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche Diagnostics GmbH, Mannheim, Germany). Primers and the MGB probe (Thermo Fisher Scientific, Waltham, MA, USA) to detect the *N* gene of ECoV were selected based on a previous report [46]. qPCR was

conducted using TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific) according to the manufacturer's instructions. The thermal cycling protocol applied was as follows: 50 °C for 5 min, 95 °C for 20 sec, 40 cycles at 95 °C for 3 sec, and 60 °C for 30 sec. All tests were performed in triplicates. Positive diagnosis was based on any one of the three Ct values being ≤ 40. To confirm that the extraction method was suitable for qPCR, a housekeeping gene (β2-microglobulin) was used as positive control in all extracted samples according to the manufacturer's instruction (Assay ID: Ec03468699_m1, TaqMan Gene Expression Assays, Thermo Fisher Scientific). qPCR was performed continuously until each horse tested negative twice in a row. The number of days from the date of the first positive result to the date of the last positive result was defined as the viral shedding period for each horse.

Molecular diagnosis was conducted for other viral pathogens causing fever in horses prior to the first detection of an ECoV case. Nucleic acid was extracted from nasal swabs and whole blood samples as described for feces. Conventional PCR using nasal swabs and blood samples was performed to detect DNA of equid alphaherpesvirus 1 and 4 [26]. qPCR (Cobas Influenza A/B & RSV; Roche Diagnostics GmbH) using nasal swabs was performed to detect equine influenza virus RNA [35]. Conventional RT-PCR using blood and nasal swabs was performed to detect Getah virus RNA [59] and equine arteritis virus RNA [1].

VN test

The VN test to detect antibodies against ECoV was performed with paired sera collected from all 41 horses. First sera were collected between March 27 and April 3, 2020, and second sera were collected from all horses on April 22. HRT-18G cells (ATCC,

#CRL-11663) derived from human colorectal adenocarcinoma were cultured in RPMI-1640 medium (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific), a mixed solution of 100 units/mL of penicillin and 100 μg/mL of streptomycin (Nacalai Tesque, Inc., Kyoto, Japan), and 1 mM of sodium pyruvate (Sigma-Aldrich Co.). HRT-18G cells were seeded at 5.5 × 10^4/well on flat-bottomed 96-well plates (AGC Techno Glass Co., Shizuoka, Japan) and were incubated for 24 h at 37 °C in 5% CO_2. Sera were diluted at a ratio of 1:4 with Minimum Essential Medium (MP Biomedicals, Santa Ana, CA, USA) containing 2% FBS and inactivated at 56 °C for 30 min. Fifty-microliter of serially diluted sera from 1:8 to 1:1024 were mixed with an equal volume of virus suspension (NC99, 200 $TCID_{50}$/50 μL) and incubated for 1 h at 37 °C in 5% CO_2. HRT-18G cells were then inoculated with 100 μL of the viral mixture and incubated at 37 °C and 5% CO_2 for 5 days. Antibody titers were expressed as the reciprocal of the highest serum dilution that completely inhibited cytopathic effects. A 4-fold or greater increase in VN antibody titer between paired sera was defined as seroconversion.

Sequence and phylogenetic analysis of ECoV

Viral RNA was extracted from the sample collected from a horse which showed mild diarrhea (#14 in Table 1). RT-PCR was performed with seven primer sets (Table 2) [33] using RT-PCR kits (PrimeScript II High Fidelity RT-PCR Kit and PrimeScript II High Fidelity One Step RT-PCR Kit, Takara, Shiga, Japan). The PCR amplicons were sequenced using Ion Torrent technology (Thermo Fisher Scientific) according to the manufacturer's instructions. The average coverage was about 9800×, and sequence analysis was performed using the Geneious Prime v. 2021.0.1 software (Biomatters,

Auckland, New Zealand). Phylogenetic trees were constructed in the Mega7 software [25] from nucleotide sequences using the maximum-likelihood method. The optimal substitution models—the Kimura 2-parameter + G (*N* gene) and Tamura-Nei (*S* gene)—were selected by the program. Statistical analysis was performed using the bootstrap test (1000 replicates) for multiple alignments.

Statistical analysis

The morbidity rate and the detection period of ECoV RNA between Thoroughbred and other breeds were compared using Fisher's exact test and Mann-Whitney U test, respectively. All statistical evaluations were performed using the JMP v. 13 software (SAS Institute Inc., Cary, NC, USA) with statistical significance set at $P < 0.05$.

Results

History of the outbreak

On March 27 and 28, 2020, three horses (#1–#3 in Table 1) developed a fever (38.8–39.5 °C) with mild lethargy or anorexia. These three horses had participated in a show jumping competition a few days earlier held at the JRA's Racing School in Chiba Prefecture, 50 km east of the Tokyo racecourse. They were considered to be the index cases of this outbreak, because they were healthy prior to departure and this stable did not have any febrile horses for at least one month before the outbreak. Another seven horses (#4–#10) developed a fever within a week, from April 2 to 3, and viral involvement was then suspected. Nasal swabs or whole blood from these pyretic horses (#4–#10) were tested for equid alphaherpesvirus 1 and 4, equine influenza virus, Getah virus, and equine arteritis virus, and all results came back negative. On April 3, another horse (#11)

presented with mild diarrhea. The qPCR performed on the feces collected from this horse resulted positive for ECoV RNA. Between April 5 and 9, four additional horses (#12–#15) were affected. Ultimately, 15 out of the 41 horses (37%) on the premise were affected. The most common clinical sign was fever (11 horses), followed by lethargy (6 horses), anorexia (4 horses), and mild diarrhea (3 horses). One horse with mild dehydration and diarrhea (#11) was treated with supportive fluid therapy. No horses died or were severely affected, and all affected horses recovered within three days of disease onset. The morbidity rate between Thoroughbreds (5/16; 31%) and non-Thoroughbreds (10/25; 40 %) was compared, but there was no statistically significant difference ($P = 0.74$).

qPCR

qPCR showed that 30 of the 41 horses (73%) were positive for ECoV at least once during the outbreak. In the positive cases, the viral shedding period ranged from 1 to 98 days (Table 1). Five non-Thoroughbred horses shed the virus for > 1 month: 98 (#19), 88 (#23), 50 (#34), 50 (#38), and 37 days (#7). In contrast, the longest shedding period in the Thoroughbreds was 19 days (#15). The viral shedding period of the non-Thoroughbreds (median 8 days) was significantly longer than that of the Thoroughbreds (median 1 day; $P = 0.036$). The housekeeping gene (β2-microglobulin) was detected in all samples, confirming that the extraction and qPCR were performed appropriately.

VN test

The VN test showed that all paired sera were seroconverted to ECoV (Table 1).

Sequence and phylogenetic analysis using a nearly complete genome sequence of ECoV

The nearly complete genome sequence of ECoV detected in this outbreak (Tokyo2020) was determined and has been deposited in the DNA Data Bank of Japan DDBJ/EMBL/GenBank database (accession number, LC592689). The nucleotide identities of 10 genes were 97.5–99.6% between Tokyo2020 and NC99, 98.2–99.4% between Tokyo2020 and Tokachi09, and 98.8–99.8% between Tokyo2020 and Obihiro12-1/-2 (excluding the *NS2* gene) (Table 3). The identity of *NS2* of Tokyo2020 was not compared with that of Obihiro12-1 and 2, because these two Obihiro strains have a 2-nucleotide deletion in the *NS2* region that truncates the open reading frame. Phylogenetic analysis of the complete *N* and partial *S* genes showed that all ECoVs, including Tokyo2020, are closely related to each other (Figure 1). The length from the putative *p4.7* to *p12.7* genes encoding non-structural proteins was 535 bp in Tokyo2020, 396 bp in Tokachi09, 541 bp in Obihiro12-1, and 2, 581 bp in NC99 and 544 bp in the ECoV detected in Ireland (Figure 2). The nucleotide identities were not compared due to multiple nucleotide insertions and deletions in the putative *p4.7* to *p12.7* region among the strains.

Discussion

To the best of my knowledge, this is the first report describing an outbreak of ECoV infection in a population composed of various breeds other than draft horses in Japan. In this outbreak, 15 of the 41 horses were visibly affected. The most common clinical sign was fever, while only three horses showed diarrhea. All affected horses showed mild signs and recovered within a few days of onset with or without treatment. These clinical

characteristics are similar to those in previously reported outbreaks of ECoV infections [40, 46]. It is suggested that ECoV infection should be suspected when several horses on the same premise show fever, even if they do not show gastrointestinal signs represented by diarrhea.

The VN test showed that all 41 horses on the premise were infected with ECoV, indicating that ECoV is highly contagious and has spread throughout the herd. The infection rate determined by qPCR has varied in the previous outbreaks, ranging from 56% to 83% [12, 15, 46]. Although qPCR has a high sensitivity and specificity for detecting viral RNA in clinical specimens, some infected horses do not necessarily shed the detectable amount of virus in the feces. Therefore, the use of serological assays such as VN test and the recently developed ECoV specific ELISA [23] is recommended in addition to qPCR for a retrospective confirmation of the true infection rate. While all 41 horses were confirmed to be infected with ECoV, 26 horses (67%) were asymptomatic. Interestingly, the four horses that shed the virus for 50 days or longer were all asymptomatic. In an outbreak of ECoV infection in the American miniature horses, even horses without any clinical signs shed the virus for relatively long periods of at least 18, 22, and 25 days [15]. These findings suggest that the viral shedding periods do not correlate with the presence or absence of clinical signs, and that subclinical horses can play an important role as ECoV spreaders. Therefore, once ECoV infection is confirmed, qPCR should be performed on as many individuals as possible in the herd, regardless of the presence or absence of clinical signs.

The three index cases in the current outbreak had participated in a show jumping competition held at the JRA's Racing School a few days before disease onset. The Racing School housed about 140 horses, and 13 of them developed fever around January–

February 2020, approximately two months before the current outbreak in the Tokyo racecourse. A retrospective VN test using paired sera performed after the current outbreak revealed that these 13 horses were also positive for ECoV seroconversion, suggesting that ECoV was circulating among horses at the Racing School during this period. These two ECoV outbreaks occurred sequentially, therefore, an epidemiological link between them was highly likely. It is speculated that the three index cases played a role in the transmission of ECoV from the Racing School to their stable.

Before the current outbreak, all ECoV outbreaks reported in Japan have occurred in a population of draft horses [28, 40, 41]. A seroprevalence study of ECoV in healthy adult horses in the USA reported that the odds ratio of seroprevalence was significantly higher in draft horses but lower in Thoroughbreds compared with other breeds, although the direct association of breed with susceptibility to ECoV infection was inconclusive [24]. In the current outbreak, the viral shedding period was significantly longer in non-Thoroughbreds than in Thoroughbreds, although the morbidity rate did not differ between them. This result suggests that there is a difference in the persistence of ECoV among horse breeds and that some breeds contribute more to the spread of the virus than other breeds. One limitation of the current study is that the data of viral shedding periods and morbidity rates in non-Thoroughbred were combined from multiple breeds due to the insufficient number of horses for individual breed. To better describe the difference in the persistence of the virus or clinical characteristics in each horse breed, it is preferable to distinguish them and compare the data. Further studies should focus on collecting clinical data for each breed to have a better understanding of the ECoV infection characteristics.

Sequence and phylogenetic analyses showed that Tokyo2020 is genetically similar to previous ECoV strains as a whole. In particular, the sequence for the structural proteins

(HE, S, E, M, and N) were highly conserved among strains (Table 3). In contrast, the nucleotide length from *p4.7* to *p12.7* in Tokyo2020 was different from that in previous ECoV strains detected in Japan, the USA, and Ireland. The deletion of nucleotides in the *NS2* region identified in Obihiro12-1 and 2 was not observed in Tokyo2020. These differences suggest that this outbreak was caused by a different ECoV variant from the one that caused the outbreaks among draft horses in 2009 and 2012 in Japan.

In conclusion, the current outbreak shows that ECoV is highly contagious and that subclinical horses may contribute to the spread of ECoV. The difference in viral shedding periods among horse breeds may indicate that some breeds excrete ECoV longer than the others and can contribute more to the spread of the virus.

Table 1

Breed, age, clinical signs, the date of onset of disease, detection period of equine coronavirus RNA, and the result of VN test

Horse No.	Breed	Age	Clinical signs	Date of onset	Detection period of ECoV RNA	VN titer 1st	VN titer 2nd
# 1	Thoroughbred	10	fever, lathargy	Mar-28	−	8	1024
# 2	Thoroughbred	11	fever, anorexia	Mar-27	−	8	512
# 3	Japanese sport horse	9	fever	Mar-28	−	16	1024
# 4	Mixed	10	fever, lethargy	Apr-2	1	< 8	512
# 5	Thoroughbred	8	fever	Apr-2	−	64	1024
# 6	Japanese bred	14	fever	Apr-2	1	10	1024
# 7	Japanese sport horse	18	fever	Apr-2	37	32	256
# 8	Japanese bred	6	fever, anorexia	Apr-2	8	< 8	64
# 9	Andalusian	14	fever, lethargy	Apr-2	1	16	512
# 10	Pony	3	fever, lethargy	Apr-3	8	< 8	512
# 11	Thoroughbred	14	diarrhea, anorexia, lethargy	Apr-3	1	32	1024
# 12	Lusitano	10	diarrhea	Apr-5	1	32	1024
# 13	Andalusian	18	lethargy, anorexia	Apr-8	13	64	1024
# 14	Andalusian	13	diarrhea	Apr-8	8	64	512
# 15	Thoroughbred	15	fever	Apr-9	19	16	1024
# 16	Thoroughbred	14	−		1	16	1024
# 17	Thoroughbred	9	−		1	< 8	512
# 18	Lusitano	19	−		11	256	1024
# 19	Andalusian	12	−		98	< 8	16
# 20	Japanese bred	14	−		1	< 8	1024
# 21	Thoroughbred	12	−		11	< 8	512
# 22	Thoroughbred	6	−		−	8	1024
# 23	Friesian	5	−		88	32	512
# 24	Andalusian	14	−		1	64	512
# 25	Andalusian	13	−		11	128	512
# 26	Thoroughbred	10	−		1	64	1024
# 27	Thoroughbred	11	−		−	< 8	256
# 28	Thoroughbred	15	−		1	16	1024
# 29	Thoroughbred	15	−		1	128	1024
# 30	Thoroughbred	12	−		1	< 8	128
# 31	Thoroughbred	11	−		−	8	512
# 32	Thoroughbred	9	−		1	< 8	1024
# 33	Friesian	5	−		−	< 8	256
# 34	Pony	6	−		50	< 8	16
# 35	Pony	11	−		−	32	512
# 36	Pony	10	−		−	< 8	1024
# 37	Pony	8	−		−	< 8	512
# 38	miniature horse	1	−		50	< 8	32
# 39	miniature horse	13	−		1	64	512
# 40	miniature horse	8	−		15	< 8	512
# 41	miniature horse	5	−		8	< 8	64

Table 2

Primer sequences used for the RT-PCR to read the genome almost completely

Primer set	name	Sequence (5'-3')	Genome position [a]	Reference
Segment 1	ECoV-90F	CTGTAATCTATGCTTGCGAG	90-109	This study
	ECoV-3651R	GCCAATCACACAAAGTAAGT	3651-3632	
Segment 2	ECoV-3084F	GATGAAGATTTTGGAGAGCC	3084-3103	This study
	ECoV-6867R	GCTTTGATGCAATTTCTGTG	6867-6848	
Segment 3	ECoV-6000F	GGACACACCGTCTGTGATAG	6000-6019	[33]
	ECoV-11999R	AGCAACACCCAGATCAGAAG	11999-11980	
Segment 4	ECoV-11301F	CCATCAGTTGAGTATACTTATACTG	11301-11325	[33]
	ECoV-17800R	AGCAGCAAAGTTCTGACTAT	17800-17781	
Segment 5	ECoV-17100F	CACATCTTGCTATCGGTTTG	17100-17119	[33]
	ECoV-22802R	AGAAGCGGTGCATTTAAAGC	22802-22783	
Segment 6	ECoV-22608F	CCTCGTAATTATTCTTATATGGACC	22608-22632	[33]
	ECoV-29009R	AACTCCACCAACTACCAGTT	29009-28990	
Segment 7	ECoV-28110F	GTTTTACTAACGGATTTAACGTCC	28110-28133	[33]
	ECoV-30935R	ACTTAACATGCTGGCTCTTC	30935-30916	

[a] Based on ECoV strain NC99 (GenBank accession number: NC_010327).

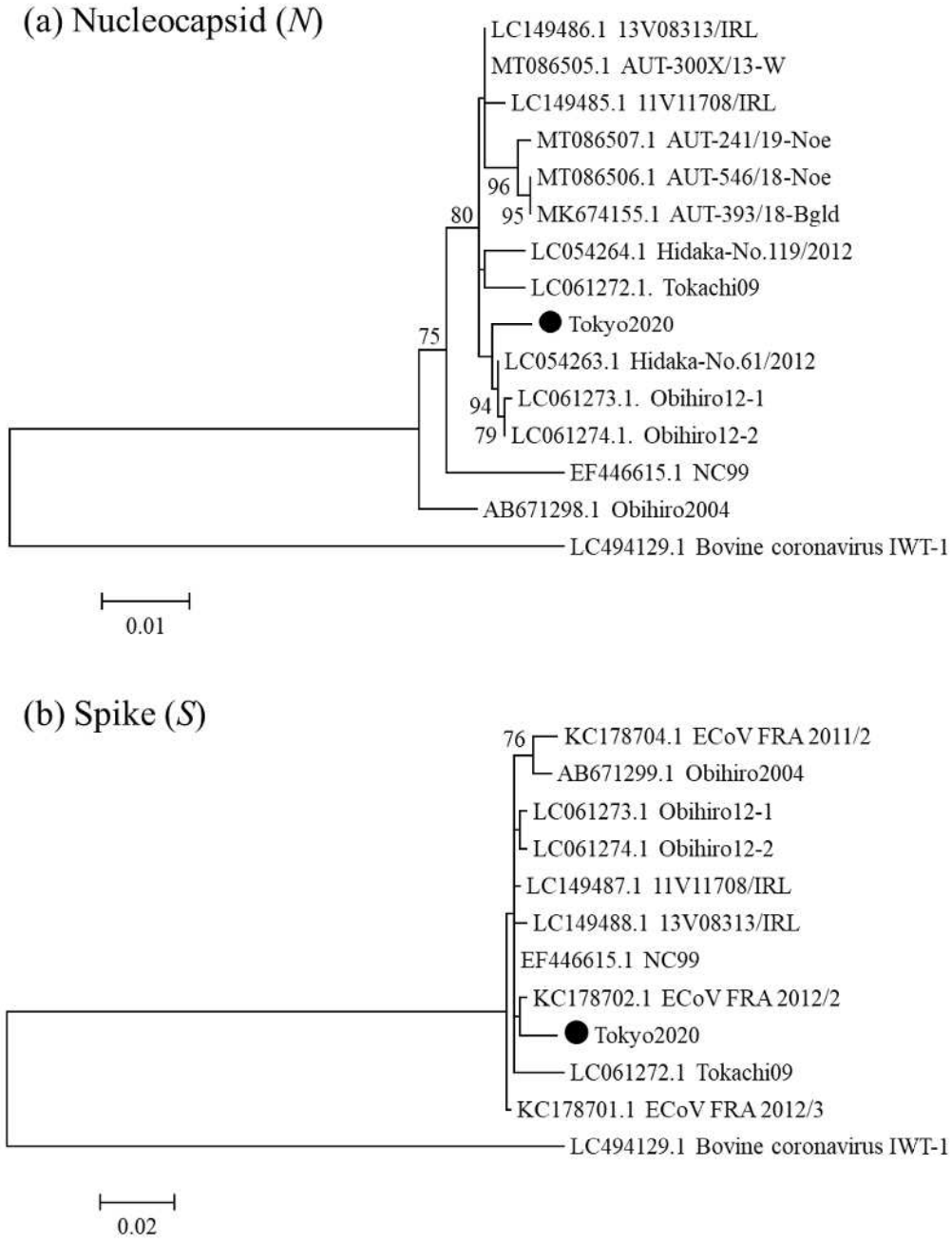
Table 3

Nucleotide sequence identities (%) of Tokyo2020 compared with those of NC99, Tokachi09, Obihiro12-1, and Obihiro12-2 strains

	NC99	Tokachi09	Obihiro12-1	Obihiro12-2
ORF1a	98.8	98.4	99.6	99.6
ORF1ab	98.8	98.5	99.4	99.4
NS2	97.5	98.2	-	-
HE	97.8	98.4	99.7	99.8
S	98.3	98.3	99.1	99.2
p12.7	99.1	99.4	98.8	98.8
E	99.6	99.2	98.8	99.2
M	99.3	98.3	99.1	99.1
N	98.1	98.9	99.3	99.4
I	97.7	98.4	99.4	99.4

Figure 1

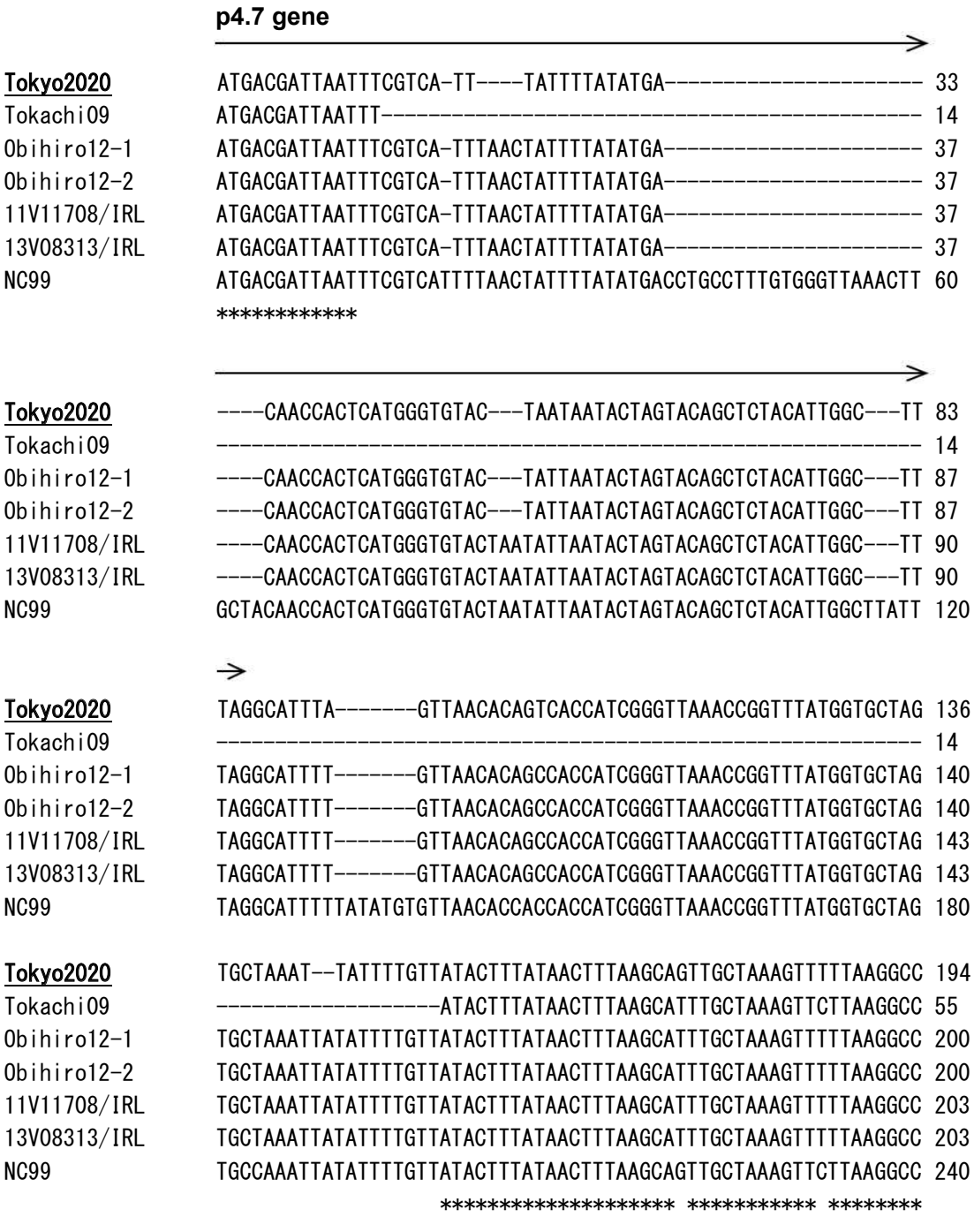
Phylogenetic analysis of the nucleotide sequences

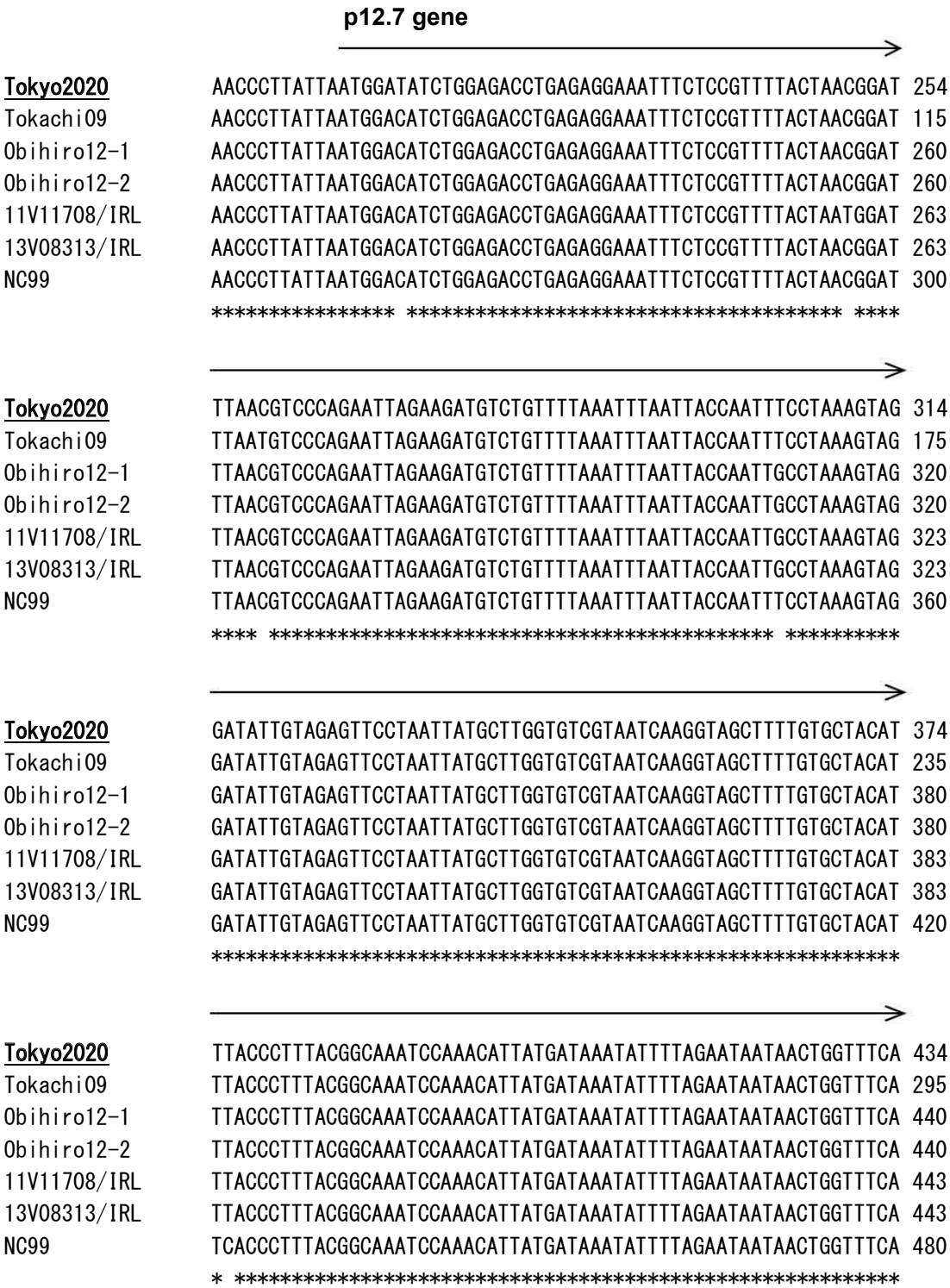


(a) the complete nucleocapsid (*N*) gene and (b) the partial spike (*S*) gene of ECoV (*N*, nt 29363–30703; *S*, nt 25527–26132; nt positions in strain NC99, EF446615.1)

●, Tokyo2020. Percentage bootstrap support is indicated by the value at each node, with values < 70% omitted. BCoV IWT-1 is used as an outgroup.

Figure 2
Nucleotide Sequences from the *p4.7* to *p12.7* genes




p12.7 gene
Tokyo2020 AACCCTTATTAATGGATATCTGGAGACCTGAGAGGAAATTTCTCCGTTTTACTAACGGAT 254
Tokachi09 AACCCTTATTAATGGACATCTGGAGACCTGAGAGGAAATTTCTCCGTTTTACTAACGGAT 115
Obihiro12-1 AACCCTTATTAATGGACATCTGGAGACCTGAGAGGAAATTTCTCCGTTTTACTAACGGAT 260
Obihiro12-2 AACCCTTATTAATGGACATCTGGAGACCTGAGAGGAAATTTCTCCGTTTTACTAACGGAT 260
11V11708/IRL AACCCTTATTAATGGACATCTGGAGACCTGAGAGGAAATTTCTCCGTTTTACTAATGGAT 263
13V08313/IRL AACCCTTATTAATGGACATCTGGAGACCTGAGAGGAAATTTCTCCGTTTTACTAACGGAT 263
NC99 AACCCTTATTAATGGACATCTGGAGACCTGAGAGGAAATTTCTCCGTTTTACTAACGGAT 300
Tokyo2020 TTAACGTCCCAGAATTAGAAGATGTCTGTTTTAAATTTAATTACCAATTTCCTAAAGTAG 314
Tokachi09 TTAATGTCCCAGAATTAGAAGATGTCTGTTTTAAATTTAATTACCAATTTCCTAAAGTAG 175
Obihiro12-1 TTAACGTCCCAGAATTAGAAGATGTCTGTTTTAAATTTAATTACCAATTGCCTAAAGTAG 320
Obihiro12-2 TTAACGTCCCAGAATTAGAAGATGTCTGTTTTAAATTTAATTACCAATTGCCTAAAGTAG 320
11V11708/IRL TTAACGTCCCAGAATTAGAAGATGTCTGTTTTAAATTTAATTACCAATTGCCTAAAGTAG 323
13V08313/IRL TTAACGTCCCAGAATTAGAAGATGTCTGTTTTAAATTTAATTACCAATTGCCTAAAGTAG 323
NC99 TTAACGTCCCAGAATTAGAAGATGTCTGTTTTAAATTTAATTACCAATTTCCTAAAGTAG 360
Tokyo2020 GATATTGTAGAGTTCCTAATTATGCTTGGTGTCGTAATCAAGGTAGCTTTTGTGCTACAT 374
Tokachi09 GATATTGTAGAGTTCCTAATTATGCTTGGTGTCGTAATCAAGGTAGCTTTTGTGCTACAT 235
Obihiro12-1 GATATTGTAGAGTTCCTAATTATGCTTGGTGTCGTAATCAAGGTAGCTTTTGTGCTACAT 380
Obihiro12-2 GATATTGTAGAGTTCCTAATTATGCTTGGTGTCGTAATCAAGGTAGCTTTTGTGCTACAT 380
11V11708/IRL GATATTGTAGAGTTCCTAATTATGCTTGGTGTCGTAATCAAGGTAGCTTTTGTGCTACAT 383
13V08313/IRL GATATTGTAGAGTTCCTAATTATGCTTGGTGTCGTAATCAAGGTAGCTTTTGTGCTACAT 383
NC99 GATATTGTAGAGTTCCTAATTATGCTTGGTGTCGTAATCAAGGTAGCTTTTGTGCTACAT 420
Tokyo2020 TTACCCTTTACGGCAAATCCAAACATTATGATAAATATTTTAGAATAATAACTGGTTTCA 434
Tokachi09 TTACCCTTTACGGCAAATCCAAACATTATGATAAATATTTTAGAATAATAACTGGTTTCA 295
Obihiro12-1 TTACCCTTTACGGCAAATCCAAACATTATGATAAATATTTTAGAATAATAACTGGTTTCA 440
Obihiro12-2 TTACCCTTTACGGCAAATCCAAACATTATGATAAATATTTTAGAATAATAACTGGTTTCA 440
11V11708/IRL TTACCCTTTACGGCAAATCCAAACATTATGATAAATATTTTAGAATAATAACTGGTTTCA 443
13V08313/IRL TTACCCTTTACGGCAAATCCAAACATTATGATAAATATTTTAGAATAATAACTGGTTTCA 443
NC99 TCACCCTTTACGGCAAATCCAAACATTATGATAAATATTTTAGAATAATAACTGGTTTCA 480

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                  ------------------------------------------------------------>
Tokyo2020         CAGCGTTCGCTAATACTATAGAGGAGGCTGTTAACAAACTGGTTTTAGAGGCTGTTGATT 494
Tokachi09         CAGCGTTCGCTAATACTATAGAGGAGGCTGTTAACAAACTGGTTTTAGAGGCTGTTGATT 355
Obihiro12-1       CAGCGTTCGCCAATACTATAGAGGAGGCTGTTAATAAACTGGTTTTAGAGGCTGTTGATT 500
Obihiro12-2       CAGCGTTCGCCAATACTATAGAGGAGGCTGTTAATAAACTGGTTTTAGAGGCTGTTGATT 500
11V11708/IRL      CAGCGTTCGCCAATACTATAGAGGAGGCTGTTAACAAACTGGTTTTAGAGGCTGTTGATT 503
13V08313/IRL      CAGCGTTCGCCAATACTATAGAGGAGGCTGTTAACAAACTGGTTTTAGAGGCTGTTGATT 503
NC99              CAGCGTTCGCCAATACTATAGAGGAGGCTGTTAACAAACTGGTTTTAGAGGCTGTTGATT 540
                  ********** ********************** *************************

                  ------------------------------------>
Tokyo2020         TTATTATCTGGCGTAGCCAGAATTTAAATGCTTATGGCTGA                     535
Tokachi09         TTATTATCTGGCGTAGCCAGAATTTAAATGCTTATGGCTGA                     396
Obihiro12-1       TTATTATCTGGCGTAGCCAGAATTTAAATGCTTATGGCTGA                     541
Obihiro12-2       TTATTATCTGGCGTAGCCAGAATTTAAATGCTTATGGCTGA                     541
11V11708/IRL      TTATTATCTGGCGTAGCCAGAATTTAAATGCTTATGGCTGA                     544
13V08313/IRL      TTATTATCTGGCGTAGCCAGAATTTAAATGCTTATGGCTGA                     544
NC99              TTATTATCTGGCGTAGCCAGAATTTAAATGCTTATGGCTGA                     581
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CHAPTER 2

Serosurveillance of equine coronavirus infection among Thoroughbreds in Japan

Summary

There has been limited information on the epidemiology of ECoV infection among Thoroughbreds. To understand the epidemiology and evaluate the potential risk of ECoV infection to the horse industry in Japan, serosurveillance was performed in the population of Thoroughbred yearlings and racehorses. The VN test was performed using sera collected three times at 4-month intervals from 161 yearlings and at 6–7-month intervals from 181 active racehorses in Japan during 2017–2018, 2018–2019, and 2019–2020. VN titer ≥ 1:8 was defined as seropositive, and a ≥ 4-fold increase in titer between paired sera was regarded as indicative of infection. The VN test showed that 44.1% (71/161) of the yearlings were seropositive in August, when they first entered the yearling farm. The infection rate was significantly higher between August and December (60.9%, 98/161) than between December and the following April (5.6%, 9/161; $P = 0.002$). Among the racehorses, the infection rate was significantly higher between November and the following May (15.5%, 28/181) than between the preceding April/May and November (0%; $P = 0.018$). The morbidity rates during the estimated periods of viral exposure were 39.2% and 4.0% in the yearlings and racehorses, respectively. No horses showed any severe clinical signs. This study shows that ECoV was substantially prevalent in Thoroughbred yearlings and racehorses in Japan, and that there was a difference in the epizootic pattern between these populations in terms of predominant periods of infection. ECoV infection was considered to be responsible for some of the pyretic cases in the yearlings. However, no sick horses were severely affected in either population, suggesting that the potential risk of ECoV infection to the horse industry in Japan is low.

Introduction

Several outbreaks of ECoV infection have occurred in foals and adult horses in the USA, Europe, and Asia including Japan [12, 15, 27, 40]. As shown in CHAPTER 1, an ECoV outbreak among 41 riding horses comprising of various breeds has recently been reported.

There are several epidemiological studies describing ECoV infections. It was previously shown that only a low proportion of adult horses tested positive by qPCR, regardless of the presence of clinical signs [5, 49]. In these reports, the positive rate detected by qPCR ranged from 1.5% to 2.6%. In contrast, Thoroughbred foals up to six months of age in central Kentucky, USA, were reported to have higher positive rates in both diseased (29.4%) and healthy (27.0%) groups, with no significant difference between the groups [52]. In Japan, the prevalence of ECoV RNA in Thoroughbred foals was investigated using qPCR or RT-LAMP [34]. In this study, no diarrheic foals tested positive, suggesting that ECoV infection is not associated with diarrhea in foals in the major breeding area of Japan. The epidemiology of ECoV in Japan is limited to this study, and the prevalence of ECoV in Thoroughbred yearlings and adult racehorses has not been investigated. Hence, the potential risk of ECoV infection to the horse industry in Japan is not well known.

It was reported that the number of patients infected with the four common types of HCoVs (HCoV-NL63, 229E, HKU1, and OC43) increased during the cold season [51]. The number of cases that suffered from winter dysentery caused by BCoV, which is most closely related to ECoV, are also known to increase during the winter season [7, 8]. A retrospective study also showed that ECoV infection rates increased during the cooler months of the year in the USA [3]. Therefore, ECoV may also has a similar epidemic

pattern with other CoVs in terms of seasonality. However, to the best of my knowledge, a comprehensive serological investigation to evaluate the seasonal nature of ECoV has not been reported.

To understand the epidemiology and evaluate the potential risk of ECoV infection to the horse industry in Japan, we performed serosurveillance of ECoV infection in the population of Thoroughbred yearlings and racehorses.

Materials and Methods

Study populations and sample collection

This study was approved by the Research Planning and Ethics Committee of the Equine Research Institute of the JRA. The owners of the horses were all notified by the racing authorities that their horses might be subjected to mandatory sampling for research purposes. Thoroughbred yearlings and racehorses from three seasons: 2017–2018, 2018–2019, and 2019–2020 were included in this study. Sera were collected from 161 yearlings (55 in 2017–2018, 56 in 2018–2019, and 50 in 2019–2020) reared at the Hidaka Training and Research Center, Hokkaido, northern Japan, a farm managed by the JRA. Most of the yearlings were purchased at the yearling sales and introduced to the farm in August, while a few horses were bred and raised at the farm. After introduction, they were grouped by sex and shared a pasture from 3:00 p.m. to 8:00 a.m. each day in August. From September to mid-October, they were usually separated into individual stalls and grazed with other horses only on weekends. After mid-October, they were completely housed in individual stalls, and they left the farm in the following April for a training sale. Sera were collected monthly from August through the following April for the purpose of other studies. Initially, sera collected in August,

December, and the following April were tested. If sera showed a ≥ 4-fold increase in VN titer in either time interval, an additional test was performed using the monthly collected sera to determine the period of viral exposure more accurately.

Sera were also collected from 181 racehorses (42 in 2017–2018, 64 in 2018–2019, and 75 in 2019–2020). The age of the subjects ranged from 2–8 years (median, 3 years). These horses were housed at the JRA's Miho Training Center in eastern Japan, which can accommodate approximately 2000 racehorses. Most racehorses first enter the center between the spring and autumn at two years old and are trained for racing. After one or more races, they are usually taken to nearby private farms for rest or conditioning and are returned to the training center a few weeks or months later. These cycles then repeat. Racehorses are housed in individual stalls at both the training center and the private farms. Sera are routinely collected from all horses at the training center in May and November every year for surveillance purposes. We analyzed the sera from the horses that remained at the center and were sampled in May, November, and the following May in each period as described above. For 2-year-old horses (n = 49), sera collected at the center's quarantine facility in April or May when they first entered the center were used for the test.

VN test

Collected sera were stored at −20°C until tested. The VN test was performed as described in CHAPTER 1. Each sample was tested in duplicates, and the lower VN titer was used when the titers differed. A VN titer of ≥ 1:8 was defined as seropositive. A 4-fold or greater increase in VN titer between sera was regarded as seroconversion, i.e., the horse was infected with ECoV during the period. To compare the results, a VN titer

of < 1:8 was defined as 1:4, and ≥ 1024 was defined as 1:1024.

Evaluation of clinical history

Since all yearlings stayed at the yearling farm during the entire study period from August to the following April of each year, all clinical records were available. On the other hand, racehorses moved between the center and private farms, and therefore, their clinical records were available only during their stay at the training center, but not when they were outside the center.

Body temperature was measured twice daily by grooms for all yearlings and racehorses. Horses exhibiting clinical abnormalities such as fever (rectal temperature ≥ 38.5 °C), anorexia, lethargy, or gastrointestinal signs such as colic or diarrhea were examined by veterinarians and treated when necessary. The infected horses that developed clinical abnormalities during the periods between seven days before pre-serum collection and seven days before post-serum collection were regarded as symptomatic, referring a previous study showing that VN titer against ECoV increases significantly at 7 dpi [36].

Statistical analysis

Mixed Poisson regression modelling with year as a random effect was used to compare infection rates between periods within populations. All statistical analyses were performed using the SPSS statistics software version 24.0 (IBM Japan, Ltd., Tokyo, Japan). $P < 0.05$ was considered statistically significant.

Results

Seroprevalence in yearlings

The VN test showed that 44.1% (range, 27.3–57.1%) of the yearlings were seropositive in August when they first entered the yearling farm, 88.8% (81.8–92.9%) in December, and 85.7% (81.8–90.0%) in the following April (Table 4).

The infection rate was significantly higher between August and December (60.9%, 54.0–69.6%) than between December and the following April (5.6%, 0–14.5%; $P = 0.002$; Table 5). Additional VN tests using monthly collected sera showed that the number of infected horses was highest in August–September in 2017–2018 and 2019–2020, and in October–November in 2018–2019 (Table 6). Of the total 107 infected yearlings over the three seasons, 42 (39.2%) horses exhibited fever during the estimated period of viral exposure. All affected horses showed mild clinical signs and recovered without needing intensive care.

Seroprevalence in racehorses

Among 2-year-old racehorses (n = 49), 83.7% (range, 70–95%) were seropositive when they first entered the training center in April or May, 81.6% (50–100%) in November, and 77.6% (60–91%) in the following May (Table 7). Among ≥ 3-year-old horses (n = 132), the seropositive rates were > 80% at all time points (Table 8).

The infection rate was significantly higher between November and the following May (15.5%, 9.4–21.3%) than between the preceding April/May and November (0%; $P = 0.018$; Table 9). No horses were infected with ECoV during the former period. Of the total 28 infected racehorses, one (4.0%) horse developed a fever during the estimated period of viral exposure, but it did not require intensive care.

Discussion

The current study showed that ECoV was substantially prevalent among Thoroughbred yearlings and racehorses in Japan and that there was a difference in the epizootic pattern between them in terms of predominant periods of infection. It was expected that few yearlings would be seropositive when they first entered at the yearling farm, because a previous study reported that Thoroughbred foals in Japan had a low qPCR/RT-LAMP-positivity detected from rectal swabs [34]. However, approximately half of the yearlings had VN antibodies against ECoV on arrival, indicating that they had been infected with ECoV before their summer as yearlings. This discrepancy in two studies may be due to the difference of tests performed or the difference in timing of exposure to ECoV. qPCR has a high sensitivity but can only detect the viral RNA when a detectable amount of RNA is shed in the clinical specimens, while the VN test can evaluate the history of infection since an elevated titer is maintained for several months post infection. The VN test then might show a higher positive rate than qPCR when the same population is tested. Alternatively, it is likely that they were infected after weaning through contact with other horses. Further investigation is needed to estimate the exact time at which ECoV spreads among foals.

More than 60% of the yearlings became infected with ECoV within four months after entering the farm, whether or not they already had antibodies on arrival. Clustered cases of ECoV infection may have been caused by the drastic change in their rearing environment rather than by the seasonal nature of the virus. The yearlings originated from discrete breeding farms in various locations in Hokkaido. After entering the yearling farm in August, they were mixed, and shared pastures with other horses until mid-October. It is likely that some of the yearlings introduced the virus into the herd, and close contact

with other yearlings during grazing caused the rapid spread of ECoV. In contrast to the period between August and December, the infection rate was much lower between December and the following April. The immunity acquired from the initial rapid spread of ECoV infection may have protected the yearlings from further infections. In addition, group grazing was discontinued in mid-October, and horses were housed individually in separate stalls, reducing opportunities for contact and subsequent virus transmission. The seropositive rate of the yearlings in April (85.7%), when they left the farm, was similar to that of the 2-year-old racehorses in April/May (83.7%). Since these two groups comprised different populations, this similarity suggests that a similar level of exposure to ECoV occurred at other yearling farms in Hokkaido.

The number of ECoV cases increased during the colder months (October–April) in the United States [45]. HCoV-OC43, also in the genus β-CoVs, has a peak prevalence in the cold season (December–March) in the United States [22]. Moreover, BCoV, which is phylogenetically close to ECoV, causes winter dysentery characterized by hemorrhagic diarrhea in adult beef and dairy cattle, and spreads widely in the colder months [7, 54, 55]. In this study, ECoV infection occurred predominantly during the cold season (November–May) among the racehorses, and no infected case was observed during the warm season (April/May–November). Racehorses are kept in individual stalls except during training, and they rarely have the opportunity to contact with each other. This rearing environment does not change greatly throughout the year, unlike that of the yearlings. Therefore, the difference in infection rate between the warm and cold seasons indicates a seasonal nature of ECoV. This study suggests that ECoV infection is more likely to occur during the cold months, much like other CoVs.

Among the yearlings infected with ECoV, 39.2% developed a fever during the

estimated period of viral exposure. It is a limitation that sera were collected at fixed time points and not when clinical signs were observed. Although it cannot be determined whether their clinical signs were necessarily caused by the ECoV infection or not, this result suggests that ECoV infection contributed to their illness to some extent. Many viral pathogens cause fever in foals and yearlings, including equid alphaherpesvirus 1 and 4, equine rhinitis viruses A and B, and equine adenovirus [14]. This study suggests that ECoV is also likely to cause fever in young horses. However, all affected horses did not show severe clinical signs; therefore, the negative impact of ECoV infection on Thoroughbred yearlings is considered to be small. ECoV is also prevalent in the racehorse population in Japan, as 15.5% of them were infected during the colder months. However, the morbidity rate in this population was substantially lower than that in the yearlings, and the one diseased horse was not severely affected. Another limitation of this study is that racehorses often leave the center for rest or conditioning, and the clinical records used here did not cover these periods. The actual morbidity rate may have been higher than that confirmed because the infected horses could have developed the disease outside the training center. However, it seems that ECoV does not threaten the racehorse population and has little negative impact on the equine industry at present, based on the current study and the lack of detectable outbreaks among Thoroughbred racehorses in Japan.

In conclusion, ECoV has been widely prevalent among Thoroughbred yearlings and racehorses in Japan. ECoV spread rapidly among the yearlings, probably due to their close contact with each other. Infection in the racehorses occurred predominantly during the cold season, indicating a seasonal nature of ECoV. The relatively high morbidity rate in the yearlings suggests a possible association of ECoV infection with pyretic cases in this

population, although this was not the case in the racehorses. No diseased horses were severely affected in either population. Therefore, the potential risk of ECoV infection to the equine industry in Japan is considered low.

Table 4. Numbers of yearlings seropositive for ECoV at each timepoint

		Number of seropositive horses (%)					
		Aug		Dec		next Apr	
2017–2018	(n = 55)	15	(27.3)	45	(81.8)	45	(81.8)
2018–2019	(n = 56)	32	(57.1)	52	(92.9)	48	(85.7)
2019–2020	(n = 50)	24	(48.0)	46	(92.0)	45	(90.0)
Total	(n = 161)	71	(44.1)	143	(88.8)	138	(85.7)

VN titer ≥1:8 was defined as seropositive.

Table 5. Numbers of infected yearlings

		Number of infected yearlings (%)				Poisson regression (per year)		
		Aug–Dec		Dec–Apr		IR	95%CI	*P*-value
2017–2018	(n = 55)	32	(58.2)	8	(14.5)			
2018–2019	(n = 56)	39	(69.6)	1	(1.8)			
2019–2020	(n = 50)	27	(54.0)	0	(0)			
Total	(n = 161)	98	(60.9)	9	(5.6) *	0.09	0.04 to 0.24	**0.002**

Four-fold or greater increase of VN titer between sera was regarded as indicating infection with ECoV.

*Statistically significant difference between Aug-Dec and Dec-Apr ($P = 0.002$)

IR: infection ratio (Dec–Apr/Aug–Dec); 95%CI: 95% confidence interval.

Table 6. Numbers of infected yearlings by additional VN test using monthly-collected sera.

	Aug–Sep	Sep–Oct	Oct–Nov	Nov–Dec	Dec–Jan	Jan–Feb	Feb–Mar	Mar–Apr	Total
2017–2018	31	10	2	1	5	2	0	0	51 †
2018–2019	2	8	34	0	0	0	0	0	44 †
2019–2020	18	8	0	1	0	0	0	0	27

Four-fold or greater increase of VN titer between sera was regarded as indicating infection with ECoV.

†Some horses show ≥ 4-fold increase multiple times.

Table 7. Numbers of 2-year-old racehorses seropositive for ECoV at each time point

		Number of seropositive horses (%)					
		Apr/May		Nov		next May	
2017–2018	(n = 10)	7	(70.0)	5	(50.0)	6	(60.0)
2018–2019	(n = 18)	14	(77.8)	14	(77.8)	13	(72.2)
2019–2020	(n = 21)	20	(95.2)	21	(100)	19	(90.5)
Total	(n = 49)	41	(83.7)	40	(81.6)	38	(77.6)

VN titer ≥ 1:8 was defined as seropositive.

Table 8. Numbers of ≥ 3-year-old racehorses seropositive for ECoV at each time point

		Number of seropositive horses (%)					
		Apr/May		Nov		next May	
2017–2018	(n = 32)	30	(93.8)	28	(87.5)	31	(96.9)
2018–2019	(n = 46)	35	(76.1)	35	(76.1)	38	(82.6)
2019–2020	(n = 54)	53	(98.1)	49	(90.7)	50	(92.6)
Total	(n = 132)	118	(89.4)	112	(84.8)	119	(90.2)

VN titer ≥ 1:8 was defined as seropositive.

Table 9. Numbers of infected racehorses

		Number of infected horses (%)				Poisson regression (per year)		
		Apr/May–Nov		Nov–May		IR	95%CI	*P*-value
2017–2018	(n = 42)	0	(0.0)	6	(14.3)			
2018–2019	(n = 64)	0	(0.0)	6	(9.4)			
2019–2020	(n = 75)	0	(0.0)	16	(21.3)			
Total	(n = 181)	0	(0.0)	28	(15.5) *	10.33	1.93 to 55.38	**0.018**

Four-fold or greater increase of VN titer between sera was regarded as indicating infection with ECoV.

*Statistically significant difference between Apr/May–Nov and Nov–May (P = 0.018).

IR: infection ratio ("Nov–May"/"Apr/May–Nov"); 95%CI: 95% confidence interval.

CHAPTER 3

Distribution of equine coronavirus RNA in the intestinal and respiratory tracts of experimentally infected horses

Summary

ECoV RNA is usually detected in the feces of horses infected with the virus. ECoV RNA is also detected in nasal swabs from naturally or experimentally infected horses. However, details on ECoV infection sites in the intestinal and respiratory tract are lacking. To identify the ECoV infection sites in these tissues, an experimental infection study was performed. The intestinal and respiratory tissue samples collected from four infected horses that were euthanized at 3, 5, 7, and 14 dpi (one horse per time point) were analyzed using qPCR and ISH. Two horses became febrile, and no horses showed diarrhea or respiratory signs. No severe cases were observed in this study. At necropsy, none of the horses showed obvious abnormalities in the intestinal or respiratory tract. qPCR and ISH showed that ECoV RNA was abundant throughout the intestinal tract, and ECoV-positive cells were detected mainly on the luminal surface of the intestine by ISH. In one horse that showed viremia on the day of euthanasia, ECoV RNA was detected in the lung by qPCR but not by ISH. These results suggest that ECoV infection was not present inside the lung cells themselves, but that qPCR detected the viral RNA in the blood contained in the lung. ECoV RNA was also detected in nasal swabs from the other three horses by qPCR but not in the tracheal and lung tissue, suggesting that the viral RNA detected was probably due to the contamination of the nasal cavity with feces containing the virus. This study concludes that ECoV broadly infects the intestinal tract and is less likely to infect the respiratory tract.

Introduction

ECoV causes pyrexia, anorexia, lethargy, and gastrointestinal signs represented by diarrhea in horses [45]. Experimental infection studies showed that horses inoculated with fecal suspension containing ECoV through a nasogastric tube or exposed to contaminated feces in their stall became infected with ECoV [36, 50]. Although clinical signs and shedding of ECoV in clinical samples were evaluated in these studies, pathological findings using tissue samples has not been reported.

In two clinicopathological studies on fatal cases, ECoV RNA was detected in the small intestine and colon tissues using IHC with monoclonal antibodies against the BCoV antigen, which is most closely related to ECoV phylogenetically [9, 13]. These studies suggest that ECoV has strong intestinal tropism, but it is unclear how ECoV is distributed through the intestinal tract during a time course. BCoV infects both the intestinal and respiratory tracts of cattle and causes enteric diseases such as calf diarrhea and winter dysentery, and respiratory disease included in a part of the bovine respiratory disease complex [56]. ECoV has also been detected in nasal swabs from horses that had been naturally or experimentally infected with ECoV [36, 44, 46]. However, it is unclear whether this results from an ECoV infection of the respiratory tract like BCoV or contamination of the nasal cavity by feces containing ECoV.

In this study, to identify the infection sites of ECoV in the intestinal and respiratory tracts, horses were experimentally infected with ECoV and intestinal and respiratory tissue samples collected from infected horses over time were analyzed using qPCR and ISH.

Materials and Methods

Horses and inoculum

The experimental protocol and all animal procedures were approved by the Animal Care Committee of the Equine Research Institute of the JRA. Four 1-year-old Thoroughbred horses were used in this study. All horses were healthy and confirmed not to have VN antibodies against ECoV (strain NC99), i.e., the antibody titers were less than 1:8 in the VN test, at 0 dpi. VN tests with strain NC99 were performed as described in CHAPTER 1. An ECoV-positive fecal sample was used as the inoculum; the inoculum consisted of watery diarrheic feces collected from a 4-year-old female draft horse during an ECoV outbreak in 2012 [40]. Bacterial culture testing confirmed that the inoculum was negative for *Clostridium perfringens*, *Clostridioides difficile*, and *Salmonella* species potentially associated with enteric diseases in horses [37]. The ECoV-positive sample was stored at −80 °C until inoculum preparation. The sample was diluted at ratio of 1:10 in PBS, and 500 mL of fecal suspension was administered via a transnasal catheter into the esophagus of each experimental horse under sedation as described previously [36]. The inoculated fecal suspension contained 3.6×10^{10} copies of the ECoV *N* gene. Viral copy numbers were determined by qPCR as described below.

Clinical observation and sampling

Clinical signs and rectal temperatures were recorded daily. Rectal temperatures exceeding 38.5 °C were defined as fever. Fecal samples, nasal swabs, serum, and whole blood samples were collected daily. Fecal samples were homogenized using a disposable homogenizer (BioMasher Standard, Takara bio, Kusatsu, Japan) at a ratio of 1:10 (w/v) in homogenization buffer comprising Dulbecco's modified Eagle's medium (Sigma-

Aldrich Co.) supplemented with 200 units of penicillin, 200 μg of streptomycin, and 0.5 μg of amphotericin B per milliliter (Antibiotic-Antimycotic; Thermo Fisher Scientific). Nasal swabs were collected and immersed in PBS supplemented with 0.6% tryptose phosphate broth (Sigma-Aldrich Co.) with 500 units of penicillin, 500 μg of streptomycin, and 1.25 μg of amphotericin B per milliliter. Fecal suspensions and nasal swabs were centrifuged at 21,500 × *g* for 5 min and 860 × *g* for 15 min, respectively, and the supernatant was used for RNA extraction. Whole blood samples were diluted at ratio of 1:1 (v/v) in PBS for extraction of viral RNA.

To perform necropsy, one horse was euthanized at each time point: 3 dpi (horse #1), 5 dpi (horse #2), 7 dpi (horse #3), and 14 dpi (horse #4). The intestinal tract (duodenum, jejunum, ileum, cecum, colon, and rectum), trachea, lungs, and lymph nodes (cranial mesenteric, cecal, colonic, caudal mesenteric, and pulmonary lymph nodes) were collected for qPCR and histopathological analysis. Tissue samples were homogenized at a ratio of 1:10 (w/v) in homogenization buffer and centrifuged in the same manner as fecal samples. For histopathological analysis, tissue samples were fixed in 10% neutral buffered formalin and then embedded in paraffin.

qPCR

Viral RNA was extracted from the fecal samples, nasal swabs, whole blood, and tissue samples (400 μL for each sample), using an automated nucleic acid isolation machine (MagLEAD; Precision System Science, Matsudo, Japan), and eluted into 100 μL of elution buffer. To detect the *N* gene, qPCR was performed using specific primers (ECoV-380f 5’-TGGGAACAGGCCCGC-3’ and ECoV-522r 5’-CCTAGTCGGAATAGCCTCATCAC-3’) and a TaqMan MGB probe (ECoV-436p 5’-6-

FAM-TGGGTCGCTAAC AAG-MGB-3') (Thermo Fisher Scientific) [46], using TaqPath 1-Step RT-qPCR Master Mix, CG (Thermo Fisher Scientific) according to the manufacturer's instructions. Thermal cycling conditions were as follows: initial hold at 25 °C for 2 min, 50 °C for 15 min and 95 °C for 2 min, and then 40 cycles of 95 °C for 3 sec and 60 °C for 30 sec. To produce a standard curve, control ECoV RNA was synthesized by Fasmac (Atsugi, Japan). This corresponded to a portion of the *N* gene including the target region of the qPCR assay. Samples containing more than 10 copies per reaction were considered positive. Ten copies per reaction corresponded to 6.3×10^3 ($10^{3.8}$) copies per gram of fecal and tissue samples, 6.3×10^2 ($10^{2.8}$) copies per milliliter of nasal swab suspension, and 1.3×10^3 ($10^{3.1}$) copies per milliliter of whole blood. qPCR reactions were performed in triplicates, and average copy numbers were determined. To confirm that the extraction from samples was properly performed, a housekeeping gene (β2-microglobulin) was used as an internal control, using specific primers and a probe kit (Assay ID: Ec03468699_m1, TaqMan Gene Expression Assays; Thermo Fisher Scientific).

Hematoxylin and eosin (HE) staining and ISH

HE staining was performed using routine procedures as described previously [38]. ISH to detect ECoV RNA in formalin-fixed, paraffin-embedded tissues was performed using RNAscope technology (Advanced Cell Diagnostics Inc., Newark, CA, USA) according to the manufacturer's instructions. The probe (Advanced Cell Diagnostics Inc.) targeting the nucleoprotein gene of the strain NC99 (accession number EF446615) was used [62]. After staining, ECoV-positive cells in 20 randomly selected fields at 400 × magnification were counted using PatholoCount ver. 1.2.0 (Mitani Corporation, Tokyo,

Japan). The average number of ECoV-positive cells per field was calculated and divided into three groups: < 1, ≥ 1, and > 50. As negative controls, intestinal samples collected from a 1-year-old horse that had not been inoculated with ECoV were also used for HE staining and ISH.

Results

Clinical signs and detection of viral RNA in clinical samples

Two horses presented with a fever at 2 dpi (39.5 °C, horse #1) and 3 dpi (38.9 °C, horse #2) (Figure 3), while the other two (horses #3 and #4) did not develop fever during the study period. None of the four horses showed other clinical signs such as diarrhea, colic, or respiratory disease. The ECoV RNA copy numbers in fecal samples, nasal swabs, and EDTA blood samples are shown in Figure 4. ECoV RNA was detected in fecal samples at 2 or 3 dpi in all horses. Horses #1, #2, and #3 shed the virus in their feces from 3 dpi to the day of euthanasia (at 3, 5, and 7 dpi, respectively), and horse #4 shed the virus at 2–11 and 13 dpi. For nasal swabs, ECoV RNA was detected in horse #2 at 4–5 dpi, horse #3 at 6–7 dpi, and horse #4 at 3–7, 9, 11 and 13 dpi. ECoV RNA was detected in whole blood samples from horse #1 at 2–3 dpi, and horse #4 at 2–9 and 11 dpi.

Detection of viral RNA in tissue samples

The ECoV RNA copy numbers in each tissue are shown in Table 10. In the intestinal tracts, ECoV RNA was detected in the jejunum, ileum, cecum, and colon at 3 dpi (horse #1). At 5 and 7 dpi (horse #2 and #3), ECoV RNA was detected in all sections of the intestinal tracts. At 14 dpi (horse #4), ECoV RNA was detected in the jejunum, ileum, cecum, and colon, although the quantities of viral RNA detected were lower than those in

the other horses. In the respiratory tracts, ECoV RNA was detected only in the lung tissue collected from horse #1 euthanized at 3 dpi. ECoV RNA was not detected in the trachea of any of the horses. In lymph nodes, ECoV RNA was detected in the cranial mesenteric and cecal lymph nodes in all infected horses. The colonic lymph node was positive for ECoV RNA in horse #2, #3, and #4, and the caudal mesenteric lymph node was positive in #1 and #3. ECoV RNA was not detected in the pulmonary lymph nodes in any of the horses.

Gross and histological evaluation

Grossly, no significant abnormality was observed in the intestinal or respiratory tissues in any of the horses. In contrast, swelling of the cranial mesenteric, cecal, and colonic lymph nodes was observed in all horses.

Microscopically, atrophy of villi, detachment of epithelial cells, and macrophage accumulation at the tips of the villi and in the lamina propria were observed in the jejunum and ileum collected at 3 dpi (Figure 5A, B). Similar findings were observed in the jejunum and ileum collected at 5 and 7 dpi. The accumulation of lymphocytes and plasmacytes in the lamina propria was observed in the jejunum and ileum at 7 and 14 dpi (Figure 5C, D). Follicular hyperplasia was observed in the cranial mesenteric, cecal, and colonic lymph nodes in all infected horses. In addition, these findings were also observed in the caudal mesenteric lymph node at 5, 7, and 14 dpi. No abnormalities were observed in the respiratory tissues, including the lungs and trachea, in any of the horses. No abnormalities were observed in the jejunum and ileum collected from a horse that were not inoculated with ECoV used as a negative control (Figure 5E, F).

The microscopic images obtained in ISH are shown in Figure 6, and the distribution

of ECoV-positive cells detected by ISH is shown in Table 11. At 3 dpi, ECoV-positive cells were detected in the tips of villi from the jejunum to the cecum (Figure 6A, B). At 5 and 7 dpi, ECoV-positive cells were detected in almost all sections in the intestinal tracts (Table 11). The microscopic images in the rectum at 5 dpi are shown in Figure 6C and D. At 3, 5, and 7 dpi, ECoV-positive signals were observed in the epithelial cells and macrophages within the lamina propria (Figure 6B, D), but there were no ECoV-positive cells in lymph nodes related to the gastrointestinal tract other than the cranial mesenteric lymph nodes at 3 dpi (Table 11). At 14 dpi, ECoV-positive cells were also observed at the tips of villi from the jejunum to the colon, although they were fewer than those observed at 3, 5, and 7 dpi. In addition, ECoV-positive cells were detected in the jejunum lymph nodes at 14 dpi (Figure 5E). No ECoV-positive cells were observed in the trachea or lung tissues in any of the horses (Figure 6F and Table 11). No ECoV positive cells were observed in the jejunum and rectum tissues from the horse used as negative control that did not undergo inoculation with ECoV (Figure 6G, H).

Discussion

In this study, the tissue tropism of ECoV was investigated using intestinal and respiratory tissue samples collected at different time points from horses that were experimentally inoculated with ECoV. qPCR and ISH showed that infected lesions were broadly distributed from the small intestine to the large intestine and enteric lymph nodes following a time course. Corresponding to the detection of ECoV RNA by qPCR, histological evaluation showed the accumulation of lymphocytes and plasmacytes and phagocytosis of ECoV by macrophages in the lamina propria of the small intestine.

All horses started to shed the virus in their feces 2–3 days after inoculation with

ECoV, although none of them showed gastrointestinal signs. This result demonstrates that all horses subjected to this study were infected with ECoV by oral administration of the virus containing feces. Although only two of the four horses showed fever, these findings also corresponded with those presented by previous infection studies [36]. It is considered that the horses in the current study reproduced the cases of natural infection.

Gross pathological evaluation showed no significant abnormalities in the intestinal tissues. Histopathological analysis showed that intestinal tissue damage was limited to the luminal surface of the small intestine, where villous atrophy and epithelial cell detachment were observed. ISH also showed that ECoV-positive cells were localized only on the luminal surface of the small intestine. These findings contrast with the previous reports describing pathological findings in fatal cases [9, 13]. In these reports, ECoV infection caused fatal necrotizing enterocolitis, and histopathological evaluation revealed that the cytoplasm of deep glandular enterocytes in the small intestine was infected with ECoV [13]. The differences in the distribution of ECoV within the intestinal tissues between the previous studies and the current study might be related to the severity of pathological changes in the tissues and clinical signs exhibited. Most horses that are naturally infected with ECoV in the field show mild clinical signs [15], and the results in the current study are likely to reproduce such cases. It is suggested that ECoV does not cause significant damage to the intestinal tissues in the most infected horses, although the virus infects the luminal surface throughout the intestinal tract. Other additional factors might be related to the worsening of clinical signs. Further studies are required to address unknown factors that cause the difference in viral distribution within the intestinal tissues and in disease severity.

None of the infected horses showed respiratory signs such as cough or nasal

discharge. They did not show any abnormalities in respiratory tissues such as lung or trachea tissues by gross pathological and microscopic evaluations. In horse #1 (euthanized at 3 dpi), ECoV RNA was detected in the lung by qPCR, suggesting the possibility that ECoV infected the lung tissue of this horse. However, no positive cells were detected by ISH. In addition, horse #1 showed viremia at 3 dpi when this horse was euthanized, and the lung was found to contain a large amount of blood during post-mortem examination. Therefore, these results indicate that the detection of ECoV RNA in the lung tissue of horse #1 by qPCR was not caused by the infection of the lung cells but was a result of viremia and blood contamination of the tissue. In horse #2, 3, and 4 (euthanized at 5, 7, and 14 dpi, respectively), ECoV RNA was detected in nasal swabs by qPCR a few days after inoculation of the virus. However, postmortem examination revealed that ECoV RNA was not detected in the trachea and lung tissues by either qPCR or ISH in these horses. Moreover, the highest copy number of ECoV RNA in nasal swabs was 5.8×10^5 copies at 5 dpi in horse #2, while that in fecal samples was 3.2×10^9 copies at 4 dpi. The copy numbers in nasal swabs were all at least ten times lower than those in fecal samples collected on the same days, which is consistent with our previous experimental infection study [36]. It is likely that these horses rubbed their noses against the stool floor or wall contaminated by feces containing ECoV, and the virus particles were introduced into their nasal cavity. An experimental infection study using calves inoculated orally with BCoV showed that BCoV infected the respiratory tract and induced epithelial damage in the nasal turbinate, trachea, and lungs, and interstitial pneumonia, even though the respiratory tract was not inoculated directly with BCoV [42]. In another study, involving experimental exposure by direct contact with infected calves, it was reported that the level of BCoV RNA in nasal swabs was more than 1×10^9 copies at

maximum and was sometimes higher than that in the fecal samples collected on the same day [39]. In contrast, the experimental ECoV infection of horses via the oral route in this study did not result in damage to the respiratory tract or massive viral replication, as described above, suggesting that ECoV is less likely to infect the respiratory tract.

In conclusion, qPCR and ISH showed that ECoV can broadly infect the intestinal tract tissues, but the virus infection did not cause significant damage. To determine the mechanism and factors related to the distribution of the virus and deteriorating the host tissue in fatal cases, further study is needed. Although ECoV RNA was detected by qPCR in respiratory specimens such as nasal swabs and postmortem lung tissue, viral infection was not confirmed by other examinations. Therefore, this study suggests that ECoV is less likely to infect respiratory tissues in horses.

Table 10. Copy numbers of ECoV RNA in each tissue collected from experimentally infected horses

	Horse no. (collection day)			
Tissue	#1 (3 dpi)	#2 (5 dpi)	#3 (7 dpi)	#4 (14 dpi)
Duodenum	—	8.5	8.2	—
Jejunum	9.8 [a]	8.6	7.0	4.2
Ileum	10.3	9.5	6.6	4.4
Cecum	9.0	11.6	10.1	5.9
Colon	6.0	10.7	7.8	6.0
Rectum	—	11.0	9.0	—
Trachea	—	—	—	—
Lung	6.2	—	—	—
Cranial mesenteric LN	9.7	6.2	6.7	6.2
Cecal LN	5.4	7.3	6.4	5.1
Colonic LN	—	4.0	7.9	5.3
Caudal mesenteric LN	5.2	—	4.1	—
Pulmonary LN	—	—	—	—

'—, negative; LN, lymph node

[a] The copy numbers of equine coronavirus RNA per gram are shown as the logarithm to the base 10. Samples that had more than 6.3×10^3 ($10^{3.8}$) copies per gram in tissues were considered positive for ECoV.

Table 11. Distribution of ECoV positive cells in intestinal and respiratory tissues analyzed by *in situ* hybridization

	Horse no. (collection day)			
Tissue	#1 (3 dpi)	#2 (5 dpi)	#3 (7 dpi)	#4 (14 dpi)
Duodenum	−[a]	+[b]	+	−
Jejunum	++[c]	+	−	+
Ileum	++	+	+	+
Cecum	+	++	++	+
Colon	−	++	++	+
Rectum	−	++	+	−
Trachea	−	−	−	−
Lung	−	−	−	−
Cranial mesenteric LN	+	−	−	−
Cecal LN	−	−	−	−
Colonic LN	−	−	−	−
Caudal mesenteric LN	−	−	−	−
Pulmonary LN	−	−	−	−

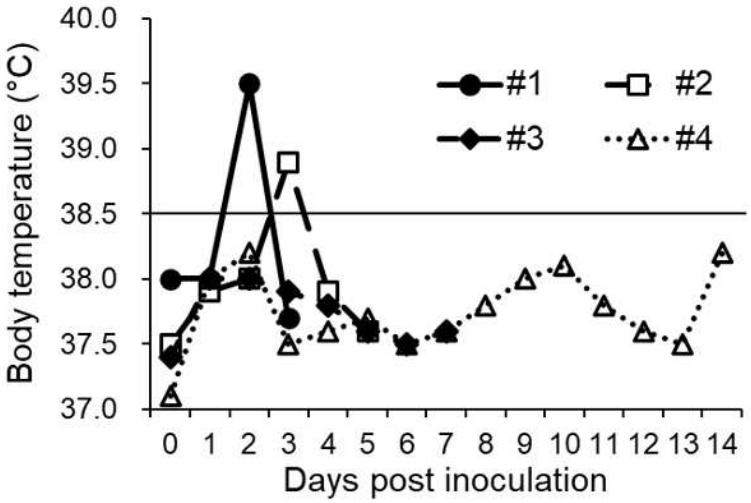
dpi, days post inoculation; LN, lymph node

[a] −, The average number of ECoV-positive cells per field was < 1.

[b] +, The average number of ECoV-positive cells per field was ≥ 1.

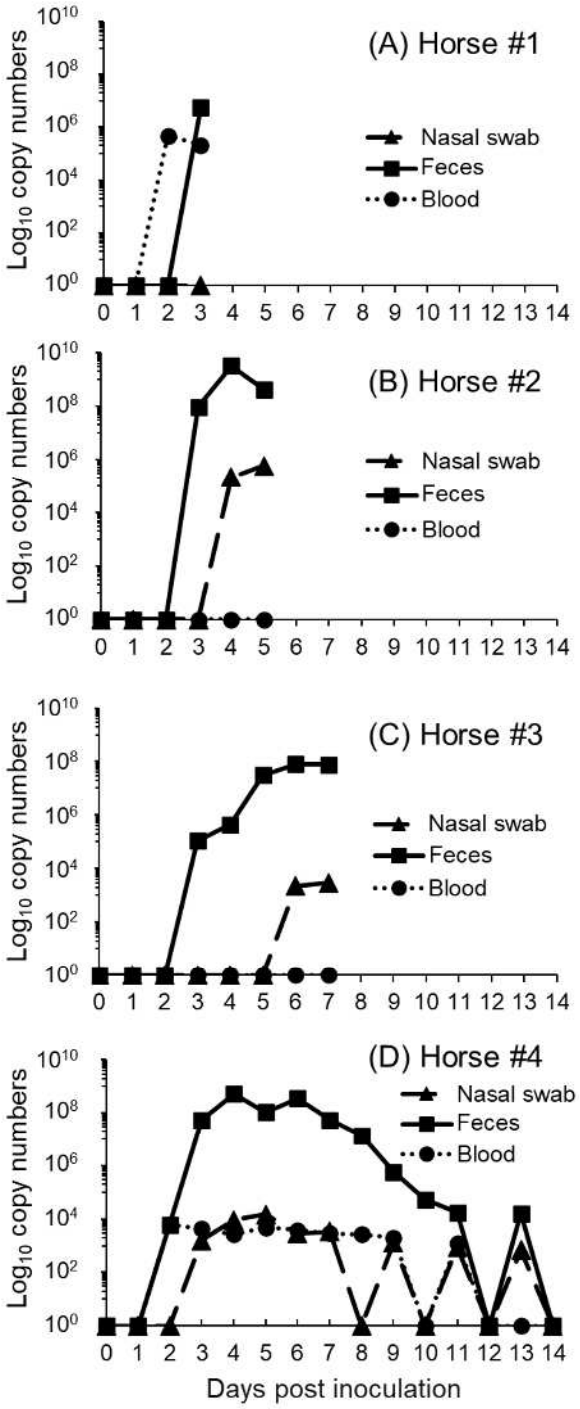
[c] ++, The average number of ECoV-positive cells per field was > 50.

Figure 3. Body temperatures of four horses experimentally inoculated with ECoV



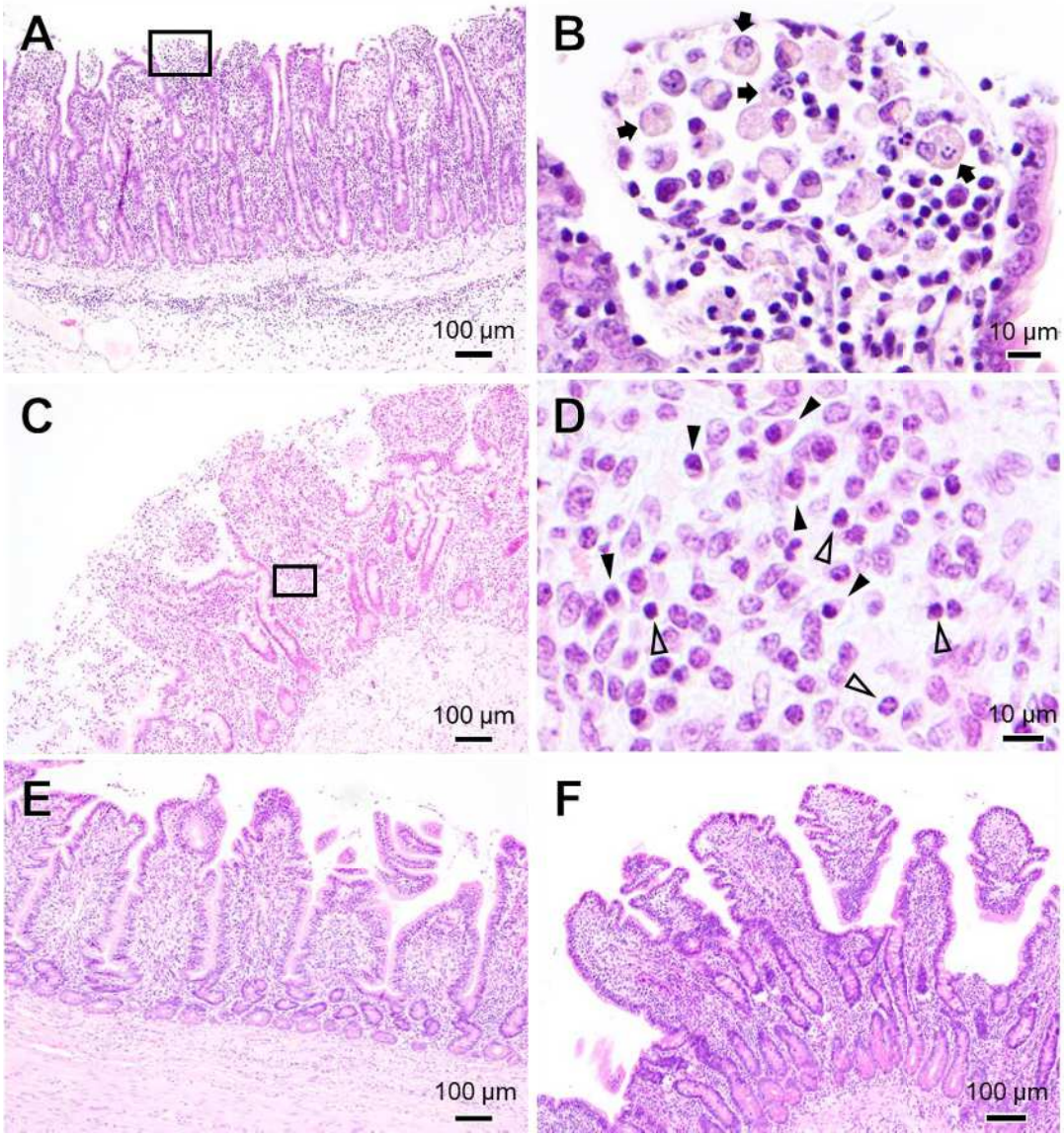
Body temperatures of horses #1, #2, #3, and #4 were recorded until 3, 5, 7, and 14 days post-inoculation, respectively. The horizontal line indicates 38.5 °C.

Figure 4. Copy number of ECoV RNA in clinical specimens



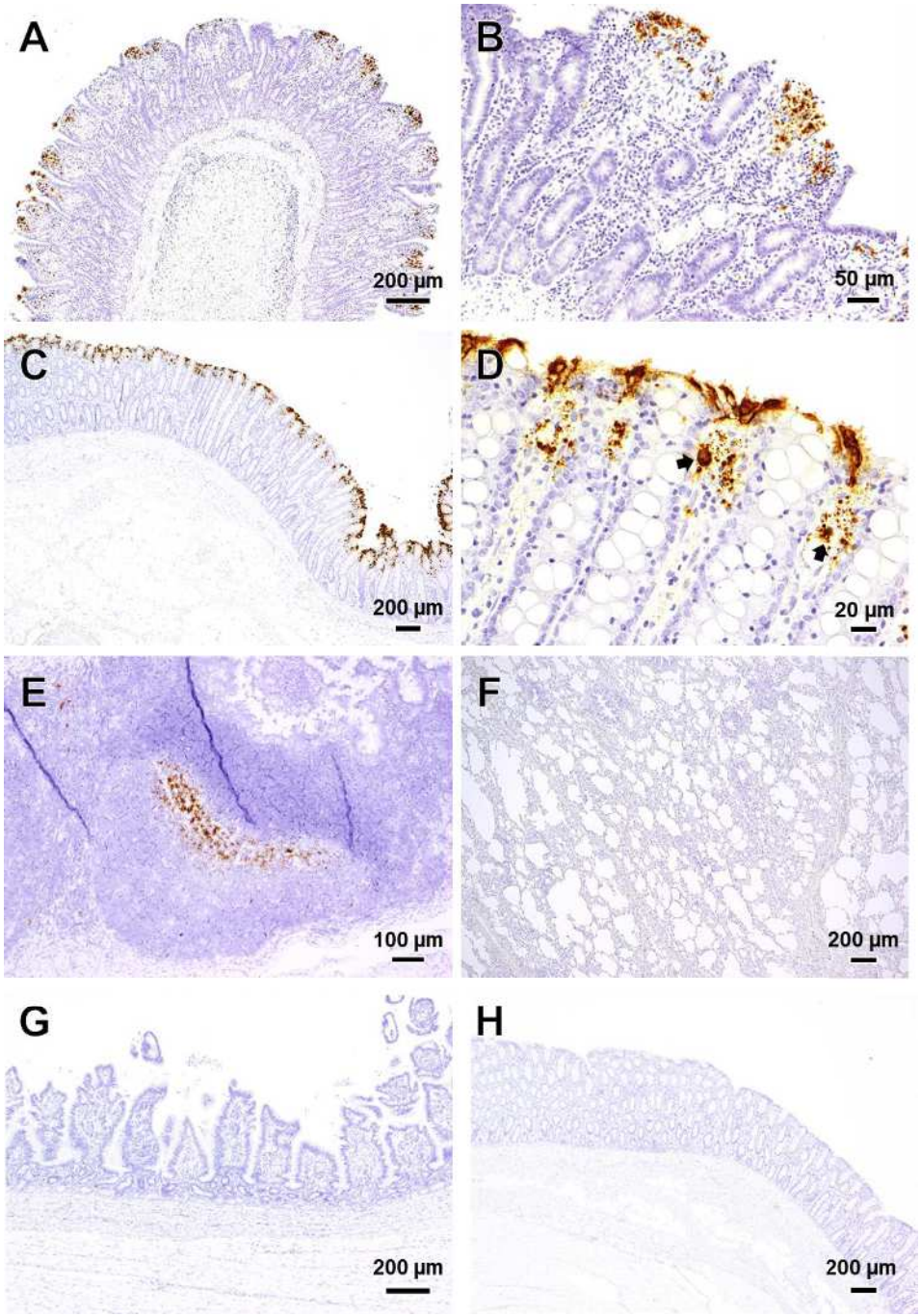
The copy numbers of ECoV RNA per gram (fecal samples) or milliliter (nasal swabs and whole blood samples) are shown. Samples that had more than 6.3×10^3 ($10^{3.8}$) copies per gram in feces, 6.3×10^2 ($10^{2.8}$) copies per milliliter in nasal swabs, and 1.3×10^3 ($10^{3.1}$) copies per milliliter in whole blood samples were considered positive.

Figure 5. Microscopic images in HE staining



(A) Representative image of the jejunum at 3 dpi (horse #1). (B) Magnification image of panel A. Atrophy of villi, detachment of epithelial cells, and macrophage accumulation (arrows) at the tips of the villi were observed. (C) Representative image of the ileum at 14 dpi (horse #4). (D) Magnification image of panel B. Lymphocytes (open arrowheads) and plasmacytes (arrowheads) accumulation in the lamina propria. (E) Jejunum and (F) Ileum of a horse that was not inoculated with ECoV as negative controls

Figure 6. Microscopic images in ISH



(A) Image of the jejunum at 3 dpi (horse #1). (B) Magnification image of panel A. (C) Image of rectum at 5 dpi (horse #2). (D) Magnification image of panel C. Macrophages are illustrated by arrows (E) Lymph node in the jejunum at 14 dpi (horse #4). (F) Lung at 3 dpi (horse #1). (D). (G) Jejunum and (H) Rectum of a horse that was not inoculated with ECoV were used as negative controls

GENERAL DISCUSSION

ECoV is an enteric pathogen of horses that causes fever, lethargy, anorexia, and diarrhea [45]. Outbreaks of ECoV infection have been reported around the world, including Asia, Europe, and North America [15, 27, 40]. In Japan, although outbreaks of ECoV infection occurred three times in a population of draft horses [28, 40, 41], there has been no reported ECoV outbreak involving other equine breeds including Thoroughbreds.

The epidemiology of ECoV has been reported sporadically around the world, and ECoV RNA has been detected in the feces collected from both sick and healthy horses [52]. A retrospective study showed that the infection rate was increased during the cold season [3], indicating ECoV is more likely to spread during cold months than warm months, as is the case with other CoVs such as BCoV and some types of HCoVs [53, 56]. Although there is one report describing the prevalence of ECoV in pre-weaned foals [34], the epidemiology of ECoV infection among Thoroughbred yearlings and racehorses in Japan has not been surveyed. Therefore, the potential risk of ECoV infection to the horse industry in Japan is not well known.

Although a few studies described clinical signs and shedding of the virus in horses experimentally inoculated with ECoV, the post-mortem pathological findings were not evaluated [36, 50]. Pathological reports in fatal cases suggested that ECoV has strong intestinal tropism [9, 13], but the distribution of the virus through the intestinal tract was not known. ECoV RNA was also detected in nasal swabs collected from both naturally and experimentally infected horses [27, 36]. However, it was not determined that the detection of ECoV RNA in nasal swabs was indicative of a viral infection to the respiratory tract like other CoVs, because it may have been due to a secondary contamination of the nasal cavity by feces containing the virus.

Thus, there is a gap in knowledge regarding epidemiological and pathological characteristics of ECoV infection, especially in Thoroughbreds in Japan. This series of studies was conducted to elucidate these characteristics, and the following findings have been obtained.

In CHAPTER 1, an outbreak that occurred among 41 riding horses comprised of various breeds has been reported. This is the first report of an ECoV outbreak involving equine breeds other than draft horses in Japan. In this outbreak, 15 horses were affected, and most of them showed fever, while only three horses developed diarrhea. All affected horses recovered within a few days without needing intensive care. The VN test showed that all horses in this stable became infected with ECoV, suggesting that ECoV is highly contagious. ECoV RNA was detected in 30 horses at least once during this outbreak. The longest viral shedding period was 98 days, which was recorded in an Andalusian showing no clinical signs, suggesting that subclinical horses can play an important role as ECoV spreaders. These results underscore the importance of quarantine measure, especially to prevent the virus transmission to other premises during the ECoV outbreak. There was also a significant difference in the viral shedding period between Thoroughbreds and non-Thoroughbreds, suggesting a potential difference in the persistence of ECoV among horse breeds and that some breeds can contribute more to the spread of the virus. In the sequence and phylogenetic analyses, the nucleotide length from *p4.7* to *p12.7* genes in the current virus was different from that in the previous ECoV strains, despite the overall similarities. In addition, the deletion of nucleotides in the *NS2* region which was identified in the strains isolated in the previous outbreak in Japan was not observed. Therefore, this outbreak might have been caused by a different ECoV from those that caused outbreaks among draft horses in Japan.

In CHAPTER 2, the epidemiology of ECoV in the Thoroughbred yearlings and racehorses in Japan was investigated. Sera collected three times at intervals of several months in each population were subjected to this study, i.e., sera were collected in August, December, and the following April in yearlings, and in April/May, November, and the following May in racehorses. The ECoV infection in the yearlings predominantly occurred between August and December with 60.9% infection rate, and 39.2% of them developed fever during the estimated period of viral exposure. Pasture sharing and close contact of the horses after their introduction into the farm in August may have contributed to the rapid spread of ECoV. In racehorses, the infection rate was significantly higher between November and the following May than between the preceding April/May and November. Since racehorses are always reared in individual stalls and do not have opportunities to closely contact with other horses like yearlings do, the difference in infection rates between the seasons should reflect the seasonal nature of ECoV. Although the previous outbreaks have occurred during both warm and cold seasons [28, 40, 41], ECoV infection is more likely to spread during cold months like other CoVs such as BCoV [7]. This study shows that ECoV is widely prevalent in the Japanese Thoroughbred population. In addition, the relatively high morbidity rate in the yearlings suggests an association of ECoV infection with pyretic cases, whereas this was not the case in the racehorses. However, no diseased horses were severely affected in either population, therefore, the potential risk of ECoV infection to the equine industry in Japan is considered low.

In CHAPTER 3, the infection site of ECoV and the pathological findings in the intestinal and respiratory tissues were investigated in four horses that were experimentally infected with ECoV and euthanized at 3, 5, 7, and 14 dpi. qPCR showed that all horses

shed the virus in their feces at 2 or 3 dpi, and two of them developed fever. No horses exhibited diarrhea and were severely affected. Histological evaluations of the small intestine revealed villous atrophy, detachment of epithelial cells, and accumulation of macrophages at the tips of the villi and in the lamina propria. ECoV RNA was detected in the wide range of the small intestine and colon by qPCR. ISH also showed that ECoV-positive cells were broadly distributed in the luminal surface of the intestinal tracts, corresponding with the qPCR results. These results suggest that ECoV can infect all sections of the intestine, which may contribute to the large amount of virus shed in the feces. ECoV RNA was also detected in nasal swabs in a few horses and in the lung tissue from one horse. However, these results were not indicative of respiratory infection with ECoV because they may have been caused by the secondary contamination with feces containing ECoV or viremia. In both the clinical and pathological findings, this experimental study reproduced the naturally infected cases that showed mild clinical signs or were asymptomatic.

In conclusion, this thesis demonstrates that ECoV is highly contagious, commonly prevalent in Thoroughbred populations in Japan, and spreads predominantly during the cold season. However, the potential risk of ECoV infection to the equine industry is considered low because all cases in these studies showed mild clinical signs or were asymptomatic. Nevertheless, it should be recognized that ECoV can be the causative agent of fever in horses and is easily transmitted to other horses. Equine practitioners, owners, and employees may have missed the ECoV infected cases without diarrhea so far, because it might be a common perception that gastrointestinal signs are representative for ECoV infection. The halt of training due to fever, even temporary, can lead to economic loss in competition horses including Thoroughbred racehorses. Therefore, the spread of

infection should be controlled although most cases show mild clinical signs. The current studies suggested that movement of horses between facilities and the change of rearing environment are most likely to be associated with the spread of ECoV. In addition, people can play a role in transmission of ECoV as suggested in a previous study [15]. ECoV is transmitted via the fecal-oral route, and boots and clothing contaminated with feces are likely to be sources of infection. The movement of horses should be restricted during an outbreak, and biosecurity management of people is also important. Although all cases showed mild clinical signs or were asymptomatic in the current studies, some previous reports described fatal cases. Since the risk factors that lead to the worsening of the disease and the consequent development of enteritis and neurological disorders are not known, further case studies are needed for clarification.

ACKNOWLEDGEMENT

I would like to express my gratitude to my chief supervisor, Professor Daisuke Hayasaka, Laboratory of Veterinary Microbiology, Joint Graduate School of Veterinary Science, Yamaguchi University, for his invaluable guidance and review of this thesis.

I am deeply grateful to Dr. Hiroshi Bannai, Dr. Manabu Nemoto, and Dr. Koji Tsujimura, Molecular Biology Division, Equine Research Institute, Japan Racing Association, as well as Dr. Minoru Ohta, Clinical Veterinary Medicine Division, Equine Research Institute, Japan Racing Association, for their enthusiastic and patient cooperation and support for my research. I am also grateful to my laboratory members, Dr. Nanako Kawanishi for her advice on my research and to Akira Kokubun, Miwa Tanaka, Akiko Kasagawa, Kaoru Watanabe, Akiko Suganuma, and Kayo Iino for their technical assistance in the laboratory. Additionally, I am thankful for the helpful cooperation and sample collection provided by all members of the Equine Research Institute and veterinarians of the Japan Racing Association.

Finaly, I would like to thank my family for their constant encouragement and support.

REFERENCES

1. Balasuriya, U. B., Leutenegger, C. M., Topol, J. B., McCollum, W. H., Timoney, P. J. and MacLachlan, N. J. 2002. Detection of equine arteritis virus by real-time TaqMan reverse transcription-PCR assay. *J Virol Methods* **101**: 21-28.

2. Bass, E. P. and Sharpee, R. L. 1975. Coronavirus and gastroenteritis in foals. *Lancet* **2**: 822.

3. Berryhill, E. H., Magdesian, K. G., Aleman, M. and Pusterla, N. 2019. Clinical presentation, diagnostic findings, and outcome of adult horses with equine coronavirus infection at a veterinary teaching hospital: 33 cases (2012-2018). *Vet J* **248**: 95-100.

4. Browning, G. F., Chalmers, R. M., Snodgrass, D. R., Batt, R. M., Hart, C. A., Ormarod, S. E., Leadon, D., Stoneham, S. J. and Rossdale, P. D. 1991. The prevalence of enteric pathogens in diarrhoeic thoroughbred foals in Britain and Ireland. *Equine Vet J* **23**: 405-409.

5. Bryan, J., Marr, C. M., Mackenzie, C. J., Mair, T. S., Fletcher, A., Cash, R., Phillips, M., Pusterla, N., Mapes, S. and Foote, A. K. 2019. Detection of equine coronavirus in horses in the United Kingdom. *Vet Rec* **184**: 123.

6. Bustin, S. A. and Mueller, R. 2005. Real-time reverse transcription PCR (qRT-PCR) and its potential use in clinical diagnosis. *Clin Sci (Lond)* **109**: 365-379.

7. Cho, K. O., Halbur, P. G., Bruna, J. D., Sorden, S. D., Yoon, K. J., Janke, B. H., Chang, K. O. and Saif, L. J. 2000. Detection and isolation of coronavirus from feces of three herds of feedlot cattle during outbreaks of winter dysentery-like disease. *J Am Vet Med Assoc* **217**: 1191-1194.

8. Collins, J. K., Riegel, C. A., Olson, J. D. and Fountain, A. 1987. Shedding of enteric coronavirus in adult cattle. *Am J Vet Res* **48**: 361-365.

9. Davis, E., Rush, B. R., Cox, J., DeBey, B. and Kapil, S. 2000. Neonatal enterocolitis associated with coronavirus infection in a foal: a case report. *J Vet Diagn Invest* **12**: 153-156.

10. Durham, P. J., Stevenson, B. J. and Farquharson, B. C. 1979. Rotavirus and coronavirus associated diarrhoea in domestic animals. *N Z Vet J* **27**: 30-32.

11. Dwyer, R. M. 1993. Rotaviral diarrhea. *Vet Clin North Am Equine Pract* **9**: 311-319.

12. Fielding, C. L., Higgins, J. K., Higgins, J. C., McIntosh, S., Scott, E., Giannitti, F., Mete, A. and Pusterla, N. 2015. Disease associated with equine coronavirus infection and high case fatality rate. *J Vet Intern Med* **29**: 307-310.

13. Giannitti, F., Diab, S., Mete, A., Stanton, J. B., Fielding, L., Crossley, B., Sverlow, K., Fish, S., Mapes, S., Scott, L. and Pusterla, N. 2015. Necrotizing Enteritis and Hyperammonemic Encephalopathy Associated With Equine Coronavirus Infection in Equids. *Vet Pathol* **52**: 1148-1156.

14. Gilkerson, J. R., Bailey, K. E., Diaz-Méndez, A. and Hartley, C. A. 2015. Update on viral diseases of the equine respiratory tract. *Vet Clin North Am Equine Pract* **31**: 91-104.

15. Goodrich, E. L., Mittel, L. D., Glaser, A., Ness, S. L., Radcliffe, R. M. and Divers, T. J. 2020. Novel findings from a beta coronavirus outbreak on an American Miniature Horse breeding farm in upstate New York. *Equine Vet Educ* **32**: 150-154.

16. Guy, J. S., Breslin, J. J., Breuhaus, B., Vivrette, S. and Smith, L. G. 2000. Characterization of a coronavirus isolated from a diarrheic foal. *J Clin Microbiol* **38**: 4523-4526.

17. Haake, C., Cook, S., Pusterla, N. and Murphy, B. 2020. Coronavirus Infections in Companion Animals: Virology, Epidemiology, Clinical and Pathologic Features. *Viruses* **12**.

18. Hemida, M. G., Chu, D. K. W., Perera, R., Ko, R. L. W., So, R. T. Y., Ng, B. C. Y., Chan, S. M. S., Chu, S., Alnaeem, A. A., Alhammadi, M. A., Webby, R. J., Poon, L. L. M., Balasuriya, U. B. R. and Peiris, M. 2017. Coronavirus infections in horses in Saudi Arabia and Oman. *Transbound Emerg Dis* **64**: 2093-2103.

19. Hierweger, M. M., Remy-Wohlfender, F., Franzen, J., Koch, M. C., Blau, D., Schoster, A., Nicholson, P., Gerber, V., Gurtner, C., Fouché, N., Unger, L. and Seuberlich, T. 2022. Outbreak of equine coronavirus disease in adult horses, Switzerland 2021. *Transbound Emerg Dis* **69**: 1691-1694.

20. Hu, B., Guo, H., Zhou, P. and Shi, Z. L. 2021. Characteristics of SARS-CoV-2 and COVID-19. *Nat Rev Microbiol* **19**: 141-154.

21. Huang, J. C., Wright, S. L. and Shipley, W. D. 1983. Isolation of coronavirus-like agent from horses suffering from acute equine diarrhoea syndrome. *Vet Rec* **113**: 262-263.

22. Killerby, M. E., Biggs, H. M., Haynes, A., Dahl, R. M., Mustaquim, D., Gerber, S. I. and Watson, J. T. 2018. Human coronavirus circulation in the United States 2014-2017. *J Clin Virol* **101**: 52-56.

23. Kooijman, L. J., Mapes, S. M. and Pusterla, N. 2016. Development of an equine coronavirus-specific enzyme-linked immunosorbent assay to determine serologic responses in naturally infected horses. *J Vet Diagn Invest* **28**: 414-418.

24. Kooijman, L. J., James, K., Mapes, S. M., Theelen, M. J. and Pusterla, N. 2017. Seroprevalence and risk factors for infection with equine coronavirus in healthy horses in the USA. *Vet J* **220**: 91-94.

25. Kumar, S., Stecher, G. and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol* **33**: 1870-1874.

26. Lawrence, G. L., Gilkerson, J., Love, D. N., Sabine, M. and Whalley, J. M. 1994.

Rapid, single-step differentiation of equid herpesviruses 1 and 4 from clinical material using the polymerase chain reaction and virus-specific primers. *J Virol Methods* **47**: 59-72.

27. Miszczak, F., Tesson, V., Kin, N., Dina, J., Balasuriya, U. B., Pronost, S. and Vabret, A. 2014. First detection of equine coronavirus (ECoV) in Europe. *Vet Microbiol* **171**: 206-209.

28. Narita, M., Nobumoto, K., Takeda, H., Moriyama, T., Morita, Y. and Nakaoka, Y. 2011. Prevalence of disease with inference of equine coronavirus infection among horses atabled in a draft-horse racecourse. *J Jpn Vet Med Assoc* **64**: 535-539, in Japanese, with English abstract.

29. Nemoto, M. and Matsumura, T. 2021. Equine rotavirus infection. *J Equine Sci* **32**: 1-9.

30. Nemoto, M., Schofield, W. and Cullinane, A. 2019. The First Detection of Equine Coronavirus in Adult Horses and Foals in Ireland. *Viruses* **11**.

31. Nemoto, M., Kanno, T., Bannai, H., Tsujimura, K., Yamanaka, T. and Kokado, H. 2017. Antibody response to equine coronavirus in horses inoculated with a bovine coronavirus vaccine. *J Vet Med Sci* **79**: 1889-1891.

32. Nemoto, M., Morita, Y., Niwa, H., Bannai, H., Tsujimura, K., Yamanaka, T. and Kondo, T. 2015. Rapid detection of equine coronavirus by reverse transcription loop-mediated isothermal amplification. *J Virol Methods* **215-216**: 13-16.

33. Nemoto, M., Oue, Y., Murakami, S., Kanno, T., Bannai, H., Tsujimura, K., Yamanaka, T. and Kondo, T. 2015. Complete genome analysis of equine coronavirus isolated in Japan. *Arch Virol* **160**: 2903-2906.

34. Nemoto, M., Oue, Y., Higuchi, T., Kinoshita, Y., Bannai, H., Tsujimura, K.,

Yamanaka, T. and Kondo, T. 2015. Low prevalence of equine coronavirus in foals in the largest thoroughbred horse breeding region of Japan, 2012-2014. *Acta Vet Scand* **57**: 53.

35. Nemoto, M., Okita, N., Kitahata, M., Bannai, H., Tsujimura, K., Kinoshita, Y., Kambayashi, Y., Cullinane, A., Yamanaka, T. and Ohta, M. 2020. Evaluation of cobas Influenza A/B & RSV Test for Diagnosis of Equine Influenza. *J Equine Vet Sci* **94**: 103249.

36. Nemoto, M., Oue, Y., Morita, Y., Kanno, T., Kinoshita, Y., Niwa, H., Ueno, T., Katayama, Y., Bannai, H., Tsujimura, K., Yamanaka, T. and Kondo, T. 2014. Experimental inoculation of equine coronavirus into Japanese draft horses. *Arch Virol* **159**: 3329-3334.

37. Nomura, M., Kuroda, T., Tamura, N., Muranaka, M. and Niwa, H. 2020. Mortality, clinical findings, predisposing factors and treatment of Clostridioides difficile colitis in Japanese thoroughbred racehorses. *Vet Rec* **187**: e14.

38. Ochi, A., Sekiguchi, M., Tsujimura, K., Kinoshita, T., Ueno, T. and Katayama, Y. 2019. Two Cases of Equine Multinodular Pulmonary Fibrosis in Japan. *J Comp Pathol* **170**: 46-52.

39. Oma, V. S., Tråvén, M., Alenius, S., Myrmel, M. and Stokstad, M. 2016. Bovine coronavirus in naturally and experimentally exposed calves; viral shedding and the potential for transmission. *Virol J* **13**: 100.

40. Oue, Y., Morita, Y., Kondo, T. and Nemoto, M. 2013. Epidemic of equine coronavirus at Obihiro Racecourse, Hokkaido, Japan in 2012. *J Vet Med Sci* **75**: 1261-1265.

41. Oue, Y., Ishihara, R., Edamatsu, H., Morita, Y., Yoshida, M., Yoshima, M., Hatama, S., Murakami, K. and Kanno, T. 2011. 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. *Vet Microbiol* **150**: 41-48.

42. Park, S. J., Kim, G. Y., Choy, H. E., Hong, Y. J., Saif, L. J., Jeong, J. H., Park, S. I.,

Kim, H. H., Kim, S. K., Shin, S. S., Kang, M. I. and Cho, K. O. 2007. Dual enteric and respiratory tropisms of winter dysentery bovine coronavirus in calves. *Arch Virol* **152**: 1885-1900.

43. Prutton, J. S. W., Barnum, S. and Pusterla, N. 2019. Evaluation of safety, humoral immune response and faecal shedding in horses inoculated with a modified-live bovine coronavirus vaccination. *Equine Vet Educ* **32**: 33-36.

44. Pusterla, N., James, K., Mapes, S. and Bain, F. 2019. Frequency of molecular detection of equine coronavirus in faeces and nasal secretions in 277 horses with acute onset of fever. *Vet Rec* **184**: 385.

45. Pusterla, N., Vin, R., Leutenegger, C. M., Mittel, L. D. and Divers, T. J. 2018. Enteric coronavirus infection in adult horses. *Vet J* **231**: 13-18.

46. Pusterla, N., Mapes, S., Wademan, C., White, A., Ball, R., Sapp, K., Burns, P., Ormond, C., Butterworth, K., Bartol, J. and Magdesian, K. G. 2013. Emerging outbreaks associated with equine coronavirus in adult horses. *Vet Microbiol* **162**: 228-231.

47. Saif, L. J. 2010. Bovine respiratory coronavirus. *Vet Clin North Am Food Anim Pract* **26**: 349-364.

48. Saif, L. J. and Jung, K. 2020. Comparative Pathogenesis of Bovine and Porcine Respiratory Coronaviruses in the Animal Host Species and SARS-CoV-2 in Humans. *J Clin Microbiol* **58**.

49. Sanz, M. G., Kwon, S., Pusterla, N., Gold, J. R., Bain, F. and Evermann, J. 2019. Evaluation of equine coronavirus fecal shedding among hospitalized horses. *J Vet Intern Med* **33**: 918-922.

50. Schaefer, E., Harms, C., Viner, M., Barnum, S. and Pusterla, N. 2018. Investigation of an experimental infection model of equine coronavirus in adult horses. *J Vet Intern*

Med **32**: 2099-2104.

51. Shah, M. M., Winn, A., Dahl, R. M., Kniss, K. L., Silk, B. J. and Killerby, M. E. 2022. Seasonality of Common Human Coronaviruses, United States, 2014-2021(1). *Emerg Infect Dis* **28**: 1970-1976.

52. Slovis, N. M., Elam, J., Estrada, M. and Leutenegger, C. M. 2014. Infectious agents associated with diarrhoea in neonatal foals in central Kentucky: a comprehensive molecular study. *Equine Vet J* **46**: 311-316.

53. Su, S., Wong, G., Shi, W., Liu, J., Lai, A. C. K., Zhou, J., Liu, W., Bi, Y. and Gao, G. F. 2016. Epidemiology, Genetic Recombination, and Pathogenesis of Coronaviruses. *Trends Microbiol* **24**: 490-502.

54. Tråvén, M., Näslund, K., Linde, N., Linde, B., Silván, A., Fossum, C., Hedlund, K. O. and Larsson, B. 2001. Experimental reproduction of winter dysentery in lactating cows using BCV -- comparison with BCV infection in milk-fed calves. *Vet Microbiol* **81**: 127-151.

55. Tsunemitsu, H. and Saif, L. J. 1995. Antigenic and biological comparisons of bovine coronaviruses derived from neonatal calf diarrhea and winter dysentery of adult cattle. *Arch Virol* **140**: 1303-1311.

56. Vlasova, A. N. and Saif, L. J. 2021. Bovine Coronavirus and the Associated Diseases. *Front Vet Sci* **8**: 643220.

57. Wang, Q., Vlasova, A. N., Kenney, S. P. and Saif, L. J. 2019. Emerging and re-emerging coronaviruses in pigs. *Curr Opin Virol* **34**: 39-49.

58. Weiss, S. R. and Navas-Martin, S. 2005. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. *Microbiol Mol Biol Rev* **69**: 635-664.

59. Wekesa, S. N., Inoshima, Y., Murakami, K. and Sentsui, H. 2001. Genomic analysis of some Japanese isolates of Getah virus. *Vet Microbiol* **83**: 137-146.

60. Woo, P. C., Lau, S. K., Huang, Y. and Yuen, K. Y. 2009. Coronavirus diversity, phylogeny and interspecies jumping. *Exp Biol Med (Maywood)* **234**: 1117-1127.

61. Woo, P. C., Lau, S. K., Lam, C. S., Lau, C. C., Tsang, A. K., Lau, J. H., Bai, R., Teng, J. L., Tsang, C. C., Wang, M., Zheng, B. J., Chan, K. H. and Yuen, K. Y. 2012. Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. *J Virol* **86**: 3995-4008.

62. Zhang, J., Guy, J. S., Snijder, E. J., Denniston, D. A., Timoney, P. J. and Balasuriya, U. B. 2007. Genomic characterization of equine coronavirus. *Virology* **369**: 92-104.

63. Zhong, N. S., Zheng, B. J., Li, Y. M., Poon, Xie, Z. H., Chan, K. H., Li, P. H., Tan, S. Y., Chang, Q., Xie, J. P., Liu, X. Q., Xu, J., Li, D. X., Yuen, K. Y., Peiris and Guan, Y. 2003. Epidemiology and cause of severe acute respiratory syndrome (SARS) in Guangdong, People's Republic of China, in February, 2003. *Lancet* **362**: 1353-1358.

64. Zhu, N., Zhang, D., Wang, W., Li, X., Yang, B., Song, J., Zhao, X., Huang, B., Shi, W., Lu, R., Niu, P., Zhan, F., Ma, X., Wang, D., Xu, W., Wu, G., Gao, G. F. and Tan, W. 2020. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N Engl J Med* **382**: 727-733.

65. Zumla, A., Hui, D. S. and Perlman, S. 2015. Middle East respiratory syndrome. *Lancet* **386**: 995-1007.