Canine Coronavirus: Not Only an Enteric Pathogen

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Canine coronavirus (CCoV) is strictly related to coronaviruses of cats and pigs, with which it is now included in a unique viral species.[1] To date, two different canine coronavirus genotypes are known, which have been designated types I and II,[2] and canine/porcine recombinant strains have been also identified in recent years.[3,4] CCoV is generally recognized as the etiologic agent of self-limiting infections of the small intestine, which can lead to mild gastroenteritis.[5] However, a few years ago a highly virulent strain (pantropic CCoV) was isolated that was responsible for an outbreak of fatal, systemic disease in pups.[6] Such a strain displayed some genetic changes with respect to extant strains circulating in the dog population. The disease induced by the strain isolated from the natural outbreak was reproduced under experimental conditions.[7–9] This article reviews the currently available literature on pantropic CCoV, providing a meaningful update on the virologic, epidemiologic, clinical, diagnostic, and prophylactic aspects of the infections caused by this emerging pathogen of dogs.

AN OVERVIEW OF CANINE CORONAVIRUSES
Coronavirus Structure, Genome, and Taxonomy

Coronaviruses (family Coronaviridae, order Nidovirales) are enveloped viruses associated mainly with enteric and respiratory diseases in mammals and birds. The round and sometimes pleomorphic coronavirion, 80 to 120 nm in diameter, contains a linear, positive-strand RNA molecule, which is complexed with the highly basic nucleocapsid phosphoprotein (N) to form a helical capsid found within the viral envelope. The coronavirus membranes contain at least three viral proteins: the spike (S), envelope (E), and membrane (M) proteins. The S glycoprotein mediates viral attachment to specific cell receptors and fusion between the envelope and plasma membrane and it is the main inducer of virus-neutralizing antibodies. The E protein plays an important role in viral envelope assembly, but it is not essential for virus propagation. The M protein, the most abundant structural component, is a type III glycoprotein consisting

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of a short amino-terminal ectodomain, a triple-spanning transmembrane domain, and a long carboxyl-terminal inner domain. Some coronaviruses possess an additional structural protein, the hemagglutinin-esterase (HE), closely related to the hemagglutinin-esterase fusion protein of influenza C virus.[10]

Among RNA viruses, coronaviruses possess the largest genome, 27.6 to 31 kb in size. The 5′-most two thirds of the genome comprise the replicase gene, which consists of two overlapping open reading frames, ORF 1a and 1b. Located downstream of ORF1b are up to 11 ORFs that code for the 4 common structural proteins and a variable set of accessory proteins. Number, nucleotide sequence, and order of these additional genes can vary remarkably among different coronaviruses. The function of the accessory proteins is in most cases unknown. Albeit not essential for virus replication, they play an important role in virus–host interactions because they are generally maintained during natural infection, and their loss—either through spontaneous mutation or reversed genetics—results in reduced virulence.[10]

Until a few years ago, three major coronavirus groups were distinguished based on phylogenetic and antigenic analyses. CCoV was included in phylogroup 1, together with feline coronaviruses (FCoVs) type I and type II, transmissible gastroenteritis virus (TGEV) of swine, porcine respiratory coronavirus (PRCoV), porcine epidemic diarrhea virus (PEDV), and human coronaviruses 229E (HCoV-229E) and NL63 (HCoV-NL63).[10] Subsequently, a ferret coronavirus[11] and viruses identified in bats[12] were proposed as tentative members of this group. Recently, the International Committee of Taxonomy of Viruses accepted the proposal of the Coronavirus Study Group to revise the family Coronaviridae to include the corona- and toroviruses as subfamilies (Corona- and Torovirinae) and to convert the coronavirus phylogroups 1, 2, and 3 into genera (*Alpha*-, *Beta*-, and *Gammacoronavirus*, respectively).[1] The new taxonomy is based upon rooted phylogeny and quantitative pairwise sequence comparison and includes a clear definition of coronavirus species demarcation in accordance with that used in other virus families. Given their close genetic relatedness (>96% amino acid identity in the key replicase 1ab domains), TGEV, CCoV, and FCoV are now considered not as separate viruses but rather as host range variants of the same species, *Alphacoronavirus-1*.

Apart from the enterotropic virus CCoV, dogs harbor a genetically and antigenically unrelated coronavirus, canine respiratory coronavirus (CRCoV).[13] This virus displays high sequence identity to bovine coronavirus (BCoV)[14,15] and is now recognized as a host variant of the unique species *Betacoronavirus-1* of the genus *Betacoronavirus*,[1] together with BCoV and BCoV-related viruses.[16,17]

Origin and Evolution of CCoV

The first report on CCoV infection appeared in 1971, when Binn and colleagues isolated a coronavirus (strain 1-71) from dogs with acute enteritis in a canine military unit in Germany.[18] The disease could be reproduced in young dogs by experimental infection with the purified virus, thus fulfilling Koch's postulates.[19] Since then, several CCoV outbreaks have been reported worldwide, showing that CCoV is an important enteropathogen of dogs. Serologic and virologic investigations demonstrated that CCoV is widespread in the dog population, and the virus is highly prevalent in kennels and animal shelters.[5] Enteric CCoV infection is characterized by high morbidity and low mortality. The virus is shed at high titers in the feces and transmitted via the fecal–oral route. CCoV infection is generally restricted to the alimentary tract, leading to the onset of clinical signs typical of gastroenteritis including loss of appetite, vomiting, fluid diarrhea, dehydration, and, only very rarely, death.[5] Although, in general, CCoV does not cause systemic disease, the virus has been isolated from

several tissues (tonsils, lungs, and liver) of experimentally infected pups.[20] Fatal disease commonly occurs as a consequence of mixed infections of CCoV with canine parvovirus type 2 (CPV-2),[21,22] with canine adenovirus type 1 (CAdV-1)[23] or with canine distemper virus (CDV).[24]

Currently, two genotypes of CCoV are known, which have been designated CCoV types I (CCoV-I) and II (CCoV-II).[25–32] These genotypes differ mainly in their spike proteins that share only 54% identity.[25] Moreover, CCoV-I strains possess a unique ORF, 624 nt in length, that is completely absent in FCoV-I strains and of which only remnants remain in the genomes of CCoV-II and TGEV.[33] In addition, CCoVs with a recombinant origin between CCoV-II and TGEV have been identified in the feces of dogs with diarrhea and have been found to be widespread in dog populations. Accordingly, CCoV-II has been further classified into two subtypes, CCoV-IIa and CCoV-IIb, including "classic" CCoVs and TGEV-like strains, respectively.[3] Subtype CCoV-IIb has been reported in several European countries,[4,34] as well as in Japan.[31]

PANTROPIC CCoV
Virus Emergence and Clinical Outbreaks

Similar to other coronaviruses, CCoV can mutate, resulting in more virulent strains and corresponding increased severity of enteric illness.[35–38]

On May 2005, a severe outbreak of fatal, systemic disease affected seven dogs housed in a pet shop in Apulia region, Italy. Clinical signs were first observed in three miniature pinschers and a cocker spaniel, 45 and 53 days of age, respectively, and were highly suggestive of canine parvovirus infection, consisting of fever (39.5°–40°C), lethargy, anorexia, vomiting, hemorrhagic diarrhea, and neurologic signs (ataxia, seizures) followed by death within 2 days after the onset of the symptoms. Veterinarians also reported a marked leukopenia, with total WBC counts below 50% of the baseline values. After a few days, the same signs were observed in two other 45-day-old miniature pinschers and in one 56-day-old Pekinese dog, which underwent a rapid fatal outcome.[6]

Necropsy on the carcasses of these three pups revealed severe lesions in the alimentary tract, tonsils, lungs, liver, spleen, and kidneys. The most prominent lesion was hemorrhagic gastroenteritis. Tonsils were enlarged and contained multifocal hemorrhages. Lobar subacute bronchopneumonia was evident both in the cranial and caudal lobes (**Fig. 1**), and accompanied effusions in the thoracic cavity. Spleens were enlarged and exhibited subcapsular hemorrhages. Necrosis and lipidosis with hemorrhages was evident in the livers. Infarction and hemorrhages were detected in the cortex and medulla of kidneys (**Fig. 2**; Ref.[6] and Buonavoglia and colleagues, unpublished data, 2006).

Histopathology in the mucosa of the small intestine consisted of atrophy and flattening of most villi, with denudating of the lining epithelium, mononuclear infiltration of the lamina propria, and cell depletion of the centers of lymphoid follicles. Severe coalescing bronchoalveolar lesions in the lungs consisted of a densely cellular fibrinopurulent exudate (**Fig. 3**). Diffuse hepatocyte degeneration was present with moderate microvacuolar fatty change and minimal random necrosis (**Fig. 4**). Splenic lesions were characterized by a diffuse fibrinoid degeneration with arteriolar necrosis. There was leukocytolysis within residual follicles and many macrophages infiltrated the hemorrhagic and hyperemic parenchyma. Diffuse lymphoid depletion was noted in the spleens. Diffuse deep and superficial areas of the renal cortex exhibited coagulative necrosis with peripheral hyperemia (**Fig. 5**) and degeneration of arteriolar walls.[39]

Fig. 2. Kidney of a dog with pantropic CCoV infection. Extensive hemorrhagic areas in the cortex.

Virologic and bacteriologic investigations on the parenchymatous organs failed to detect common canine pathogens, whereas CCoV-I and CCoV-II were identified in the intestinal content of all pups by genotype-specific real-time reverse transcriptase-polymerase chain reaction (RT-PCR) assays. Unexpectedly, CCoV-II RNA was also detected at high titers in lungs, spleen, liver, kidney, and brain. A coronavirus strain (CB/05) was isolated on A-72 cells from all the examined tissues but brain. Immuno-histochemistry using an *Alphacoronavirus-1* monoclonal antibody detected viral antigen in all tissues, including lungs (**Fig. 6**).

Other outbreaks of pantropic CCoV infection occurred in France, Belgium,[40] and Belgium. J Small Anim Pract. Submitted for publication), and Greece (V. Ntafis and E. Xylouri-Fragkiadaki, personal communication, 2009). Between March 2008 and August 2009, five outbreaks of a fatal systemic disease occurred in breeding kennels or pet shops of the north of France and Belgium, involving a total of 21 pure-bred pups, 20 of which died. Clinical signs were the same in all outbreaks and consisted of lethargy, vomiting, anorexia, diarrhea, and convulsions.
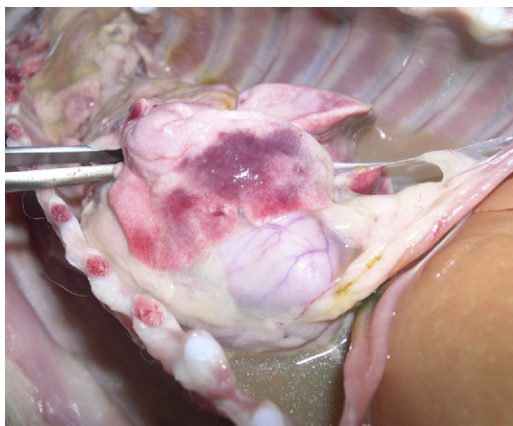

Fig. 1. Lung of a dog with pantropic CCoV infection. Pneumonia in the caudal lobe.
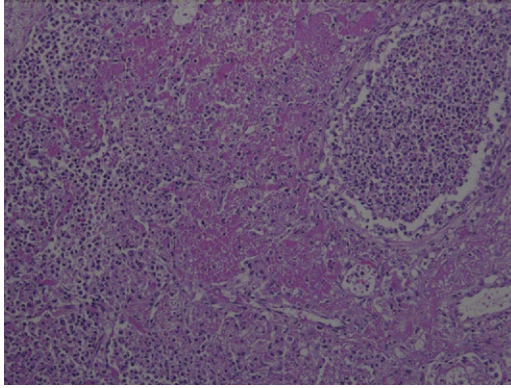
Fig. 3. Photomicrograph of the lung of a dog with pantropic CCoV infection. Densely cellular fibrinopurulent exudates, septae disruption, and diffuse edema (H&E). (*Courtesy of* Prof. M. Castagnaro, University of Padua.)

Post mortem examination revealed minimal to severe changes in the intestines and major organs. Lesions of discrete to moderate enteritis were generally confined to the small intestine, the serosa of which was often rough and pitted, and pale to old-pink. Loco-regional lymph nodes were enlarged and occasionally congested. Many puppies presented light to severe hepatic degeneration, with a yellow-brown discoloration. The spleen was slightly thickened and congestive and lungs were congested.

Histopathologically, apart from the changes in the intestinal mucosa (denudation and atrophy of the intestinal villi and necrosis of the crypts), edema and depletion of lymphoid tissues were evident. Other peculiar changes included discrete to severe hepatocyte degenerative changes with cytoplasm microvacuolization and extensive subacute interstitial pneumonia with intense vascular congestion.

In four outbreaks, a CCoV-II strain was detected in all internal organs, although in three cases there was a coinfection with CPV-2c. In one outbreak, lethal disease was
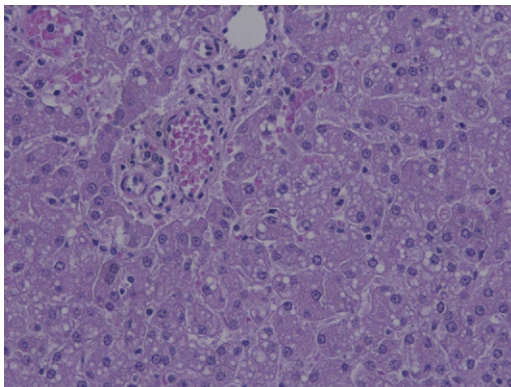

Fig. 4. Photomicrograph of the liver of a dog with pantropic CCoV infection. Diffuse hepatocyte degeneration and microvacuolar fatty change (H&E). (*Courtesy of* Prof. M. Castagnaro, University of Padua.)
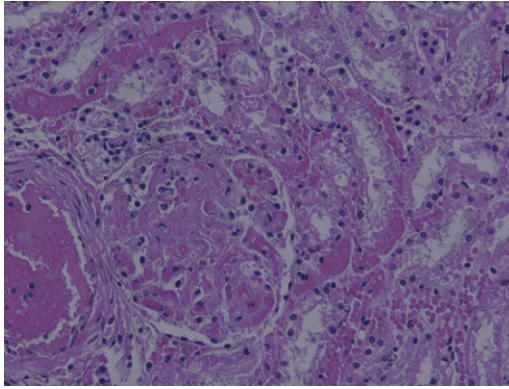
Fig. 5. Photomicrograph of the kidney of a dog with pantropic CCoV infection. Coagulative necrosis associated with marked hyperemia (H&E). (*Courtesy of* Prof. M. Castagnaro, University of Padua.)

associated to single pantropic CCoV infection in all examined organs (gut, spleen, liver, lung, brain). In the remaining case, a coinfection with CPV-2c and CCoV-I was identified, but the systemic spread of CCoV-I was associated to a possible synergistic effect of the CPV-induced enteritis.[40]

To date, limited information is available about the Greek pantropic CCoV outbreak, but preliminary data seem to confirm the same clinical and pathologic findings observed in the Italian, French, and Belgian cases (V. Ntafis and E. Xylouri-Fragkiadaki, personal communication, 2009).

Additional cases of pantropic CCoV disease, with or without concurrent CPV infection, have been observed in recent years, but these outbreaks are still under study (Decaro and colleagues, unpublished data, 2011).

Experimental Infections

Experimental infection of five CCoV-seronegative pups with strain CB/05 reproduced the disease with occurrence of severe clinical signs, including pyrexia, anorexia,
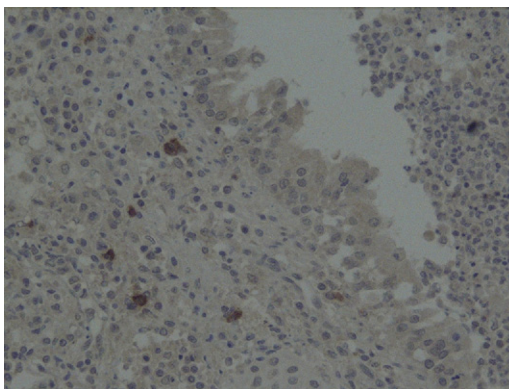

Fig. 6. Immunohistochemical staining with monoclonal anti-CCoV antibody of a lung section from a dog with pantropic CCoV infection. CCoV-infected cells are brown stained. (*Courtesy of* Prof. M. Castagnaro, University of Padua.)

depression, vomiting, diarrhea, and leucopenia.[7] A different clinical course was observed according to the age of the infected pups. The older dogs, 6 months of age, slowly recovered from the disease, whereas two out of three 2.5-month-old dogs were euthanized due to the severity of the CB/05-induced disease. The pantropism of the virus was confirmed by the presence of gross lesions in the internal organs of the dead dogs, as well as by the detection of viral RNA in those tissues, including brains, albeit at lower titers with respect to those detected in dogs that succumbed to natural infection. Traces of viral RNA were detected in the blood of a single dog, although further unpublished studies have demonstrated that detectable viremia can occur easily during CB/05 experimental infection (Decaro and colleagues, unpublished data, 2010).

Subsequently, strain CB/05 was proven to be able to infect even dogs recovered from a recent infection caused by enteric CCoV, inducing the occurrence of mild clinical signs.[8] Although the dogs used in that study had a strong humoral immunity to enteric CCoV at the time of challenge, experimental infection with strain CB/05 was successful in all pups irrespective of the viral dose administered. Exposure to even low amounts of virus would have a similar pattern of infection on seropositive animals, as dogs inoculated with different viral loads displayed the same duration of the viral shedding and not so very different viral titers in the feces. The duration of viral shedding was shorter and the clinical signs milder with respect to previous observations in seronegative dogs,[7] attributed mainly to the cross-protection induced by antibodies against enteric CCoV. Lymphotropism of strain CB/05 was clearly demonstrated by the occurrence of moderate lymphopenia in several infected pups. However, despite the moderate lymphopenia and the presence of the virus in the lymphoid tissues, the viral RNA was not detected in the blood at any time.

A further experiment aimed to evaluate the effects of pantropic CCoV infection on circulating monocytes and lymphocyte populations.[9] Infection of 11-week-old pups with strain CB/05 resulted in a profound depletion of T cells and a slight loss of B cells in the first week postinfection. In particular, while the $CD8^+$ and the B lymphocytes returned to baseline levels by day 7 postinfection, the $CD4^+$ T cells remained significantly low for 1 month and recovered completely after only 2 months. Monocytosis was also observed after CB/05 infection with a peak at day 5 postinfection. In this study, the polyclonal production of serum IgG or IgM against CCoV was not altered. However, the prolonged depletion of circulating $CD4^+$ T cells may affect humoral as well as cell-mediated immunity, thus compromising the ability to generate or maintain an effective immune response.

In contrast with findings observed in natural outbreaks, most recent experimental studies demonstrated that the outcome of pantropic CCoV infection is not invariably fatal. Indeed, the main effect of this new pathogen is the long-term lymphopenia, which could determine a severe impairment of the dog immune response against concurrent pathogens or vaccinal antigens. In fact, in environmental conditions of kennels and animal shelters, pups are exposed to multiple pathogens and a concurrent infection with pantropic CCoV may exacerbate the clinical course of other viruses, thus leading to a rapid death of the affected pups. In addition, routine vaccinations are usually carried out in pups at the age of 40 to 60 days, when CCoV infection reaches the maximal frequency.

Molecular Virology

Sequence analysis of the 3′ genome end, including ORFs 2 (S gene), 3a, 3b, 3c, 4 (E gene), 5 (M gene), 6 (N gene), 7a and 7b, showed that strain CB/05 had a high degree of amino acid identity to the cognate ORFs of CCoV-II, although the S protein

displayed the highest identity to FCoV-II strain 79-1683. A genetic marker was identified in the CB/05 genome, consisting of a 38-nt deletion in ORF3b, which was responsible for a predicted truncated nonstructural protein 3b.[41]

The further pantropic CCoV strains identified so far have not been extensively analyzed at the genetic level. However, preliminary data seem to indicate that those strains are highly similar to prototype virus CB/05, but most of them lack the ORF3b deletion that was proposed as a genetic marker for pantropic CCoV.

At present, the genetic changes associated to the pantropism of the virus are far to be determined, representing a challenge for the future, analogously to what described for the strictly related feline infectious peritonitis virus (FIPV).[42] Therefore, there is the need to develop a reverse genetics system similar to that established for FIPV,[43] which could be useful to understand the molecular basis of the change of virulence and tropism.

Diagnosis

Diagnosis of pantropic CCoV infection cannot be made on the basis of clinical and post mortem findings, considering that the course of infections caused by other canine pathogens, such as CPV and CAdV, may be undistinguishable. Thus, making a definitive diagnosis of pantropic CCoV-induced disease is difficult. In the absence of a clear genetic marker, that should be common to all pantropic strains identified so far, the detection of a CCoV-II strain in the internal organs is the essential condition required for a definitive diagnosis.

Considering the widespread circulation of enteric CCoV and the cross reactions existing between this virus and the pantropic strains, serologic tests such as enzyme-linked immunosorbent assay, virus neutralization,[44] and Western blotting[45] are not suitable to diagnose a pantropic CCoV infection. In contrast, virologic methods developed for detection of enteric CCoV are also employed for diagnosis of systemic infections.

Viral isolation on cell lines of canine (A-72) or feline (Crandel feline kidney) origin using tissue homogenates is usually followed by detection of viral antigens by immunofluorescence assay. However, CCoV is quite unstable in the environment, so that virus isolation succeeds only if samples contain high viral titers and are stored and transported in the cold chain. In contrast, methods based on nucleic acid amplification are highly sensitive, even in the presence of low amounts of viral RNA. Several RT-PCR assays have been developed to detect CCoV in fecal specimens,[46–48] the majority of which can be easily conducted on RNA extract from internal organs.

Quantitative PCR using TaqMan chemistry has allowed for sensitive detection of strain and amount of virus[49] and for rapid discrimination between the CCoV genotypes.[27]

Pantropic CCoV antigens can be also detected in tissue sections by immunochemical staining within macrophages in inflammatory sites and within arterial walls.[6,39]

Treatment and Prevention

There is no specific treatment for infections caused by pantropic CCoV. As for other viral diseases of dogs, management must emphasize supportive treatment to maintain fluid and electrolyte balance. Although rarely indicated, broad-spectrum antimicrobial agents can be given to treat secondary bacterial infections.

To date, no homologous vaccines against pantropic CCoV are available on the market. Inactivated vaccines that are currently used against enteric CCoV were shown to be poorly effective, as they induced high serum antibody levels but no

protection after experimental infection with enteric CCoV.[50] Analogously, a killed, MF59-adjuvanted vaccine recently developed against TGEV-like strains (CCoV-IIb) was not able to prevent fecal shedding of the challenge virus.[51] Only an experimental modified-live virus vaccine administered oronasally has been able to induce complete protection from disease as well as from infection.[52] Considering that the immunity induced by natural infection with enteric CCoV is not able to protect pups from challenge with strain CB/05, the efficacy of currently used vaccines prepared with enteric CCoV strains may be poorer against pantropic CB/05-like viruses. According to this scenario, dogs vaccinated with enteric CCoV may acquire subclinical infections with pantropic CCoV resulting in lymphopenia that may represent a predisposing factor for opportunistic pathogens and for a more severe disease induced by "true" pathogens (CPV, CAdV, CDV, and others).

Extensive epidemiologic surveys would assess whether the pantropic CCoV infection is widespread in dog populations. Systematic vaccination programs using homologous live vaccines would seem important in environments such as kennels, shelters, and pet shops that are at high risk of exposure to this newly identified virus.

SUMMARY

Canine coronavirus (CCoV) is an enteric pathogen, which is currently included in the new species *Alphacornavirus-1* of the *Alphacoronavirus* genus. To date, two genotypes of CCoV have been described, CCoV-I and CCoV-II, with the latter including two different subtypes, CCoV-IIa and CCoV-IIb. Usually, CCoV causes mild to severe diarrhea in pups, whereas fatal infections have been associated mainly with concurrent infections by other canine pathogens. However, a few years ago, an outbreak of fatal, systemic disease caused by a highly virulent CCoV-II strain (CB/05) was reported. To date, pantropic CCoV outbreaks have occurred in different parts of Europe, with clinical presentations and post mortem findings similar to those observed in the first outbreak. The pantropic CCoV-induced disease was also reproduced under experimental conditions, although the most prominent finding was a severe, long-lasting lymphopenia (mainly associated to a dramatic reduction of $CD4^+$ cells), rather than the death of the infected pups. Lacking any specific vaccine against this emerging pathogen of dogs, further studies should carefully evaluate (1) the worldwide distribution of the virus in dog populations and (2) the efficacy of existing vaccines, based on enteric CCoV, against pantropic viruses.

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