IRACEMA NUNES DE BARROS

Diversidade molecular e evolução *in vitro* de coronavírus canino (CCoV)

São Paulo

2015

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Tese apresentada ao Programa de Pós-Graduação em Epidemiologia Experimental Aplicada às Zoonoses da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção do título de Doutor em Ciências

Departamento:
Medicina Veterinária Preventiva e Saúde Animal

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Orientador:
Prof. Dr. Paulo Eduardo Brandão

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UNIVERSIDADE DE SÃO PAULO

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

Comissão de Ética no uso de animais

CERTIFICADO

Certificamos que o Projeto intitulado "Diversidade molecular e evolução in vitro de coronavírus canino (CCoV)", protocolado sob o nº 2188/2011, utilizando 100 (cem) cães, sob a responsabilidade do(a) Prof. Dr. Paulo Eduardo Brandão, está de acordo com os princípios éticos de experimentação animal da "Comissão de Ética no uso de animais" da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e foi aprovado em reunião de 18/5/2011.

We certify that the Research "Molecular diversity and in vitro evolution of canine coronavirus (CCoV)", protocol number 2188/2011, utilizing 100 (one hundred) dogs, under the responsibility Prof. Dr. Paulo Eduardo Brandão, agree with Ethical Principles in Animal Research adopted by "Ethic Committee in the use of animals" of the School of Veterinary Medicine and Animal Science of University of São Paulo and was approved in the meeting of day 05/18/2011.

São Paulo, 19 de maio de 2011.

Profa. Dra. Denise Tabacchi Fantoni
Presidente

Av. Prof. Dr. Orlando Marques de Paiva, nº87
Cidade Universitária "Armando de Salles Oliveira"
São Paulo/SP – Brasil
05508-270

Fax: +55 11 3032-2224 / 3091-7757
Fone: + 55 11 3091-7671/7676
E-mail: fmvz@usp.br
http://www.fmvz.usp.br

FOLHA DE AVALIAÇÃO

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Prof. Dr. _____ _____

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Prof. Dr. _____ _____

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À minha mãe, *Rejane* ou mais conhecida por *Jane*, exemplo de força, superação, e amor incondicional, que sempre me apoiou quando todos achavam que era impossível.

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“Nem tudo o que pode ser contado conta, e nem tudo o que conta pode ser contado.”

(Albert Einstein)

PREFÁCIO

O trabalho aqui apresentado como Tese de Doutorado foi subdivido em três artigos já no formato dos periódicos com opção *Open-Access* às quais serão posteriormente submetidos. O trabalho foi assim escrito para incentivar a produção científica e valorizar o trabalho acadêmico e de acordo com as normas do novo regimento do Programa de Pós-Graduação e Epidemiolgia Experimetal Aplicada da FMVZ USP.

Cada artigo contém resumo, palavras-chave, materiais e métodos, discussão, conclusão e referências de acordo com a revista selecionada. Entretanto, os mesmos ainda não foram submetidos aos respectivos periódicos em função da necessidade de aprimoramento das análises de dados, sobretudo em termos evolutivos, ainda não concluídas em função do longo tempo laboratorial tomado pela execução do trabalho, além da intenção de se submeter os mesmos a revisão profissional de Inglês.

Em suma, o trabalho apresenta os seguintes tópicos:

- Resumo em português e inglês (*abstract*) contendo os objetivos, principais aspectos e resultados da tese e conclusões.
- Sumário
- Artigo 1 – "Canine coronavirus (CCoV), a neglected pathogen: association with Canine parvovirus (CPV) and molecular diversity of genes S, M, N and 3b."
- Artigo 2 – "Isolation of a putatively pantropic type II canine coronavirus strain in A72 cells"
- Artigo 3 – "*In vitro* evolution of CCoV-II based on partial spike gene markers"

RESUMO

BARROS, I. N. **Diversidade molecular e evolução *in vitro* de coronavírus canino (CCoV).** [Molecular diversity and *in vitro* evolution of canine coronavirus (CCoV)]. 2015. 73 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2015.

O coronavírus canino (CCoV) causa gastroenterite em cães jovens, podendo ser letal, sobretudo quando há coinfecção com parvovírus canino (CPV). Os objetivos do presente projeto foram investigar a presença de CCoV e CPV em amostras fecais de cães jovens; estudar a diversidade molecular das amostras de CCoV com base em sequenciamento parcial dos genes M, S, 3b e N, incluindo amostras vacinais, e estudar a evolução *in vitro* de CCoV em células A72 de fibroma canino. Foram detectados 40,17% (47/117) animais positivos para CCoV e 13,68% (16/117) para CPV. Estudos filogenéticos demonstraram que oito amostras foram classificadas como CCoV-II, vinte e cinco como CCoV-I. Análises para o gene M destacaram alta identidade de CCoV-I com amostras de coronavírus da peritonite infecciosa felina (PIF) e uma possível amostra pantrópica foi demonstrada pela análise do gene S. O gene do nucleocapsídeo de CCoV é altamente conservado entre os tipos I e tipo II, com uma resolução mais baixa em relação a árvores para os genes M e S. Amostras dos tipos I e II apresentam um polimorfismo baixo para o gene 3b, sem marcadores estáveis para diferenciação dos tipos de CCoV. Uma amostra de CCoV-II putativamente pantrópica foi isolada em células A72, resultando em efeito citopático no 5º dia da 5a passagem. No estudo evolutivo, a amostra vacinal CCV 1-71 e nove passagens desta em células A72 foram submetidas a amplificação parcial e clonagem molecular do gene S seguida de sequenciamento de DNA. Os resultados mostraram mutações não silenciosas, silenciosas e três deleções de aminoácidos, mas nenhuma mutação compartilhada entre as diversas passagens. Amostras vacinais de CCoV-II adaptadas em células podem ser altamente geneticamente estáveis após passagens em série em uma mesma linhagem celular, acumulando substituições de nucleotídeos principalmente sinônimas no gene S devido a relação célula - hospedeiro estável. Estes resultados de epidemiologia molecular e processos evolutivos de CCoV podem servir para uma melhor compreensão da virologia básica e ser base de dados para estudos em outros coronavírus.

Palavras-chave: CCoV. Molecular. Sequenciamento. Isolamento. Evolução.

ABSTRACT

BARROS, I. N. **Molecular diversity and *in vitro* evolution of canine coronavirus (CCoV)** [Diversidade molecular e evolução *in vitro* de coronavírus canino (CCoV).]. 2015. 73 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2015.

Canine coronavirus (CCoV) causes gastroenteritis in young dogs and can be lethal, especially when there is co-infection with canine parvovirus (CPV). The objectives of this project were to investigate the presence of CCoV and CPV in stool samples from young dogs, the molecular diversity of CCoV strains based on partial sequencing of genes *M, S, N* and *3b*, and the *in vitro* evolution of CCoV in A72 canine fibroma. Of the fecal samples studied, 40.17% animals (47/117) were positive for CCoV and 13.68% (16/117) for CPV. Phylogenetic studies have shown that eight strains were CCoV-II and twenty five CCoV-I. Phylogenetic analysis for the *M* gene highlighted the high identity of CCoV-I strains with a feline coronavirus strain (FCoV) that causes feline infectious peritonitis (FIP). Further analysis based on the spike gene showed a putative pantropic CCoV strain (CCoV-II/dog50). CCoV nucleocapsid gene is highly conserved among type I and type II, with a lower resolution relative to trees based on *M* and *S* genes. CCoV Types I and II had a low polymorphism for 3b gene, without any stable markers to differentiate thee types. Regarding the virus isolation trial, a putative pantropic CCoV-II strain was successfully isolated in A72 cells from, resulting in cytopathic effect on the 5th day of the 5th passage. In the evolutionary study, the vaccine strain CCV 1-71 and nine passages of this strain in A72 were submitted to partial S gene amplification and molecular cloning followed by DNA sequencing. Missense, silent, and three amino acids deletions were found amongst diverse clones of each passage, but no mutation was repeatedly found among passages. Cell culture-adapted CCoV-II vaccine strains can be highly genetically stable upon serial passage in a same cell line, accumulating primarily synonymous nucleotide substitutions in the *S* gene due to a stable cell-host relationship. In short, all data gathering herein on molecular epidemiology and evolutionary processes of CCoV can serve for a better understanding of basic virology and as a basis for studies on other coronaviruses.

Keywords: CCoV. Molecular. Sequencing. Isolation. Evolution.

SUMÁRIO

I. Canine coronavirus (CCoV), a neglected pathogen: association with Canine parvovirus (CPV) and molecular diversity of genes S, M, N and 3b.

Barros, I.N.B. [1,2]; Pinto, L.D.[3]; Kuroda, R.B.S. **[1]**; Silva, S.O.S.[1]; Richtzenhain, L.J. [1,2], Canal, C.W.[3]; Brandão, P.E. [1,2].

[1]Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine, USP, Av. Prof. Dr. Orlando Marques de Paiva, 87, CEP 05508 270 - Cidade Universitária São Paulo/SP – Brasil; [2]Coronavirus Research Group, USP, Av. Prof. Dr. Orlando Marques de Paiva, 87, CEP 05508 270 - Cidade Universitária São Paulo/SP – Brazil; [3] Laboratory of Virology, School of Veterinary Medicine, University of Rio Grande do Sul, Brazil; E-mail: cemavet@usp.br

ABSTRACT

Gastroenteritis is common in young dogs and is caused chiefly by canine coronavirus (CCoV) and canine parvovirus (CPV). CPV is considered the most important cause of diarrhea by veterinarian clinicians. However, studies have shown an increasing prevalence and importance of CCoV around the world, and fatal CCoV infections have been described, but there's a gap on the information regarding the molecular diversity of CCoV in many parts of the world. In this study, canine fecal samples from diverse States of Brazil were submitted to PCRs screening for CCoV and CPV, and CCoV positive samples were submitted to partial sequencing of membrane (M), spike (S), nucleotide (N), non-structural protein 3b (NS3b) genes. Of the samples collected, 40.17% (47/117) were positive for CCoV and 13.68% (16/117) for CPV; 57.45% (27/47) of CCoV infected animals showed enteritis and most of them were younger than 3 months (36/47) and unvaccinated animals (35/47). Regarding CVP,

13.68% (16/117) were positive. On these CPV positive animals, 37.5% (6/16) were co-infected with CCoV and 87.5% (14/16) had enteritis Distance genealogy and sequence identity matrix using CCoV sequences from GenBank for *M* gene showed the strain of the positive control and eight strains from fecal samples were included in the same group of CCoV-II strains, and twenty six were included in CCoV-I group for nt and aa trees. Results of this study can highlight the high identity of CCoV-I strains with feline coronavirus (FCoV) and showed a putatively pantropic CCoV-II strain (CCoV-II/dog50).

Keywords: Canine; coronavirus; parvovirus; enteritis; detection; sequencing;

1. Introduction

Symptomatic enteritis is common and the most important etiologic agents are canine parvovirus (CPV) and canine coronavirus (CCoV) (Parrish et al., 1991; Tupler et al., 2012). Fatal enteritis might also occur as a result of co-infections by these two viruses (Pratelli et al., 1999; Evermann et al., 1988; Zicola et al., 2012). However, mortality associated with CCoV in the absence of CPV infection has been reported in young dogs (Evermann et al., 2005), and later in 2007 and a highly virulent CCoV strain (CB/05) is extremely lethal as a result of systemic spread Decaro et al (2007).

CCoV (*Nidovirales: Coronaviridade: Coronavirinae: Alphacoronavirus: Alphacoronavirus 1*) is a single-stranded positive-sense RNA virus and has a genome consisting of genes encoding the structural proteins spike (S), envelope (E), membrane (M) and nucleocapsid (N); in addition to ORFs translated into non structural proteins, as replicase polyprotein (ORF1 - open read frame 1), 3a, 3b, 3c, 7a and 7b (Masters and Perlman, 2013; ICTV, 2014).

CPV (*Parvoviridae*: *Parvovirinae:* for CPV type I *Bocavirus: Carnivore bocaparvovirus 1;* for CPV type II *Protoparvovirus: Carnivore protoparvovirus 1*) has single-stranded DNA genome of negative polarity, about 5200 nucleotides in length; the icosahedral viral capsid is 26 nm diameter, consisting of three proteins VP1, VP2 and VP3. The VP1 and VP2 proteins constitute the majority of the viral capsid (Reed et al., 1988; Park et al., 2007; ICTV, 2014).

Two different genotypes of CCoV have been recognized, CCoV type-I, with a high identity with feline coronavirus (FCoV), and CCoV type-II, divided in two subtypes, CCoV-IIa and CCoV-IIb, the former being the pantropic type (Decaro et al., 2007; Decaro et al. 2009, Decaro 2010; Pratelli et al., 2001; Pratelli et al., 2002). Later, other authors reported infection with pantropic CCoV IIa and IIb in Greek, Ireland, Japan, France and Belgium (Ntafis et al., 2010; McEligott et al., 2011; Soma et al., 2011; Zicola et al, 2012), demonstrating the widespread of this CCoV infection.

However, there are few studies about the frequency of CCoV in feces of dogs (Castro et al, 2010; Castro et al, 2013) and little data on the molecular diversity of CCoV strains is available from many parts of the world, including Brazil (Guirao et al., 2013; Pinto et al., 2014; Costa et al, 2014; Gizzi et al., 2014).

This study aimed to investigate the presence of CCoV and CPV in canine fecal samples using RT- PCR assays and to the molecular diversity of CCoV strains based on the genes of membrane (M), spike (S) and nucleocapsid (N) structural proteins and non-structural protein 3b (NS3b).

2. Materials and Methods

2.1. Fecal samples and controls

A total of 117 fecal samples from dogs (Table 1) were collected from 2009 to 2014 and stored at - 80°C until the time of testing with or without symptomatic enteritis, different breeds, genders and ages, from five Brazilian States (Pernambuco n=3; 27 from Mato Grosso n=27; Rio Grande do Sul n=18 and São Paulo n=72) (Fig. 1).

CCV 1-71 (ATCC VR 809) and CPV-2 Cornell 780916 (ATCC VR-953) strains ($10^{4.03}$ TCID50/mL and $10^{6.66}$ TCID50/mL, respectively), were kindly provided by the Biovet Laboratories, Brazil, and used as positive controls for RT-PCR. As negative control, ultra-pure water treated with 0.1% diethylpyrocarbonate (DEPC water) was used.

Fig.1. Map of Brazil showing the Sates in which fecal samples of dogs were collected during this study

2.2. RNA and DNA Extraction

Fecal samples were prepared as 20% (v/v) suspensions in DEPC water and clarified at 5,000 × g / 15 min at 4 ° C, taking the supernatant as a sample. Total RNA and DNA from fecal samples supernatants and controls was performed with TRIzol Reagent™ (Life Technologies) as per manufacturer's instructions and by the phenol/chloroform methods as described by (Mesquita et al., 2001).

2.3. Synthesis of complementary DNA (cDNA) of CCoV

For cDNA synthesis, 3.5 µL of the extracted RNA were denatured at 95ºC for 5 minutes, and then added to the reverse transcription mix containing 1 x First Strand Buffer (Life Technologies), 1 mM of each dNTP, 10 mM DTT, 25ng Random Primers™ (Life Technologies) 100U of M-MLV Reverse Transcriptase™ (Life Technologies ™) and DEPC water q.s.p 10µL and incubated at 37°C for 60 minutes.

2.4. Polymerase chain reaction

PCRs were carried out with 12.5 µL of GoTaq® Green Master Mix™ (Promega), and 2.5 µL of each cDNA for CCoV or DNA for CPV in separate reactions, 1.25 µL of each respective primer at 10µ M (Tables 2 and 3) and 7.5 µL of DEPC water to a final volume of 25µL.

Cycles for CCoV were as follows: *M* gene, 94°C/10min, followed by 35 cycles of 94ºC/30sec, 55ºC/1min 72ºC/1min, followed by 72ºC/10min for final extension. For both reaction for *S* gene (one for CCoV-I and another separated for CCoV-II): 94ºC/5min, 35 cycles of 94°C/1min, 50ºC/1min and 72ºC/2min, followed by 72ºC/10min; For *N* gene, 94°C/5min and 35 cycles of 95ºC/1min, 53ºC/40sec and 72ºC/30sec, followed by

72ºC/10min. For *3b* gene 94ºC/10min, 35 cycles of 94ºC/30sec, 55ºC/1min e 72ºC/1min, followed by 72ºC/10min final extension

For CPV VP2 protein gene detection, the cycle was 94°C/10min, followed by 34 cycles of 94ºC/30sec, 55ºC/1min and 72ºC/1min, followed by 72ºC/10min for final extension of the gene encoding the.

A samples were considered if the correspondent amplicons for CCoV M and CPV VP-2 genes (Table 2 and 3) were detected after electrophoresis on 1.5% agarose gel stained with ethidium bromide at 0.5 μg/mL and observed under ultra-violet light.

Table 2 - Primers used for amplification and partial sequencing of the genes encoding the M, S, N and 3b proteins of canine coronavirus.

Gene	*Primers*	Strand	Sequence (5´- 3´´)	Amplicon size	References
M	CCV1	Forward	TCCAGATATGTAATGTTCGG	410 bp	Pratelli et al. (1999)
	CCV2	Reverse	TCTGTTGAGTAATCACCAGCT		
S (3´)	EL1F	Forward	CAAGTTGACCGTCTTATTATTACTGGTAG	CCoV Type I 346 bp	Pratelli et al., (2004),
	EL1R	Reverse	TCATATACGTACCATTATAGCTGAAGA		
	S5	Forward	TGCATTTGTGTCTCAGACTT	CCoV type II 694 bp	
	S6	Reverse	CCAAGGCCATTTTACATAAG		
N	CENP1	Forward	CTCGTGGYCGGAAGAATAAT	280 bp	Erles e Brownlie (2009)
	CENP2		GCAACCCAGAMRACTCCATC		
3b	NSP3B-S	Forward	CTTGGTCTCTCTATTGTTGAAG	200 bp	This study
	NSP3B-A	Reverse	GCGTTGCGTTTAGAATGG		

Table 3 - Primers used for detection of VP2 of canine parvovirus.

Primers	Strand	Sequence (5’ -3’)	Amplicon size	Reference
555F	Forward	CAGGAAGATATCCAGAAGGA	583 pb	McElligott et al., 2011
555R	Reverse	GGTGCTAGTTGATATGTAATAAACA		

2.5. Statistical analysis

CCoV and CPV results data were registered on a calculation sheet and analyzed for vaccination and enteritis presence using Chi-square analysis in the IBM SPSS (Statistical Package for Social Sciences, 2012) version 20.

2.6. DNA Sequencing

Amplicons of the *M, S, N* and *3b* genes of CCoV were purified from agarose gels with GFX™ PCR DNA and GEL BAND Purification Kit™ (GE Healthcare) and submitted to bi-directional DNA sequencing using BigDye 3.1™ (Applied Byosystems) and ABI-3500 Genetic Analyzer™ (Applied Byosystems) as per manufacturer's instructions.

The chromatograms obtained for each DNA strand sequences of each sample were subjected to Phred application online (http://asparagin.cenargen.embrapa.br/phph/ to evaluate the quality of the same ones). Only positions with scores higher than 20 (less than 1 % of error probability) were used and the chromatograms were also manually checked with the program Finch TV © (©Geospiza) to search for interpretation errors and discrepancies between each DNA strand sequenced.

The CAP sequence assembly of each sequence was obtained with the program BioEdit version 7.2.5 and submitted to BLASTn (http://www.ncbi.nlm.nih.gov/BLAST).

2.7. Phylogenetic analysis

The final nucleotide and putative amino acids sequences *M, S, N* and *3b* genes of CCoV were aligned with homologous sequences of *Alphacoronavirus-1* retrieved from GenBank (accession numbers of common sequences at the phylogenetic analysis*:* DQ201447; GQ477367; KC175340; JQ404410; JQ404409; EU924790; AY342160; F682842; EU924791; EU856361; EU856362; HQ462571; GQ152141; NC_002306; DQ112226; in addition for *M, S* and *N*, but not for *3b* trees: D13096; DQ811789; DQ112226; and only for *M*: GU300113; GU300120; GU300129; GU300127; JX442376; JX442377; JX442378; JF682842; for *S*: JF682842; AY170345; KF312719; JX446572; for *N*: JF682842; for *3b:* JQ408980; AY426983), using CLUSTAL/W application running in BioEdit v. 7.2.5.

The alignments were used to build Neighbor-Joining (NJ) nucleotide (with the Maximum Composite Likelihood model) and amino acids (with the Poisson model) trees with 1000 bootstrap replicates using MEGA 6.0 (Tamura et al., 2013).

All sequences generated in this study were deposited in the GenBank under the accession numbers: KP322041 to KP322050 for *S* gene from CCoV-I; KP322051 to KP322053 for *S* gene from CCoV-II; KP322054 to KP322088 for *M* gene; KP322089 to KP322111 for *N* gene;

Though the 3b amplicon had an expected size of 200 bp, when the respective primers were exclude during sequence editing, sequences were below the minimum size for Genbank submission, but they are available upon request to the authors.

3. Results

Regarding CCoV, 40.17% (47/117) of the samples were positive for M gene (Table 1), with frequencies of positives being of 37.5% (27/71) and 44.45% (20/45) amongst animals with and without diarrhea, respectively, while 57.45% (27/47) of CCoV-positive animals showed enteritis. Most of the positive animals were younger than 3 months (36/47). CCoV was detected in 54.69% (35/64) of unvaccinated animals, 33.34% (2/6) of those with complete vaccine schedule, 100% (8/8) in those with incomplete vaccine schedule, and in 5.1 % (2/39) in those animals for with no vaccination data was available.

Concerning CPV, 13.68% (16/117) of the total samples resulted positive by PCR, with frequencies of positives being of 19.45% (14/72) and 4.5% (2/45) amongst animals with and without enteritis, respectively; 37.5% (6/16) of the CPV-positive animals were co-infected with CCoV and 87.5% (14/16) had enteritis.

Chi-square tests using SPSS20.0 software comparing enteritis x CPV (p=0.02) and vaccine x CPV (p=0.01) was considered statistically significant. However, enteritis x vaccine (p=0.30), enteritis x CCoV (p = 0.45) and vaccine x CCoV (p = 0.25) were without statistical significance.

Only high-quality DNA sequences (Phred score> 20) were included for the analyses of *M* (n=35), *S* (n=10 for CCoV-I and 3 for CCoV-II), *N* (n=23) and *3b* (n=27) genes.

Phylogenetic analysis for *M* gene showed that the strain of the positive control and eight strains from fecal samples were included in the same group of CCoV-II strains, and the other ones were included in CCoV-I group for both nt and aa trees (Fig. 1). The non-synonymous between CCoV types I and II in the amino acid alignment are located at positions: 150 (Val → Ile/ Phe/ Thr), 154 (Ala → Val / Ile), 200 (Thr →Val), 220 (Met → Ile), 227 (Glu → Asp), 228 (His →Asn) and 250 (Gln → Lys) regarding *M* gene of the strain 1-71 (VR-809 ATCC) GenBank access number JQ404409 (Fig. 2). Amino acids substitutions in M of CCoV-I strains from te present study are also found in the FIPV USA strain, GenBank number NC002306, with 82.5% - 83.1% nt and 90% aa identities amongst them, while CCoV-II strains showed 77.7% - 78.5% nt and 82.7% - 83.6% aa identity with the FIPV strain.

In agreement to what was found for M gene trees, *S* gene phylogenetic analysis resulted in two major clusters, *i.e.*, one for CCoV type I and another one for CCoV type II strains (Fig. 3), with amino acids substitutions between types I and II (Fig. 4) at positions: 1161 (Ala → Thr), 1166 (Tyr → Phe), 1168 (Ala → Ser), 1173 (Gln → Arg), 1174 (Tyr →Gln), 1175 (Thr →Ala), 1178 (Lys → Arg), 1185 (Met → Lys), 1186 (Glu → Asp), 1193 (Lys→Arg), 1197 (Asp →Gln), 1199 (Tyr →Phe), 1217 (Asp→ Asn), 1219 (Leu →Met), 1220 (Leu → Ile), 1222 (Leu → Phe), 1230 (Glu → Ala), 1231 (Trp → Tyr), 1232 (Glu → Thr), 1242 (Val → Ala), 1243 (Asn →Leu/Ser), 1245 to 1247 (deletion of the three aa – Gly,

Asp and Arg), 1249 (Tyr → Phe), 1250 (Ala → Gly), 1251 (Tyr → Leu), 1253 (Leu→ Val), 1256 (Phe → Val), 1257 (Lys → Gln), 1258 (Ser → Leu), 1259 (Ser→ Thr) and 1260 (deletion) with reference *S* gene of the strain 1-71 (VR-809 ATCC) GenBank access number JQ404409 (Fig. 4). One Brazilian CCoV-I strain (KF312719), not from this study, showed the amino acid Arg at position 1178 instead of Lys. For *S* gene the identity amongst CCoV-I strains from this study and FIPV strain (NC_002306) were 65.5%for nt and 67.3% for aa. All the CCoV-I strains from this study had 100% nt and aa identities with each other, 96.5% nt and 100% amino acid identity with CCoV-I Elmo/02 strain (AY170345), 99.3% nt and 98.9% with a CCoV-I Brazilian strain from another study (GenBank Accession number KF312719). Regarding CCoV-II, TGEV and FCoV-II from GenBank (see Fig.s 3 and 4 for GenBank accession numbers) the identities ranged from 64.5% to 66.5% for nt and 65.3% to 67.3% for aa for *S*.

On the CCoV-II *S* gene tree (Fig. 5), CCoV-II/dog50 and CCoV-II/dog57 strains segregated in the same cluster with the Brazilian CCoV-IIa Cao4 strain (GenBank accession No. JX446572) also from Rio Grande do Sul State (Fig. 5). The identity ranged from 97.8% to 98% for nt and 99% to 99.5% for aa, respectively, among them and this other Brazilian strain. Taking into account the other alphacoronaviruses included in the analysis, there was 92.8% to 94.3% nt and 92.9% to 99% aa identity amongst CCoV-II and FIPV, and TGEV). Comparing the CCoV-II strains from this study and the CCoV-I Elmo/02 strain (AY170345), nt identities were 61.8% - 62.1% and, for aa, 57.5%.

In the trees for the N gene (nt and aa, Fig. 6), though a lower resolution was found when compared to M trees, two CCoV-II strains from this study (CCoV-II/dog50 and CCoV-II/dog 56) clustered with one CCoV-II (GenBank GQ477367), with 95.8% to 97% nt and 95% to 96.2% aa identities. All CCoV-I strains of this study segregated in a unique cluster and showed 93.3% to 100% nt and 95% to 98.7% aa identities among them. Amino acids

substitutions could also be noticed in the aa alignment of *N* gene (Fig. 7). For *N* gene the identities among CCoV-I strains from this study and FIPV strain (NC_002306) were 80.8% - 83.7% for nt and 81.2% - 83.7% for aa.

Finally, the phylogenetic trees for *3b* gene (Fig. 8), shows that, except for the strain CCoV-II dog50 from this study, which clustered in the CCoV II cluster, one CCoV- II (CCoVII/dog 56) and all CCoV-I strains segregated in a cluster containing CCoVs type I and one FCoV serotype II (GenBank, accession numbers AY170345 (Elmo/02 strain), AY426984 (23/03 strain), and DF-2 R3i (JQ408980), respectively). Amino acids substitutions for *3b* gene are shown in Fig. 8.

The strain used as positive control in this study showed 100% identity for nt and aa for *M, S* and *3b* gene, 99.1% nt and 98.7% aa for *N* gene, in the alignments with the strain 1-71 and was included in the same cluster of this vaccine strain. Nucleotide and amino acid identities among CCoV strains sequenced in this study and two CCoV vaccines strains, TN-449 (VR-2068 ATCC; GenBanK access number) and 1-71(VR-809 ATCC; GenBank access number JQ404410), for partial regions of *M, S, N* and *3b* genes are shown in Table 4.

Table 4 – Nucleotide and amino acid identity among CCoV strains sequenced in this study and two strains, TN-449 (VR-2068 ATCC; GenBanK access number) and 1-71(VR-809 ATCC; GenBank access number JQ404410), for partial regions of *M, S, N* and *3b* genes.

Gene (nt region for gene in the reference sequence)	**Reference strain**	**Strains from this study**	**nt %**	**aa%**
M	TN-449	CCoV-I	88.2% - 89.1%	94.5%
		CCoV-II	95.7% - 96.3%	98.1% - 99%
		Positive Control	96.3%	96.3%
	1-71	CCoV-I	87.6% - 88.2%	92.7%
		CCoV-II	95.7% - 96.9%	97.2% - 98.1%
		Positive Control	100%	99%
S (CCoV-I)	TN-449	CCoV-I	64.4%	67.3%
	1-71		65.5%	67.3%
S	TN-449	CCoV-II	92.9% - 94%	97.2% - 98.6%
		Positive Control	92.9%	97.6%
	1-71	CCoV-II	91.4% - 92.5%	96.7% - 98.1%
		Positive Control	100%	100%
N	TN-449	CCoV-I	84.5% - 87.5%	83.7% - 86.2%
		CCoV-II	87.5% - 92.9%	87.5% - 92.5%
		Positive Control	93.3%	91.2%
	1-71	CCoV-I	85.4% - 88.3%	86.2% - 88.7%
		CCoV-II	87.5% - 94.1%	85% - 96.2%
		Positive Control	99.1%	98.7%
3b	TN-449	CCoV-I	77% - 82.1%	73% - 78.8%
		CCoV-II	78.3% - 87.8%	76.9% - 86.5%
		CCoV dog 51	81.5%	78.8%
		Positive Control	91%	88.4%
	1-71	CCoV-I	79.6% - 82.1%	76.9% - 80.7%
		CCoV-II	81.5% - 91.7%	80.7% - 88.4%
		CCoV dog 51	83.4%	82.6%
		Positive Control	100%	100%

Table 1 - Fecal samples from dogs collected in this study and results for PCRs detection of CCoV and CPV. (NI) not informed data ; (PE) Pernambuco State; (MT) Mato Grosso State; (RS) Rio Grande do Sul State; (SP) Sao Paulo State; (m) months; (d) days; (F) Female; (M) male; (NB) No breed; (Neg) negative; (**Pos**) positive;

Sample ID	Collection	State	Age	Sex	Breed	Enteritis	Vaccine	CCoV Gene M	CPV
1	2010	PE	NI	NI	NI	Present	NI	Neg	Neg
2	2010	PE	NI	NI	NI	Present	NI	Neg	Neg
3	2010	PE	NI	NI	NI	Present	NI	Neg	Neg
4	-	MT	2m	M	Pit Bull	Present	NI	Neg	**Pos**
5	-	MT	2m	F	NB	Present	NI	Neg	Neg
6	-	MT	3m	F	Pinscher	Present	NI	Neg	Neg
7	-	MT	3m	M	Pinscher	Present	NI	Neg	**Pos**
8	-	MT	2m	F	Pinscher	Present	NI	Neg	Neg
9	-	MT	3m	F	Pit Bull	Present	NI	Neg	Neg
10	-	MT	2m	M	Rottweiler	Present	NI	Neg	Neg
11	-	MT	3m	F	Poodle	Present	NI	Neg	Neg
12	-	MT	3m	M	Pit Bull	Present	NI	Neg	**Pos**
13	-	MT	1m18d	F	Pit Bull	Present	NI	Neg	**Pos**
14	-	MT	1m	M	Rottweiler	Present	NI	Neg	**Pos**
15	-	MT	2m10d	F	NB	Present	NI	Neg	**Pos**
17	-	MT	3m	M	NB	Present	NI	Neg	Neg
18	-	MT	2m	F	NB	Present	NI	Neg	Neg
19	-	MT	3m	F	Pit Bull	Present	NI	Neg	Neg
21	-	MT	3m	M	Shi Tzu	Present	NI	Neg	Neg
23	-	MT	2m	F	NB	Present	NI	Neg	Neg
24	-	MT	2m21d	F	NB	Present	NI	Neg	Neg
25	-	MT	3m	M	NB	Present	NI	Neg	Neg
26	-	MT	2m	M	NB	Present	NI	Neg	Neg
27	-	MT	3m	F	NB	Present	NI	Neg	**Pos**
28	-	MT	4m	F	Dalmatian	Present	NI	Neg	**Pos**
29	-	MT	3m	F	Shar Pei	Present	NI	Neg	**Pos**
30	-	MT	2m	F	Shar Pei	Present	NI	Neg	**Pos**

31	-	MT	4m	M	NB	Present	NI	Neg	Neg
32	-	MT	3m	F	NB	Present	NI	Neg	Neg
33	-	MT	2m	M	Bull Terrier	Present	NI	Neg	Neg
40	2011	SP	12m	M	Basset Hound	Absent	NI	Neg	Neg
41	2011	SP	5m	M	Lhasa Apso	Absent	NI	Neg	Neg
42	2009	RS	4m	M	Border Collie	Absent	Absent	**Pos**	Neg
43	2009	RS	2m	F	NB	Absent	Absent	**Pos**	Neg
44	2009	RS	2m	F	NB	Absent	Absent	**Pos**	Neg
45	2009	RS	2m	F	NB	Absent	Absent	**Pos**	Neg
46	2009	RS	5m	M	NB	Absent	Complete	**Pos**	Neg
47	2009	RS	5m	M	NB	Absent	Complete	**Pos**	Neg
48	2009	RS	2m	F	NB	Absent	Incomplete	**Pos**	Neg
49	2010	RS	2m	M	German Shepherd	Absent	Incomplete	**Pos**	**Pos**
50	2010	RS	2m 15d	F	Shi Tzu	Present	Incomplete	**Pos**	**Pos**
51	2009	RS	2m	F	NB	Absent	Absent	**Pos**	Neg
52	2010	RS	1m	M	Golden	Present	Incomplete	**Pos**	**Pos**
53	2008	RS	4m	M	Akita	Absent	Absent	**Pos**	**Pos**
54	2009	RS	7m	M	NB	Absent	Absent	**Pos**	Neg
55	2010	RS	6m	M	Pinscher	Present	Incomplete	**Pos**	**Pos**
56	2009	RS	3m	F	NB	Absent	Absent	**Pos**	Neg
57	2009	RS	3m	F	NB	Absent	Absent	**Pos**	Neg
58	2010	RS	3m	F	NB	Absent	Absent	**Pos**	Neg
59	2009	RS	2m	F	NB	Present	Absent	**Pos**	**Pos**
60	2012	SP	4m	F	Jack Russel	Present	Incomplete	**Pos**	Neg
61	2012	SP	2m	M	Pinscher	Absent	Absent	Neg	Neg
62	2012	SP	2m	M	Jack Russel	Present	Incomplete	**Pos**	Neg
63	2012	SP	2m	M	Jack Russel	Absent	Absent	Neg	Neg
64	2012	SP	12m	F	Bernese	Absent	Absent	Neg	Neg
65	2012	SP	3m	F	French Bulldog	Present	Absent	**Pos**	Neg
66	2012	SP	10m	F	Australian Sheppherd	Present	Absent	Neg	Neg

67	2012	SP	3m	M	Jack Russel	Present	Absent	**Pos**	Neg
68	2012	SP	25d	M	Pinscher	Absent	Absent	Neg	Neg
69	2012	SP	25d	M	Pinscher	Absent	Absent	Neg	Neg
70	2012	SP	25d	F	Pinscher	Absent	Absent	Neg	Neg
71	2012	SP	16d	F	Pinscher	Absent	Absent	Neg	Neg
72	2012	SP	3m	M	Jack Russel	Absent	Absent	**Pos**	Neg
73	2012	SP	2m	M	Pinscher	Present	Absent	**Pos**	Neg
74	2012	SP	2m	F	Lhasa Apso	Absent	Absent	Neg	Neg
75	2012	SP	3m	F	Jack Russel	Present	Absent	**Pos**	Neg
76	2012	SP	3m	F	Jack Russel	Present	Absent	**Pos**	Neg
77	2012	SP	12m	F	Pinscher	Present	Complete	Neg	Neg
78	2012	SP	4m	F	Italian Greyhouy	Present	Complete	Neg	Neg
79	2012	SP	4m	F	Italian Greyhouy	Present	Absent	Neg	Neg
80	2012	SP	9m	F	French Bulldog	Present	Absent	**Pos**	Neg
81	2012	SP	4m	M	Pinscher	Present	Absent	**Pos**	Neg
82	2012	SP	12m	M	Pug	Present	Absent	Neg	Neg
83	2012	SP	12m	F	Jack Russel	Absent	Complete	Neg	Neg
84	2012	SP	12m	F	Jack Russel	Absent	Complete	Neg	Neg
88	2012	SP	4m	M	Golden	Present	NI	**Pos**	Neg
89	2012	SP	2m	F	Yorkshire Terrier	Present	NI	**Pos**	Neg
90	2012	SP	50d	F	Yorkshire Terrier	Absent	Absent	Neg	Neg
91	2012	SP	50d	F	Yorkshire Terrier	Absent	Absent	Neg	Neg
92	2012	SP	45d	F	French Bulldog	Present	Absent	**Pos**	Neg
93	2012	SP	45d	F	French Bulldog	Present	Absent	**Pos**	Neg
94	2012	SP	45d	M	French Bulldog	Present	Absent	**Pos**	Neg
95	2012	SP	45d	M	French Bulldog	Present	Absent	**Pos**	Neg
96	2012	SP	45d	F	French Bulldog	Absent	Absent	Neg	Neg
97	2012	SP	45d	M	French Bulldog	Present	Absent	**Pos**	Neg
98	2012	SP	45d	M	French Bulldog	Present	Absent	**Pos**	Neg
99	2012	SP	40d	F	Pinscher	Present	Absent	**Pos**	Neg

100	2012	SP	40d	M	Pinscher	Absent	Absent	**Pos**	Neg
101	2012	SP	40d	M	Pinscher	Present	Absent	**Pos**	Neg
102	2012	SP	40d	F	Pinscher	Absent	Absent	Neg	Neg
103	2012	SP	40d	F	Pinscher	Absent	Absent	Neg	Neg
104	2012	SP	40d	F	Pinscher	Absent	Absent	**Pos**	Neg
105	2012	SP	40d	M	Pinscher	Present	Absent	Neg	Neg
106	2012	SP	50d	M	Lhasa Apso	Absent	Absent	Neg	Neg
107	2012	SP	50d	M	Lhasa Apso	Absent	Absent	Neg	Neg
108	2012	SP	50d	M	Lhasa Apso	Absent	Absent	Neg	Neg
109	2012	SP	50d	M	Lhasa Apso	Absent	Absent	Neg	Neg
110	2012	SP	37d	F	Golden	Absent	Absent	Neg	Neg
111	2012	SP	50d	F	Lhasa Apso	Absent	Absent	Neg	Neg
112	2012	SP	3m	M	Chihuahua	Present	Absent	**Pos**	Neg
113	2012	SP	8d	F	French Bulldog	Present	Absent	**Pos**	Neg
114	2012	SP	37d	M	Golden	Present	Absent	**Pos**	Neg
115	2012	SP	37d	M	Golden	Absent	Absent	Neg	Neg
116	2012	SP	37d	M	Golden	Absent	Absent	**Pos**	Neg
117	2012	SP	37d	F	Golden	Absent	Absent	**Pos**	Neg
118	2012	SP	37d	M	Golden	Absent	Absent	**Pos**	Neg
119	2012	SP	37d	M	Golden	Absent	Absent	Neg	Neg
120	2012	SP	2m	F	NB	Present	Absent	Neg	Neg
121	2012	SP	5m	F	Jack Russel	Present	Absent	Neg	Neg
122	2012	SP	3m	F	German Shepherd	Present	Absent	Neg	Neg
123	2012	SP	4m	M	NB	Present	Incomplete	**Pos**	Neg
124	2012	SP	NI	-	-	Present	NI	Neg	Neg
125	2012	SP	NI	-	-	Present	NI	Neg	Neg
126	2012	SP	6m	M	Yorkshire Terrier	Present	Absent	Neg	Neg
128	2012	SP	NI	-	-	Present	NI	Neg	Neg
131	2012	SP	5m	M	NB	Present	NI	Neg	Neg
132	2012	SP	2m	F	Spitz Alemão	Present	NI	Neg	Neg

Fig. 1. Phylogenetic trees for partial sequences of nt and aa for *M* gene; strains from this study are in bold and with an arrow. Each node represents the values of 1000 bootstrap replicates and has been demonstrated only bootstrap values above 50%. The bar represents the number of substitutions per site.

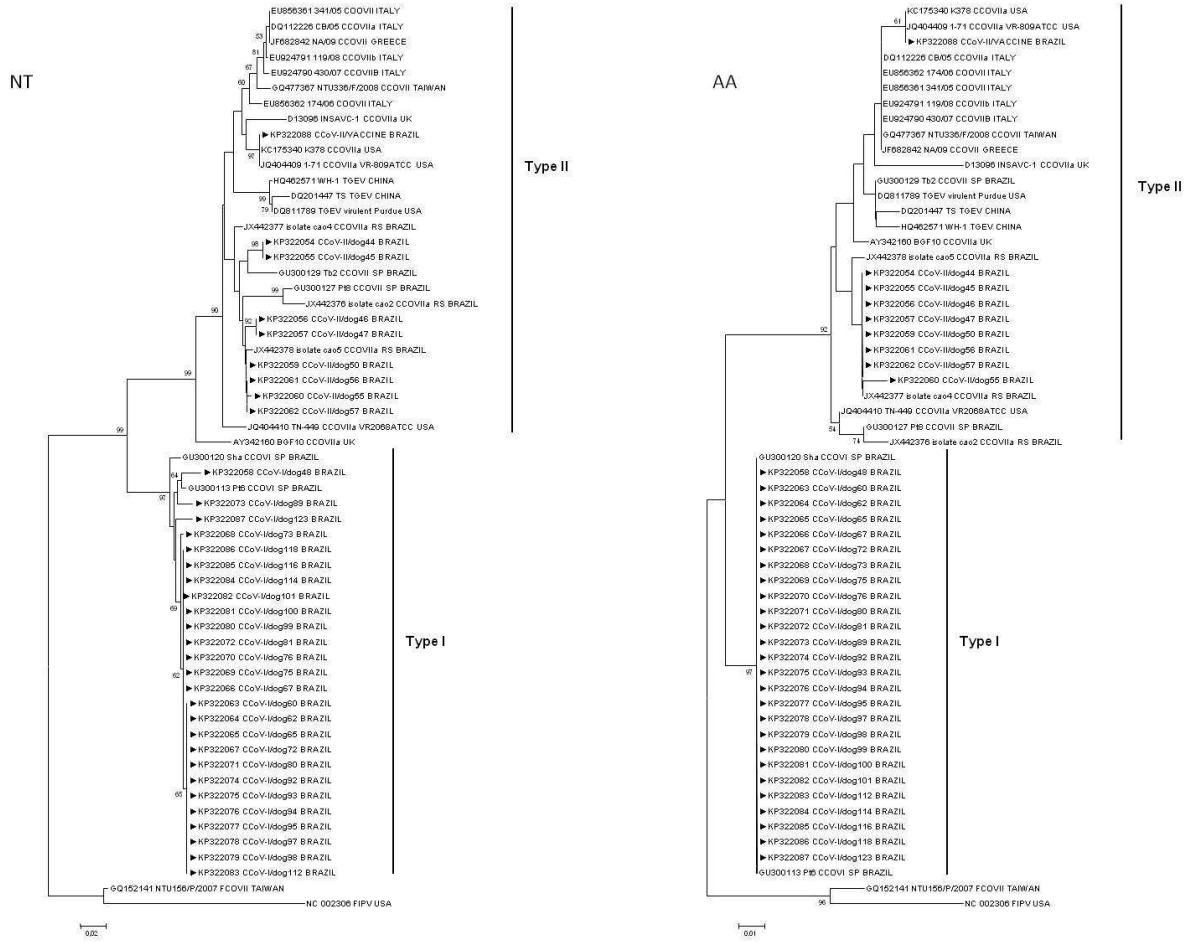


Fig. 2. Differences in amino acid alignment of CCoV *M* gene (reference strain 1-71 (VR-809 ATCC), genbank JQ404409).

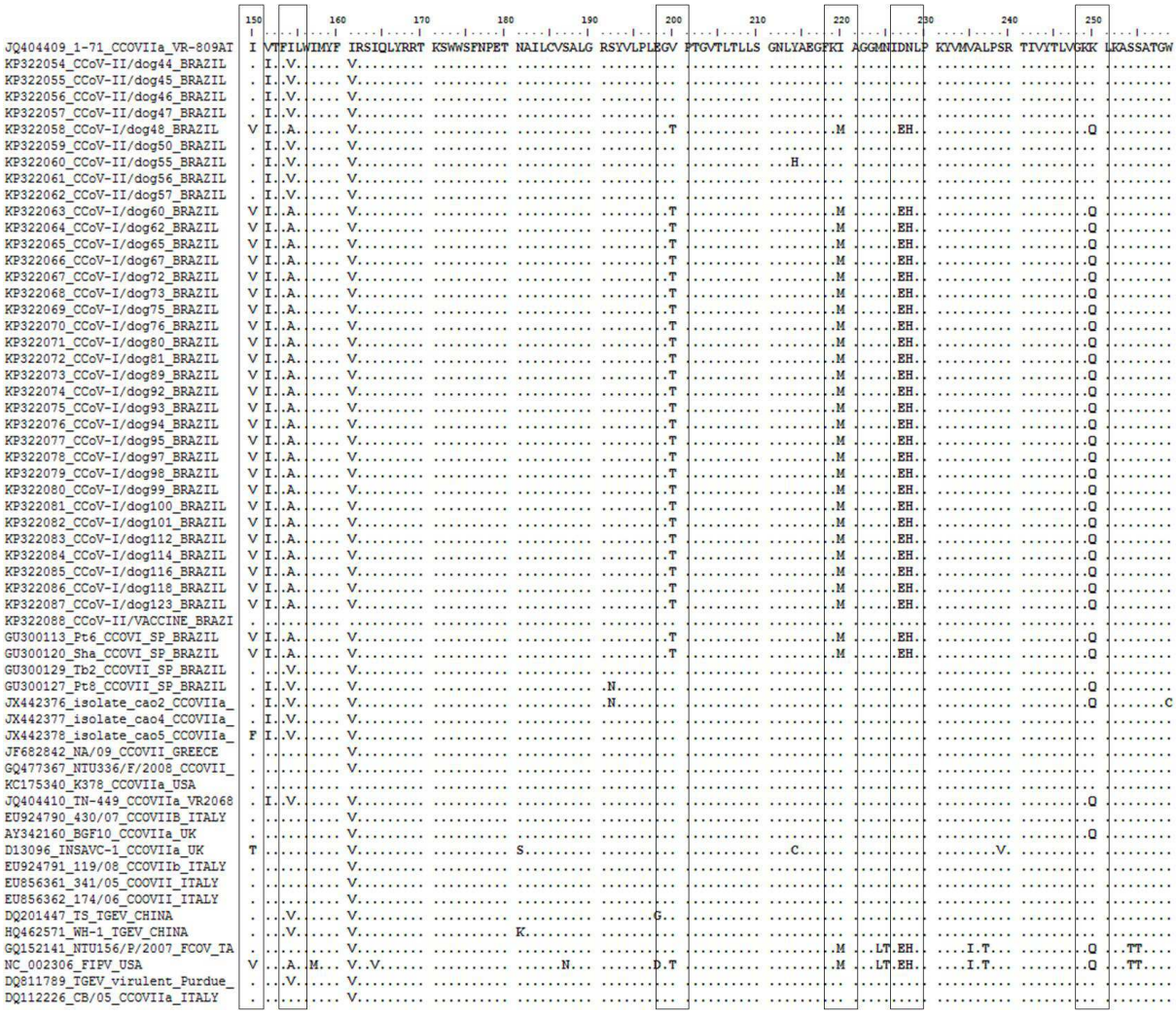


Fig. 3. Phylogenetic trees for partial nt and aa sequences of *S* gene comparing CCoV-I with CCoV-II; strains from this study are in bold and with an arrow. Each node represents the values of 1000 bootstrap replicates and has been demonstrated only bootstrap values above 50%. The bar represents the number of substitutions per site.

Fig. 4. Differences in amino acid alignment of CCoV *S* gene (reference strain 1-71 (VR-809 ATCC), genbank JQ404409).

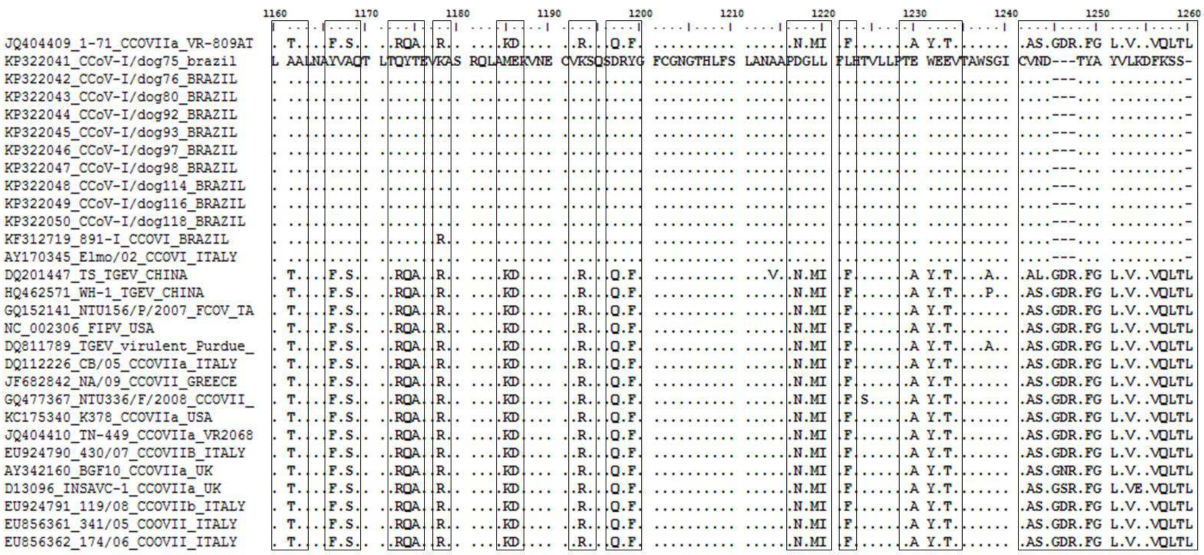


Fig. 5. Phylogenetic tree for partial nt sequences of *S* gene for CCoV-II (with optimization criterion, NJ algorithm and evolutionary model MCL). Strains from this study are in bold and with an arrow. Each node represents the values of 1000 bootstrap replicates and has been demonstrated only bootstrap values above 50%. The bar represents the number of substitutions per site.

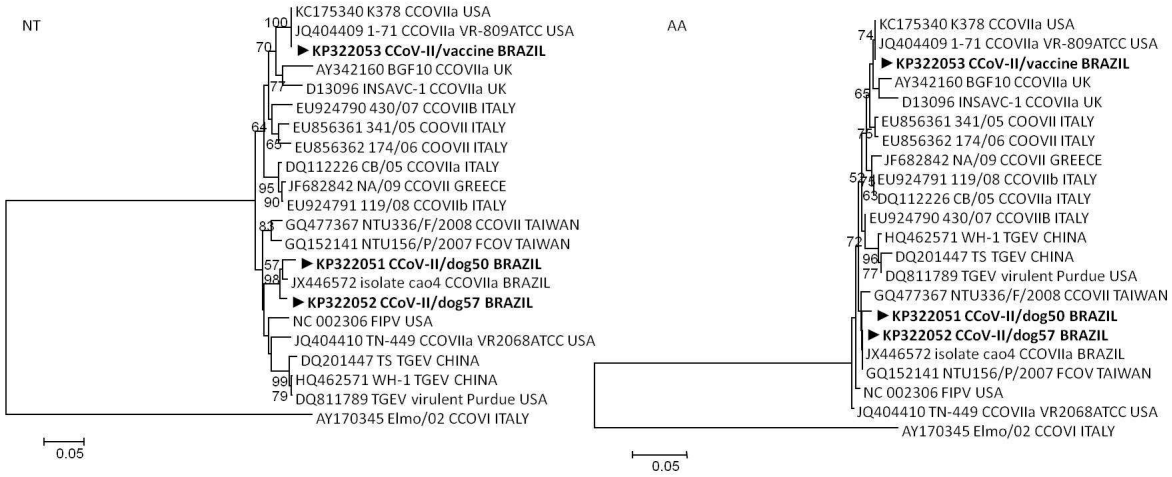


Fig. 6. Phylogenetic trees for partial sequences of nt and aa for *N* gene; strains from this study are in bold and with an arrow. Each node represents the values of 1000 bootstrap replicates and has been demonstrated only bootstrap values above 50%. The bar represents the number of substitutions per site.

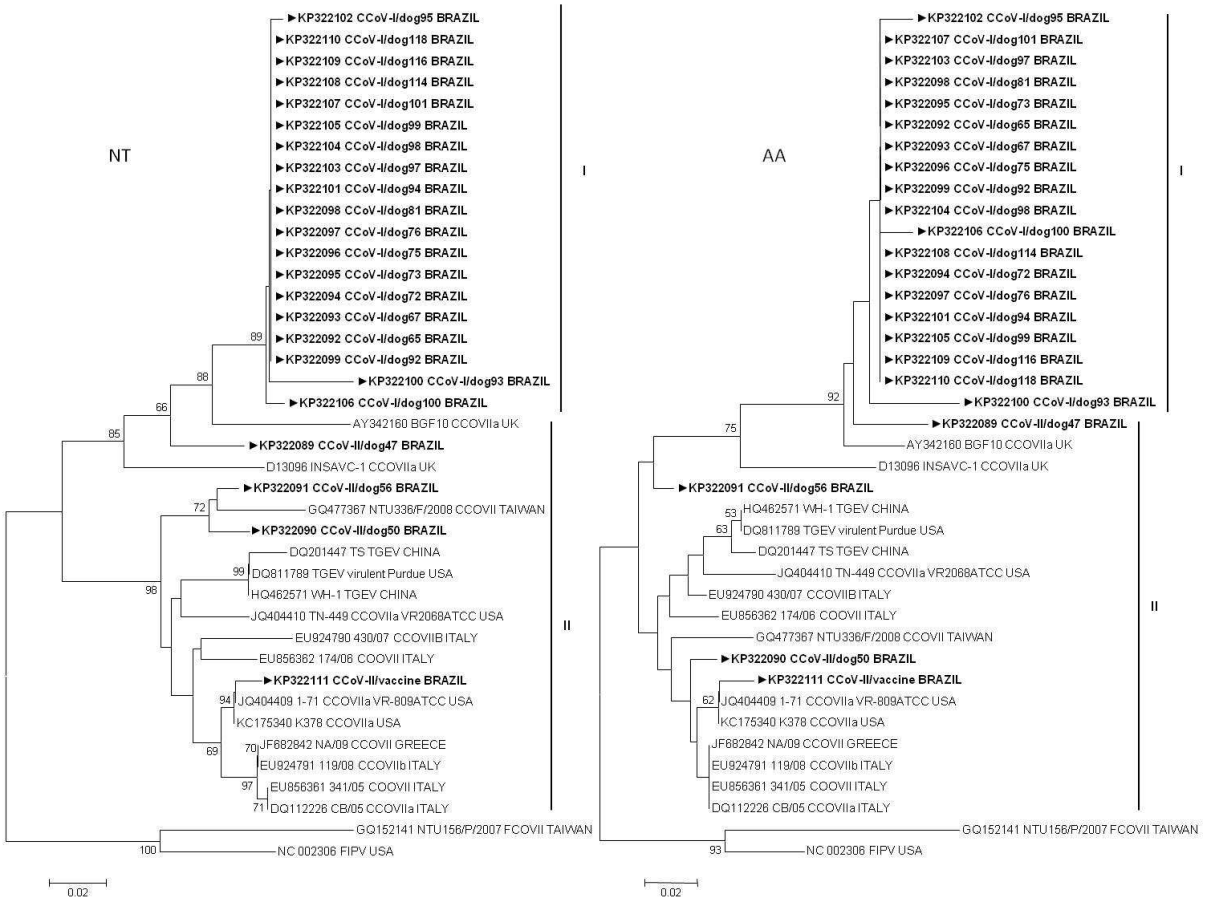


Fig. 7. Differences in amino acid alignment of CCoV *N* gene (reference strain 1-71 (VR-809 ATCC), genbank JQ404409).

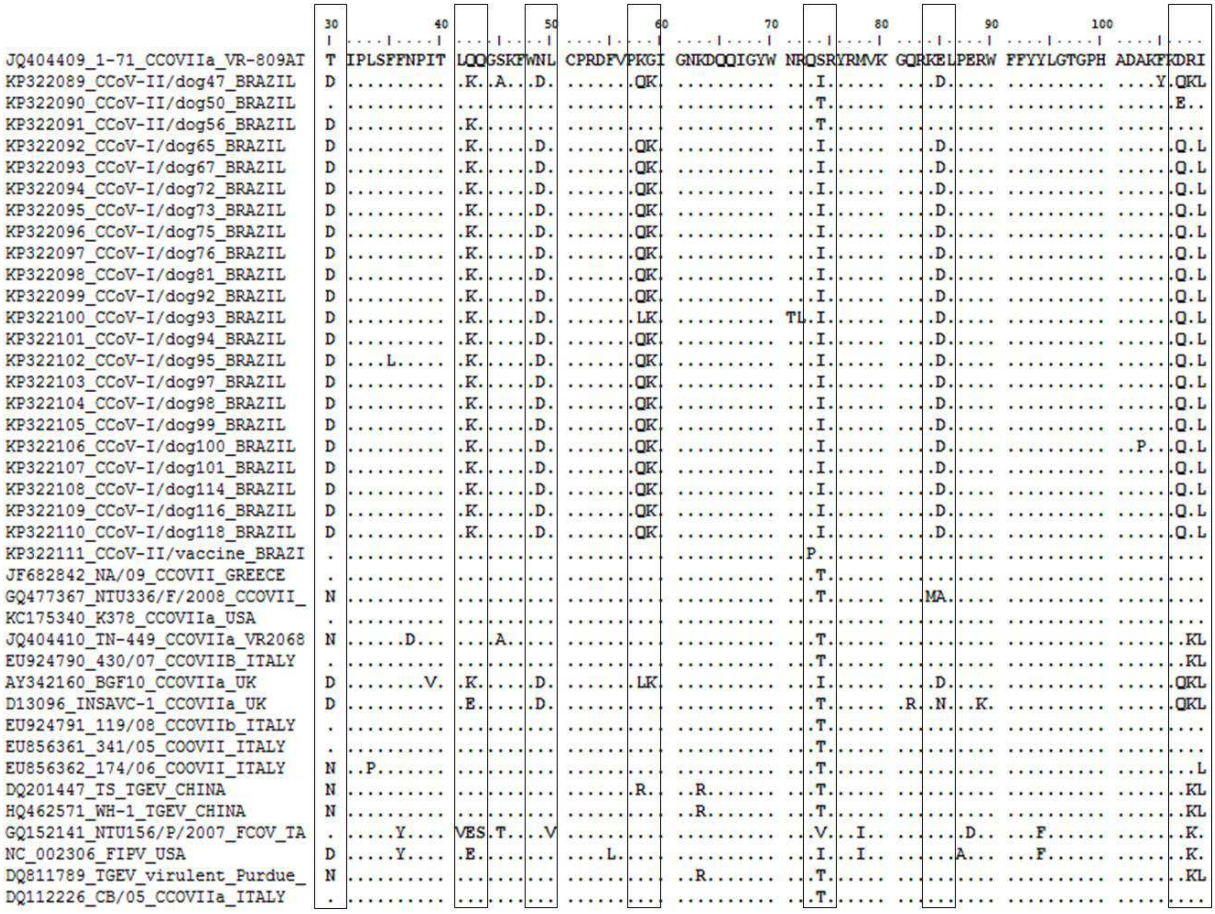


Fig. 8. Phylogenetic trees for partial sequences of nt and aa for *3b* gene; strains from this study are in bold and with an arrow. Each node represents the values of 1000 bootstrap replicates and has been demonstrated only bootstrap values above 50%. The bar represents the number of substitutions per site.

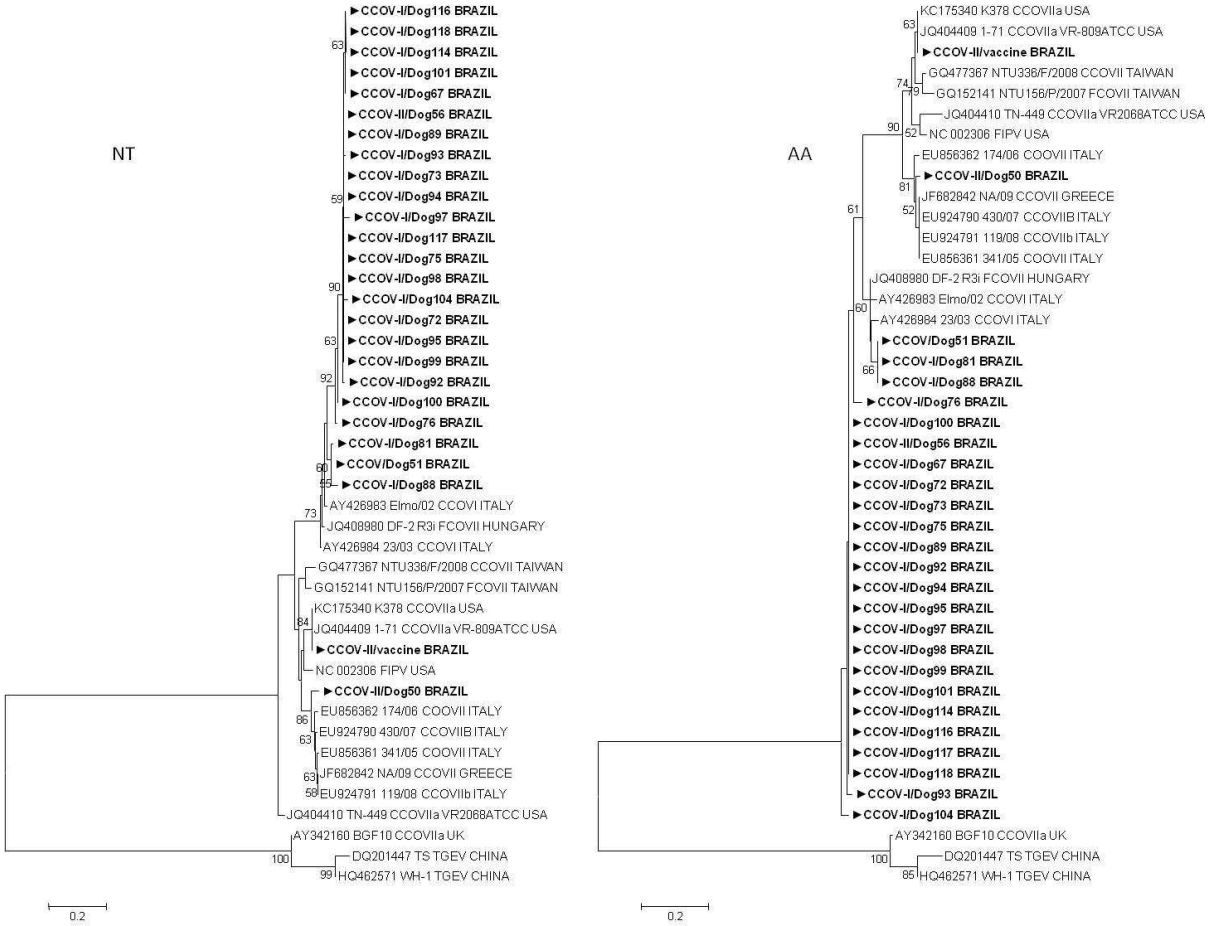
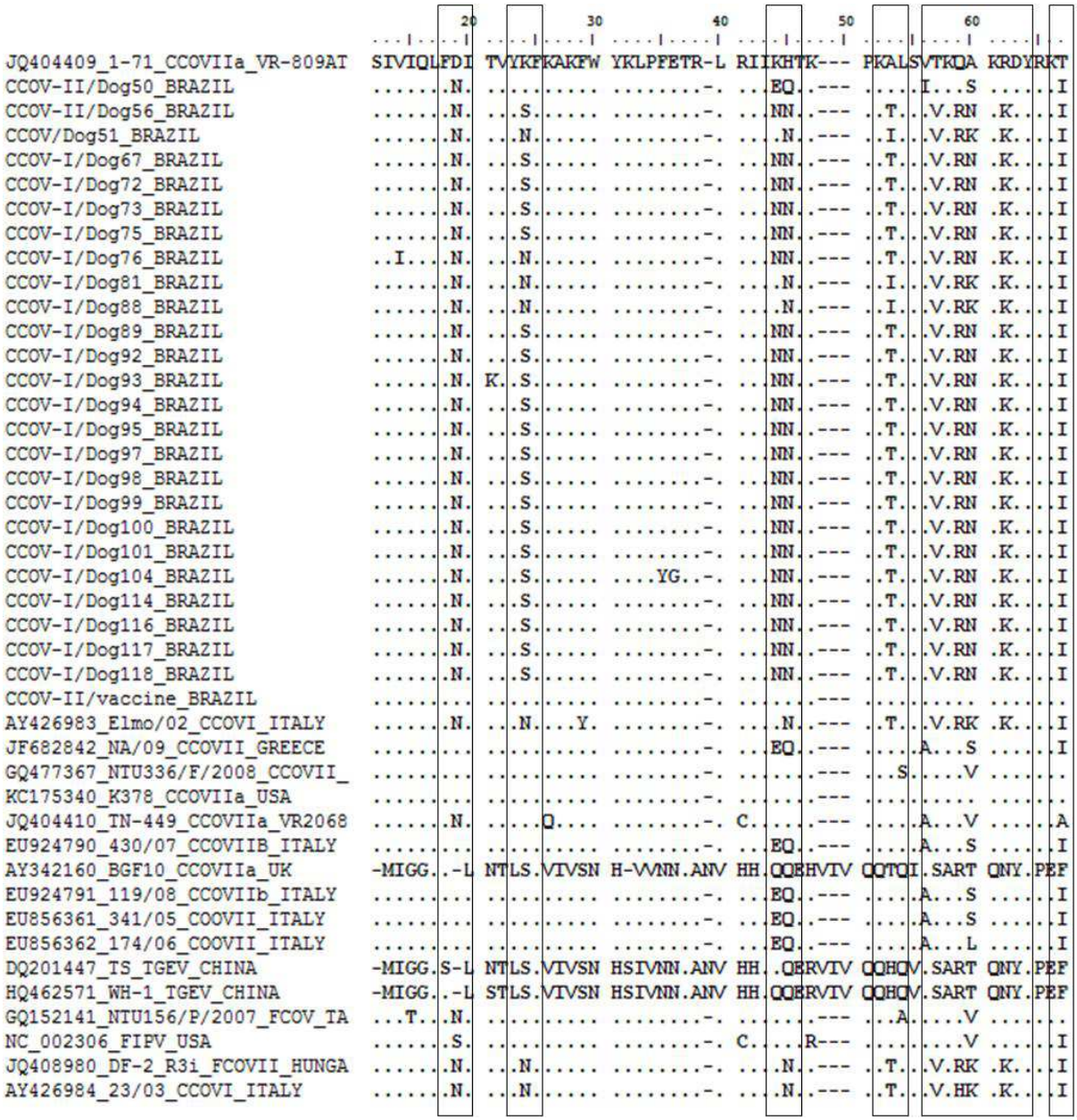


Fig. 9. Differences in amino acid alignment of CCoV *3b* gene (reference strain 1-71 (VR-809 ATCC), genbank JQ404409).



4. Discussion

In this study, nucleotide and amino acids diversity was found for all CCoV genes studied. Phylogenetic analysis for *M* gene showed that CCoV-I and CCoV-II separated in different cluster. CCoV-I presented similar aa substitutions as a FIPV strain, what is in agreement with results reported by Pratelli et al. (2002). However, for S gene, the same FIPV

strain was closer to CCoV-II strains than CCoV-I, probably because *M* is highly conserved due to its role on virion assembly (Arndt et al., 2010), in opposition to the spike protein, a target for neutralizing antibodies (Schultze et al., 1991) in which the variation of the amino acids between CCoV-I and CCoV-II can be up to 46% (Pratelli, 2006).

Difference between types I and II were clearly shown on the trees for *S* gene. The region used for typing CCoV-I strains from this study had different substitution for types of CCoVs. However, a wider part of this gene is still needed to survey for subtyping and more accurate amino acid markers.

For *N* gene trees, CCoV-I and CCoV-II a lower resolution was found when comparing to M analyses, probably because the *N* gene is a largely conserved region with little variability (Lai and Cavanagh 1997; Master, 2006), being associated with the complex of viral transcription and replication (Verheije et al., 2010), mainly considering the small portion on N studied herein.

In relation to phylogenetic trees for *3b* gene, stable markers could be found to differentiate types I and I of CCoV, also because of small fragment. Since this gene is conserved for CCoV-I strains and tropism may support the occurrence of mutations and deletions (Lorusso et al., 2008; Le Poder et al, 2013).

The efficacy of the immunity provided by vaccines can be controversial with different types or subtypes of CCoV (Pratelli et al. 2004; Pratelli, 2007; Decaro et al, 2011) and major envelope proteins as the spike proteins are determinant (Masters and Perlman, 2013). Thus, taking into account the relatively high diversity of nucleotides and amino acid among CCoV strains sequenced in this study and two CCoV vaccine strains, TN-449 (VR-2068 ATCC) and 1-71(VR-809 ATCC), one could speculate that a low crossed-protection is in curse among then Brazilian canine population with regard o CCoV infection and disease.

Conventionally, the most important canine enteric pathogen for veterinarian clinicians is CPV. However, the frequency of CCoV in this study was higher than CPV. Most CCoV positive samples from this study were CCoV-I and were from a commercial kennel from São Paulo State. Enteritis in this Kennel also was correlated with stress factors, animal agglomeration, dietary changes and different pathogens.

PCR positivity for CCoV indicates the presence of the etiologic agent, but not necessarily the disease. However, studies have shown that co-infection with other pathogens, as canine parvovirus, can exacerbate the symptoms and be fatal in some cases (Pratelli et al., 1999; Yesilbag et al., 2007). Besides, some CCoV strains can cause severe gastroenteritis and systemic disease, being fatal for puppies (Buonavoglia et al., 2006; Decaro et al., 2007; Zicola et al., 2012). In the present study, as 12.76% (6/47) of CCoV positive animals were also positive for CPV. It is worth noting that most of them (4/6) had clinical signs.

Hence, this work showed that CCoV infection is spread out among canine in Brazil, and more studies about the molecular diversity are necessary to predict the risk of infection, and perhaps an effective vaccine is needed. Nevertheless, Castro et al. (2010) found 52.2% (36/69) seronegative vaccinated dogs, indicating that immunizations to CCoV may not be effective, so preventive measures should be better evaluated due to viral spread and failure of CCoV vaccine.

Thus, isolation of Brazilian strains, cloning, sequencing analysis and comparison of mutations in wider portions of the genome, especially *S* gene, are important to understand selection and recombination between minor variants among different types of *Alphacoronavirus*-1 and are the issue of future publications by the authors.

5. Conclusion

The nucleocapsid gene of CCoV is highly conserved among types I and type II, with a lower tree resolution regarding trees based on the matrix and spike genes. Strains of types I and II show a low polymorphism for the 3b gene and no stable markers for type differentiation is found in the 3b area under analysis in this study.

Conflict of interest

None

Acknowledgmentss

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References

Arndt, A.L., Larson, B.J., Hogue, B.G. 2010. A conserved domain in the Coronavirus Membrane protein tail is important for virus assembly. Journal of Virology, 84, 11418-11428.

Buonavoglia, C., Decaro, N., Martella, V., Elia, E., Campolo, M., Desario, C., Castagnaro, M., Tempesta, M. 2006. Canine coronavirus highly pathogenic for dogs. Emerging Infectious Diseases, 12 (3), 492-494.

Castro, C.C., Johann, J.M., Finger, P.F., Nunes, C.F., Vargas, G. D'a., Fischer, G., Hübner, S.O. 2010. Canine coronavirus (CCoV) in dogs vaccinated and unvaccinated domiciliated in Pelotas, RS, Brazil. Ciências Agrárias, Londrina, 31 (4), 995-1000.

Castro, T.X., Garcia, R.C.N.C., Gonçalves, L.P.S., Costa, E.M., Marcello, G.C.G., Labarthe, N.V., Mendes-Almeida, F., 2013. Clinical, hematological, and biochemical findings in puppies with coronavirus and parvovirus enteritis. Can. Vet. J. 54, 885-888.

Costa, E.M., Castro, T.X., Bottino, F.O., Garcia, R.C.N.C., 2014. Molecular charactezation of canine coronavirus strains circulating in Brazil. Veterinary Microbiology. 168, 8-15.

Decaro, N., Martella, V., Elia, G., Campolo, M., Desario, C., Cirone, F., Tempesta, M., Buonavoglia, C. 2007. Molecular characterization of the virulent canine coronavirus CB/05 strain. Virus Research, 125, 54–60.

Decaro, N. Mari V, Campolo M, Lorusso A, Camero M, Elia G, Martella V, Cordioli P, Enjuanes L, Buonavoglia C. 2009. Recombinant canine coronaviruses related to transmissible gastroenteritis virus of Swine are circulating in dogs. Journal of Virology, 83 (3), 1532-1537.

Decaro, N., Mari, V., Elia, G.; Addie, D.D., Camero, M., Lucente, M.S., Martella, V., Buonavoglia, C. 2010. Recombinant canine coronaviruses in dogs, Europe. Emerg Infect Dis., 16 (1), 41-47.

Decaro, N., Mari, V., Sciarretta, R., Colao, V., Losurdo, M., Catella, C., Elia, G., Martella, V., Del Giudice, G., Buonavoglia, C. 2011. Immunogenicity and protective efficacy in dogs of an MF59TM-adjuvanted vaccine against recombinant canine/porcine coronavirus Vaccine, 29, 2018–2023.

Decaro, N., Pratelli,A., Tinelli, A., Martella, V., Camero, M., Buonavoglia, D., Tempesta, M., Caroli, A.M., Buonavoglia, C. 2004. Fecal Immunoglobulin A Antibodies in Dogs Infected or Vaccinated with Canine Coronavirus. Clinical and diagnostic laboratory immunology, jan, 102–105.

Evermann, J.F., Mc Keirman, A.J., Eugster, A.K., Solozano, R.F., Collins, J.K., Black, J.W., Kim, J.S. 1988. Update on canine coronavirus infections and interactions with other enteric pathogens of the dog. Compan. Anim. Pract.(Canine Pract.), 19 (2), 6-12.

Evermann, J.F., Abbott, J.R., Han, S. 2005. Canine coronavirus-associated puppy mortality without evidence of concurrent canine parvovirus infection. Journal of Veterinary Diagnostic Investigation, 17 (6), 610-614.

Gizzi, A.B.R., Oliveira, S.T., Leutenegger, C.M., Estrada, M., Kozemjakin, D.A., Stedile, R., Marcondes, M., Biondo, A.W., 2014. Presence of infectious agents and co-infections in

diarrheic dogs determined with a real-time polymerase chain reaction-based panel. BMC Veterinary Reasearch. 10:23, 1-8.

Guirao, M.P., Souza, S.P., Jerez, J.A., Richtzenhain, L.J., Brandao, P.E., 2013. Phylogeny of canine coronavirus (CCoV) from Brazilian dogs based on membrane protein partial sequences. Arq. Bras. Med. Vet. Zootec**.** 65, 1887-1890.

ICTV - International Committee on Taxonomy of Viruses [http://ictvonline.org/virusTaxonomy.asp], accessed in January, 2015.

Lai, M. M. C., Cavanagh, D. 1997. The molecular biology of coronavirus. Advances in Virus Research, 48, 1-100.

Le Poder, S., Pham-Hung D'alexandry D'orangiani, Al, Duarte, L., Fournier, A, Horhogea, C, Pinhas, C, Vabret, A, Eloit, M. 2013. Infection of cats with atypical feline coronaviruses harbouring a truncated form of the canine type I non-structural ORF3 gene. Infect Genet Evol. Dec, 20, 488-494.

Lorusso, A, Decaro, N., Schellen, P., Rottier, P.J.M., Buonavoglia, C., Haijema, Bj, De Groot, R.J., 2008. Gain, Preservation, and Loss of a Group 1a Coronavirus Accessory Glycoprotein. J Virol.; 82 (20), 10312–10317.

Master, P.S. 2006. The molecular biology of coronaviruses. Advances in Virus Research, 66, 193-292.

Masters, P.S., Perlman, S., 2013 *Coronaviridae*. *In:* KNIPE, D.M.; HOWLEY, P.M.; Fields Virology, Lippincott Williams & Wilkins, Philadelphia, Pa. 1, 825-858.

McElligott, S., Collins, P. J., Sleator, R. D., Martella, V., Decaro, N., Buonavoglia, C., O'shea, H., 2011. Detection and genetic characterization of canine parvoviruses and coronaviruses in southern Ireland. Archives of Virology, 156, 495-503.

Mesquita, R.A., Anzai, E.K., Oliveira, R.N., Nunes, F.D. 2001. Avaliação de três métodos de extração de DNA de material parafinado para amplificação de DNA genômico pela técnica da PCR. Pesquisa Odontológica Brasileira, 15 (4), 314-319.

Ntafis, V., Mari, V., Danika, S., Fragkiadaki, E., Buonavoglia, C. 2010. An outbreak of canine coronavirus in puppies in a Greek Kennel. J Vet Diagn Invest, 22, 320-323.

Park, J.S., Choi, B.K., Vijayachandran, L.S., Ayyappan, V., Chong, C., Lee, K., Kim, S.C., Choi, C.W. 2007. Immunodetection of canine parvovirus (CPV) in clinical samples by polyclonal antisera against CPV-VP2 protein expressed in *Esherichia coli* as an antigen. Journal of Virological Methods, 146, 281-287.

Parrish, C. R., Aquadro, C. F., Strassheim, M. L., Evermann, J. F., Sgro, J.-Y.; Mohammed, H. O. 1991. Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. Journal of Virology, 65 (12), 6544-6552.

Pinto, L.D., Barros, I.N., Budaszewski, R.F., Weber, M.N., Mata, H., Antunes, J.R., Boabaid, F.M., Wouters, A.T.B., Driemeier, D., Brandao, P.E., Canal, C.W., 2014

Characterization of pantropic canine coronavirus from Brazil. The Veterinary Journal. 202, 659-662.

Pratelli, A., Tempesta, M., Greco, G., Martella, V., Buonavoglia, C. 1999. Development of a nested PCR assay for the detection of canine coronavirus. Journal of Virological Methods, v. 80 (1), 11-15.

Pratelli, A., Martella, V., Elia, G., Decaro, N., Aliberti, A., Buonavoglia, D., Tempesta, M., Buonavoglia, C. 2001. Variation of the sequence in the gene encoding for transmembrane protein M of canine coronavirus (CCV). Mol. Cell Probes, 15, 229–233.

Pratelli, A., Elia, G., Martella, V., Tinelli, A., Decaro, N., Marsilio, F., Buonavoglia, D., Tempesta, M., Buonavoglia, C., 2002. M gene evolution of canine coronavirus in naturally infected dogs. Vet. Rec., 151, 758–761.

Pratelli,A.; Tinelli, A., Decaro, N., A; Martella ,V.; Camero, M.; Tempesta, M., Martini, M., Carmichael, L.E., Buonavoglia, C. 2004. Safety and efficacy of a modified-live canine coronavirus vaccine in dogs. Veterinary Microbiology, 99 , 43–49.

Pratelli, A. 2006. Genetic evolution of canine coronavirus and recent advances in prophylaxis. Veterinary Research, 37 (2), 191-200.

Pratelli, A. 2007. High-cell-passage canine coronavirus vaccine providing sterilising immunity Journal of Small Animal Practice, 48, 574–578.

Reed, A. P., Jones, E.V., Miller, T. J. 1998. Nucleotide sequence and genome organization of canine parvovirus. Journal of Virology, 62, 266-276.

Schultze, B., Gross, H.-J., Brossmer, R., Herrler, G., 1991. The S protein of bovine coronavirus is a hemagglutinin recognizing 9-*O*-acetylated sialic acid as a receptor determinant. Journal of Virology. 65, 6232-6237.

Soma, T., Ohinata, T., Ishii, H., Takahashi, T., Taharaguchi, S., Hara, M., 2011. Detection and genotyping of canine coronavirus RNA in diarrheic dogs in Japan. Research in Seterinary Science. 90, 205-207.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution, 30, 2725-2729.

Tupler, T., Levy, J.K., Sabshin, S.J., Tucker, S.J., Greiner, E.C., Leutenegger, C.M., 2012. Enteropathogens identified in dogs entering a Florida animal shelter with normal feces or diarrhea. Journal of the American Veterinary Medical Association, 241 (3), 338-343.

Verheije, M.H., Hagemeijer, M.C., Ulasli, M., Reggiori, F., Rottier, P.J.M., Masters, P.S., De Haan, C.A.M. 2010. The Coronavirus Nucleocapsid protein is dynamically associated with the replication-transcription complexes. Journal of Virology, 84 (21), 11575-11579.

Yesilbag, K., Yilmaz, Z., Ozkul, A., Pratelli, A., 2007. Aetiological role of viruses in puppies with diarrhoea. Vet Rec. 161, 169-70.

Zicola, A., Jolly, S., Mathijs, E., Ziant, D., Decaro, N., Mari, V., Thiry, E., 2012 Fatal outbreaks in dogs associated with pantropic canine coronavirus in France and Belgium. J Small Anim Pract. 53, 297-300.

II. Isolation of a putatively pantropic type II canine coronavirus strain in A72 cells

Barros, I.N.B. [1,2*]; Pinto, L.D.[3]; Silva, S.O.S. [1]; Filippsen, P. [1]; Menezes, W.B. [4]; Sanches, A. A.[1,2]; Richtzenhain, L.J. [1,2], Canal, C.W.[3]; Brandão, P.E. [1,2].

[1]Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, CEP 05508 270 - Cidade Universitária São Paulo/SP – Brasil; [2]Coronavirus Research Group, University of São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87, CEP 05508 270 - Cidade Universitária São Paulo/SP – Brazil; [3] Laboratory of Virology, School of Veterinary Medicine, University of Rio Grande do Sul, Brazil; [4] Department of Animal Science, Federal University of the Semi-Arid, Mossoró, Rio Grande do Norte, CEP 59625 900, Brazil.

*E-mail: cemavet@usp.br

ABSTRACT

Canine enteric coronavirus (CCoV) is an enveloped RNA virus causing gastroenteritis in dogs, with highly lethal strains reported. Two genotypes (CCoV type-I and CCoV type-II, with subtypes CCoV-IIa and CCoV-IIb) have been described, being CCoV-IIa considered a pantropic variant. Most studies focus on the molecular diversity of CCoV but only a few have described the isolation of this virus in cell culture, which is of most importance for phenotypical characterization and evolution studies. This study describes the isolation of a putatively pantropic canine coronavirus strain type II in A72 canine cells from a fecal sample from a dog, resulting in cytopathic effect on the 5th day of the 5th passage. Further cloning and sequence analysis are ongoing in order to understand the *in vitro* evolution and molecular epidemiology of canine coronavirus.

Keywords: Canine coronavirus; A72 cells; isolation.

1. Introduction

Canine coronavirus (CCoV) is a member of the order *Nidovirales*, family *Coronaviridae*, genus *Alphacoronavirus*, species *Alphacoronavirus-1*, together with transmissible gastroenteritis virus (TGEV) and feline coronavirus (FCoV) (Carstens, 2010). The genome of *Alphacoronavirus-1* is approximately 29kb (Dye and Siddell, 2008). CCoV is an enveloped virus with positive-stranded RNA usually responsible for gastrointestinal infection in dogs (Masters and Perlman, 2013), but in some cases this virus can cause systemic disease and outbreaks with high mortality in young dogs (Evermann, et al. 2005; Buonavoglia et al. 2006).

CCoV has a genome consisting of genes encoding spike (*S*), nucleocapsid (*N*), membrane (*M*) and envelope (*E*) structural proteins and ORFs translated into non structural proteins, as replicase polyprotein (ORF1 - open read frame 1), 3a, 3b, 3c, 7a and 7b (Masters and Perlman, 2013). The *S* protein is the most polymorphic in coronaviruses (Bosch et al. 2003) and the main target of neutralizing antibodies (Schultze et.al. 1991).

Currently, two different genotypes of CCoV have been recognized, CCoV-I and CCoV-II. The former shares high similarity with FCoVs, and the latter has been considered a pantropic CCoV variant (Pratelli et al. 2004; Pratelli, 2006) for presenting tropism for the lungs, kidneys, liver, spleen and lymph nodes (Buonavoglia et al. 2006). CCoV-II is further divided in two subtypes, CCoV-IIa, associated with fatal diarrhea, and CCoV-II b, as a result of recombination event between CCoV-II and TGEV (Decaro et al. 2009, 2010).

Canine coronaviruses are spread worldwide. The first isolation of canine coronavirus was in 1971 during an outbreak of gastroenteritis in military dogs (Binn et al. 1974). CCoV has been reported in Australia, Italy, Turkish, U.S.A, United Kingdom, Greece, Ireland, Japan, France and Belgium (Naylor et al. 2001; Pratelli et al. 2004; Yesilbag et al. 2004; Evermann, et al. 2005; Erles and Brownlie, 2009; Ntafis et al. 2010; McEligott et al. 2011; Soma et al. 2011; Zicola et.al 2012), including only a few isolation reports (Buonavoglia et al. 2006; Ntafis et al. 2011).

In Brazil, studies of detection and molecular characterization of CCoV have been published (Guirao et al. 2013; Castro et al. 2013; Pinto et al. 2014; Costa et al. 2014; Gizzi et al. 2014). However, no isolation for this virus has been reported so far for strains from this country.

This manuscript reports the isolation of a putatively pantropic canine coronavirus strain (CCoV-IIa) from a fecal sample from a dog from Brazil, including the description of the molecular typing and the isolation protocol.

2. Materials and methods

2.1. Fecal samples and CCoV molecular typing

A total of thirteen fecal samples of puppies with gastroenteritis were used for virus isolation. Total RNA extraction was performed with TRIzol Reagent™ (Life Technologies) following the manufacturer's instructions. PCR for M gene was carried out using primers described by Pratelli et al. (1999) and M-MLV Reverse Transcriptase™ (Life Technologies) plus GoTaq (Promega) Green Master Mix as per manufacturers' instructions.

The amplicons(410 bp) were purified from agarose gels with GFX™ PCR DNA and GEL BAND Purification Kit™ (GE Healthcare), followed by sequencing reactions performed with BigDye 3.1™ (Applied Biosystems) according to the manufacturer's instructions, and subjected to bi-directional DNA sequencing in an ABI-3500™ Genetic Analyzer (Applied Biosystems). Evaluation of the quality of chromatograms obtained for each DNA strand sequences was performed using Phred application online (http://asparagin.cenargen.embrapa.br/phph/). Only positions with scores higher than 20 (less than 1 % of error probability) were used and the chromatograms were also manually checked using the program Finch TV© (Geospiza) to search for interpretation errors and discrepancies between each DNA strand sequenced. The final sequence of each strain was obtained using Cap-Contig in the program Bioedit version 7.2.5 and submitted to BLASTn in http://www.ncbi.nlm.nih.gov/BLAST.

The sequences were then aligned with homologous sequences of *Alphacoronavirus* retrieved from GenBank (GU300112; GU300113; GU300114; GU300115; GU300116; GU300120; GU300121; GU300123; GU300125; GU300126; GU300127; GU300128; GU300129; GU300130; GU300132; GU300133; GU300134; EU924790; EU924791; DQ112226; AY342160; D13096; EU856361; EU856362; HQ462571; DQ201447; JF682842; NC_002306; GQ152141) using CLUSTAL/W application/algorithm running in Bioedit 7.2.5 and the alignment was used to build a Neighbor-Joining nucleotide distance tree with the Maximum Composite Likelihood model and 1000 bootstrap replicates using MEGA 6 (Tamura et al. 2013).

2.2. Virus isolation

Canine Fibroma (A72, ATCC CRL 1542) cell line was used for CCoV isolation. A72 cells and the CCV 1-71 CCoV strain (ATCC VR 809) used as positive control in this study were kindly provided by Biovet Laboratories, Brazil. Cells were propagated in Eagle's MEM supplemented with 10% fetal bovine serum (FBS) and 25mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). Each fecal sample was homogenized (10%v/v) in Eagle's MEM with gentamicin (50mg/mL) and kept at 4°C for 12 hours.

Next, the suspensions were centrifuged at 5,000g for 15 minutes at 4°C. Supernatants were filtered through 0.45 μm filters (Millipore™) and 1 ml of the filtrate was inoculated on partially (~70 per cent) confluent A72 cell cultures and incubated at 37°C / 1 hour.

In parallel, 1 ml of serum-free MEM (negative control) or 1mL of CCV 1-71 strain (positive control, $10^{4.03}$ $TCID_{50}$/mL) diluted 1:10 in MEM medium without serum were inoculated in parallel flasks as negative and positive controls, respectively.

After one hour for virus attachment, the inocula (fecal suspensions and both negative and positive controls) were discarded from the monolayers and 7ml of Eagle's MEM supplemented with 2% FBS was added to each flask. Cells were daily observed with a microscope for cytopathic effect (CPE) for 5 days. After this observation period, the monolayers were frozen at -80 ° C. The inoculation process was repeated for a total of 10 serial passages. Same A72 cells passages were used to inoculate controls and fecal samples in order to avoid interference of cell senescence. The presence of CCoV in each passage was assessed using the PCR for the M gene as described above.

3. Results

Eight strains clustered with CCoV type I strains and were named CCoVI/dog followed by 48, 60, 62, 65, 67, 80 and 81 (GenBank accession numbers KP322058, KP322063,

KP322064, KP322065, KP322066, KP3220067, KP322071 and KP322072, respectively). Five strains clustered with CCoV type II, supposedly pantropic strains, and were named CCoVII/dog followed by 44, 45, 46, 47 and 50 (GenBank accession numbers KP322054, KP322055, KP322056, KP322057 and KP322059, respectively).

Only cells inoculated with CCoVII/dog50 strain and the positive control showed cytopathic effect, beginning on the fifth day post-inoculation of the fifth passage, consisting of syncytia (Fig. 1). No other strain showed any CPE. All ten passages of CCoVII/dog50 and of the positive control were subjected to screening for CCoV using RT-PCR target to *M* gene, resulting all positive, while the ten passages of the negative control caused no changes in the monolayers.

4. Discussion

A CCoV strain (CCoVII/dog50) typed as type II was successfully isolated in A72 cells, since cytopathic effects were detected on the 5^{th} day post-inoculation of the 5^{th} passage and all the 10 passages resulted positive for CCoV after PCR for the M gene. The lack of isolation of the other four CCoV-II strains may be due to insufficient amount of virus for infection and the presence of non-viable virions but with genetic material enough to be detected by PCR, since RT-PCR has been shown to be more sensitive than isolation in cell culture for coronavirus detection (Mahony and Richardson, 2005).

The possible presence of other viruses in the fecal samples that might be competitors for replication factors might also inhibit the replication of CCoV (Condit, 2013).

The success on isolating CCoV-II and not CCoV-I can be explained by differences on the binding affinity of these viruses to cell receptors. It is known that spike protein is the major target for neutralizing antibodies (Schultze et al. 1991) and the most common

component of the receptor for *S* gene in the surface of permissive cells membranes is sialic acids, which might act also as a receptor on susceptible cell cultures (Schultze and Herrler, 1992).

In a review, Cavanagh (2005) suggests that the binding of coronaviruses might occur by as synergic effect of two different receptors. The first receptor (a sialic acid) brings the virus and cell membranes closer, and the second receptor (e.g. aminopeptidase N, APN) is responsible for the membrane fusion (Schwegmann-Wessels et al. 2002). However, some receptors might carry out both functions for some coronaviruses (Cavanagh, 2005).

There are a number of differences amongst APNs as receptor of coronavirus even in the same viral species, as *Alphacororavirus*-1, as a consequence of virus-host co-evolution. For example, the spike protein of FCoV-II (Feline infectious peritonitis virus type II (FIPV-II) and feline enteric coronavirus type II (FECV-II)) has been derived from a recombination event between FCoV-I and CCoV-II (Herrewegh et al. 1998), while CCoV-I is genetically more similar to FCoV-I than to CCoV-II (Pratelli et al. 2003), leading to differences in the ability to bind to different APNs.

Hohdatsu et al. (1998) have shown that a monoclonal antibody to feline APN blocked the infection of primary feline cells by FCoV-II, CCoV and TGEV, showing that this is a receptor for FCoV type II but not for type I. This suggests that FCoV-II may be easier to isolate *in vitro* than FCoV-I, as well as CCoV-II when compared to CCoV-I, what can be used to explain the reason CCoV vaccines strains are all type II and the lack of success in isolating CCoV-I.

5. Conclusion

As a conclusion, a putatively pantropic canine coronavirus strain type II was isolated in canine cells causing a syncytial CPE, further supporting that CCoV type II is more readily isolated when compared to type I. Further cloning and sequence analysis are ongoing in order to understand the in vitro evolution and molecular epidemiology of canine coronavirus.

Conflict of interest

None

Acknowledgments

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References

Binn, L., Lazar, E.C., Keenan, K.P., Huxsoll, D.L., Marchwicki, R.H., Strano, A.J., 1974. Recovery and characterization of a coronavirus from military dogs with diarrhea. Proc. Annu. Meet. U. S. Anim. Health Assoc. 78, 359–366.

Bosch, B.J., Van Der Zee, R., De Haan, C.A.M., Rottier, P.J.M, 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. Journal of Virology. 77, 8801-8811.

Buonavoglia, C., Decaro, N., Martella, V., Elia, E., Campolo, M., Desario, C., Castagnaro, M., Tempesta, M., 2006. Canine coronavirus highly pathogenic for dogs. Emerging Infectious Diseases.12, 492-494.

Cavanagh, D., 2005. *Coronaviridae*: a review of coronaviruses and toroviruses. In: Schmidt A and Wolff MH (eds.) Coronaviruses with Special Emphasis on First Insights Concerning SARS. Basel: Birkhäuser, 1-54.

Carstens, E.B., 2010. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2009). Archives of Virology. 155, 133–146.

Castro, T.X., Garcia, R.C.N.C., Gonçalves, L.P.S., Costa, E.M., Marcello, G.C.G., Labarthe, N.V., Mendes-Almeida, F., 2013. Clinical, hematological, and biochemical findings in puppies with coronavirus and parvovirus enteritis. Can. Vet. J. 54, 885-888.

Condit, R.C., 2013. Principles of Virology. In: Knipe DM, Howley PM, editors. Fields virology. Philadelphia: Lippincott, Williams & Wilkins.1, 21-51.

Costa, E.M., Castro, T.X., Bottino, F.O., Garcia, R.C.N.C., 2014. Molecular charactezation of canine coronavirus strains circulating in Brazil. Veterinary Microbiology. 168, 8-15.

Decaro N., Mari V., Campolo M., Lorusso A., Camero M., Elia G., Martella V., Cordioli P., Enjuanes L., Buonavoglia C., 2009. Recombinant canine coronaviruses related to

transmissible gastroenteritis virus of Swine are circulating in dogs. Journal of Virology. 83, . 1532-1537.

Decaro, N., Mari, V., Elia, G., Addie, D.D., Camero, M., Lucente, M.S., Martella, V., Buonavoglia, C., 2010. Recombinant canine coronaviruses in dogs, Europe. Emerg Infect Dis. 16, 41-47.

Dye, C., Siddell, S.G. 2005. Genomic RNA sequence of *Feline coronavirus* strain FIPV WSU-79/1146 Journal of General Virology 86, 2249-2253.

Erles, K., Brownlie, J., 2009. Sequence analysis of divergent canine coronavirus strains present in a UK dog population. Virus Research. 141, 21-25.

Evermann, J.F., Abbott, J.R., Han, S., 2005. Canine coronavirus-associated puppy mortality without evidence of concurrent canine parvovirus infection. Journal of Veterinary Diagnostic Investigation. 17, 610-614.

Gizzi, A.B.R., Oliveira, S.T., Leutenegger, C.M., Estrada, M., Kozemjakin, D.A., Stedile, R., Marcondes, M., Biondo, A.W., 2014. Presence of infectious agents and co-infections in diarrheic dogs determined with a real-time polymerase chain reaction-based panel. BMC Veterinary Reasearch. 10:23, 1-8.

Guirao, M.P., Souza, S.P., Jerez, J.A., Richtzenhain, L.J., Brandao, P.E., 2013. Phylogeny of canine coronavirus (CCoV) from Brazilian dogs based on membrane protein partial sequences. Arq. Bras. Med. Vet. Zootec. 65, 1887-1890.

Herrewegh, A.A.P.M., Smeenk, I., Horzinek, M.C., Rottier, P.J.M., Degroot, R J., 1998. Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. Journal of Virology. 72, 4508–4514.

Hohdatsu, T., Izumiya, Y., Yokoyama, Y., Kida, K., Koyama, H., 1998. Differences in virus receptor for type I and type II feline infectious peritonitis virus. Archives of Virology. 143, 839–850.

Mahony, J.B., Susan Richardson, S., 2005. Molecular Diagnosis of Severe Acute Respiratory Syndrome. Journal of Molecular Diagnostics. 7, 5.

Masters, P.S., Perlman, S., 2013 *Coronaviridae*. *In:* KNIPE, D.M.; HOWLEY, P.M.; Fields Virology, Lippincott Williams & Wilkins, Philadelphia, Pa. 1, 825-858.

Mcelligott, S., Collins, P. J., Sleator, R. D., Martella, V., Decaro, N., Buonavoglia, C., O'shea, H., 2011. Detection and genetic characterization of canine parvoviruses and coronaviruses in southern Ireland. Archives of Virology. 156, 495-503.

Naylor, M.J., Monckton, R.P., Lehrbach, P.R., Deane, E.M., 2001. Canine coronavirus in Australian dogs. Aust. Vet. J.79, 116-119.

Ntafis, V., Mari, V., Danika, S., Fragkiadaki, E., Buonavoglia, C., 2010. Na outbreak of canine coronavirus in puppies in a Greek Kennel. J Vet Diagn Invest. 22, 320-323.

Pinto, L.D., Barros, I.N., Budaszewski, R.F., Weber, M.N., Mata, H., Antunes, J.R., Boabaid, F.M., Wouters, A.T.B., Driemeier, D., Brandao, P.E., Canal, C.W., 2014 Characterization of pantropic canine coronavirus from Brazil. The Veterinary Journal. 202, 659-662.

Pratelli, A., Tempesta,M., Greco, G., Martella, V., Buonavoglia, C., 1999. Development of a nested PCR assay for the detection of canine coronavirus. Journal of Virological Methods, 80, 11-15.

Pratelli, A., Martella, V., Decaro, N., Tinelli, A., Camero, M., Cirone, F., Elia, G., Cavalli, A., Corrente, M., Greco, G., Buonavoglia, D., Gentile, M., Tempesta, M., Buonavoglia C., 2003. Genetic diversity of a canine coronavirus detected in pups with diarrhoea in Italy. Journal of Virological Methods. 110, 9-17.

Pratelli, A., Decaro, N., Tinelli, A., Martella, V., Elia, G., Tempesta, M., Cirone, F., Buonavoglia, C., 2004. Two genototypes of canine coronavirus simultaneously detected in the fecal camples of dogs with diarrhea. Journal of Clinical Microbiology, 42, 1797-1799.

Pratelli, A., 2006. Genetic evolution of canine coronavirus and recent advances in prophylaxis. Veterinary Research. 37, 191-200.

Schultze, B., Gross, H.-J., Brossmer, R., Herrler, G., 1991. The S protein of bovine coronavirus is a hemagglutinin recognizing 9-*O*-acetylated sialic acid as a receptor determinant. Journal of Virology. 65, 6232-6237.

Schultze, B., Herrler, G., 1992. Bovine coronavirus uses N-acetyl-9-O-acetylneuraminic acid as a receptor determinant to initiate the infection of cultured cells. J Gen Virol. 73, 901–906.

Schwegmann-Wessels, C., Zimmer, G., Laude, H., Enjuanes, L., Herrler, G., 2002. Binding of transmissible gastroenteritis coronavirus to cell surface sialoglycoproteins. Journal of Virology. 76, 6037–6043.

Soma, T., Ohinata, T., Ishii, H., Takahashi, T., Taharaguchi, S., Hara, M., 2011. Detection and genotyping of canine coronavirus RNA in diarrheic dogs in Japan. Research in Seterinary Science. 90, 205-207.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Molecular Biology and Evolution, 30, 2725-2729.

Yesilbag, K., Yilmaz, Z., Ozkul, A., Pratelli, A., 2007. Aetiological role of viruses in puppies with diarrhoea. Vet Rec. 161, 169-70.

Zicola, A., Jolly, S., Mathijs, E., Ziant, D., Decaro, N., Mari, V., Thiry, E., 2012 Fatal outbreaks in dogs associated with pantropic canine coronavirus in France and Belgium. J Small Anim Pract. 53, 297-300.

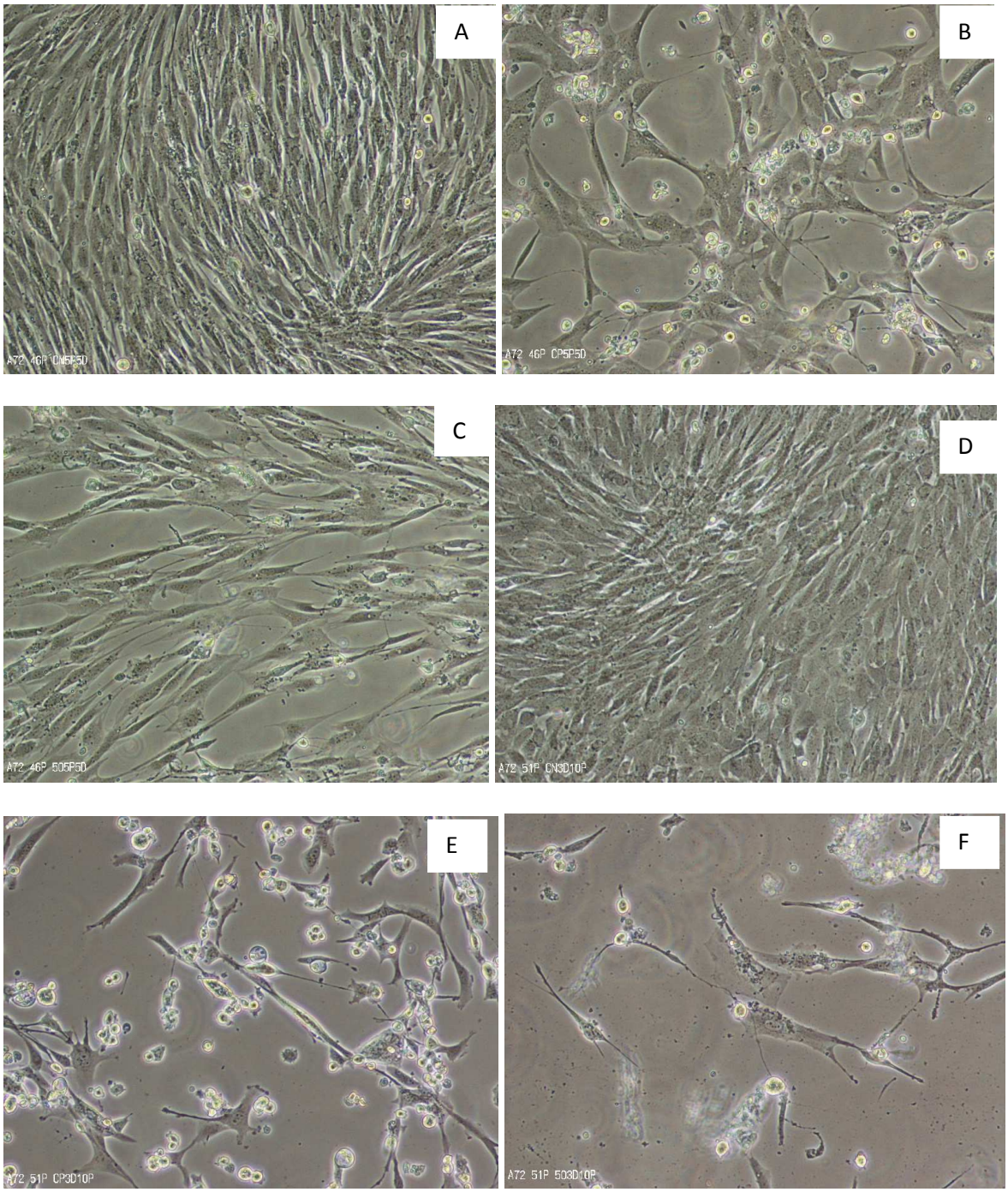


Fig. 1. **A72 cells, 5th day of the 5th viral passage**. **A**. Negative control; **B**. CCoV positive control (CCV 1-71 strain (ATCC VR 809)); **C**. CCoVII/dog50 strain; **3rd day of the 10th viral passage. D.** Negative control; **E.** CCoV positive control (CCV 1-71 strain (ATCC VR 809); **F.** CCoVII/dog50 strain; cytopathic effects characterized by syncytia, rounding and cell death detected in B, C, E and F.

III. *In vitro* evolution of CCoV-II based on partial spike gene markers

Barros, I.N.B. [1,2]; Silva, S.O.S.[1,2]; Taniwaki, S.A. [1,2]; Brandão, P.E. [1,2].

[1]Department of Preventive Veterinary Medicine and Animal Health, School of Veterinary Medicine, USP, Av. Prof. Dr. Orlando Marques de Paiva, 87, CEP 05508 270 - Cidade Universitária São Paulo/SP – Brasil; [2]Coronavirus Research Group, USP, Av. Prof. Dr. Orlando Marques de Paiva, 87, CEP 05508 270 - Cidade Universitária São Paulo/SP – Brazil; E-mail: cemavet@usp.br

ABSTRACT

Coronaviruses (CoVs) have a large positive-strand nonsegmented RNA and due to the genetic complexity and high RNA recombination events, CoVs have high diversity. Those mutations may help viral evolution in new host, new genotypes emerging, tropism or increasing virulence of viruses, for example. As canine coronavirus (CCoV), feline coronavirus (FCoV) and transmissible gastroenteritis virus (TGEV), which are the same species *Alphacoronavirus-1*, emerged as a result of recombination events among them. Changes correlated with highly pathogenic strains are usually found in *S* protein, the main target of neutralizing antibodies and the most polymorphic protein in coronaviruses. This report aimed to deep study a strain 1-71 adapted in A72 cells and its passages by cloning and sequencing based on partial *S* gene for analyzing mutations events in vitro. Results showed nonsynonymous mutations, silent and three aa deletion in the subunit S2 of *S* gene, but in a low number and without a passage-association pattern. Thus, cell-adapted strains may be highly genetically stable after serial passages in a same cell line, accumulating mainly synonymous nucleotide substitutions in the spike gene due to a stable cell-host relationship.

However, only a portion of the gene was analyzed here. Further studies as cloning and sequencing different genes, isolation and evolution studies of a natural strain are needed to understand the recombination events affecting coronaviruses. Considering that any documentation of CCoV evolutionary processes may serve for better understanding of basic virology and data base for other coronaviruses.

Keywords: Canine coronavirus; adaptation; recombination; selection; variants; A72 cells

1. Introduction

Coronaviruses are enveloped, pleomorphic viruses, approximately 220nm in diameter, with positive-sense single-stranded nonsegmented RNA as a genome with 27-32kb in length (Masters And Perlman, 2013). The large RNA virus has high diversity mainly due to the genetic complexity and high RNA mutation events (Makino et.al., 1986; Decaro and Buonavoglia, 2008).

Canine coronavirus (CCoV), feline coronavirus (FCoV) and transmissible gastroenteritis virus (TGEV) are currently classified in the same species *Alphacoronavirus-1*, (*Nidovirales: Coronaviridae: Coronavirinae*) (Carstens, 2010) and evolutionary studies on *Alphacoronavirus-1* have demonstrated that CCoV, FVoV and TGEV emerged as a result of recombination events among each other (Pratelli et al., 2004; Pratelli et al., 2006; Decaro et al. 2009).

CCoV has a genome consisting of genes encoding spike (*S*), nucleocapsid (*N*), membrane (*M*) and envelope (*E*) structural proteins and ORFs translated into non structural proteins, as replicase polyprotein (ORF1 - open read frame 1), 3a, 3b, 3c, 7a and 7b (Masters And Perlman, 2013). The *S* protein is the main target of neutralizing antibodies and the most

polymorphic protein in coronaviruses (Bosch et al., 2003). Recombination events in *S* gene have been shown in a high lethal emerged CCoV, the pantropic strains (Decaro et al., 2007). New virulent viruses usually emerge due a incomplete adaption to the host that can take long duration to stabilize (Truyen et al., 1995).

CCoV is of special interest as a model for virus molecular evolution as it occurs as two types (I and II) and as low and high virulence lineages (Pratelli et al, 2004; Buonavoglia et al. 2006). Type II is the basis for vaccine strains currently in use worldwide and produced in A72 cells; despite a high occurrence of CCoV-I is reported (Guirao et al, 2013). Furthermore, no data on the molecular diversity of the vaccine strains in successive passage cell cultures and the mechanism of molecular evolution of CoV in vitro is available.

The aim of this investigation was to assess the clonal diversity of a CCoV-II vaccine strain after serial passages in A-72 cells based on the molecular diversity of the spike gene and to gather insights on coronaviruses molecular evolution

2. Material and Methods

2.1. CCoV strain and cell line

CCoV 1-71 type II vaccine strain (ATCC VR 809) and canine fibroma A72 (ATCC CRL 1542) cell line were used for this study and were kindly provided by Biovet Laboratories, Brazil.

Cells were propagated in Eagle's MEM supplemented with 10% fetal bovine serum (FBS) and 25mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid).

2.2. Serial passages of CCoV

For the first passage, 1mL of CCV 1-71 strain ($10^{4.03}$ $TCID_{50}$/mL) diluted 1:10 (v/v) in MEM was inoculated in 25 cm^2 flasks containing partially (~70%) confluent A72 cells and incubated at 37°C / 1 hour for virus attachment. Next, the virus suspension was discarded and 7ml of Eagle's MEM supplemented with 2% FBS was added to the flask.

Cells were daily observed with a microscope for cytopathic effect (CPE) for five days. After this observation period, the monolayers were frozen at -80°C. Then, 1mL of the respective lower passage was used for the next following the same protocol described above up to the 9^{th} passage.

For each passage, another 25 cm^2 with A72 was mock-infected (negative control) with MEM without virus under the same conditions described for virus inoculation.

2.3. RNA Extraction and RT-PCR

For the original CCoV 1-71 and each passage (CCoV and negative control), total RNA extraction was extracted with TRIzol Reagent™ (Life Technologies) following the manufacturer's instructions. cDNA was reserve transcribed using 3.5 µL of the extracted RNA denatured at 95ºC for 5 minutes, and then added to the reverse transcription mix containing 1 x First Strand Buffer™ (Life Technologies), 1 mM of each dNTP, 10 mM DTT, 50ng of Random Primers™ (Life Technologies ™) 100U of M-MLV Reverse Transcriptase™ (Life Technologies™), DEPC-treated water to 10µL, performing and incubating at 37°C for 60 minutes.

Primers used for *S* gene PCR were S5 (Forward – 5` TGCATTTGTGTCTCAGACTT 3`) and S6 (Reverse – 5` CCAAGGCCATTTTACATAAG 3`) with amplification of 694 bp

(Pratelli et al., 2004) (nucleotides 3492 to 4185 of *S* gene in the reference strain 1-71 (VR-809 ATCC), genbank JQ404409)).

PCR was carried out with 6 ml of cDNA in a reaction mix containing 1 x High Fidelity PCR buffer ™ (Life Technologies), 0.2 µM of each dNTP, 2mM of $MgSO_4$, 0.2 µM of each primer, 1U of Platinum Taq Polymerase High Fidelity ™ (Life Technologies) and water DEDEPC-water to 50µL. PCR cycling conditions were 94 °C for 5 min then 10 cycles of 94°C for 30 sec, 58.5°C for 30sec, 68°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55.5°C for 30sec, 68°C for 5 min, followed by a final extension step at 68°C for 10 min.

2.4. DNA Cloning and Sequencing

The 694bp S gene amplicons were purified using ExoSAP-IT ™ (Affymetrix) and the total DNA visually quantified with Low Mass DNA Ladder ™ (Life Technologies) according to the manufacturer's instructions.

The purified amplicons was inserted into the plasmid vector pTZ57R / T™ (InsTAclone), according to the insert: vector proportion of 3:1 according to the following formula: Insert Quantity (ng) = vector (ng) x insert size (bp) x 3 / array size (bp).

The ligation reaction was performed at 15°C for 16 hours and was followed by the addition of 5µL of ligation reaction to 100 µL of competent DH5α *E. coli* cells grown in LB (Luria-Bertani) liquid medium and incubated on ice for 20 to 30 min. After a thermal shock (42°C / 2 min and ice / 2min), 900µL of LB medium without antibiotics was added to the reaction and incubated at 37°C / 150rpm / 1h30min.

The cells were then plated on solid LB medium containing 100 µg/ml ampicillin, IPTG (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) and X-Gal (isopropylthio-β-Dgalactoside) and incubated up to 16 hours at 37ºC.

Individual white colonies, predicted to have inserts, were selected and subjected to a reaction PCR to confirm the insertion of an amplicon. using 12.5 µL of GoTaq Green Master Mix™ (Promega), 0.5 µL of each primer target to the plasmid (M13F – forward 5`- GTTTTCCCAGTCACGAC-3`; and M13R – reverse 5`- CAGGAAACAGCTATGAC – 3`) and 11.5 µL of DEPC water to a final volume of 25µL followed by cycles of 94°C / 5 min and 30 cycles of 94°C / 30 sec, 45°C / 30 sec and 72°C / 1 min and a final extension of 72°C / 5min.

A number of 10 clones of each passage and of the original virus confirmed as carrying the inserted amplicon were subjected to DNA sequencing with BigDye 3.1™ (Applied Byosystems) and ABI-3500 Genetic Analyzer™ (Applied Byosystems) as per manufacturer's instructions using primers M13F and M13R. Evaluation of the quality of chromatograms obtained for each DNA strand sequences was performed using Phred application online (http://asparagin.cenargen.embrapa.br/phph/) and manual checked using the program Finch TV © (©Geospiza) to search for interpretation errors and discrepancies between each DNA strand sequenced. The final sequence of each clone was obtained using Cap-Contig, with phred score >20, in the program Bioedit version 7.2.5 and submitted to BLASTn in http://www.ncbi.nlm.nih.gov/BLAST for homology checking.

2.5. Sequences Analysis

Sequences of clones of each passage and the original virus (*e.g.* intra-passage analysis) were aligned using Clustal/W in Bioedit version 7.2.5 and compared in terms of nucleotide and amino acids identities.

Identical sequences that predominated in the intra-passage comparison were herein named as the Master Sequence for that passage, while those in lower frequencies were named Mutants.

Next, the Mutants and Master Sequences for all passages and the original virus were aligned together including *Alphacoronavirus* homologous sequences retrieved from GenBank (JQ404409; JF682842; GQ477367; KC175340; JQ404410; EU924790; AY342160; D13096; EU924791; EU856361; EU856362; DQ201447; HQ462571; GQ152141; NC_002306; DQ811789; DQ112226) using CLUSTAL/W running in Bioedit 7.2.5.

All sequences generated in this study were deposited in the GenBank under the accession numbers: KP281485 to KP281596.

3. Results

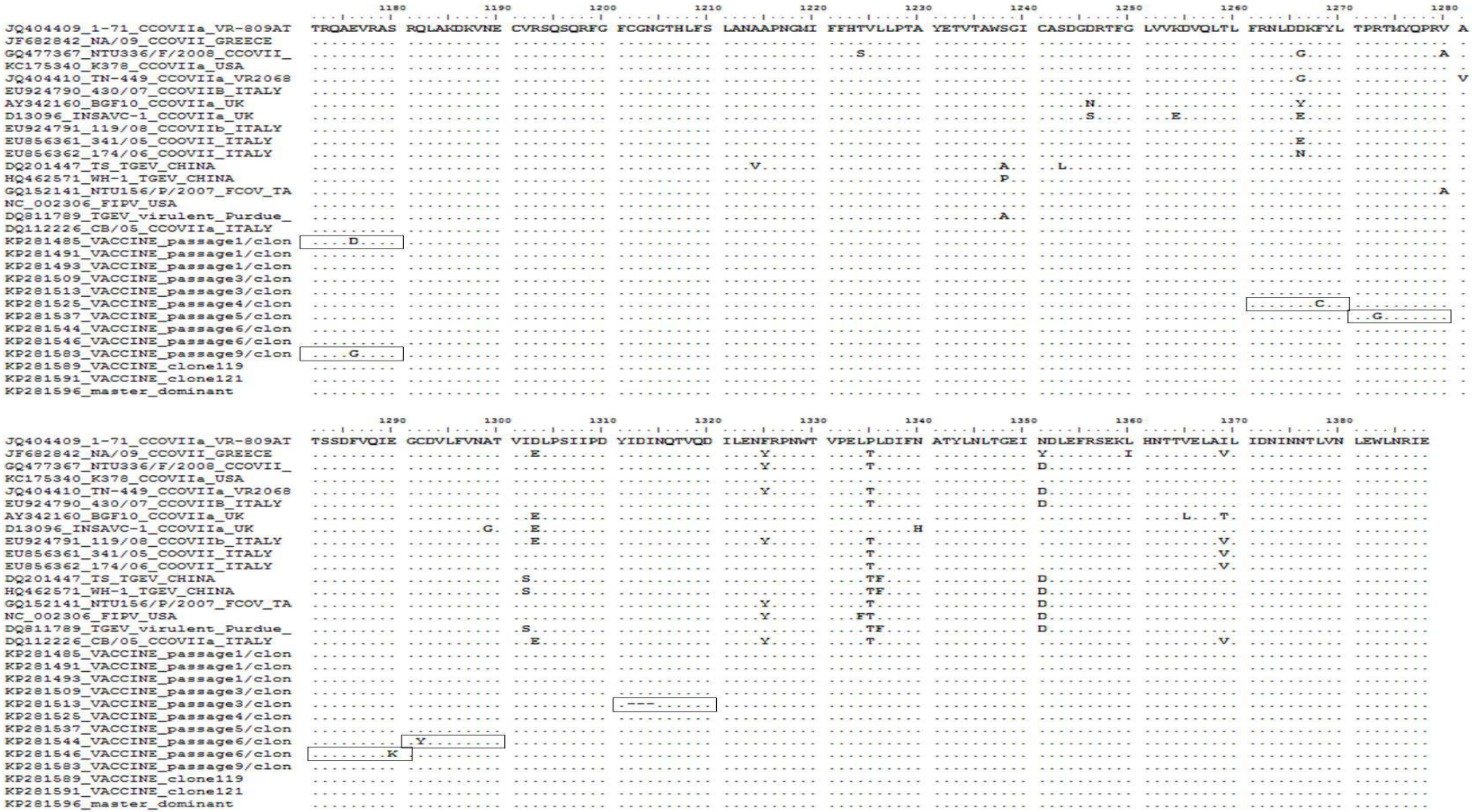
A total of 112 viable DNA sequences were obtained to passages 1 to 9 and the original CCoV 1-71 strain and only 12 nucleotide mutations were found amongst these sequences, ranging from 0 to 3 per passage (Table 1) but in a passage-exclusive way.

From these mutations the total of point mutations eight were transitions, three were transversions and one deletion. Of these mutations: six were missense leading to amino acid mutations, especially in the central region; five were synonymous codon mutations and there was a deletion of 9 nt / 3aa at passage three, in position 3935-3943 of the alignment with clones sequences and the reference strain 1-71 (VR-809 ATCC, genbank JQ404409) The deletion were in a Mutant variant of the third passage, and was not found in any of the other clones.

Table 1. Nucleotide and putative amino acids mutations of the spike gene of CCoV stain 1-71 passaged in A72 cells. P- passage number (0 refers to the original virus); NC - number of clones sequenced per each passage; MU - number of mutations per passage regarding the Master Sequence. Numbers in cells of each mutation represent the number of occurrences of that specific mutation. Positions at *S* gene in the reference strain 1-71 (VR-809 ATCC), genbank JQ404409).

P	SC	MU	A3519G Arg/Arg	T3813C Thr/ Thr	G3528T Glu/Asp	C3699T Thr/Thr	A4165C Thr/Thr	TTGATATTA 3935 to 3943 Deletion (Ile+Asp+Ile)	T4155C Asn/Asn	T3803G Phe/Cys	A3817G Arg/Gly	G3868A Cys/Tyr	G3875A Glu/Lys	A3527G Glu/Gly
0	9	2	1	1	0	0	0	0	0	0	0	0	0	0
1	10	3	0	0	1	1	1	0	0	0	0	0	0	0
2	11	0	0	0	0	0	0	0	0	0	0	0	0	0
3	11	2	0	0	0	0	0	1	1	0	0	0	0	0
4	10	1	0	0	0	0	0	0	0	1	0	0	0	0
5	13	1	0	0	0	0	0	0	0	0	1	0	0	0
6	13	2	0	0	0	0	0	0	0	0	0	1	1	0
7	12	0	0	0	0	0	0	0	0	0	0	0	0	0
8	11	0	0	0	0	0	0	0	0	0	0	0	0	0
9	12	1	0	0	0	0	0	0	0	0	0	0	0	1

Fig. 1. Mutations in amino acid of clones from this study and CCoV-II *S* protein at position 1172 to 1388 in reference to strain 1-71 (VR-809 ATCC Genbank JQ404409).



4. Discussion

The 1-71 strain of CCoV adapted in A72 cells showed mutations transitions, transversions and deletions but in a low number and without a passage-association pattern, what might indicate that this strain in this study may have reached a quite stable virus-cell relationship with no further mutations being fixed even after serial passages, as it had been previously adapted to grow in A72 cells.

Selection pressure can be indicated by frequency of silent and non-silent mutations. Usually surface proteins present mostly non-silent mutations as a result of immunological pressure, while internal proteins mostly show silent changes (Truyen et al., 1995).

Taking this into account, the amplified region of the *S* gene (S2) in this study is responsible for membrane fusion and syncytia formation and has a higher mutation pressure than region S1, responsible for binding to cellular receptors (Lai And Cavanagh 1997). Nevertheless, mutations were found also in S2 region (Gallagher and Buchmeier, 2001) and evolutionary studies of other coronavirus showed that mutations in the S2 subunit can allow virus entry into a variety of types of cells in a trypsin-independent way (Belouzard, Chu and Whittaker 2009; Borucki et al, 2013). Amino acid substitutions occurred mainly at the center of the sequence, and the deletion, which may suggest that this area has a lower selective pressure.

Other factors that could justify not repeat mutants are these could already be preselected and were extinct, or gained in strength and after weighed, or may have been biased sampling of clones. Instability of spike protein is a disadvantage in vitro because stabilized variant are selected (Gallagher and Buchmeier, 2001).

Since the dominant strain is stable and adapted to a new host cell, adaptation is a rapid process (Fang et al., 2005) and thus the master sequences may have been as a result of this

stability. Conversely, in the case of field strains, on e need to take into account that there's interference during co-infection with CCoV I, Feline coronavirus (FCoV) and Transmissible Gastroenteritis Coronavirus (TGEV) as inter-types recombination is a well known phenomenon for these viruses (Decaro et al. 2009, Decaro 2010; Vennema, 1999).

Far beyond the clinical level, evolution and genetic studies of coronavirus modeled the CCoV allow the understanding of basic virology and the evolution of these viruses more broadly, generating data that can serve as a basis for studies in other coronavirus species including those that affect humans, as cold-causing coronavirus OC43 and 229E and the coronavirus associated with the syndrome severe acute (SARS) respiratory.

5. Conclusion

Cell-adapted CCoV-II vaccine strains may be highly genetically stable after serial passages in a same cell line, accumulating mainly synonymous nucleotide substitutions in the spike gene due to a stable cell-host relationship.

Conflict of interest

None

Acknowledgments

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References

Belouzard, S., Chu, V.C., Whittaker, G.R. 2009. Activation of the SARS coronavirus spike protein via sequential proteolytic cleavage at two distinct sites. Proceedings of the National Academy of Sciences of the United States of America. 106 (14), 5871–5876.

Borucki, M. K., Allen, J. E., Chen-Harris, H., Zemla, A., Vanier, G., Mabery, S., Torres, C., Hullinger, P., Slezak, T. 2013. The role of viral population diversity in adaptation of bovine coronavirus to new host environments, PLoS ONE, 8 (1), 1-11.

Bosch, B.J., Van Der Zee, R., De Haan, C.A.M., Rottier, P.J.M, 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. Journal of Virology, 77, 8801-8811.

Buonavoglia, C., Decaro, N., Martella, V., Elia, E., Campolo, M., Desario, C., Castagnaro, M., Tempesta, M., 2006. Canine coronavirus highly pathogenic for dogs. Emerging Infectious Diseases.12, 492-494.

Carstens, E.B., 2010. Ratification vote on taxonomic proposals to the International Committee on Taxonomy of Viruses (2009). Archives of Virology. 155, 133–146.

Decaro, N., Martella, V., Elia, G., Campolo, M., Desario, C., Cirone, F., Tempesta, M., Buonavoglia, C. 2007. Molecular characterization of the virulent canine coronavirus CB/05 strain. Virus Research, 125, 54–60.

Decaro, N., Buonavoglia, C. 2008. An update on canine coronaviruses: viral evolution and pathobiology. Veterinary Microbiololgy 132 (3-4), 221–234.

Decaro N., Mari V., Campolo M., Lorusso A., Camero M., Elia G., Martella V., Cordioli P., Enjuanes L., Buonavoglia C., 2009. Recombinant canine coronaviruses related to transmissible gastroenteritis virus of Swine are circulating in dogs. Journal of Virology. 83, 1532-1537.

Decaro, N., Mari, V., Elia, G., Addie, D.D., Camero, M., Lucente, M.S., Martella, V., Buonavoglia, C., 2010. Recombinant canine coronaviruses in dogs, Europe. Emerg Infect Dis. 16, 41-47.

Fang, S.G., Shen, S., Tay, F.P.L., Liu, D.X. 2005. Selection of and recombination minor variants lead to the adaptation of an avian coronavirus to primate cells. Biochemical and Biophysical Research Communications, 336, 417-423.

Gallagher, T.M., Buchmeier, M.J., 2001. Minireview Coronavirus spike proteins in viral entry and pathogenesis. Virology, 279, 371-374.

Guirao, M.P., Souza, S.P., Jerez, J.A., Richtzenhain, L.J., Brandao, P.E., 2013. Phylogeny of canine coronavirus (CCoV) from Brazilian dogs based on membrane protein partial sequences. Arq. Bras. Med. Vet. Zootec. 65, 1887-1890.

LAI, M. M. C.; CAVANAGH, D. 1997. The molecular biology of coronavirus. Advances in Virus Research, 48, 1-100.

Makino, S.; Keck, J. G.; Stohlman, S. S.; Lai, M. M. C. 1986. High frequency RNA recombination of murine coronavirus. Journal of Virology, 57, 729-739.

Masters, P.S., Perlman, S., 2013 *Coronaviridae*. *In:* KNIPE, D.M.; HOWLEY, P.M.; Fields Virology, Lippincott Williams & Wilkins, Philadelphia, Pa. 1, 825-858.

Pratelli, A., Decaro, N., Tinelli, A., Martella, V., Elia, G., Tempesta, M., Cirone, F., Buonavoglia, C., 2004. Two genototypes of canine coronavirus simultaneously detected in the fecal camples of dogs with diarrhea. Journal of Clinical Microbiology, 42, 1797-1799.

Pratelli, A., 2006. Genetic evolution of canine coronavirus and recent advances in prophylaxis. Veterinary Research. 37, 191-200.

Vennema, H. 1999. Genetic drift and genitc shift during feline coronavirus evolution. Veterinary Microbiology. 69, p. 139-141.

Truyen, U., Parrish, C.R., Harder, T.C., Kaaden, O-R. 1995. There is nothing permanent except change. The emergence of new virus diseases. Veterinary Microbiology, 45, 103-122.

IV. Genera Conclusions

Phylogenetic analysis based of *M* gene of CCoV is a useful tool for typing, however not for sub typing CCoV strains. *N* gene of CCoV is highly conserved with a lower tree resolution regarding trees based on the *M* and *S* genes. Regarding the *3b* gene, CCoVs types I and II show a low polymorphism and no stable markers for type differentiation in the *3b* segment under analysis in this study.

A putatively pantropic Brazilian CCoV type II strain was isolated in canine cells causing a syncytial CPE, with no CCoV-I isolation, suggesting that CCoV type II is more readily isolated when compared to type I.

Cell culture-adapted CCoV-II vaccine strains may be highly genetically stable upon serial passage in a same cell line, accumulating primarily synonymous nucleotide substitutions in the spike gene due to a stable cell-host relationship.

In short, all data gathered herein on molecular epidemiology and evolutionary processes of CCoV can serve for a better understanding of basic virology and as a basis for studies on other coronaviruses. Further cloning and sequence analysis of more genes of CCoV are ongoing in order to understand the *in vitro* evolution and molecular epidemiology of canine coronavirus.