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Original article

EPIDEMIOLOGY AND MOLECULAR DIAGNOSIS OF CANINE CORONAVIRUS IN EGYPT: EVALUATION OF DIFFERENT TESTS USED FOR ITS DIAGNOSIS

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Summary

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This work aimed to study the epidemiology and molecular detection of existing canine coronavirus (CCoV) strain circulating in Egypt. A total number of 86 dogs with clinical signs suggestive for CCoV infection was subjected to clinical examination and quick immunochromatography (IC) on faecal swabs to detect viral antigen. To identify CCoV viral RNA and S protein gene in blood and faeces, conventional PCR and quantitative RT-PCR were used. All examined dogs showed clinical signs suggestive of CCoV infection. Only 32 out of 86 dogs were positive for IC. Of all samples, 36 showed positive results in PCR and the amplification products from these 36 samples were confirmed as CCoV-S protein partial gene by the analysis of nucleotide sequence. However, the qRT-PCR analysis detected 45 positive samples e.g. more than those of IC or conventional polymerase chain reaction. Statistical evaluation of IC and conventional PCR to the results of qRT-PCR performance showed sensitivity, specificity, accuracy, positive and negative predictive values of 71%, 100%, 84.9%, 100%, 75.9% for IC and 80%, 100%, 89.5%, 100%, 82% for PCR, respectively. Sex and age had no effects on IC and PCR results. The prevalence of CCoV infection among the population of this study was 52.3%. Sequence analysis results proved that CCoV strain 59/08 was the strain, circulating in Egypt among dog populations. PCR products of the CCoV cDNA were closely identical to published CCoV-S partial gene. The NCBI Genbank accession number of sequence of the studied gene (CCoV-S partial gene) in this study was KY655745.

Key words: canine coronavirus, dogs, Egypt, epidemiology, qRT-PCR, sequencing

INTRODUCTION

Canine corona enteritis is a highly infectious disease that affects dogs of different ages and breeds. The disease is manifested clinically by anorexia, depression, vomiting, watery to mucoid diarrhoea (Gaskell *et al.*, 1996; German, 2005; De-

caro & Buonavoglia, 2011; Decaro *et al.*, 2012).

Infection occurs via the faecal-oral route or intranasally. After infection, the virus invades mucosal epithelial cells covering the tip and upper third of intestinal villi and to lesser extent, colonic mucosa resulting in subclinical form mostly, but in some cases progressed to severe clinical signs (Gaskell *et al.*, 1996; German, 2005; Licitra *et al.*, 2014). The disease is more severe in younger animals. CCoV is shed in faeces of infected dogs but cannot survive under external environmental condition for more than 48 hours (Gaskell *et al.*, 1996; German, 2005; Licitra *et al.*, 2014).

Canine corona virus (CCoV) belongs to *Coronaviridae* family, subfamily: *Coronavirinae,* genus: *Alphacoronavirus*, *Betacoronavirus* and *Gammacoronavirus*. *Alphacoronavirus* has positive single strand RNA with helical nucleocapside, the outer envelope carries many protein projections arranged from outer to inner as spike projection (Spike protein [S]), membrane protein projection (m), small envelope protein (E) and nucleocapside protein (N) (Denison & Becker, 2014; Licitra *et al.*, 2014).

Recently, canine coronaviruses were classified into CCoV type I which are enteric strains and CCoV type II – pantropic strains causing multisystemic infection with foetal illness showing clinical signs similar to CCoV infection (Erles *et al.*, 2003; Han *et al.*, 2004; Erles *et al.*, 2007; Decaro & Buonavoglia, 2008; Woo *et al.*, 2010; King *et al.*, 2012).

CCoV enteritis emerges as a major epidemiological problem with fatal illness in dogs populations e.g dog houses and kennels (Cavalli *et al.*, 2014). To the best of our knowledge, no data are published regarding the diagnosis, epidemiology, molecular identification of the CCoV strain. Therefore, the present study was aimed at molecular diagnosis, identification of CCoV strain circulating in Egypt in addition to comparison of different methods used for the diagnosis and detection of prevalence of CCoV infection in Egypt.

MATERIALS AND METHODS

Ethical approval and informed consent

The authors of the current study informed and received permission from the dog owners to take samples for analyses. Samples were collected as per standard sample collection procedure harmless to the animals. The proposal of this study was approved from National Research Center Committee no: 1/3/2016.

Clinical examination and sampling

This study has been conducted in Giza province, Egypt between March 1, 2016 and September 1, 2018. Eighty-six diseased dogs (2–5 months old), from different sex and breeds were checked clinically for detection of clinical signs suggestive for CCoV enteritis.

History of the examined dogs including breed, sex, age, past medical data history, and registered vaccination was recorded. Dogs were subjected to general and specific clinical examination according to Gaskell *et al.* (1996). The severity of the clinical signs observed in this study was recorded as mild, moderate, and severe (German, 2005).

Faecal and blood samples were collected from all clinically affected dogs with clinical signs of canine coronavirus gastroenteritis (n=86) and checked by a rapid IC test (rapid CCoV Ag test kit for qualitative detection of viral antigen in

faeces) (Song *et al.*, 2015) and virus detection. Collected samples were stored in 2 mL microtubes at a temperature of –80 °C (Awad *et al.*, 2018).

Immunochromatography (IC)

Direct IC (rapid CCoV Ag test kit, Bionote Inc., Korea) for the qualitative detection of canine coronavirus and canine parvoviral antigen in canine faeces was carried out on 86 faecal samples of dogs showing clinical signs of CCoV infection (Song *et al.*, 2015).

Extraction of total RNA and cDNA synthesis

The PCR cycling parameters were one cycle of 94 °C for 3 min, 35 cycles of 94 °C for 60 s, 58 °C for 1 min, 72 °C for 90 s, and a final cycle of 72 °C for 7 min. The relative quantification of the target genes to the reference gene GAPDH (Wesley, 1999) was determined by using the $2^{-\Delta\Delta CT}$ method (Decaro *et al.*, 2004).

Sequencing and phylogenetic analyses

The positive PCR products for CCoV (RNA; DNA coronavirus) were selected and purified using a QIAGEN purification kits (QIAGEN, Germany). Sequencing was carried out based on the amplified segment using a standard ABI Big Dye terminator version 3.1 sequence kit (Applied Biosystem). The obtained sequences were analysed for homology using the NCBI Basic Local Alignment Search Tool (BLAST: http://www.ncbi.nlm.nih.gov). Multiple sequence alignment was carried out using ClustalW2 and the percentage nucleotide identity was determined using DNA identity matrix. A neighbour-joining (NJ) phylogenetic tree was constructed based on the canine coronavirus sequences using MEGA5 software. The tree reliability was assessed using 26 bootstrap replicates. All nucleotide sequences were deposited in NCBI GenBank (Table 1) (Wesley, 1999; Gallagher & Buchmeier, 2001; Larkin *et al.*, 2007; Jeoung *et al.*, 2014).

Comparison between RT-PCR and IC

RT-PCR is considered the gold standard test of this study to which IC and conventional PCR were compared. The sensitivity, specificity, accuracy, positive predictive value and negative predictive values of used diagnostic tests and prevalence of the disease in population of this study were calculated according to Smith (2005) as follows: sensitivity = a/(a+c); specificity = d/(b+d); accuracy value = a+d/(a+b+c+d); positive predictive value (PPV) = a/(a+b) and negative predictive value (NPV) = d/(c+d); where a=true positive, b= false positive, c=false negative, d=true negative result.

Table 1. CCoV primers used in the study

Primers	Oligonucleotide sequence (5′-3′)	Estimated product size[a]
CCoV-S	F: ACCACCCAGTGTCAAGGAAA R: TGCCTCAGTGTACGATGTGT	275 bp
GAPDH	F: GAGAAAGCTGCCAAATATG R: CCAGGAAATGACCTTGACA	193 bp

[a]Based on available canine corona virus (CCoV-S) genome sequences.

Table 2. Clinical signs detected in examined dogs

Breed	Sex		Clinical signs				
	Male	Female	Anorexia	Depression	Vomiting*	Diarrhoea**	Diarrhoea type
Golden Retriever	6	6	Severe	Moderate	Severe	Severe	Watery
Rottweiler	6	8	Severe	Severe	Severe	Severe	Haemorrhagic
German shepherd	8	10	Severe	Severe	Severe	Severe	Watery
Belgium shepherd	8	6	Severe	Severe	Severe	Severe	Watery
White shepherd	5	5	Severe	Severe	Severe	Severe	Watery
Siberian Husky	4	2	Severe	Severe	Severe	Severe	Mucoid
Pitbull	6	6	Moderate	Moderate	Mild	Moderate	Watery

*Vomiting: mild: once per 12 h, moderate: 2–5 times per 12 h, severe: >6 times per 12 h; **diarrhoea: mild: soft or pasty, moderate: watery diarrhoea, severe: watery and bloody.

Statistical analysis

The statistical analysis of the obtained results was done according to Smith (2005), using Chi-square test to study the effect of age, sex and breeds on results of different tests.

RESULTS

Most prominent clinical signs recorded in examined dogs were anorexia, depression, vomiting and diarrhoea. The severity of clinical signs varied from moderate to severe while diarrhoea varied from watery to mucoid and sometimes haemorrhagic (Table 2).

Immunochromatography results showed that 32 out of 86 faecal samples of examined dogs were positive. Table 3 presents the obtained results according to the gender of the affected animals. Fig. 1 shows a snip kit of IC positive for CCoV presented in the form of 2 red columns in infected samples.

Conventional PCR succeeded in identifying 36 infected cases among 86 examined dog samples (Table 3). The cDNA template isolated from faeces and blood samples of dogs showed clinical symptoms suggestive for canine corona virus infection was amplified by a specific CCoV primer. The former was previously designed according to the Spike protein (S) gene of CCoV genome. The cDNA of all checked CCoV samples had produced distinctive DNA bands using specific primers sequence of 275 bp amplicon for CCoV-S protein gene (Fig. 2).

Fig. 1. Immune chromatography test kit showing positive results for CCoV infection (arrow) in a Rottweiler puppy.

The results showed that the qRT-PCR assay detected more infected dogs compared to the PCR and IC tests. The qRT-PCR succeeded in detecting 45 infected cases vs 36 and 32 cases identified by PCR and IC assays respectively. Fig. 3 represents the expression levels of *CCoV-S* gene in different samples (blood and faeces) of infected dogs. The expression levels of *CCoV-S* gene in control dog samples were under the limit of detection. However, expression values of *CCoV-S* gene in different samples of infected dogs were higher when compared to control

Table 3. Dogs examined for CCoV using immunochromatography and conventional PCR

Case	Immunochromatography – number (%)	
	Male	Female
Positive	15 (34.88)	17 (39.53)
Negative	28 (65.11)	26 (60.46)
Total	43 (100.00)	43 (100.00)
	Conventional PCR - number (%)	
Positive	17 (39.53)	19 (44.18)
Negative	26 (60.46)	24 (55.81)
Total	43 (100.00)	43 (100.00)

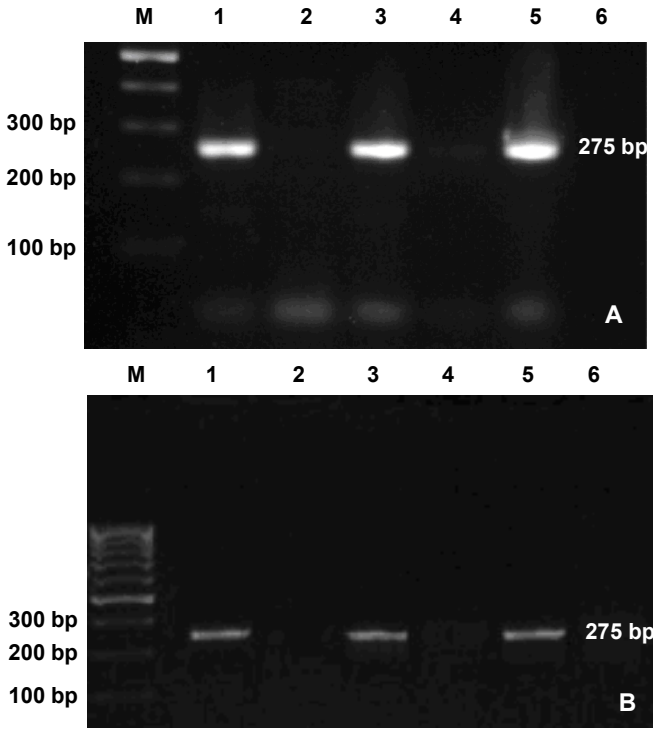

Fig. 2. Positive (lanes 1, 3, and 5) and negative (lanes 2, 4, and 6) samples from dog faeces (A) and blood (B) identified by *CCoV-S* gene (275 bp).

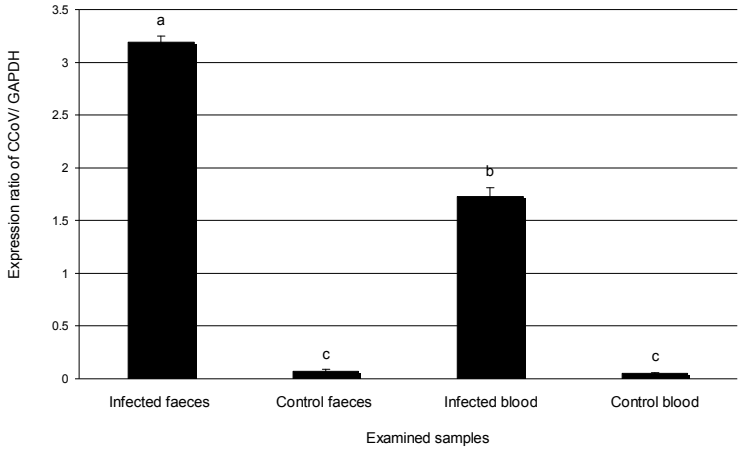

Fig. 3. Expression levels of *CCoV-S* gene in different dog samples. Data are presented as mean ± SEM. [a,b,c] different superscripts are significantly different (P≤0.05).

Table 4. Comparison between IC and PCR vs qRT-PCR according to findings of examined cases of CCoV infection

Test		qRT-PCR		Total
		Positive	Negative	
IC*	Positive	32	0	32
	Negative	13	41	54
	Total	45	41	86
PCR**	Positive	36	0	36
	Negative	9	41	50
	Total	45	41	86

*sensitivity=71%, positive predictive values (PPV)= 100%; specificity= 100%; negative predictive value (NPV)=75.9%; accuracy= 84.9%.

**sensitivity=80%, positive predictive value (PPV)=100%; specificity=100%, negative predictive value (NPV)= 82%; accuracy= 89.5%.

dogs. Additionally, the expression values of *CCoV-S* gene increased significantly (P<0.01) in infected samples of faeces in comparison to infected blood samples (Fig. 3).

The results from the statistical analysis demonstrated that age and sex had no significant effect on IC and PCR results.

qRT-PCR is considered as gold standard test in this work. The performance of IC showed 71%, 100%, 84.9%, 100% and 75.9% for sensitivity, specificity, accuracy, positive predictive value and negative protective value (Table 4) while for conventional PCR: 80%, 100%, 89.5%, 100% and 82% on sensitivity, specificity, accuracy, positive predictive value and negative protective value. The comparison of results of tests used to identify the infected dogs with CCoV indicated that

qRT-PCR exhibited highest level of identification. The prevalence of CCoV enteritis was 52.3% among the dogs population included in this study.

The PCR products analysis of the studied *CCoV-S* protein gene was conducted by specific primers. The PCR products were sequenced and designed in phylogenetic tree and compared with other published gene homologues in the NCBI Genbank. The phylogenetic tree revealed closely related homologues using BLASTN programs version 2.5.1. The sequence of the studied *CCoV-S* protein gene (accession number KY655745.1) was identical to the sequence of *CCoV-S* gene in NCBI Genbank (accession number JQ929044.1 as reference strain). The identity of the present *CCoV-S* protein gene (KY655745.1) and the reference

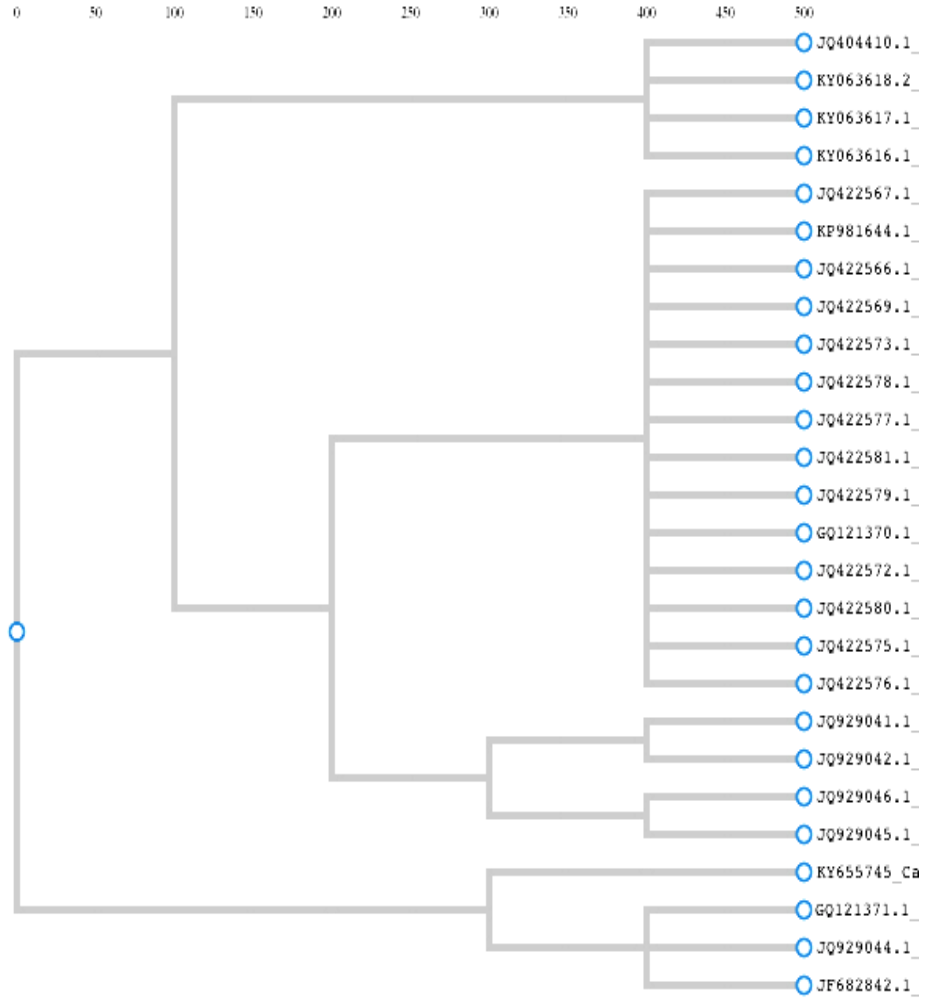

Fig. 4. Phylogenetic tree constructed from the spike protein gene nucleotide sequences of the canine coronavirus strain generated in this study and other sequences from the NCBI GenBank database.

CCoV-S gene (JQ929044.1) was 99.7%. The phylogenetic tree of the isolate of the present study with other sequences are depicted in Fig. 4.

The MEGA model (version 4.0 software) was used to analyse the sequence of CCoV-S gene resulted in the current study and the available sequences of the CCoV-S genome related to several countries published globally in the GenBank (Fig. 4). The phylogenetic tree presented on Fig. 4 shows various clusters formed which revealed that the clade of the isolated sequence of *CCoV-S* gene from this study was very close to the CCoV–S strain isolated from Italy (JQ929044.1) with 99.7% similarity.

DISCUSSION

Canine coronavirus (CCoV) infection which is known to cause mild gastroenteritis in dogs, changed to a more severe pantropic infection affecting several systems in infected dogs (Erles *et al.*, 2003; Decaro *et al.*, 2008; Zicola *et al.*, 2012; Masters & Perlman, 2013).

The IC test applied on faecal samples of diseased dogs was successful in detection of 32 out of 86 examined animals. The IC test used in this study appeared to be reliable, easy to perform and gave a quick detection of the infection in suspected dogs. These findings were also reported by Song *et al.* (2015).

Confirmation of the results of IC test was done by examination of all the examined dogs using conventional PCR technique. PCR assay identified 36 out of 86 examined dogs as infected with CCoV. Higher number of cases (36/86) identified by PCR, than those detected by IC test (32/86) can be attributed to higher sensitivity of PCR than IC as reported by many reserachers (Naylor *et al.*, 2001; Han *et al.*, 2004; Sanchez-Morgado *et al.*, 2004). The higher sensitivity of PCR is due to its ability to detect low log of viraemia (Decaro *et al.*, 2004; Dye *et al.*, 2007; Belouzard *et al.*, 2012; Gizzi *et al.*, 2014).

Different measures used for estimating the performance of the used tests – sensitivity, specificity, accuracy, positive and negative predictive values were studied vs the gold standard qRT-PCR (Decaro *et al.*, 2004; Smith, 2005). In this study, IC showed 71%, 100%, 84.9%, 100% and 75.9%, while conventional PCR showed 80%, 100%, 89.5%, 100% and 82% for above mentioned parameters, respectively. These results agree with those of Naylor *et al.* (2001), Sanchez-Morgado *et al.* (2004) and Costa *et al.* (2013).

qRT-PCR (gene expression analysis test) in this study was used as a qualitative confirmatory test for detection of S gene of CCoV strains in samples of infected cases (Decaro *et al.*, 2004; Gizzi *et al.*, 2014). The gene expression analysis test proved that it is not only a qualitative test but also a molecular quantitative diagnostic technique with higher detectable power than conventional PCR. Detection of low concentration of *CCoV-S* protein gene in faecal samples of infected dogs was achieved in this study using qRT-PCR. It could detect low log of virus from one virus particle to 10^3 and more viral cDNA in microgram of faeces, as also states by Naylor *et al.* (2001), Decaro *et al.* (2004) and Zicola *et al.* (2012).

Statistical analysis results proved that there was no significant difference between male and female dogs when assayed by either IC or PCR. Age also had no significant effect on results of IC and PCR. These findings were in agreement with reports by Song *et al.* (2015) and Awad *et al.* (2018). However the present study found a significant difference between

different dog breeds detected by calculated accuracy of IC and PCR as also recorded by Gaskell *et al.* (1996); German (2005); Awad *et al.* (2018) and Smith (2005).

Sequencing of all PCR products revealed that they were CCoV strain 59/08, meaning that this was the main strain circulating in Egypt. The analysis of phylogenetic clusters was performed, and the current phylogenetic tree among 26 genetically close isolates exhibited 98–100% genetic assembly with the S protein sequence in the current isolated strain (accession number of the present study: KY655745.1- s-Romane, Egypt) (Wesley, 1999; Gallagher & Buchmeier, 2001; Larkin *et al.*, 2007; Jeoung *et al.*, 2014).

In conclusion, the prevalence of CCoV strain 59/08 infection was 52.35% among the examined animals. IC, conventional PCR and qRT-PCR proved to be reliable tests for diagnosis of CCoV infection in dogs. The results of the sequence analysis showed that PCR products of the CCoV-S cDNA had very low variation in their nucleotide sequence of all isolates of this study in comparison with published *CCoV-S* gene. The gene sequence of CCoV-S strain in this study was deposited under Acc. No: KY655745.1 in GenBank.

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