



An ELISA based on recombinant spike protein S for the detection of antibodies to transmissible gastroenteritis virus of swine-like canine coronaviruses

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Recombinant canine coronaviruses, resembling the transmissible gastroenteritis virus of swine (TGEV) in a 5' fragment of the S glycoprotein, have been detected recently and showed to be present in canine populations. The 5' fragment of the S protein (S') of a TGEV-like canine coronavirus (CCoV), strain 174/06, was expressed in an *Escherichia coli* cell-free system. The purified recombinant polypeptide was employed to develop an ELISA test for the detection of TGEV-like CCoV-specific antibodies in dog sera. Four canine sera positive for TGEV-like CCoV, six sera positive to classical CCoV-II strains and 10 negative control sera were examined. The recombinant S' was not recognized by antibodies to classical CCoV-II, as only sera from dogs infected experimentally with TGEV-like CCoV reacted strongly with the recombinant S' polypeptide whereas dog sera with antibodies to classical CCoV-II did not react. As classical CCoV-II and TGEV-like CCoVs are related antigenically, the recombinant S' ELISA is a useful method to investigate serologically the prevalence of TGEV-like CCoVs in dogs.

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1. Introduction

Canine coronavirus (CCoV) is a common pathogen of dogs belonging to the *Coronaviridae* family, in the order of *Nidovirales*. Coronaviruses are enveloped, positive-sense single-stranded RNA particles having the largest genome (27–32 kb) among RNA viruses (Enjuanes et al., 2000). Currently, based on their antigenic and genetic relatedness, coronaviruses are classified into three groups. CCoV is included in group I, along with TGEV, porcine epidemic diarrhoea virus (PDEV), porcine respiratory coronavirus (PRCoV), feline coronaviruses (FCoVs) and human coronavirus 229E (HCoV 229E).

The spike (S) gene of the coronavirus family is reported to be important in virus–host attachment and is involved in antigenic differences between strains (Naylor et al., 2002).

Usually, coronaviruses have a strong potential for mutation which accounts for the number of new viral genotypes or mutants with different tissue or host tropism that have emerged in the last few years. Two CCoV genotypes, CCoV type 1 and CCoV type 2, have been identified so far (Pratelli et al., 2003a). CCoV-I has higher genetic relatedness to feline coronavirus type I (FCoVI) in the S gene than does CCoV type II; it also has an accessory gene (ORF3, 624 nucleotides in length) which is absent in other coron-

aviruses (Lorusso et al., 2008). Dogs with acute gastroenteritis may be detected with only CCoV-1 in their faeces, or with both CCoV-I and CCoV-II (Pratelli et al., 2004; Decaro et al., 2005). The identification of ORF3 remnants in the CCoV-II and TGEV platforms has led to hypothesis that these viruses recognize CCoV-I as their possible ancestor (Lorusso et al., 2008). CCoV-II strains with high virulence have been described (Sanchez-Morgado et al., 2004; Evermann et al., 2005; Escutenaire et al., 2007), including a pantropic variant that caused systemic disease in pups and virus spread to internal organs (Buonavoglia et al., 2006; Decaro et al., 2007; Decaro and Buonavoglia, 2008). In addition, recombination has been reported between CCoV-II and CCoV-I (Escutenaire et al., 2007) or TGEV (Wesley, 1999; Decaro et al., 2009). Sequence and phylogenetic analyses of the 3' end of the viral genome (8.8 kb) have showed that some CCoV-II strains are strictly related to TGEV in the N-terminal domain of the S protein, whereas in the C-terminus of the S protein and in other genes encoding for structural and accessory protein, they resemble classical CCoV-II isolates. Classification of CCoV-II into two different sub-types, CCoV-IIa and CCoV-IIb has been suggested because of the genetic and antigenic differences between classical CCoV and TGEV-like viruses (Decaro et al., 2009).

The aim of this study was to establish a serological tool with high specificity for antibodies raised to such newly recognized canine–porcine recombinant viruses (CCoV-IIb). To achieve this, the S gene fragment of CCoV-IIb was expressed and used as recombinant protein in an enzyme-linked immunosorbent assay (ELISA).

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2. Materials and methods

2.1. DNA technique and protein expression strategy

The CCoV-IIb strain 174/06 was employed in this study (Decaro et al., 2009). In order to express a region of the S gene which is genetically/antigenically distinct between CCoV-IIa and CCoV-IIb, a pair of primers (S' for: 5'-ATGAAATTTTGGTGTTTTA-3'; S' rev: 5'-TTAATCTACATAACCGGCACATTGA-3') was designed to amplify the 5' portion of the S gene (S'), of 807 nucleotides in length (Fig. 1). The amplified product was cloned into pEXP-NT/TOPO expression vector (pEXP-NT/TOPO TA Expression kit, Invitrogen srl, Milan, Italy) and the construct transformed into *E. coli* TOP10F cells (Invitrogen srl, Milan, Italy). The destination vector was used to express the recombinant protein controlled by the T7 promoter with an N-terminal 6× His-tag. The nucleotide sequence of the insert was verified to confirm that no mutations had occurred during the amplification reaction.

The plasmid DNA from an individual clone was used for protein expression in a cell-free *E. coli* system (Expressway Cell-free *E. coli* Expression System, Invitrogen srl, Milan, Italy). As a pilot experiment, 100 µl reaction was prepared according to the manufacturer's instructions. Briefly, 1 µg of DNA template was added to a reaction volume of 50 µl containing *E. coli* slyD-Extract, 2.5× IVPS *E. coli* Reaction Buffer, 50 mM amino acids, 75 mM Methionine and T7 Enzyme Mix. The protein synthesis reaction was then incubated at 30 °C in a thermo-mixer at 1200 rpm. After 30 min of incubation an optimized feed buffer (50 µl) was added to replenish the components depleted or degraded during protein synthesis, thus enhancing the protein yield.

Following incubation, a 5 µl aliquot was removed and added to 20 µl of SDS sample buffer. Ten microlitres of sample were then loaded onto a gel for Western blotting analysis.

2.2. Western blotting

The immunoblotting was carried out as described previously (Elia et al., 2002). The apparent molecular mass of the recombinant polypeptide S' (r-S') was determined using the marker Precision Plus Protein Standard, All Blue (Bio-Rad Laboratories srl, Milan, Italy). Briefly, the protein sample was subjected to SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked overnight at 4 °C in 5% (w/v) non-fat dry milk. Following 3× 15 min washes in Tris Buffered Saline (TBS; Tris 25 mM, NaCl 200 mM, pH 7.4), each membrane was incubated with the anti-histidine mouse monoclonal antibody with horseradish peroxidase (Anti-HisG-HRP, Invitrogen srl, Milan, Italy) at room temperature. DAB (3,3'-diaminobenzidine tetrahydrochloride, Sigma-Aldrich, Milan, Italy) was used to visualize the reaction.

2.3. Dog serum samples

Twenty canine serum samples were used. As representatives of serum samples positive to CCoV-IIb, four sera (nos. 1–4) were collected from dogs infected experimentally with CCoV-IIb strain 174/06 (Decaro et al., 2009). Representative serum samples positive to CCoV-IIb strain 174/06 were obtained from infected experimentally seronegative dogs (Decaro et al., 2009). In addition, four sera were from seropositive dogs (nos. 5–8) with antibodies only to 'classical strains' that were collected from dogs vaccinated with a commercially available inactivated CCoV-IIa vaccine (Duramune PC, Fort Dodge Animal Health, Lot No. 288AY1004) (Pratelli et al., 2003b) and then challenged with a field CCoV-IIa strain (144/01). Also included were sera (nos. 9–10) from two non-vaccinated pups that had been challenged with strain 144/01. As negative controls, 10 serum samples (nos. 11–20) were included. The negative sera

tested negative for CCoV by an ELISA based on the whole-virus antigen and by Western blotting (Pratelli et al., 2002; Elia et al., 2002).

2.4. r-S'-based ELISA

Recombinant ELISA was performed as described previously except for the antigen preparation (Elia et al., 2003). After large scale production, the recombinant polypeptide S' was purified by using nickel-nitrilotriacetic acid (Ni-NTA) Spin Kit (Qiagen SpA, Milan, Italy), according to the manufacturer's instructions. Protein concentration was determined by the Bradford method using the Bio-Rad protein assay kit (Bio-Rad Laboratories srl, Milan, Italy) and bovine serum albumin (BSA) as a standard.

Initial assays were carried out to determine the optimal r-S' working dilutions. Polysorb microtiter plates (Nunc, Milan, Italy) were coated with serial dilutions of r-S' (50–250 ng/well) and kept overnight at +4 °C. Plates were then washed three times with PBS containing 0.05% Tween 20 (PBS-T) and 100 µl of blocking solution (0.2% gelatin in carbonate buffer) added to each well for 90 min at 37 °C. After repeated washes, 100 µl of each dog serum diluted 1:50 in PBS-T was added in duplicate and the plates incubated at 37 °C for 90 min. The wash cycle was repeated, then goat anti-dog IgG antibody labeled with peroxidase (Sigma-Aldrich, Milan, Italy) were added and the plates incubated for 60 min at 37 °C. After a final three washes, ABTS [2,2'-azino-di-(3-ethylbenzothiazoline sulfonate)] substrate solution (Sigma-Aldrich, Milan, Italy) was added to each well and the plate incubated at room temperature for 25 min. The optical density (OD) was read at 405 nm using an automatic ELISA reader.

Absorbance values were considered positive if they exceeded the mean value plus two times the standard deviation obtained with control negative sera.

2.5. Whole CCoV based ELISA

The ELISA based on CCoV whole-virus antigen was performed as described previously (Pratelli et al., 2002). A cell culture adapted CCoV-IIa strain, 45/93 (Buonavoglia et al., 1994), was used to produce the antigen.

3. Results

The recombinant-tagged polypeptide S' was detected by WB immunoassay by using monoclonal antibody specific for 6× histidine tag. r-S' reacted strongly with the anti-histidine monoclonal antibody and appeared as a single band of 30 kDa. The optimal r-S' antigen concentration in ELISA (150 ng/well) was selected to give maximum discrimination between positive and control negative sera.

By the r-S'-bases ELISA, the mean and the standard deviation obtained using 10 CCoV negative sera (#11–20) were 0.021 and 0.0074, respectively. Therefore, the cut-off value of ODs was determined as 0.044. The same sera by the whole-antigen ELISA displayed an OD mean of 0.028, with a cut-off value of 0.040. The four sera positive for CCoV-IIb (#1–4) displayed an OD mean of 0.152, confirming the immunogenicity of r-S' (Table 1). The whole-antigen CCoV ELISA, carried out on the positive TGEV-like sera, confirmed the presence of antibodies to CCoV, with OD values related to those obtained in the r-S'-based ELISA (mean OD value: 0.199). As expected, the sera positive for CCoV-IIa classical strains (#5–10) tested strongly positive by the whole-antigen ELISA (mean OD value: 0.219) but they were negative by the r-S'-based ELISA, with an OD median value of 0.031.

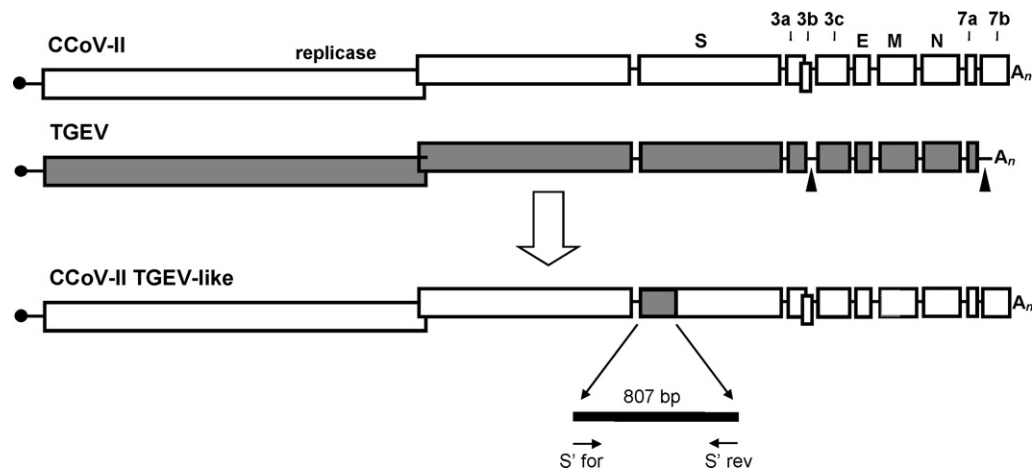


Fig. 1. Genetic features of the recombinant TGEV-like CCoV strain 174/06. The position of the primer pair *S'* for and *S'* rev, used to amplify the 5' end of the S gene, is shown.

Table 1

Detection of antibodies to CCoVs in dog sera by ELISAs based on the recombinant polypeptide *S'* and whole-virus antigen.

Serum samples	Positivity to CCoV	r <i>S'</i> based ELISA ^a		Whole CCoV ELISA ^b	
		OD ^c	Mean	OD ^c	Mean
1	CCoV-IIb	0.145	0.152	0.195	0.199
2	CCoV-IIb	0.134		0.170	
3	CCoV-IIb	0.126		0.201	
4	CCoV-IIb	0.205		0.232	
5	CCoV-IIa	0.035	0.031	0.228	0.219
6	CCoV-IIa	0.029		0.198	
7	CCoV-IIa	0.033		0.184	
8	CCoV-IIa	0.037		0.205	
9	CCoV-IIa	0.029		0.236	
10	CCoV-IIa	0.026		0.263	
11–20	Negative		0.021		0.028

^a Cut-off value: 0.044.

^b Cut-off value: 0.040.

^c Optical density.

4. Discussion

TGEV-like CCoVs appear to have a recombinant nature since they resemble TGEV of swine in a 5' fragment of the S glycoprotein, in the genomic background of classical CCoV-II. Antigenic differences have been observed between TGEV-like CCoV (sub-type IIb) and classical type II CCoVs (sub-type IIa) by cross-neutralization assays and by using a panel of monoclonal antibodies (Decaro et al., 2009). The identification of TGEV-like CCoV strains in dogs raises a number of questions as to whether CCoV-IIb strains are of epidemiological relevance in dogs and these viruses biologically differ from classical CCoVs. Also, it is uncertain whether and to which extent the observed antigenic differences between CCoV-IIa and -IIb (Decaro et al., 2009) may impact on CCoV prophylaxis, e.g. if dogs immunized with CCoV-IIa-based vaccines may be effectively protected against infection and disease by TGEV-like CCoVs.

Since CCoV-IIb strains have been detected in different years and from different geographical areas, it is possible to hypothesize that such viruses are successfully circulating in the dog population (Decaro et al., 2009). In order to assess precisely the diffusion of CCoV-IIb viruses, investigations based on specific molecular and serological tools would be more appropriate, since both the ELISA based on CCoV-II whole-virus antigen and the serum neutralization test do not indicate whether the antibodies were raised to CCoV-IIa or CCoV-IIb viruses.

The objective of this work was to develop a rapid and simple assay for the serological diagnosis of CCoV-IIb (TGEV-like), able to distinguish clearly CCoV-IIb-elicited immunity. The recent development of ELISAs based on recombinant proteins has allowed the development of serological tests of high specificity. In the present study the initial portion of the S protein of a TGEV-like CCoV was expressed in order to avoid cross-reactions between classical and TGEV-like CCoV strains. This polypeptide spans the cross-over region where recombination is thought to have occurred between TGEV and CCoV-II, leading to the generation of a chimerical S protein, and more likely accounts for the observed antigenic differences. The recombinant polypeptide was successfully produced in *E. coli* cell-free system with a poly-histidine tag that was used for protein identification and purification. The r-*S'* was then used in an ELISA test for the detection of antibodies specific for CCoV-IIb in dogs. The negative control sera did not react with the r-*S'*, while all the serum samples obtained from animals infected experimentally with the CCoV-IIb strain 174/06 displayed high OD values. As expected, the sera of the dogs vaccinated or infected experimentally with CCoV-IIa viruses did not react in the r-*S'*-based ELISA, displaying negative OD values. By converse, these sera tested highly positive in the whole-virus antigen ELISA. Accordingly, the ELISA based on the r-*S'* of CCoV-IIb was able to recognize specifically the antibodies raised to the homologous virus. Since mixed infections or sequential exposure to various CCoV strains might occur under field conditions (Pratelli et al., 2004; Decaro et al., 2005), this test cannot distinguish between the antibodies elicited by a CCoV-IIb single infection and the antibodies elicited by mixed CCoV-IIa/IIb infections, since in both cases the whole-antigen ELISA and r-*S'*-based ELISA would test positive. If necessary, a recombinant ELISA test specific for classical CCoV strains (CCoV-IIa) should be set up in order to confirm or rule out exposure to double infections.

The recombinant ELISA represents a useful tool for large sero-epidemiological studies in order to assess the prevalence of CCoV-IIb in canine populations and to gain insights into the pathological features of CCoV-IIb infection in dogs.

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