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Short Communication

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Canine Coronavirus Infection in Turkish Dog Population

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With 1 table

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Summary

Canine coronavirus (CCoV) is one of the most important viral agents affecting the gastrointestinal system of dogs. In this study virological and serological investigations were performed to demonstrate the existence and prevalence of CCoV infection in a Turkish dog population. A total of 269 animals were subjected to the study. Of 179 dogs tested for CCoV antibodies, 112 (62.5%) were found to be positive by serum neutralization test, while 133 (74.3%) were positive by ELISA. The highest prevalence (94.2%) was detected in kennel dogs. Detection of CCoV genome in faeces was performed in samples from 90 diarrhoeic puppies by reverse transcription-polymerase chain reaction. Fourteen (15.5%) faeces were positive for CCoV RNA, five of which were characterized as CCoV type I. The widespread CCoV infection in the Turkish dog population may be attributed as an important cause of viral diarrhoea in dogs.

Introduction

The genus *Coronavirus*, classified in the family *Coronaviridae*, includes three antigenically distinct groups (Spaan et al., 1988), one of which covers only genetically related mammalian viruses such as canine coronaviruses (CCoVs), feline coronaviruses (FCoVs), coronaviruses of pigs and human coronavirus 229E (Murphy et al., 1999). Coronaviruses have enveloped virion, 80–220 nm in diameter, containing a non-segmented ssRNA genome with positive polarity, 27–32 kb in length.

Canine coronavirus infection was first described in diarrhoeic dogs in 1974 (Binn et al., 1974). In young pups, or in combination with other pathogens, CCoV is responsible for diarrhoea, vomiting, dehydration, loss of appetite and occasional death. It has been observed that CCoV faecal shedding may be longer than reported (Keenan et al., 1976). Pratelli et al. (2001) have detected CCoV in the faeces of naturally infected pups for a period of up to 180 days. Hence, it can be concluded that asymptomatic dogs could serve as a continuous source of the virus and may induce an immune response in kennel population without obvious clinical disease.

Genetic investigations carried out on different regions of the genome revealed a high level of diversity in genome organisation of CCoV strains. Some field isolates are more closely related to FCoV strains (*FCoV-like CCoVs*) than to CCoV reference strains. Due to that genetic similarity a new genotype has been tentatively classified as CCoV type I (Pratelli et al., 2003).

The importance of CCoV as an enteric agent in Turkish dogs has not been investigated. In this study we aimed at evaluating the presence and the prevalence of CCoV infections in Turkish dog populations.

Materials and Methods

Animal population and specimens

A total of 269 dogs bred in seven different provinces of Turkey were included in the study. Serum samples were taken from 179 dogs for serological examination (Table 1). The dogs, aged from 5 months to 7 years, were not examined for their clinical situation at the sampling time. The animals sampled for serological controls were from private owners (112 dogs), from municipality shelter (11 dogs), from a breeding kennel (35 dogs) and stray dogs (21 dogs). Faecal samples were collected from 90 diarrhoeic puppies aged from 2 weeks to 4 months, selected from clinical cases of the Small Animal Clinics of Veterinary Faculty, Uludağ University, Bursa. None of dogs had been previously vaccinated against CCoV. Blood samples were collected into clot activator serum tubes by venepuncture. Serum was separated, heat inactivated and stored at (−30)°C. Faecal samples were directly taken in sample collection tubes and stored at (−30)°C until transferred to the Faculty of Veterinary Medicine, Bari, Italy.

Serological examinations

Serum neutralization test

Two-fold dilutions of serum samples were incubated with 100 $TCID_{50}$ of the virus (CCoV strain 45/93) in 96-well microtitre plates at room temperature for 90 min. Consequently, CrFK cells grown in Dulbecco's MEM supplemented with 10% foetal calf sera were added to plates as being 20 000 cells per well. The results were evaluated in an inverted microscope after 4 days of incubation at 37°C in 5% CO_2 atmosphere.

ELISA for CCoV antibodies

For preparation of antigen used in ELISA, the supernatants of CrFK cells infected with CCoV strain 45/93 were centrifuged at

Table 1. Distribution of serum samples to the locations and seropositivity rates according to these locations and type of breeding

Location*	Type of breeding	Serum samples	Positives in ELISA (%)	Seroprevalence by breeding type (%)	Seroprevalence by regions (%)
Bursa[w]	Privately owned	100	70 (70)	68.7	74.8[w]
Çanakkale[w]	Privately owned	8	5 (62.5)		
Yalova[w]	Privately owned	4	2 (50)		
Balıkesir[w]	Kennel	35	33 (94.2)	94.2	
Urfa[se]	Stray dog	6	4 (66.6)	71.4	66.6[se]
Adana[s]	Stray dog	15	11 (73.3)		
Antalya[s]	Municipality shelter	11	8 (72.7)	72.7	73.0[s]
Total		179	133 (74.3)		

*Provinces are located in western (w), southern (s) and south-eastern (se) regions of Turkey.

140 000 ***g*** at +4°C for 1 h. The pellet was resuspended in PBS in a dilution of 1/80 and used in the test. Negative antigen controls were prepared after the same procedures from mock-infected cultures. Negative and positive control serums were previously yielded from a dog before and after vaccination with a modified live CCoV vaccine (Pratelli et al., 2004). ELISA plate wells were coated with 100 μl of antigen suspension in carbonate buffer (pH 9.6) overnight, and treated with blocking buffer. Serum samples, 1/50 diluted in Tween-PBS, were placed into wells as duplicates and incubated for 90 min at 37°C. After washing steps 100 μl of peroxidase-conjugated goat antibody specific to canine IgG (Sigma Chemicals, St Louis, MO, USA) were added, incubated for 1 h at 37°C, washed and 100 μl of freshly prepared substrate solution (10 mg ABTS in 50 ml 0.05 M phosphate citrate buffer, pH 5.0, containing 25 μl/100 ml H_2O_2) was added afterwards. Following the stop solution (SDS 1%) adding in 50 μl volume, optical densities (OD) were determined at 405 nm. Sera having an adjusted OD value over 0.040 were considered to be positive for CCoV-specific antibodies. The sensitivity and specificity of the ELISA were previously determined to be 100 and 83.3%, respectively, when compared with SNT (Pratelli et al., 2002a).

RT-PCR and sequence analysis

A commercially available extraction kit (Qiagen GmbH, Hilden, Germany) was used to extract RNA from faecal samples. Reverse transcription-polymerase chain reaction (RT-PCR) was applied as described by Pratelli et al. (1999). In the test, the primer pair CCoV1-CCoV2, which amplified a 409-bp fragment of M gene, was employed. On the positive faecal samples, a second round of RT-PCR using primer pair CCoV1a-CCoV2 (Pratelli et al., 2002b) was applied to confirm whether CCoV type I (atypical CCoV strains) was present or not in the samples. Primer sequences and their positions (accession no. D13096) were as follows: *CCoV1*: 5′-TCCA-GATATGTAATGTTCGG-3′ (position 6729–6748); *CCoV2*: 5′-TCTGTTGAGTAATCACCAGCT-3′ (position 7138–7118); *CCoV1a*: 5′-GTGCTTCCTCTTGAAGGTACA-3′ (position 6900–6920). Moreover, the PCR products were subjected to sequence analysis (Genome Express, Labo Grenoble, France). For the sequence analysis, the NCBI and EMBL tools for molecular analysis were used. Phylogenetic and molecular evolutionary analyses were performed using MEGA version 2.1 (Kumar et al., 2001).

Statistical analysis

Chi-square analysis (Minitab, 2000) and Fisher's exact test (Graphpad, 2.02) were employed for comparison of serological methods and for evaluating the statistical significance of differences among seroprevalence values ($P < 0.05$).

Results

Serological examinations

Of 179 sera tested, 112 (62.5%) were positive by SNT, while 133 sera (74.3%) were positive by ELISA. All of the samples positive by SNT were also positive by ELISA. Twenty-one samples were negative by SNT but positive by ELISA, and 46 samples were negative by both methods. Distribution of seroprevalence values, obtained from ELISA, according to provinces and breeding types, is shown in Table 1.

RT-PCR and sequence analysis

In the first round of RT-PCR, a CCoV-specific genome fragment of 409 bp in length, was detected in 14 (15.5%) of 90 faecal samples tested. In the second round, five of 14 positive faecal samples gave positive results and a CCoV type I-specific 239 bp fragment was amplified. The specificity of the PCR typing assay was confirmed by sequence analysis of the PCR products (data not shown). In laboratory applications, six faecal samples from non-diarrhoeic dogs had been tested, and none of them gave a positive result.

Statistical analysis

The difference between the positivity rates detected by SNT and ELISA was found to be statistically significant ($P < 0.05$). There was no statistical difference among seroprevalence values of different geographical regions. Seroprevalence value detected in the kennel dogs was significantly higher than the other types of breeding ($P < 0.05$). No statistical difference was found regarding seroprevalence rates detected in privately owned, stray or shelter dogs.

Discussion

The main purpose of this study was to identify the prevalence and distribution of CCoV infections in the Turkish dog population and to demonstrate the role of the virus in diarrhoeic puppies.

The prevalence of CCoV antibodies in different countries seems to be highly variable. Seroprevalence of infection has been reported to be 15.8% in Australia (Naylor et al., 2001), 90.8% in Italy (Pratelli et al., 2002a), 44.1% in Japan (Bandai et al., 1999) and 76% in England (Tennant et al., 1993). High

level of differences could be affected by social interactions among dogs and sensitivity of methods used.

In serological examinations carried out in the present study, the positivity rates were 62.5% by SNT and 74.3% by ELISA. Previous investigations demonstrated that, in harmony with Western blotting (Pratelli et al., 2002a), the ELISA test has higher sensitivity than the SNT. In the present study, 21 sera detected as positive by ELISA, were negative in SNT. On the contrary, there was no serum sample positive by SNT/negative by ELISA. Thus, the results obtained from ELISA (74.3%) could possibly be accepted as true prevalence of CCoV antibodies in Turkish dogs (Table 1). Because of a statistically important difference between the results of the two methods, it is thought that ELISA is advantageous for serological diagnosis of CCoV infection. Such results had also been found for different agents (Paessler and Pfeffer, 2003).

No statistical difference has been observed in different geographical regions among infection rates, concluding that homogeneity of infection in Turkish dog population is present. Infection was most prevalent in kennelled dogs (94.2%) when compared with privately owned (68.7%), stray (71.4%) and shelter (72.7%) dogs. Undoubtedly, faecal–oral transmission between closely connected individuals is the most plausible reason for the high prevalence of infection in the kennel conditions.

The RT-PCR results pointed out the role of CCoVs in clinical diarrhoea cases that none of these animals were positive for CPV-2 (data not shown). These results also suggest the occurrence of CCoV type I infections in Turkish dogs. The diarrhoea cases that were negative for CCoV might have been caused by other viral, bacterial or parasitic agents.

From the results of this study it can be concluded that CCoV infection is widespread in the Turkish dog population and the virus may be attributed to be one of the important agents causing diarrhoea in puppies.

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