

**Review article**

## **Genetic evolution of canine coronavirus and recent advances in prophylaxis**

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**Abstract** – Since the first identification of the virus in 1971, the disease caused by canine coronavirus (CCoV) has not been adequately investigated and the role that the virus plays in canine enteric illness has still not been well established. In the last decade, as a consequence of the relatively high mutation frequency of RNA positive stranded viruses, CCoV has evolved and a new genotype has been identified in the faeces of infected dogs. The several studies carried out by different researchers have focused upon the epidemiological relevance of these viruses and, considering the wide diffusion of CCoV infections among dog populations, the author underlines the need for further investigation on the biology of CCoV and on the pathogenetic role of their infections.

**dog / coronavirus / genetic evolution / prophylaxis**

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### **1. INTRODUCTION**

Coronaviruses cause a variety of diseases ranging from bronchitis to gastroenteritis, hepatitis, infectious peritonitis and encephalitis in both mammals and birds. Canine coronavirus (CCoV) is a positive, single-stranded RNA virus which is responsible for mild to severe enteritis in

dogs. The infected dogs generally undergo a rapid recovery, whereas fatal infections are unusual unless mixed infections by other pathogens occur. CCoV infection was first described after isolation of the virus from sentry dogs with diarrhoea during an epizootic in Germany in 1971 [1]. Since that time, most attention has been paid to the more serious canine parvovirus type 2 (CPV2)

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enteritis. Today CCoV appears to be enzootic worldwide and dogs of all breeds and ages seem to be susceptible to the infection. The disease caused by CCoV has not been adequately investigated and the actual role that this virus plays in canine enteric illness has still not been well established. Since first identified in 1971, CCoV has evolved and new genotypes have recently been found in the faeces of infected dogs [30]. The demonstrated wide diffusion of this new viral type in the dog population [33] emphasises both the need for more in-depth investigation of the pathogenetic role of the new CCoV genotype and the epidemiological relevance of immunisation of dogs.

## 2. CANINE CORONAVIRUS GENOMIC STRUCTURE AND EVOLUTIONARY CHANGES

Coronaviruses comprise a genus in the family Coronaviridae. They are large, enveloped, positive-stranded RNA viruses that are highly prevalent in humans and domestic animals and responsible for diseases of the enteric and respiratory systems. Coronaviruses have the largest genomes (27 to 32 kb) of all the RNA viruses and replicate by a unique mechanism that results in a high frequency of recombination [15]. The genome includes 7 to 10 open reading frames (ORF) that encode both structural and non-structural proteins. Gene 1 consists of two overlapping regions (ORF1a and ORF1b) that are translated into a polyprotein, the precursor of the viral replicase (*Rep*). There are four structural proteins, (5'-*Rep*-S-E-M-N-3') downstream from the replicase gene, interspersed with several ORF encoding various non-structural proteins and, in some strains, the HE glycoprotein. The ORF differ markedly among coronaviruses in number, nucleotide (nt) sequence, gene order and in methods of expression [17].

The most important biological and immunological functions reside in the S and M proteins. The S glycoprotein (150–200 kd)

forms the large petal-shaped spikes on the surface of the virions and can be divided into three structural domains: a large external domain, which is further divided into two sub-domains S1 and S2, a transmembrane domain and a short carboxyl-terminal domain. The S1 sequence is variable and mutations in this sequence have been associated with altered pathogenicity and antigenicity; in contrast, the S2 sequence is more highly conserved [17]. The S glycoprotein, the major inducer of virus-neutralising antibodies, regulates several other important biological functions: attachment to cells, fusion of the viral envelope with host cell membranes and, sometimes, cell to cell fusion [17]. The M glycoprotein (about 29 kd) differs from the other proteins, since only a short amino-terminal domain is exposed outside of the viral envelope. This domain is followed by a triple-membrane-spanning domain and a large carboxyl-terminal domain inside the envelope [17]. Although the major immunological role has been attributed to the S protein, both the amino- and the carboxyl-terminal of the M protein elicit strong immune responses, inducing antibody-dependent, complement-mediated virus neutralisation [9]. The nucleocapsid also elicits a strong immune response.

Coronaviruses can be divided into three distinct antigenic groups; within each group, the viruses are classified according to their natural hosts, nt sequences and serologic relationships. There are two groups of mammalian coronaviruses, groups I and II; a third group includes the two avian coronaviruses, infectious bronchitis virus (IBV) and turkey coronavirus (TCoV).

Group I includes the prototype, human coronavirus (HCoV) strain 229E, and coronaviruses of pigs (transmissible gastroenteritis virus, TGEV, porcine respiratory coronavirus, PRCoV, and porcine epidemic diarrhoea virus, PEDV), cats (feline coronaviruses, FCoV) and dogs (canine coronaviruses, CCoV). On the basis of their relationship to CCoV, FCoV comprise two serotypes, FCoV type I and FCoV type II.

By sequence analysis and comparison, it was demonstrated that the two prototype strains of FCoV type II (strains 79-1146 and 79-1180) originated from double and separate recombination events between FCoV type I and CCoV [13]. Since it was found that the spike protein had been acquired from CCoV, it is understandable why FCoV type II viruses are serologically related to CCoV. The two feline serotypes also have different *in vitro* characteristics. FCoV type I grows poorly in cell cultures while FCoV type II proliferates well in tissue cultures and is widely used for the study of FCoV infection [22].

Group II mammalian coronaviruses include the prototype murine hepatitis virus (MHV), and the types that infect humans (*HCoV-OC43* and *HECoV-4408*), rats (RCoV), cattle (BCoV), pigs (PHEV) and other species [17]. Recently, a group II coronavirus was detected in the respiratory tract of some dogs during a virological survey of the cause of canine infectious respiratory diseases. This canine respiratory coronavirus, provisionally called *CRCV*, represents a novel coronavirus of dogs with high similarity to BCoV and *HCoV-OC43*, both of which are known to cause respiratory disease [10].

In 2002 a novel human coronavirus was discovered in association with outbreaks of severe acute respiratory syndrome (SARS) in Asia and elsewhere. However, complete genomic sequence analysis of several SARS-CoV isolates revealed that this coronavirus is not closely related to any of the previously characterized animal or human coronaviruses [18]. Recent studies have demonstrated that SARS-CoV might be the first member of a newly recognized group IV [37, 39] or a distinct member of the group II coronaviruses [12]. Van der Hoek et al. [43] earlier last years have identified another human pathogen from this family, *HCoV-NL63*. The virus, that has been assigned to group I, occurs frequently in young children causing lower respiratory tract infection.

CCoV is difficult to isolate in cell cultures [25, 41] and, for a long time, CCoV infections were underestimated because of the limited number of strains isolated *in vitro*. As a consequence, there are few reports on the pathogenesis of CCoV infection in dogs [1, 40] and its antigenic and genetic properties have not been largely investigated. Since then the development of a RT-PCR assay has provided important information on the distribution and the biology of this virus.

In the last decade several researchers have started to focus attention on the genomic variability of CCoV strains to gain insight into the genetic relationship among coronaviruses. Horsburgh and Brown [14] cloned and sequenced the spike protein from two distinct CCoV strains (American and British isolates). By sequence analysis and pairwise comparison of aa sequences from the two isolates with other reference CCoV strains, conservative substitutions distributed throughout the S sequences and, in particular, 5 variable regions, were identified. The pairwise comparison was also carried out with feline, porcine, murine, chicken and human coronavirus sequences, demonstrating that the canine sequences are more closely related to the S gene of the feline strains, rather than to the porcine S gene, even though they belong all to the same antigenic group I. This datum was partially confirmed later in 1999, when, by sequence analysis of the variable domain at the 5' end of the S gene of 6 CCoV strains, 5 of these 6 viruses revealed a high degree of identity with feline sequence, while only one field isolate resembled the 5' end sequence of TGEV [44].

Afterwards these preliminary observations, the first report of the possible diffusion of a novel subtype of CCoV among dog population, was from an outbreak of fatal gastroenteritis in Australia. A new CCoV, strain *UWSMN-1*, was identified in the faeces of the infected dogs and by sequence analysis of some regions of the S and *replicase* genes, it was observed that the virus

is divergent from reference CCoV and FCoV strains. Therefore, the nt variation of the *replicase* and S gene sequences observed between *UWSMN-1* and other CCoV reference strains, were only 3.9% and 7%, respectively and based on sequence data from a small part of the highly variable and large 5' terminal spike region [20, 21].

In the meantime, by sequence analysis of CCoV detected in faecal samples collected from diarrheic dogs in the South of Italy, multiple nt substitutions accumulating over a fragment of the M gene, were observed [26]. These preliminary observations gave a meaningful impulse to the study of the CCoV genetic evolution. Subsequently, a genetic drift to FCoV type II was also observed in the sequence of CCoV detected in the faeces of two naturally infected pups during the late stages of long-term viral shedding. It was thus postulated that the dogs might have been infected by a mixed population of genetically different CCoV, or that the viruses detected in both the pups were the result of mutation/recombination events [29]. Later, extensive sequence analysis on multiple regions of the viral genome, including replicase and M genes, of several CCoV positive faecal samples, provided strong evidence for the existence of two separate genetic clusters of CCoV. The first cluster includes CCoV intermingled with reference CCoV strains, such as *Insavc-1* and *K378*, while the second cluster segregates separately from CCoV and, presumably, represents a genetic outlier referred to as FCoV-like CCoV [31].

All this data were confirmed by a recent study that has demonstrated the presence of a novel CCoV type, *Elmo/02*, that circulates in dogs. The virus diverges to a great extent from the reference CCoV strains and is more closely related to FCoV type I [30]. Since the *Elmo/02* virus has a high rate of nt and aa identity of with FCoV type I, it was proposed to designate this genotype as CCoV type I and the reference classical CCoV strains as CCoV type II [30]. The two genotypes diverge dramatically in the

ORF2 (S gene), where there is more than 39% nt and about 46% aa variation from reference CCoV. Analysis of the S gene of different CCoV strains *Elmo/02-like*, reveals only slight variation (4–8%), probably as consequence of their different geographical origins. Most of the sequence changes observed were conservative, demonstrating that there is some heterogeneity in the S gene of these new viruses. Some phenotypic properties also have been studied. The high divergence in the aa composition and the loss or gain of potential glycosylation sites was similar to those in the most closely related coronaviruses (FCoV and typical CCoV), strongly suggesting that the *Elmo/02* strain is poorly related, antigenically, to the other coronaviruses of carnivores. Moreover, the presence of the stretch of basic residues RRXRR is indicative of a potential cleavage of the S protein [30]. A similar basic motif is present, approximately in the same position in all group II and group III coronaviruses identified and classified to date. However, the spike glycoprotein of typical CCoV, like other representative of the same antigenic group I, lacks this proteolytic cleavage site [45]. Cleavage of the S protein of coronaviruses has been correlated to cell-fusion activity in vitro, [17] but the potential implications in the viral pathobiology have not been determined. These data suggest that the two genotypes of CCoV underwent a linear evolution rather than a sudden shift originating from a recombinant event analogous to those leading to the appearance of FCoV type II. A possible explanation for this phenomenon is that, under natural conditions, homologous recombination events between highly homologous coronaviruses, occurs frequently. This implies that there is frequent interspecies circulation of either CCoV in cats or FCoV in dogs, since mixed infections are required to give rise to recombination. Where the recombination takes place is unknown, but it is known that CCoV is able to use the feline aminopeptidase (fAPN) glycoprotein as a cellular receptor [36, 42] and that, under experimental

conditions, cats can be infected with CCoV [2]. Moreover, the possibility that FCoV might infect dogs cannot be ruled out. According to this hypothesis, restricted sites of recombination (i.e. regions of very high nt identity between two highly homologous coronaviruses) may exist in the genome of coronaviruses and these sites may readily undergo template switches. This explanation assumes that coronaviruses possess a sort of “dynamic” genome.

This characteristic has been also strengthened by the isolation of a new coronavirus, strain *BGF*, from the faeces of dogs during an epizootic outbreak of diarrhoea occurred in a Beagle breeding colony in Great Britain [38]. The virus revealed a full-length non structural protein 3b, associated with virulence in other coronaviruses and a highly divergent region at the amino-terminal domain of the M gene.

Another examples of this evident evolution of dog coronaviruses as consequence of the accumulation of point mutations, small insertions and deletions in coding and non-coding regions of the genome, is the recent identification of a novel coronavirus, strain *CRCV*, in tissue samples collected from the respiratory tract of diseased dogs [10]. Interestingly, sequence analysis of replicase and spike genes revealed a high similarity to BCoV and *HCoV-OC43*, both of which are members of the group II of the Coronaviridae family, whereas there was a low similarity to the enteric CCoV. Moreover, the presence of the HE gene, which is a characteristic protein gene of the members of the group II coronaviruses, has been demonstrated in the *CRCV* genome.

### 3. CANINE CORONAVIRUS PATHOGENESIS

The factors that determine the course of the natural disease caused by CCoV are not well understood. CCoV is responsible for mild or moderate enteritis in dogs. The symptoms may vary, but are more severe in

young pups, or in combination with other pathogens; common signs are soft faeces or fluid diarrhoea, vomiting, dehydration, loss of appetite and, occasionally, death. Dual infections by CCoV and CPV2 and/or other pathogens are especially severe when infections occur simultaneously [3, 11, 27, 46]. It has also been shown that CCoV enhances the severity of a sequential CPV2 infection [24].

Serological investigations carried out on a large number of dogs suggest that CCoV infection is widespread in pets and in kennel populations where seroprevalence may be particularly high (approximately 70–90%) [28, 47]. In contrast, few data of gastroenteritis in dogs clearly attributable to CCoV have been reported<sup>1</sup> [1, 19], and only a few strains have been adapted to grow in vitro. The low detection frequency of virus isolation from faecal samples of infected dogs may also be due to the low stability of CCoV in normal environmental conditions or the low number of virus particles in the faeces at the time of sampling [25].

The natural route of transmission is faecal-oral and virus in faeces is the major source of infection. In neonatal dogs the virus appears to replicate primarily in the villus tips of the enterocytes of the small intestine causing a lytic infection followed by desquamation and shortening of the villi. Malabsorption and deficiency of digestive enzymes follow, resulting in diarrhoea which can be seen in some dogs by 18–72 h post infection [1]. Production of local antibodies (IgAs) restricts the spread of the virus within the intestine and arrests the progress of infection. Infected dogs generally shed CCoV in the faeces for 6–9 days post infection [16], but some naturally infected dogs have shed virus for a period as long as 6 months after clinical signs had ceased [26, 29].

<sup>1</sup> Buonavoglia C., Marsilio F., Cavalli A. et al., L'infezione da coronavirus nel cane: indagine sulla presenza del virus in Italia, Not. Farm. Vet. 2, 1994.

The incubation period is short and vomiting and diarrhoea may be seen by 1–3 days post infection [1]. Faeces may be mucoid or watery, sometimes streaked with blood for several days, but vomiting usually subsides after the first day of illness. Pups become dehydrated, depressed and anorexic even though the infection is generally afebrile. Leucopenia has not been observed, but some dogs have shown lymphopenia after experimental infection with high doses of virus [1].

A recent study has established the common occurrence of both CCoV type I and II in infected dogs [33]. In that study, 69 samples from diarrhoeic dogs were tested with specific primers for type II and I genotypes, respectively. Ten samples were recognised as CCoV type I (14.5%) and 6 samples as CCoV type II (8.7%). Both genotypes were identified in 53/69 samples (76.8%). Interestingly, only a few RT-PCR-positive samples (13/69) have been adapted to growth in vitro, which is in accordance with our previous report [25]. The faecal samples positive to both the viruses yielded only CCoV type II in cell cultures. Failure to isolate CCoV type I in vitro prevents an authentic evaluation of the immunological characteristics of this new genotype of CCoV and hinders the acquisition of key information on its pathogenetic role in dogs. The significance of these data is unclear; however, it raises several questions regarding the new CCoV genotype. Therefore, since it is difficult to understand why the two genotypes occur simultaneously in dogs, such data provides further information on the epidemiology of dog coronaviruses.

#### 4. DIAGNOSTIC PROCEDURES AND VACCINATION

Detection of CCoV antibodies can be performed by virus neutralisation (VN) tests and ELISA. It has been shown that VN tests may fail to detect antibodies in some positive sera and thus provide misleading infor-

mation on the epidemiology of the infection [28]. A recently developed ELISA was found to be more sensitive than the VN test, even though the antigen prepared from CCoV infected cells might yield variable results, depending on the method of antigen preparation [28]. Recently, it was demonstrated that antibodies against the M protein are detected consistently in seropositive dog sera after CCoV infection [7]. Based on those findings, the authors developed an ELISA with a recombinant M protein (rMP) of CCoV type II and have proposed this test as an alternative diagnostic method for antibody detection. The cloned rMP, expressed in *Escherichia coli*, was demonstrated to be antigenically similar to the natural protein [8].

The demonstrated wide dissemination of CCoV type I in the dog population [33] emphasised the need for detailed investigation of the serological relationship between the two genotypes. To this purpose, two recombinant polypeptides of the S glycoprotein of CCoV type I, were expressed in a prokaryotic system and employed to develop an ELISA for the detection of CCoV type I antibodies [34]. Canine sera collected from dogs vaccinated with an inactivated commercial CCoV type II vaccine and subsequently challenged with a type II field virus, were examined together with negative control sera and sera from dogs naturally infected with type I CCoV. All sera positive to CCoV type I reacted strongly with both polypeptides, whereas, the sera from the vaccinated dogs had low reactivity. Since CCoV type I has not been cultivated in cell cultures [33], the recombinant polypeptides represent a novel method to study the immunological and the pathogenetic characteristics of this virus [34].

The virological diagnosis of CCoV requires laboratory confirmation. The diagnostic techniques employed for the detection of CCoV in faecal samples include electron microscopy (EM), isolation in cell cultures (VI) and RT-PCR. EM appears to

be a valuable diagnostic tool for the detection of coronaviruses. However, the common presence of *coronavirus-like* particles in the faeces of dogs requires confirmation by other techniques. With respect to other methods, VI in cell cultures is difficult, time-consuming and less sensitive. The virus grows on several cell lines of canine and feline origin and it is possible to observe cytopathic effects after about 2 days of incubation at 37 °C. In addition, the identification of an isolate requires neutralisation of the cytopathic effects and/or immunofluorescence tests with a reference sera or monoclonal antibodies. Considering such difficulties, the frequency of CCoV disease has probably been underestimated and the acquisition of basic information on the pathogenetic role of CCoV in dogs has been hindered. An n-RT-PCR assay that targeted a segment of the gene encoding for the M protein of CCoV has been developed for the diagnosis of CCoV infection [23]. It has high specificity and sensitivity and allows diagnosis more rapidly than the traditional methods.

A taqMan<sup>®</sup> fluorogenic RT-PCR assay was developed for the detection and quantification of CCoV RNA in the faeces of dogs. The test, which targeted the M gene too, is more sensitive than a conventional RT-PCR assay, with a detection limit of about 10 copies of standard CCoV RNA. This method allows quantification of samples with a wide range of CCoV RNA loads [4]. Recently, two genotype-specific fluorogenic RT-PCR assays were developed for the detection and quantification of CCoV type I and type II RNA in the faeces of dogs with diarrhoea. Both the fluorogenic assays allowed the quantification of specific RNA in the faecal samples collected from dogs naturally or experimentally infected with CCoV type I, CCoV type II or both CCoV genotypes [6]. The high sensitivity, simplicity and reproducibility of the fluorogenic RT-PCR assays make these methods especially suitable for efficacy trials on CCoV vaccines [4, 6].

CCoV is highly contagious and once the virus has become established in the environment, the spread of the infection is difficult to control. Avoiding contact with infected dogs and their excretions is the only way to ensure disease prevention. Crowding, unsanitary conditions, stress during training and other conditions appear to favour development of clinical disease. CCoV is inactivated by most germicidal agents but they do not prevent dog to dog transmission. The value of CCoV vaccines in providing adequate immunity, under field conditions, is controversial. In a recent study, Pratelli et al. [32] demonstrated the low efficacy of a widely used inactivated commercial vaccine (Duramune PC, Fort Dodge) in reducing faecal shedding after challenge with a field virus. Although the efficacy and the duration of immunity engendered by inactivated vaccines have not been substantiated, only killed vaccines are licensed for the control of the infection. Modified live (ML) vaccines have been licensed in the past, but they often resulted in a high frequency adverse of post-vaccinal reactions<sup>2</sup>.

Prevention of CCoV infection is related to the production of protective levels of IgAs in the intestine [1]. Considering the long period of CCoV shedding after infection, effective control of CCoV, especially in animal shelters, requires the prevention of infection. Recently, the safety and the efficacy of an ML CCoV vaccine was evaluated in three groups of dogs: two groups were vaccinated by the intramuscular and oronasal routes, respectively, and their responses were compared with a third group of unvaccinated dogs [35]. After challenge, none of the vaccinated dogs had clinical signs. However, dogs inoculated by the intramuscular route, as well as the control dogs, shed the challenge virus for 10

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<sup>2</sup> Pratelli A., Canine coronavirus infection, in: Carmichael L.E. (Ed.), Recent Advances in Canine Infectious Diseases, International Veterinary Information Service, Ithaca NY [on line] (2000) <http://www.ivis.org>. Last updated: 29 April 2005.

and 23 median days, respectively. In contrast, virus shedding was not observed in the dogs vaccinated by the oronasal route. Even though the immune mechanisms of protection from CCoV infection are unclear, a relationship between the levels of faecal IgA to CCoV and the degree of protection against the challenge virus was observed using an ELISA test [5]. The experimental study has demonstrated a correlation between intestinal IgA antibodies and protection against infection. Since IgAs appear to play a fundamental protective role in CCoV infections, it seems important to evaluate vaccines in regards to both the vaccine type (ML versus inactivated) and the inoculation route.

## 5. CONCLUSION

All the data acquired on the biology of CCoV have dealt with factors that focus upon important epidemiological outcomes in the field in terms of both prophylaxis and virus evolution. Although CCoV infections appear to be a minor cause of life-threatening enteritis in dogs, severe illness as a consequence of dual infections has been observed [3, 11, 27, 46]. Since polymicrobial infections are common in high-density populations, such as in unvaccinated kennels, and knowing that some dogs shed the virus for periods as long as 6 months after clinical signs have ceased [26, 29], it seems likely that the immunisation of dogs that would produce a sterilising immunity against CCoV, would have beneficial epidemiological effects. Moreover, as a consequence of the relatively high mutation frequency, the RNA viruses have the potential to rapidly adjust to certain negative pressures, such as those presented by the immune system. Epidemiological monitoring of the evolution of CCoV is particularly important for scientific purposes and for prophylaxis. Recombination events affecting CCoV could make understandable the evolutionary processes leading to the proliferation of new virus strains, serotypes

and subtype, as happened for SARS-CoV. Notwithstanding the several studies carried out on CCoV, there are a lot of aspects to clarify yet: the meaning of the simultaneous infection by the two genotypes, the real pathogenetic role of the two viruses, the immune response against CCoV type I and CCoV type II, etc.

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