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# Molecular surveillance of traditional and emerging pathogens associated with canine infectious respiratory disease

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ABSTRACT

A molecular survey for traditional and emerging pathogens associated with canine infectious respiratory disease (CIRD) was conducted in Italy between 2011 and 2013 on a total of 138 dogs, including 78 early acute clinically ill CIRD animals, 22 non-clinical but exposed to clinically ill CIRD dogs and 38 CIRD convalescent dogs. The results showed that canine parainfluenza virus (CPIV) was the most commonly detected CIRD pathogen, followed by canine respiratory coronavirus (CRCoV), *Bordetella bronchiseptica*, *Mycoplasma cynos*, *Mycoplasma canis* and canine pneumovirus (CnPnV). Some classical CIRD agents, such as canine adenoviruses, canine distemper virus and canid herpesvirus 1, were not detected at all, as were not other emerging respiratory viruses (canine influenza virus, canine hepacivirus) and bacteria (*Streptococcus equi* subsp. *zooepidemicus*). Most severe forms of respiratory disease were observed in the presence of CPIV, CRCoV and *M. cynos* alone or in combination with other pathogens, whereas single CnPnV or *M. canis* infections were detected in dogs with no or very mild respiratory signs. Interestingly, only the association of *M. cynos* (alone or in combination with either *CRCoV* or *M. canis*) with severe clinical forms was statistically significant. The study, while confirming CPIV as the main responsible for CIRD occurrence, highlights the increasing role of recently discovered viruses, such as CRCoV and CnPnV, for which effective vaccines are not available in the market.



## 1. Introduction

Canine infectious respiratory disease (CIRD), previously known as kennel cough, is an endemic respiratory syndrome, which is frequently observed in densely housed environments, such as kennels and animal shelters, due to overpopulation and continuous introduction of pathogens. CIRD has a multi-agent aetiology, with more than one viral or bacterial agent being involved sequentially or synergistically to cause disease (Buonavoglia and Martella, 2007). Pathogens commonly associated with CIRD development include canine adenovirus 2 (CAdV-2), canine parainfluenza virus (CPIV) and *Bordetella bronchiseptica*. Less commonly, canid herpesvirus 1 (CaHV-1) can cause respiratory disease (Decaro et al., 2008b; Kawakami et al., 2010). Canine adenovirus 1 (CAdV-1) and canine distemper virus (CDV) infections are also involved in the development of respiratory disease, but they are usually characterized by multi-organ involvement (Decaro et al., 2008b, 2007a). Apart from these pathogens, a plethora of emerging agents have been recently associated to CIRD, including canine respiratory coronavirus (CRCoV) (Erles et al., 2003; Decaro et al., 2007b), canine pneumovirus (CnPnV) (Renshaw et al., 2010; Decaro et al., 2014), non-primate canine hepacivirus (NPCHV) (Kapoor et al., 2011; El-Attar et al., 2015), canine bocaviruses (CBoV) (Kapoor et al., 2012) and the bacterial species *Mycoplasma cynos* (Chalker et al., 2004) and *Streptococcus equi* subsp. *zooepidemicus* (Chalker et al., 2003; Priestnall et al., 2010). Equine-derived canine influenza virus (CIV) H3N8 caused a large respiratory outbreak in the US in previous years (Crawford et al., 2005), but it has been now replaced by the avian-like virus H3N2 which has originated in southeastern Asia (Zhu et al., 2015) and is now spreading in the US (unpublished

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data). While there are some reports about the circulation of classical CIRD agents, data about new and emerging respiratory pathogens in dogs are scarce (Priestnall et al., 2014). Therefore, in order to obtain new insights into the epidemiology of canine respiratory agents, we have conducted an epidemiological survey using molecular methods in CIRD clinically ill, exposed and convalescent dogs in Italy between 2011 and 2013.

## 2. Materials and methods

### 2.1. Dogs and sampling criteria

Clinical samples were sourced from diagnostic and pathology laboratories, private practitioners, animal shelters, boarding kennels and commercial dog brokers in different parts of Italy. Nasal and/or oropharyngeal swabs were collected from a total of 138 dogs meeting at least one of the following three clinical criteria: i) early acute clinically ill CIRD dogs with onset of respiratory signs at 0–3 days at the time of sample collection ($n = 78$); ii) non-clinical but exposed to clinically ill CIRD dogs ($n = 22$); iii) convalescent dogs that had clinical onset of CIRD more than 10–12 days at the time of sample collection ($n = 38$).

A clinical score was developed to evaluate the presence and severity of respiratory disease (Table 1) and signalment and anamnesis of each dog data were reported in a sample capture form. The sampled animals were client-owned ($n = 86$, 62.32%) or shelter dogs ($n = 52$, 37.68%). Dogs were aged from 1 month to 14 years (mean ± standard deviation [SD], 4.65 ± 4.00 years, 95% CI [3.97; 5.34]). Eighty-seven dogs (63.04%) were mixed-breed and the purebred animals (51/138, 36.96%) included a wide range of large and small breeds.

### 2.2. Sample processing

Swabs were immersed in 1.5 mL viral transport medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% foetal calf serum (FCS), 1000 IU/ml penicillin, 1000 μg/ml streptomycin and 10 μg/ml amphotericin B. Aliquots of the nasal and oropharyngeal swab extracts were combined and subsequently 140 μL of each sample homogenate were used for RNA and DNA extraction by means of QIAamp *cador* Pathogen Mini Kit (Qiagen S.p.A., Milan, Italy), following the manufacturer's protocol. The nucleic acid templates were stored at −70 °C until their use.

### 2.3. Molecular analyses

A panel of real-time (RT-)PCR assays, based on the TaqMan technology, was used for detection of some CIRD-associated common and emerging viral agents, including CAdV-1 and CAdV-2 (Dowgier et al., 2016), CDV (Elia et al., 2006), CaHV-1 (Decaro et al., 2010), CRCoV (Decaro et al., 2008a). CIV was searched for by means of a minor groove binder (MGB) probe real-time RT-PCR assay able to detect all influenza viruses of human and animal origin (Di Trani et al., 2006). TaqMan and MGB probe assays were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Srl) with iTaq Universal Probes Supermix (Bio-Rad Laboratories Srl, Milan, Italy). Samples were considered positive if the amplification curves were higher than the threshold line generated by the software on the basis of the background fluorescence. Gel-based (RT-)PCR assays were employed for detection of CPIV (Erles et al., 2004), CnPnV (Renshaw et al., 2010), NPCHV (Kapoor et al., 2011), *B. bronchiseptica* (Hozbor et al., 1999), *S. equi* subsp. *zooepidemicus* (Alber et al., 2004) and *M. cynos* (Chalker et al., 2004). RT-PCR and PCR assays were performed using SuperScript™ One-Step RT-PCR for Long Templates (Life Technologies, Monza, Italy) and LA PCR Kit Ver. 2.1 (TaKaRa Bio Inc., Shiga, Japan), respectively. Samples were considered positive if amplicons of the expected size were visualized after gel electrophoresis and staining with ethidium bromide.

### 2.4. Data analysis

Clinical scores were evaluated in order to result in 3 categories that were defined as follows: a total score of 1 resulted in category 1 (no clinical signs), a total score of 2–3 resulted in category 2 (mild to moderate clinical signs) and a total score of 4–5 resulted in category 3 (severe clinical signs). For each pathogen the association with single clinical scores and with the categories was explored. Categorical variables were examined using Chi-square test or Fisher's exact test, as appropriate. Logistic regression was used to identify possible bivariate associations between different pathogens in the same samples.

Statistical analysis of the risk factors were performed using a web-based software program (R version 3.3.0) (http://www.r-project.org/) and setting statistical significance to $p < 0.05$.

## 3. Results

The results of the molecular detection of CIRD-associated agents are reported in Tables 2–4. A total of 48 (34.78%) out of 138 sampled dogs were found to be infected by one or more CIRD agents, including 37/78 (47.44%) clinically ill, 5/22 (22.73%) exposed and 6/38 (15.79%) convalescent animals (Table 2). Positive samples were obtained with higher frequency in purebred (25/51 dogs, 49.02%) than mixed-breed (23/87 dogs, 26.44%) animals. Accordingly, the majority of dogs infected by CIRD agents were client-owned (38/86, 44.18%) rather than kenneled dogs (10/52, 19.23%).

As depicted in Table 3, the most frequently detected agent was CPIV, which was detected in 18 dogs, including 16 (20.52%) ill, one (4.55%) exposed and one (5.27%) convalescent animals. Detection of CRCoV was achieved in 10 animals, of which 7 (8.98%) were clinically affected, one (4.55%) had been exposed to clinically ill dogs and two (5.27%) were CIRD convalescent. Other pathogens present in CIRD clinically ill dogs were *B. bronchiseptica* (8 animals, 10.26%), *M. canis* (7 animals, 8.98%), *M. cynos* (6 animals, 7.70%), and CnPnV (5 animals, 6.41%).

Coinfections with more than one CIRD agent occurred in 9 animals, all of which were early acute clinically ill, and included two triple infections with *B. bronchiseptica*, *M. cynos* and CPIV, two dual infections with *B. bronchiseptica* and *M. canis* and single cases

**Table 1**
Clinical score used to evaluate the respiratory disease of the samples dogs.

| Score | Respiratory score description |
|---|---|
| 1 | No respiratory signs |
| 2 | Mild cough |
| 3 | Cough and nasal discharge |
| 4 | Cough and nasal discharge with depression and/or inappetance |
| 5 | Cough and nasal discharge with depression and/or inappetance and clinical signs of lower respiratory disease |