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.

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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

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

Table 2
Number of samples positive and negative for CIRD agents.

Dog category	Number (%) of positive samples	Number (%) of negative samples	Total
Early acute clinically ill CIRD dogs	37 (47.44)	41 (52.56)	78
Exposed to clinically ill CIRD dogs	5 (22.73)	17 (77.27)	22
CIRD convalescent dogs	6 (15.79)	32 (84.21)	38
Total	48 (34.78)	90 (65.22)	138

Table 3
CIRD agents detected in diseased, exposed and convalescent dogs.

Microorganism	Number (%) of early acute clinically ill CIRD dogs (n = 78)	Number (%) of exposed to clinically ill CIRD dogs (n = 22)	Number (%) of CIRD-convalescent dogs (n = 38)	Total (%) (n = 138)
B. bronchiseptica	**8 (10.25)**	**1 (1.28)**	0 (0)	**9 (6.51)**
S. equi subsp. zooepidemicus	0 (0)	0 (0)	0 (0)	0 (0)
M. cynos	**6 (7.69)**	0 (0)	**2 (5.26)**	**8 (5.79)**
M. canis	**7 (8.97)**	0 (0)	**1 (2.63)**	**8 (5.79)**
CRCoV	**7 (8.97)**	**1 (1.28)**	**2 (5.26)**	**10 (7.24)**
CPIV	**16 (20.51)**	**1 (1.28)**	**1 (2.63)**	**18 (13.04)**
CAdV-1	0 (0)	0 (0)	0 (0)	0 (0)
CAdV-2	0 (0)	0 (0)	0 (0)	0 (0)
CaHV-1	0 (0)	0 (0)	0 (0)	0 (0)
CIV	0 (0)	0 (0)	0 (0)	0 (0)
CnPnV	**5 (6.41)**	**2 (2.56)**	0 (0)	**7 (5.07)**
NPCHV	0 (0)	0 (0)	0 (0)	0 (0)
CDV	0 (0)	0 (0)	0 (0)	0 (0)

CRCoV, canine respiratory coronavirus; CPIV, canine parainfluenza virus; CAdV-1, canine adenovirus 1; CAdV-2, canine adenovirus 2; CaHV-1, canid herpesvirus 1; CIV, canine influenza virus; CnPnV, canine pneumovirus; NPCHV, non-primate canine hepacivirus; CDV, canine distemper virus.

Table 4
Coinfections of CIRD agents detected in diseased, exposed and convalescent dogs.

Microorganisms	Number (%) of early acute clinically ill CIRD dogs (n = 78)	Number (%) of exposed to clinically ill CIRD dogs (n = 22)	Number (%) of CIRD-convalescent dogs (n = 38)	Total (%) (n = 138)
B. bronchiseptica + *M.cynos* + CPIV	2 (2.56)	0 (0)	0 (0)	2 (1.44)
B. bronchiseptica + *M. canis*	2 (2.56)	0 (0)	0 (0)	2 (1.44)
B. bronchiseptica + *M. canis* + CnPnV	1 (1.28)	0 (0)	0 (0)	1 (0.72)
M. cynos + CRCoV	1 (1.28)	0 (0)	0 (0)	1 (0.72)
M. cynos + *M. canis*	1 (1.28)	0 (0)	0 (0)	1 (0.72)
CPIV + CRCoV	1 (1.28)	0 (0)	0 (0)	1 (0.72)
CnPnV + CRCoV	1 (1.28)	0 (0)	0 (0)	1 (0.72)
Samples with coinfections	9 (11.53)	0 (0)	0 (0)	9 (11.53)

CPIV, canine parainfluenza virus; CnPnV, canine pneumovirus; CRCoV, canine respiratory coronavirus.

of coinfections with *B. bronchiseptica*, *M. canis* and CnPnV, *M. cynos* and CRCoV, *M. cynos* and *M. canis*, CPIV and CRCoV, or CnPnV and CRCoV (Table 4).

Association of CIRD-agent positive results with the respiratory scores and clinical categories (Table 5) revealed that most microorganisms were detected in dogs with mild to moderate respiratory distress (scores 2 and 3), while more severe clinical signs were observed in the presence of single infections by *M. cynos* (p = 0.002) and of coinfections with *M. cynos* and *CRCoV* (p = 0.000) and with *M. cynos* and *M. canis* (p = 0.000). *M. canis* and CnPnV alone were able to induce only no or mild respiratory distress, but the difference between clinical categories was not statistically significant. For none of the other pathogens and co-infections a significant association was found.

4. Discussion

For a long time, kennel cough, presently known as CIRD, has been regarded as a common disease syndrome of limited clinical significance. However, in recent years a plethora of novel agents have been associated to this infectious, multifactorial disease of dogs, thus leading to a resurgence of interest in canine respiratory pathogens (Priestnall et al., 2014). Epidemiological investigations aiming to identify the causes of CIRD, carried out in recent years, have yielded contrasting results. A longitudinal study on respiratory viruses in a rehoming kennel in UK with a history of CIRD demonstrated the circulation of CPIV, CRCoV and, at a lesser extent, of CaHV-1, while CAdVs and CDV were not detected (Erles et al., 2004). A survey for viral and bacterial agents in household dogs presented at various animal hospitals in Japan confirmed CPIV as the main agent of CIRD, although several ill dogs were infected with *B. bronchiseptica* and other pathogens including CRCoV, CAdV-2 and CDV, were detected in few animals (Mochizuki et al., 2008). Joffe et al. (2015) conducted a similar study in CIRD dogs submitted to Canadian veterinary clinics, finding a high prevalence of CPIV and *M. cynos*, while few dogs tested positive for CRCoV. A large survey carried out on 503 asymptomatic dogs of animal shelters in the US showed active circulation of *M. cynos* (61.3%), *B. bronchiseptica* (40.8%), CAdV-2 (26.3%), and CDV (15.4%), whereas CPIV, CRCoV, CaHV-1, *S. equi* subsp. *zooepidemicus* and CIV H3N8

Table 5
Association of CIRD agents with the respiratory score.

Microorganism(s)	Clinical score					Total	P-value
	Category 1	Category 2		Category 3			
	1	2	3	4	5		
B. bronchiseptica	3.1%(1/32)	2%(1/49)	2.7%(1/37)	0.2%(1/5)	0/15	3%(4/138)	P = 0.832
M. cynos	0/32	0/49	2.7%(1/37)	0.2%(1/5)	13.3%(2/15)	3%(4/138)	**P = 0.002**
M. canis	6.2%(2/32)	2%(2/49)	0/37	0/5	0/15	3%(4/138)	P = 0.372
CRCoV	0/32	4%(4/49)	2.7%(1/37)	0/5	13.3%(2/15)	5%(7/138)	P = 0.244
CPIV	0/32	6.1%(6/49)	18.9%(7/37)	0.2%(1/5)	6.6%(1/15)	10.9%(15/138)	P = 0.063
B. bronchiseptica + *M. canis*	0/32	2%(1/49)	2.7%(1/37)	0/5	0/15	1.4%(2/138)	P = 0.541
B. bronchiseptica + *M. cynos* + CPIV	0/32	0/49	2.7%(1/37)	0/5	6.6%(1/15)	1.4%(2/138)	P = 0.318
M. cynos + CRCoV	0/32	0/49	0/37	0.2%(1/5)	0/15	0.7%(1/138)	**P = 0.000**
M. cynos + *M. canis*	0/32	0/49	0/37	0.2%(1/5)	0/15	0.7%(1/138)	**P = 0.000**
CRCoV + CPIV	0/32	0/49	2.7%(1/37)	0/5	0/15	0.7%(1/138)	P = 0.737
CnPnV	6.2%(2/32)	4%(2/49)	2.7%(1/37)	0/5	0/15	3.6%(5/138)	P = 0.499
CnPnV + CRCoV	0/32	2%(1/49)	0/37	0/5	0/15	0.7%(1/138)	P = 0.737
CnPnV + *B. bronchiseptica* + *M. canis*	0/32	0/49	0/37	0/5	6.6%(1/15)	0.7%(1/138)	P = 0.512
Overall positive samples	15.6%(5/32)	34.7%(17/49)	37.8%(14/37)	5/5	46.6%(7/15)	34.8%(48/138)	

CPIV, canine parainfluenza virus; CnPnV, canine pneumovirus; CRCoV, canine respiratory coronavirus.
P-value = overall p-value for the differences among the categories (chi-square test for single infections and logistic regression for coinfections).

were detected only sporadically (Lavan and Knesl, 2015). It is noteworthy that CDV, one of the most serious pathogens of dogs (Decaro et al., 2004), was present in a so high proportion of asymptomatic animals.

In the present study, three groups of dogs were sampled, i.e., early acute clinically ill CIRD animals, non-clinical but exposed to clinically ill CIRD dogs and CIRD convalescent dogs. While the first group was the most important, aiming to assess which microorganism is associated with CIRD in Italian dogs, the other two categories were included to investigate whether any pathogens could be detected in healthy animals and whether CIRD pathogens are shed for a long time period, respectively. However, only few animals of these two groups were found to be infected by CIRD pathogens and no coinfection with more than one microorganism was detected.

In clinically ill dogs, CPIV was the most commonly identified agent of CIRD and it was associated mainly with mild to moderate respiratory disease, although this was not statistically significant. In contrast, the virus was detected only in few dogs with severe disease, alone or in combination with other pathogens.

Other CIRD agents identified in this study were *B. bronchiseptica*, *M. cynos*, *M. canis*, CRCoV and CnPnV. *B. bronchiseptica* was detected mainly in dogs coinfected with other viral and bacterial agents, which resulted in higher clinical scores. It is well know that this bacterium can act as primary CIRD agent, but its pathogenic potential is increased with simultaneous viral and bacterial infections (Priestnall et al., 2014). As for the detected mycoplasmas, *M. cynos* is a well recognized cause of respiratory distress in dogs, while the pathogenetic role of *M. canis* is generally limited (Chalker et al., 2004). These findings were confirmed by the present study, where the former agent was significantly associated to high clinical scores alone or in co-infections with CRCoV and *M. canis*, whereas the latter induced no or very mild respiratory signs. Surprisingly, other classical CIRD pathogens, including CDV, CAdV-1 and mainly CAdV-2 were not identified in any sampled animals. CDV and CAdV-1 are responsible for very severe clinical forms, involving multiple organs and leading to high mortality of infected dogs (Martella et al., 2008; Decaro et al., 2008b). In contrast, CAdV-2 infection is generally restricted to the respiratory tract and, even in the case of systemic spreading, the induced disease is confined to this apparatus (Decaro et al., 2008b). The lack of detection of these severe or mild classical CIRD agents even in kenneled dogs may be related to the extensive vaccination programs, which have

enormously reduced their circulation in the canine population. However, sporadic re-emergence of canine distemper and canine infectious hepatitis in Italy has been observed in recent years as a consequence of uncontrolled, often illegal importation of purebred pups from eastern European countries. (Decaro et al., 2007a; Martella et al., 2008). CaHV-1 was not detected in any respiratory samples collected during the 2011–2013 epidemiological survey. Although this virus is more often associated with neonatal mortality in 2–3-week-old pups, the present findings are in contrast with those of previous studies reporting active circulation of CaHV-1 in CIRD dogs (Erles et al., 2004; Kawakami et al., 2010). In some circumstances, CaHV-1 was even proven to be responsible for fatal outbreaks of respiratory disease (Kumar et al., 2015).

As for the emerging CIRD agents, only CRCoV and CnPnV were found to circulate in Italian dogs, as previously shown (Decaro et al., 2007b, 2014). However, neither single nor mixed infections caused by these pathogens were statistically associated to any clinical category.

A striking finding of this study is that CIRD agents were mainly detected in client-owned, purebred animals. Generally, kenneled dogs are more prone to be infected by CIRD pathogens due to continuous introduction of animals, especially pups, lack of prophylactic measures of the handling personnel and high density of population that facilitates pathogen spreading among housed dogs. A possible explanation for our findings is that, in most instances, the CIRD agents were recovered from dogs displaying mild respiratory distress, which is often misrecognised in overcrowded environments presenting other, more severe clinical issues.

Currently, vaccine formulations are available only against some classical CIRD agents. According to the most recent WSAVA guidelines (Day et al., 2016), only those against systemic viral infections caused by CDV and CAdV-1, the latter being prevented using cross-reacting CAdV-2 modified live virus (MLV), are considered to be core vaccines. MLV or killed formulations are available also against CPIV and *B. bronchiseptica*, but these are considered as non-core vaccines and do not prevent active infection but only the induced disease (Day et al., 2016). Considering the increasing pathogenetic role of emerging viral and bacterial CIRD agents, specific vaccines are needed in the field. However, the recent studies have displayed spotted epidemiological situations on the basis of the geographic areas, so that vaccine formulations should be prepared taking into account the different pathogen distribution in the different countries.

Conflict of interest

Acknowledgements

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