

# 1 European surveillance of emerging pathogens associated with canine 2 infectious respiratory disease.

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## 40 Abstract

41 Canine infectious respiratory disease (CIRD) is a major cause of morbidity in dogs worldwide, and is  
42 associated with a number of new and emerging pathogens. In a large multi-centre European study the  
43 prevalences of four key emerging CIRD pathogens; canine respiratory coronavirus (CRCoV), canine  
44 pneumovirus (CnPnV), influenza A, and *Mycoplasma cynos* (*M. cynos*); were estimated, and risk factors for  
45 exposure, infection and clinical disease were investigated.

46 CIRD affected 66% (381/572) of the dogs studied, including both pet and kennelled dogs. Disease  
47 occurrence and severity were significantly reduced in dogs vaccinated against classic CIRD agents, canine  
48 distemper virus (CDV), canine adenovirus 2 (CAV-2) and canine parainfluenza virus (CPIV), but substantial  
49 proportions (65.7%; 201/306) of vaccinated dogs remained affected.

50 CRCoV and CnPnV were highly prevalent across the different dog populations, with overall seropositivity  
51 and detection rates of 47% and 7.7% for CRCoV, and 41.7% and 23.4% for CnPnV, respectively, and their  
52 presence was associated with increased occurrence and severity of clinical disease. Antibodies to CRCoV  
53 had a protective effect against CRCoV infection and more severe clinical signs of CIRD but antibodies to  
54 CnPnV did not.

55 Involvement of *M. cynos* and influenza A in CIRD was less apparent. Despite 45% of dogs being seropositive  
56 for *M. cynos*, only 0.9% were PCR positive for *M. cynos*. Only 2.7% of dogs were seropositive for Influenza  
57 A, and none were positive by PCR.

58

59 **Keywords:** canine respiratory coronavirus (CRCoV), canine pneumovirus (CnPnV), Canine influenza  
60 (CIV), *Mycoplasma cynos*, canine infectious respiratory disease (CIRD), Kennel cough.

61

## 62 Introduction

63 Canine infectious respiratory disease (CIRD) is a major cause of morbidity and an important welfare issue  
64 for kennelled dog populations worldwide. Characterised by clinical signs such as coughing, nasal discharge  
65 and dyspnoea, it can persist for several weeks, often resulting in severe disease, such as  
66 bronchopneumonia, and on occasions, lead to death or result in euthanasia (Appel and Binn, 1987).

67 CIRD is a complex disease of multifactorial aetiology, where environmental, host and pathogen interactions  
68 influence disease susceptibility, severity and persistence. The pathogens traditionally associated with CIRD  
69 include canine parainfluenza virus (CPIV)(Appel and Percy, 1970), canine adenovirus type 2 (CAV-  
70 2)(Ditchfield et al., 1962), and *Bordetella bronchiseptica* (Bb) (Bemis, 1992), which act sequentially or  
71 synergistically to cause disease. Several multivalent vaccines targeting these agents are available for  
72 routine vaccination (Day et al., 2016). However outbreaks of respiratory disease continue despite their use,  
73 resulting in expensive treatment costs and delays in rehoming or training (Erles et al., 2004).

74 In recent years a number of newly emerging pathogens, including canine respiratory coronavirus (CRCoV)  
75 (Erles et al., 2003), canine pneumovirus (CnPnV)(Renshaw et al., 2010), canine influenza H3N8  
76 (CIV)(Crawford et al., 2005), and *Mycoplasma cynos* (*M. cynos*)(Chalker et al., 2004), have been implicated  
77 in the development and persistence of CIRDC, and are the subject of a comprehensive review (Priestnall et  
78 al., 2014).

79 Whilst it is widely recognised that these agents are all likely to play an important role in the onset or  
80 persistence of CIRDC, the exact nature of their contribution to the pathogenesis of disease is yet to be  
81 determined. Most, if not all, studies to date have either been limited to the investigation of individual  
82 pathogens, or have been geographically restricted to a small number of kennels or a single outbreak, and  
83 no comprehensive survey of pet dogs has been completed. Thus the true extent of the prevalence of these  
84 novel agents, and the risk factors associated with exposure, infection and disease amongst different  
85 cohorts, have not been fully investigated.

86 To help address this we present data from a large multi-centre European study in which the prevalences  
87 of four of the most important and recently identified emerging CIRDC pathogens (CRCoV, CnPnV, CIV/  
88 Influenza A and *M. cynos*) were estimated in both client-owned and kennel dogs, and risk factors for  
89 exposure, infection and clinical disease were investigated.

## 90 **Methods**

### 91 ***Study design***

92 Samples and signalment data were collected from dogs in animal shelters, breeding kennels and academic  
93 or private veterinary clinics across Europe by collaborating Investigator Centres between October 2011 and  
94 August 2013. Dogs were actively recruited onto the study and sampled upon presentation to the  
95 veterinarian if they fell into one of three different clinical groups: (A) clinically unaffected but exposed to  
96 acute CIRDC-affected dogs, (B) acute CIRDC-affected dogs (0 to 3 days post onset of CIRDC), or (C) convalescent  
97 CIRDC-affected dogs (10-12 days post onset of CIRDC). Details of the clinical grouping, source (i.e. client-  
98 owned, shelter/ rehoming kennel, hospital, breeding kennel), recorded or estimated age, vaccination  
99 history for Bb, CPIV, CAV-2 and CDV, and clinical respiratory score (1-5) at the time of sample collection  
100 were recorded. Briefly the clinical scores described previously (Erles et al., 2003) were defined as: 1) no  
101 respiratory signs, 2) mild cough, 3) cough and nasal discharge, 4) cough and nasal discharge with depression  
102 and/ or inappetence, 5) cough and nasal discharge with depression and/ or inappetence and clinical signs  
103 of lower respiratory tract disease.

104  
105 ***Sampling:*** Viral nasal swabs (VNS) (Sterilin, UK), oropharyngeal Amies swabs (OAS) (Sterilin, UK) and serum  
106 samples were collected by local veterinary practitioners. All samples were collected as part of the standard  
107 veterinary diagnostic work-up for respiratory disease, and with informed consent for clinical research  
108 purposes (obtained by the attending veterinarian or collaborating centre). Samples were sent to

109 investigator centres across Europe, where they were appropriately stored, batched and shipped to the  
110 Royal Veterinary College for analysis. Duplicate samples were retained by collaborating investigator centres  
111 for their own research purposes and to date one study relating to a subset of these has been published  
112 (Decaro et al., 2016). The study was approved by the Royal Veterinary College's Ethical Review Board.

113

#### 114 ***Detection of viral pathogens:***

115 The tip of each VNS was immersed in 1mL of DMEM medium (Sigma, Dorset, UK) and vigorously mixed.  
116 RNA was extracted from 200µl of the resulting swab fluid using the RNeasy Mini Kit (Qiagen, Crawley, UK)  
117 as recommended by the manufacturer, and eluted in 30µl of RNase free water. Five microliters of RNA  
118 was reverse transcribed into cDNA using Random Hexameres (Qiagen, Crawley, UK) and Improm II reverse  
119 transcriptase (Promega, Southampton, UK) according to the manufacturer's protocol. CRCoV and CnPnV  
120 were detected as described previously by PCR (Erles et al., 2003) and real-time PCR (Mitchell et al., 2013b)  
121 respectively. Influenza A was detected using primers targeting a conserved region of the matrix gene (Ellis  
122 and Zambon, 2001), and the GoTaq polymerase system (Promega, Southampton, UK).

123

#### 124 ***Detection of Mycoplasma Spp:***

125 The tip of each OAS was placed in 1mL of Mycoplasma Experience (ME) liquid media (Mycoplasma  
126 Experience Ltd, Bletchingley UK), vigorously mixed and incubated at 37°C with 5% CO<sub>2</sub>. Cultures were  
127 monitored daily (for up to 3 days) for signs of mycoplasma growth, as indicated by the presence of turbidity  
128 and or colour change. Once growth was observed, or at 3 days post inoculation with no apparent growth,  
129 the cells from 0.7mL of the liquid culture were pelleted and the DNA extracted using the DNeasy Mini Kit  
130 (Qiagen, Crawley, UK) as recommended by the manufacturer. DNA was eluted in 100µl of elution buffer  
131 and analysed using a pan-*Mycoplasma Spp.* PCR (Kobayashi et al., 1995), followed by a specific *M. cynos*  
132 PCR (Chalker et al., 2004) as previously described.

#### 133 ***Detection of antibodies:***

134 The antibody status of each dog for CRCoV, CnPnV and *M. cynos* was determined by ELISA using the  
135 following antigens:

136 *CRCoV ELISA Antigen:* Recombinant CRCoV hemagglutinin esterase (HE) protein. The CRCoV HE protein  
137 (strain 4182) was expressed using the BacMagic™ recombinant baculovirus expression system (Merck, UK)  
138 at 5MOI in *Spodoptera frugiperda 9* (SF9) cells. The antigen was prepared by lysing infected cells with lysis  
139 buffer (1% Igepal, 50mM NaH<sub>2</sub>PO<sub>4</sub>, 300mM NaCl, pH8) at 3 days post infection. The CRCoV HE antigen was  
140 standardised to a mock infected SF9 lysate which served as the control antigen, and 0.8 micrograms of total  
141 protein per well was used to coat the plate.

142

143 *CnPnV ELISA Antigen*: Freeze-dried murine pneumovirus and control antigen (Churchill Applied  
144 Biotechnology Ltd, Huntingdon) was prepared and used as described previously (Mitchell et al., 2013b).

145  
146 *M. cynos ELISA Antigen*: *M. cynos* (UK strain 491) cell pellets were washed three times in PBS, then  
147 resuspended in 5mL PBS, and freeze thawed. Six nano-grams per well of total *M. cynos* protein in 50µl of  
148 PBS was used to coat the plate. Wells coated with PBS only were used as a background control.

149  
150 *Briefly for each ELISA*: Antigen was adsorbed overnight at 4°C onto 96 well Nunc Maxisorb™ ELISA plates,  
151 and blocked with 5% milk in 0.05% PBS Tween. Serum samples were diluted 1:100 in blocking buffer and  
152 50µl dispensed in duplicate onto positive and control antigens and incubated at 37°C for 1 hour. Following  
153 three washes with 0.05% PBST, the anti-dog IgG peroxidase conjugate (Sigma-Aldrich, Poole) secondary  
154 antibody was diluted 1:2500 (CRCoV, CnPnV) or 1:5000 (*M. cynos*) in blocking buffer, and 50µl dispensed  
155 into each well and incubated. The plates were washed as before and bound antibody was detected using  
156 Sigma-Fast™ OPD peroxidase substrate (Sigma Aldrich, UK) according to the manufacturer's instructions,  
157 and stopped with 2M H<sub>2</sub>SO<sub>4</sub>. Optical densities (OD) were measured at 490nm (Optimax automatic plate  
158 reader Molecular Devices, Wokingham). Positive and negative control sera were included in duplicate on  
159 each plate. For each serum sample, the average OD value for the negative antigen was subtracted from  
160 that of the positive antigen to give the average corrected OD value. The positive cut-off was defined as the  
161 average corrected OD value for a panel of reference serum (IgG negative for the antigen used) plus three  
162 times the standard deviation. Samples were considered positive if the 'average corrected OD value' was  
163 greater than or equal to the positive cut-off value for the ELISA.

164  
165 *Influenza A ELISA*: The influenza antibody status was determined via two independent methods:  
166 1) Freeze-dried equine influenza A (H3N8 Miami) and control antigen (Churchill Applied B Biotechnology  
167 Ltd, Huntingdon) were used according the manufacturers recommendation and validated for use with  
168 canine samples using a panel of CIV (H3N8) positive and negative control serum (Zoetis Animal Health,  
169 Kalamazoo). The ELISA was performed as described above using a 1:2500 dilution of secondary antibody.  
170 2) The commercially available ID Screen Influenza A Antibody competition assay. The assay was performed  
171 according to the manufacturer's protocol using a 1:10 dilution of canine serum as previously described  
172 (Pratelli and Colao, 2014). The assay was validated using the controls supplied with the kit and a panel  
173 of CIV (H3N8) positive and negative control serum (Zoetis Animal Health, Kalamazoo).

#### 174 **Statistical analysis**

175 Data were recorded in Excel spreadsheets (Microsoft) and imported into Stata/SE 13 software (Statacorp)  
176 for analysis. Continuous variables were summarised using mean (standard deviation) if normally distributed  
177 and median (minimum, maximum) if non-normally distributed. Categorical variables were summarised  
178 using numbers and percent in each category and 95% confidence intervals (CI) around prevalence estimates

179 calculated using the Clopper-Pearson method. For the purposes of these analyses the clinical score variable  
180 was collapsed from the original categorisation of 1-5 (as detailed above) to a three-point 'severity score' of  
181 'no disease' (clinical score 1), 'mild to moderate disease' (clinical score 2 and 3) and 'severe disease' (clinical  
182 score 4 and 5). The presence or absence of disease was also assessed, where the original clinical score of  
183 2-5 indicated the presence of disease and 1 indicated no disease.

184 Variations in disease presence and severity by different factors (source, age, country and vaccination  
185 status), and variations in individual pathogen prevalence and seroprevalence in association with source,  
186 clinical group, disease presence and severity were examined using Chi-square/Fisher's exact tests and  
187 univariable logistic regression. Multivariable logistic regression was used to examine relationships where  
188 more than one factor was significantly associated with the outcome at the 25% level (univariable screening  
189  $p$ -value <0.25).

## 190 **Results**

### 191 **Descriptive statistics**

192 Descriptive statistics relating to dogs and samples included in the study are summarised in Table 1. Samples  
193 and signalment data were obtained from 572 dogs (Table 1). Of these, 45.6% (n=261) were client-owned  
194 dogs, 46.2% (n=264) were from shelters and 8.2% (n=47) were from other sources (predominantly a single  
195 veterinary hospital). Overall, 24.1% (n=138) were clinically unaffected dogs that had been exposed to acute  
196 CIRDC-affected dogs, 49.3% (n=282) were acute CIRDC-affected dogs and 26.6% (n=152) were convalescent  
197 (10 days post onset of acute disease and may or may not have still been showing clinical signs of CIRDC).  
198 Approximately equal proportions (~20%) of samples came from Italy, Greece, Hungary and France  
199 respectively with smaller proportions (~10%) from Spain and Netherlands. Estimated age was recorded for  
200 542 dogs and ranged from 5 weeks to 15 years with a median of 3 years.

201 Clinical signs of respiratory disease were observed in 66.6% (n=381) of the 572 dogs. Just over half of the  
202 dogs (55.4%; n=317) showed signs of mild to moderate disease and 11.2% (n=64) showed signs of more  
203 severe disease.

204 In total, 525 serum samples were examined for the presence of CRCoV, CnPnV, Influenza A or *M.cynos*  
205 antibodies and 559 viral nasal swabs (VNS) and 566 oropharyngeal Amies swabs (OAS) were analysed for  
206 the presence of the pathogens.

207 The overall estimated seroprevalence in this study population was 47% (247/525; 95% CI: 42.7-51.4%) for  
208 CRCoV, 41.7% (219/525; 95% CI: 37.4-46.1%) for CnPnV, 45% (236/525; 95% CI: 40.6-49.3%) for *M. cynos*,  
209 and 2.7% (6/220; 95% CI: 1.0-5.8%) for Influenza A .

210 The overall estimated prevalence of each pathogen detected by PCR was 7.7% (43/559; 95% CI: 5.6-10.2%)  
211 for CRCoV, 23.4% (130/555; 95% CI: 20-27.2%) for CnPnV, 0.9% (n=5/566, CI) for *M. cynos*, and 0% (0/511)  
212 for Influenza A.

### 213 **Disease presence and severity was associated with source and a history of vaccination.**

214 Significant univariable variations in the presence and severity of disease were associated with source,  
215 country, vaccination and age (data not shown). Dogs with clinical signs were significantly younger (median  
216 3 years; range 5 weeks to 15 years) than those without (median 4 years; range 5 weeks to 15 years)  
217 ( $p=0.003$ ), with an inverse relationship between age and disease severity. Whilst clinical disease was clearly  
218 prevalent amongst dogs both with and without a history of vaccination (Bb, CPIV, CAV-2 and CDV) the odds  
219 of disease were significantly reduced in dogs vaccinated against CPIV, CAV-2, and CDV, but not Bb.

220 Variations in disease presence in association with country and age were no longer significant in  
221 multivariable analysis. However, reduced odds of disease remained significantly associated with a history  
222 of vaccination for CPIV, CAV-2 or CDV with an independent effect of reduced odds of disease in shelter dogs  
223 and dogs from other sources compared with client-owned dogs (Table 2). Further examination of the  
224 vaccination effect indicated that the proportion of dogs with a history of vaccination against CPIV, CAV-2  
225 and CDV reduced significantly as the severity of disease increased (Figure 1).

### 226 **Significant variations in pathogen seroprevalence were associated with country, source, age, antibody 227 status and clinical group, but varied by the pathogen studied.**

228 Significant univariable variations in CRCoV seroprevalence were detected by country, age, source, disease  
229 status, clinical score, and CnPnV seropositivity (data not shown). Dogs with antibodies to CRCoV were  
230 significantly less likely to develop respiratory disease (OR: 0.5,  $p<0.001$ ), with odd ratios of 0.5 ( $p<0.001$ )  
231 and 0.4 ( $p=0.003$ ) for dogs with clinical scores of 2 and 3 respectively compared to dogs with no clinical  
232 signs. Following multivariable analysis, independent effects of country, age and CnPnV antibody status  
233 remained significant (Table 3). Dogs from France and Spain had significantly increased odds of being  
234 seropositive (OR 4.8,  $p<0.001$  and OR 4.1,  $p<0.001$  respectively) compared with dogs from Italy. Dogs were  
235 also significantly more likely to be CRCoV seropositive with increasing age (OR 1.1;  $p=0.001$ ), or if they were  
236 also seropositive for CnPnV (OR 2.7,  $p<0.001$ ).

237 Significant univariable variations in CnPnV seroprevalence were detected by country, source, clinical group,  
238 disease status and clinical score (data not shown). Dogs with antibodies to CnPnV were significantly less  
239 likely to develop respiratory disease than those without (OR: 0.4,  $p<0.001$ ), with odds ratios of 0.5 ( $p<0.001$ )  
240 and 0.4 ( $p=0.002$ ) for dogs with clinical scores of 2 and 3 respectively compared to those with no clinical  
241 signs of disease. In multivariable analysis, independent effects of country, source and CRCoV and *M. cynos*  
242 antibody status remained significant (Table 3). Dogs from France and the Netherlands had 3.4 ( $p=0.001$ )  
243 and 3.7 ( $p=0.002$ ) times the odds of being seropositive compared with dogs from Italy. Dogs from shelters

244 were almost 3 times (OR 2.8,  $p < 0.001$ ) and dogs from other sources were 8 times (OR 8.0,  $p < 0.001$ ) as likely  
245 to be CnPnV seropositive than client-owned dogs. The odds of being CnPnV seropositive were also  
246 significantly increased in association with CRCoV (OR 2.7,  $p = < 0.001$ ) and *M. cynos* (OR 1.8,  $p = 0.005$ )  
247 seropositivity.

248 Significant univariable variations in *M. cynos* seroprevalence were detected in association with country,  
249 source, clinical group, and CRCoV and CnPnV antibody status. No significant association between *M. cynos*  
250 antibody status and the presence or severity of clinical disease was observed (data not shown).  
251 Independent effects of country, source, clinical group and age remained significant in multivariable analysis  
252 (Table 3). Dogs from Greece, France and Spain were 2.3 ( $p = 0.008$ ), 2.5 ( $p = 0.012$ ) and 2.2 ( $p = 0.028$ ) times  
253 as likely to be seropositive for *M. cynos* as those from Italy, respectively. Shelter dogs were almost twice as  
254 likely as pet dogs to be seropositive for *M. cynos* (OR 1.8;  $p = 0.024$ ), and convalescent dogs almost three  
255 times as likely to be seropositive than clinically unaffected but exposed dogs (OR 2.8;  $p < 0.001$ ). The odds  
256 of *M. cynos* seropositivity also increased with age (OR 1.06;  $p = 0.024$ ).

257 The overall seroprevalence of Influenza A was 2.7% (6/220; 95% CI: 1.0-5.8%) with good correlation  
258 between the two ELISA protocols used. The small number of seropositive dogs precluded further statistical  
259 analysis. However, of the six positive dogs five were from a shelter (5/114; 4.4%) and one was client-owned  
260 (1/91; 1.1%). Three were acute with mild signs of clinical disease, two convalescent with no clinical signs of  
261 disease, and one healthy but exposed.

262 **Significant variations in pathogen presence were associated with country, source, clinical score and**  
263 **antibody status, but varied by the pathogen studied.**

264 Significant univariable variations in CRCoV presence were detected with country, age, clinical score, CRCoV  
265 and CnPnV seropositivity (data not shown). Independent associations with country, clinical score and the  
266 presence of CRCoV antibody remained significant in multivariable analysis (Table 4). Dogs from Greece (OR  
267 12.2;  $p = 0.02$ ), Hungary (OR 8.9;  $p = 0.045$ ), France (OR 28;  $p = 0.002$ ) and Spain (OR 22.5;  $p = 0.004$ ), at  
268 significantly increased odds of CRCoV positivity compared with dogs from Italy. Dogs with severe clinical  
269 signs were 3.5 times as likely to be positive for CRCoV as those with no signs of clinical disease ( $p = 0.029$ ),  
270 but were almost half as likely to be positive for CRCoV if they had antibodies to the virus (OR 0.4;  $p = 0.015$ ).

271 Significant univariable variations in CnPnV presence were detected with country, source and clinical score,  
272 but not with the presence or absence of CnPnV antibody (data not shown). Independent effects of country,  
273 clinical score and CRCoV status remained significant in multivariable analysis (Table 4). Dogs from Hungary  
274 (OR 2.3;  $p = 0.007$ ) had twice the odds of CnPnV positivity as dogs from Italy. The presence of CRCoV was  
275 associated with a two-fold increase in the odds of CnPnV presence (OR 2.0,  $p = 0.040$ ) and dogs with severe  
276 clinical disease had more than twice the odds of CnPnV positivity as those with no clinical disease ( $p = 0.029$ ).



277 Overall 47.9% (n=271/566, CI) of dogs were positive for *Mycoplasma Spp.* and 0.9% (n=5/566, CI) were  
278 positive for *M. cynos* (data not shown). The small number of *M. cynos* positive dogs (n=5) precluded further  
279 analysis. However, four were shelter dogs and one was client-owned. Three had severe respiratory signs  
280 (one requiring euthanasia) whilst two dogs were non-clinical. The trachea, lung, bronchial lymph node,  
281 palatine tonsil and thymus from the euthanized dog were analysed and all, with the exception of the lung,  
282 were positive for *M. cynos*.

283 None of the 511 dogs screened for the presence of Influenza A by PCR were positive (data not shown).

## 284 Discussion

285 This study examined the occurrence of CIRDC, and four of the most important emerging pathogens  
286 associated with the disease in different dog populations across Europe. Key variables (e.g. clinical  
287 presentation, housing, vaccination etc.) and their relationship with disease occurrence, severity, pathogen  
288 exposure and presence were also analysed.

289 Overall clinical disease occurred in approximately two-thirds of the dogs, with variations in the rate of  
290 occurrence and severity predominantly associated with housing and vaccination history. Whilst CIRDC is  
291 generally regarded as a disease of kennelled dogs as shown here, we have also provided substantial  
292 evidence of CIRDC in pet (client-owned) dogs, many of which had moderate to severe signs of disease,  
293 highlighting the need for further consideration of pet dogs in the occurrence and spread of CIRDC, and the  
294 potential risk factors associated with this group (e.g. multi-dog households, attendance at dog training,  
295 doggy day care, recent boarding or kennelling). However, the high rate of CIRDC observed in client-owned  
296 dogs, compared to kennelled dogs, is most likely explained by an inherent sampling bias. Healthy client-  
297 owned dogs are unlikely to have been presented or sampled as part of a normal veterinary investigation,  
298 except in some cases of multi-dog households, doggy day care etc. where CIRDC has been problematic. In  
299 contrast, sampling of apparently healthy dogs in kennelled environments often forms part of a standard  
300 outbreak investigation.

301 Vaccination therefore is likely to have had the biggest influence on the occurrence and severity of disease  
302 in this study. Within the EU it is recommended that dogs are routinely vaccinated against CAV-2 and CDV  
303 as part of the core vaccinations they receive from puppyhood. The modified-live virus vaccines are  
304 administered parenterally, and are highly efficacious (Day et al., 2016). Some core vaccine formulations  
305 include CPIV but not Bb, and whilst separate modified-live intranasal vaccines for Bb and CPIV are available  
306 they tend to be administered only to high risk dogs (i.e. during periods of kennelling). Studies of CPIV and  
307 Bb vaccine efficacy are regulated by European monographs, requiring specific efficacy and safety  
308 requirements including the onset and duration of immunity. However, published studies are limited and  
309 there is debate on the differing efficacies demonstrated (Day et al., 2016; Ellis, 2015; Ellis and Krakowka,  
310 2012; Mitchell and Brownlie, 2015) .

311 Here, a history of vaccination against the classic CIRDC-associated pathogens CDV, CAV-2 and CPIV,  
312 significantly reduced the occurrence and severity of disease. However, vaccination against Bb did not,  
313 although this could be due to the small number of Bb vaccinated dogs (n=66) in this study. Despite evidence  
314 of a protective effect substantial proportions of CDV, CAV-2 and CPIV vaccinated dogs remained affected  
315 by CIRDC, many with severe clinical signs, supporting the widely accepted view that vaccination against these  
316 key agents may reduce disease occurrence and severity in some individuals or outbreaks, but is often poorly  
317 effective in others. Differences may be a result of vaccine formulation or protocol used, the biological  
318 properties and characteristics of the agent and host, or the involvement of newly identified or emerging  
319 pathogens (Erles et al., 2004; Priestnall et al., 2014)

320 Whilst these first two points fall beyond the scope of this study, in examining four of the most important  
321 emerging CIRDC pathogens it is clear their role in CIRDC may be significant for both kennelled and pet dogs.  
322 In particular CRCoV and CnPnV were highly prevalent across the different dog populations studied and their  
323 presence was positively associated with an increased occurrence and severity of clinical disease. The  
324 presence and involvement of *M. cynos* and influenza A in CIRDC was however less apparent.

325 Almost half the dogs in this study were seropositive for CRCoV and 7.7% were positive for the virus,  
326 consistent with published data (Erles et al., 2003; Priestnall et al., 2006). Multivariable analysis showed  
327 that the likelihood of dogs having antibodies to CRCoV increased with age, and those with antibodies were  
328 significantly less likely to be positive for the virus. Dogs with increasing disease severity were significantly  
329 more likely to be positive for CRCoV. Dogs with antibodies to CRCoV that developed CIRDC were less likely  
330 to develop the more severe clinical signs of disease. Younger dogs (less likely to have antibodies to CRCoV)  
331 were more likely to be infected with CRCoV, and had a greater rate of occurrence of CIRDC, and developed  
332 more severe clinical signs. These findings are consistent with previously published data (Erles et al., 2004;  
333 Erles et al., 2003; Mitchell et al., 2013a), and strengthen the evidence for a causal relationship between  
334 CRCoV infection and CIRDC, as well as for the protective effect of CRCoV antibodies against both CRCoV  
335 infection and clinical signs of CIRDC in general. Whilst evidence of CRCoV in pet dogs has been reported  
336 previously (Mochizuki et al., 2008; Priestnall et al., 2006) this is the first study to link infection to clinical  
337 disease in this group.

338 Compared to CRCoV similar overall levels of CnPnV seropositivity (41.7%), but higher detection rates  
339 (23.4%) of CnPnV were observed and are consistent with published data (Mitchell et al., 2013b). Antibodies  
340 to CnPnV were detected in dogs from all three sources, demonstrating the susceptibility of both pet and  
341 kennelled dogs to this virus. Although shelter dogs and dogs from 'other sources' had significantly increased  
342 odds of being positive compared to pet dogs, the higher seroprevalence observed in dogs from 'other sources'  
343 is most likely due to the samples being derived predominantly from an outbreak of CIRDC in a single  
344 veterinary hospital, where, 96% of dogs were seropositive and 33% were positive for the virus.

345 Dogs were significantly more likely to be CnPnV seropositive if they were also seropositive for CRCoV (and  
346 vice versa) and *M. cynos*, suggesting frequent co-infection, or co-circulation of one or more of these  
347 pathogens. In further support of this, dogs were significantly more likely to be positive for CnPnV (by PCR)  
348 if they were also positive for CRCoV (by PCR). Although only 17 such cases were observed in this study,  
349 precluding further analysis, CIRDC is increasingly being considered as a complex infection where multiple  
350 agents act sequentially or synergistically to cause disease, and similar disease complexes in humans are the  
351 subject of several reviews Eg:(Bosch et al., 2013). It is likely therefore that co-infections with both the classic  
352 and novel agents of CIRDC will contribute the increased likelihood of disease onset, severity and duration  
353 of CIRDC. Given the limited amount work in the field that currently addresses this point however, this should  
354 be an important focus of future.

355 Dogs with increasing disease severity were significantly more likely to be positive for CnPnV, supporting a  
356 causal relationship with CIRDC (Glineur et al., 2013; Mitchell et al., 2013b). Following multivariable analysis  
357 no significant relationship between the presence of CnPnV antibodies and infection or disease occurrence  
358 was observed. This relationship was however significant at the univariable level consistent with other  
359 published data (Mitchell et al., 2013b), suggesting that whilst CnPnV specific antibodies may confer some  
360 degree of protection, this was confounded in this instance by the effects of other factors. That said, it is  
361 well documented that antibody mediated immunity for related human and bovine respiratory syncytial  
362 viruses is poor, and individuals may be repeatedly re-infected throughout their lifetime with clinical signs  
363 of respiratory disease (Falsey, 2007; Hall et al., 1976; Van der Poel et al., 1993). A number of key  
364 pneumovirus features enable them to evade and modify host immune responses as reviewed by (Collins and  
365 Melero, 2011), and as such it would be vital to assess the genetic and antigenic diversity of circulating  
366 CnPnV strains to better understand CnPnV ecology and inform vaccine design and development.

367 Although studies are limited, *M. cynos* also represents a potentially important agent in the development  
368 and persistence of CIRDC (Chalker et al., 2004; Rosendal, 1972). This is the first study to examine the  
369 seroprevalence of *M. cynos*, and overall 45% of dogs were seropositive, comparable to that of CRCoV and  
370 CnPnV. In multivariable analysis increased odds of *M. cynos* seropositivity were seen in shelter dogs  
371 (compared to pet dogs), and convalescent (compared to clinically unaffected) dogs and with age.

372 Whilst the seropositivity suggests exposure, very few dogs with *M. cynos* itself (n=5) were detected. Of  
373 those that were, three had severe respiratory signs but two were clinically unaffected. Given the limited  
374 number of positive cases in this study it is not possible to comment on the prevalence of *M. cynos* or its  
375 relationship with clinical disease. However it is worth considering the limitations of the study which may  
376 have influenced this finding. Firstly, several mycoplasma species are carried by dogs, often as part of their  
377 normal respiratory flora (Chalker, 2005), however very limited data is available regarding their serological  
378 cross-reactivity (Rosendal, 1975). The possibility exists therefore that the use of whole cell ELISA antigen in  
379 this study may have resulted in an overestimate in *M. cynos* seropositivity. Secondly, oropharyngeal swabs

380 collected as part of a standard veterinary investigation of CIRDC were analysed. Previously respiratory tissues  
381 were shown to yield a much higher recovery rate of *M. cynos* (99.7-23.9%) compared to oropharyngeal  
382 swabs (0-0.9%) (Chalker et al., 2004), and indeed studies that have implicated *M. cynos* in CIRDC were based  
383 on tissue sampling, particularly of the lower respiratory tract (Hong and Kim, 2012; Zeugswetter et al.,  
384 2007), the level of *M. cynos* detected in this study may therefore have been vastly underestimated and  
385 highlights an important consideration for the study of *M. cynos* in living dogs.

386 Influenza A (H3N8) caused significant respiratory disease in dogs in north America (Anderson et al., 2013;  
387 Crawford et al., 2005) with an estimated seroprevalence of 49% in high risk populations (Anderson et al.,  
388 2013). Subsequent studies from North and Central America and Asia have also identified a number of other  
389 influenza A subtypes which infect and cause disease in dogs, some of which have undergone cross-species  
390 transmission, including possible transmission between dogs and humans (Lin et al., 2012; Song et al., 2008;  
391 Song et al., 2012; Songserm et al., 2006). Assays were therefore selected on the basis that they would allow  
392 for the broad detection of Influenza A rather than specific subtypes (Damiani et al., 2012; Ellis and Zambon,  
393 2001). In agreement with other recently published European studies (Damiani et al., 2012; Dundon et al.,  
394 2010) however very little evidence of influenza A was found in this study. All of the dogs were negative for  
395 Influenza A by PCR, and only 2.7% had evidence of exposure via antibody detection. Whilst influenza A  
396 appears relatively insignificant in the European dog population at present, given its rapid spread across  
397 north America and Asia, the increasing number of influenza A subtypes detected in dogs, and the increased  
398 movement of dogs into and across Europe, it may be only be a matter of time before it makes its  
399 appearance. Moreover, the co-existence of multiple types of influenza in dogs, with the potential to cross-  
400 species, should underline the importance of continued vigilance by both the veterinary and medical  
401 communities.

402 Our current understanding of the true prevalence and complexity of CIRDC is limited by a lack of  
403 comprehensive investigations into causative agents and associated risk factors. This study, which has begun  
404 to unpick some of these key areas, is one of the largest studies undertaken in the field to date. Findings  
405 highlight the need for a far greater consideration of pet dogs and other dog populations, the role of current  
406 vaccine formulations and strategies in preventing and managing disease outbreaks, and the impact of  
407 classic and newly identified pathogens on disease onset, development and persistence. For pathogens such  
408 as CRCoV and CnPnV, the emerging data provide a clearly identifiable link with CIRDC. However continued  
409 effort is required to characterise the epidemiology and pathogenesis of more elusive pathogens such as *M.*  
410 *cynos*, to ensure that they are not underestimated or overlooked.

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520 Table 1: Study population and sample distribution

Variable	Seropositive								Pathogen detected				
			CRCoV		CnPnV		<i>M. cynos</i>		CRCoV		CnPnV		
	Dogs		(n=525)				(n=559)				(n=555)		
	n	%	n	%	n	%	n	%	n	%	n	%	
<b>Total*</b>	<b>572</b>	<b>100</b>	<b>247</b>	<b>47</b>	<b>219</b>	<b>41.7</b>	<b>236</b>	<b>45</b>	<b>43</b>	<b>7.7</b>	<b>130</b>	<b>23.4</b>	
Source	Client	261	45.6	86	36.7	51	21.8	92	39.3	20	7.9	46	18.3
	Shelter	264	46.2	139	55.6	137	54.8	137	54.8	21	8.1	73	28.2
	Other	47	8.2	22	53.7	31	75.6	7	17	2	4.4	11	24.4
Clinical Group	Unaffected	138	24.1	73	55.3	67	50.8	55	41.7	8	5.9	28	20.6
	Acute	282	49.3	109	42.9	90	35.4	97	38.2	27	9.8	60	22.2
	Convalescent	152	26.6	65	46.8	62	44.6	84	60.4	8	5.4	42	28.2
Country	Italy	130	22.7	37	31.9	25	21.5	38	32.8	4	3.1	22	16.9
	Greece	112	19.6	37	38.5	26	27.1	50	52.1	9	8.6	23	21.9
	Hungary	109	19	38	39.2	42	43.3	50	51.5	8	7.4	34	32.4
	France	101	17.7	70	72.2	68	70.1	60	61.9	11	11.5	24	25.3
	Spain	61	10.7	39	63.9	23	37.7	26	42.6	3	4.9	9	14.8
	Netherlands	59	10.3	26	44.8	35	60.3	12	20.7	8	13.6	18	30.5
Severity Score	1 (healthy)	191	33.4	108	59.3	100	55	87	47.8	11	5.9	42	22.5
	2 (mild-moderate)	317	55.4	119	41.3	102	35.4	121	42	22	7	67	21.5
	3 (severe)	64	11.2	20	36.4	17	30.9	28	51	10	17	21	37.5
Disease	N	191	33.4	108	59.3	100	54.9	87	47.8	11	5.9	42	22.5
	Y	381	66.6	139	40.5	119	34.7	149	43.4	32	8.6	88	23.9
Vaccination	CDV (n=403)	N	72	17.9	-	-	-	-	-	-	-	-	-
		Y	331	82.1	-	-	-	-	-	-	-	-	-
	CAv (n=405)	N	87	21.5	-	-	-	-	-	-	-	-	-
		Y	318	78.5	-	-	-	-	-	-	-	-	-
	CPIV (n=381)	N	149	39.1	-	-	-	-	-	-	-	-	-
		Y	232	60.9	-	-	-	-	-	-	-	-	-
	Bb (n=307)	N	241	78.5	-	-	-	-	-	-	-	-	-
		Y	66	21.5	-	-	-	-	-	-	-	-	-

522 **Table 2: Final multivariable model for presence of clinical disease (N=406).**

Variable		OR	<i>p</i>	95% CI	
Source	Client	Ref	-	-	-
	Shelter	0.1	<0.001	0.06	0.19
	Other	0.22	<0.001	0.1	0.45
Vaccination (CPIV, CAV-2, CDV)	N	Ref	-	-	-
	Y	0.3	0.002	0.15	0.66

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547 **Table 3: Final multivariable models for pathogen seropositivity.**

Pathogen	Variable	OR	<i>p</i>	95% CI		
CRCoV (N=496)	Country	Italy	<i>Ref</i>			
		Greece	1.6	0.126	0.87	2.97
		Hungary	1.4	0.331	0.73	2.51
		France	4.8	<0.001	2.34	9.69
		Spain	4.1	<0.001	2.06	8.34
		Netherlands	1.5	0.289	0.72	3.00
	CnPnV IgG	Negative	<i>Ref</i>			
		Positive	2.7	<0.001	1.77	4.00
	Age		1.1	0.001	1.04	1.17
	CnPnV (N=525)	Country	Italy	<i>Ref</i>		
Greece			1.4	0.331	0.71	2.81
Hungary			1.6	0.151	0.83	3.17
France			3.4	0.001	1.67	6.93
Spain			1.7	0.192	0.75	3.92
Netherlands			3.7	0.002	1.64	8.22
Source		Client	<i>Ref</i>			
		Shelter	2.8	<0.001	1.61	4.87
		Other	8.0	<0.001	3.33	19.26
CRCoV IgG		Negative	<i>Ref</i>			
		Positive	2.7	<0.001	1.79	4.08
<i>M. cynos</i> IgG		Negative	<i>Ref</i>			
		Positive	1.8	0.005	1.20	2.76
<i>M. cynos</i> (N=496)	Country	Italy	<i>Ref</i>			
		Greece	2.3	0.008	1.23	4.16
		Hungary	1.6	0.171	0.82	3.03
		France	2.5	0.012	1.22	5.27
		Spain	2.2	0.028	1.08	4.46
		Netherlands	0.7	0.334	0.29	1.51
	Source	Client	<i>Ref</i>			
		Shelter	1.8	0.024	1.08	3.15
		Other	0.5	0.126	0.18	1.23
	Clinical Group	Clinically unaffected	<i>Ref</i>			
		Acute	1.3	0.287	0.79	2.17
		Convalescent	2.8	<0.001	1.60	4.87
Age		1.07	0.024	1.00	1.13	

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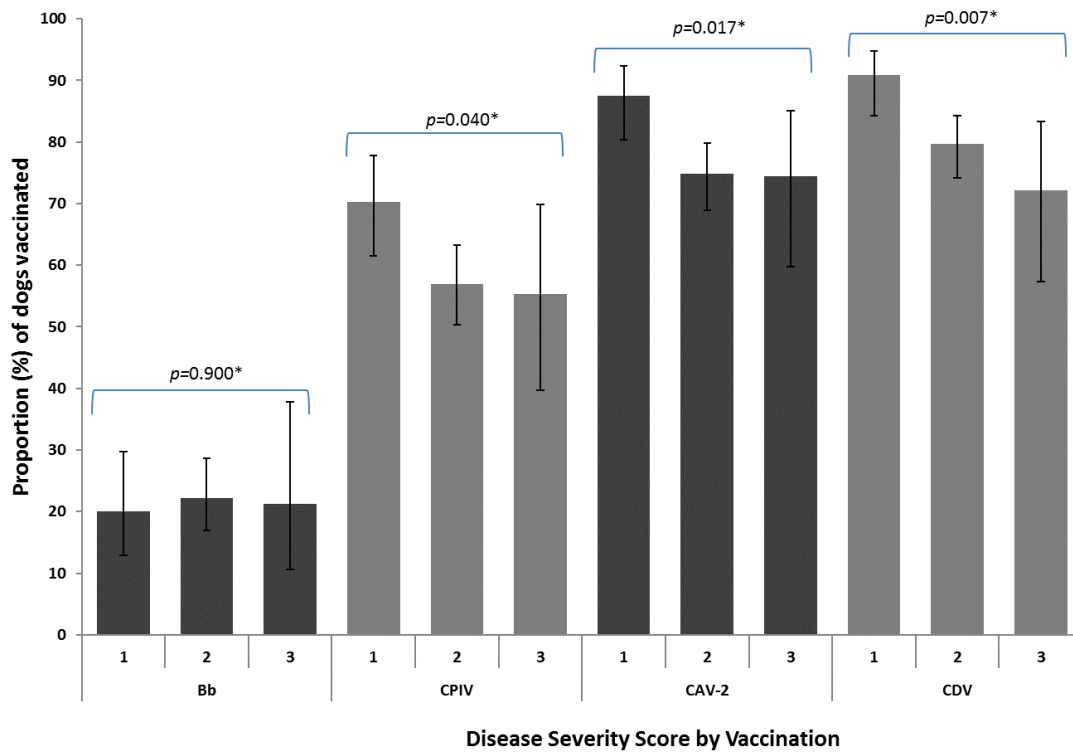
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555 **Table 4: Final multivariable models for pathogen detection.**

Pathogen	Variable	OR	<i>p value</i>	95% CI		
CReCoV (N=517)	Country	Italy	<i>Ref</i>			
		Greece	12.2	0.02	1.48	100.27
		Hungary	8.9	0.045	1.05	76.62
		France	28	0.002	3.32	236.08
		Spain	8.7	0.066	0.86	87.34
		Netherlands	22.5	0.004	2.71	187.35
	Severity Score	1	<i>Ref</i>			
		2	1.9	0.155	0.78	4.51
		3	3.5	0.029	1.13	10.59
	CReCoV IgG	Negative	<i>Ref</i>			
		Positive	0.4	0.015	0.17	0.82
	CnPnV (N=555)	Country	Italy	<i>Ref</i>		
Greece			1.3	0.409	0.68	2.55
Hungary			2.3	0.007	1.26	4.40
France			1.7	0.127	0.85	3.40
Spain			0.9	0.762	0.37	2.05
Netherlands			2.0	0.061	0.97	4.21
Severity Score		1	<i>Ref</i>			
		2	1.1	0.803	0.66	1.69
		3	2.1	0.029	1.07	4.20
CReCoV PCR		Negative	<i>Ref</i>			
		Positive	2.03	0.040	1.03	3.98

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570 **Figure 1: Proportion of vaccinated dogs with different clinical disease severity scores.**  
571 Vaccinations: (Bb) Boredetella bronchiseptica, (CPIV) canine parainfluenza, (CAV-2) canine adenovirus-2,  
572 (CDV) canine distemper virus. The disease severity score is indicted on the x-axis by 1) Healthy, 2) mild to  
573 moderate, and 3) Severe respiratory disease. Error bars show 95% confidence interval. \*Chi-squared/  
574 Fisher’s exact test *p-value*.

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