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European surveillance of emerging pathogens associated with canine 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 CIRD, 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 CIRD, 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 CIRD pathogens (CRCoV, CnPnV, CIV/
88 Influenza A and *M. cynos*) were estimated in both client-owned and kenneld 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 CIRD-affected dogs, (B) acute CIRD-affected dogs (0 to 3 days post onset of CIRD), or (C) convalescent
97 CIRD-affected dogs (10-12 days post onset of CIRD). 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_2. 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_2PO_4, 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_2SO_4. 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 CIRD-affected dogs, 49.3% (n=282) were acute CIRD-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 CIRD).
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 CIRD, 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 CIRD is
291 generally regarded as a disease of kennelled dogs as shown here, we have also provided substantial
292 evidence of CIRD 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 CIRD, 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 CIRD 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 CIRD 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 availaible
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 requirmemnts 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 CIRD-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 CIRD, 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 CIRD pathogens it is clear their role in CIRD 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 CIRD 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 CIRD 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 CIRD, 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 CIRD, as well as for the protective effect of CRCoV antibodies against both CRCoV
335 infection and clinical signs of CIRD 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 seroprevelce observed in dogs from 'other sources'
343 is most likely due to the samples being derived predominantly from an outbreak of CIRD 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, CIRD 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 CIRD will contribute the increased likelihood of disease onset, severity and duration
353 of CIRD. 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 CIRD (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 consistant 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 reviwed 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 CIRD (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 CIRD 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 CIRD 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 CIRD 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 CIRD. 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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416 ## References

417 Anderson, T.C., Crawford, P.C., Dubovi, E.J., Gibbs, E.P., Hernandez, J.A., 2013. Prevalence of and
418 exposure factors for seropositivity to H3N8 canine influenza virus in dogs with influenza-like
419 illness in the United States. J Am Vet Med Assoc 242, 209-216.
420 Appel, M.J., Binn, L.N., 1987. Canine infectious tracheobronchitis short review: kennel cough. In: Virus
421 infections of Carnivors

422 Elseiver Science Publishing Co, New York, 201-211 pp.
423 Appel, M.J., Percy, D.H., 1970. SV-5-like parainfluenza virus in dogs. J Am Vet Med Assoc 156, 1778-1781.
424 Bemis, D.A., 1992. Bordetella and Mycoplasma respiratory infections in dogs and cats. Vet Clin North Am
425 Small Anim Pract 22, 1173-1186.
426 Bosch, A.A., Biesbroek, G., Trzcinski, K., Sanders, E.A., Bogaert, D., 2013. Viral and bacterial interactions in
427 the upper respiratory tract. PLoS pathogens 9, e1003057.
428 Chalker, V.J., 2005. Canine mycoplasmas. Res Vet Sci 79, 1-8.
429 Chalker, V.J., Owen, W.M., Paterson, C., Barker, E., Brooks, H., Rycroft, A.N., Brownlie, J., 2004.
430 Mycoplasmas associated with canine infectious respiratory disease. Microbiology 150, 3491-
431 3497.
432 Collins, P.L., Melero, J.A., 2011. Progress in understanding and controlling respiratory syncytial virus: still
433 crazy after all these years. Virus Res 162, 80-99.
434 Crawford, P.C., Dubovi, E.J., Castleman, W.L., Stephenson, I., Gibbs, E.P., Chen, L., Smith, C., Hill, R.C.,
435 Ferro, P., Pompey, J., Bright, R.A., Medina, M.J., Johnson, C.M., Olsen, C.W., Cox, N.J., Klimov, A.I.,
436 Katz, J.M., Donis, R.O., 2005. Transmission of equine influenza virus to dogs. Science 310, 482-
437 485.
438 Damiani, A.M., Kalthoff, D., Beer, M., Muller, E., Osterrieder, N., 2012. Serological survey in dogs and cats
439 for influenza A(H1N1)pdm09 in Germany. Zoonoses and public health 59, 549-552.
440 Day, M.J., Horzinek, M.C., Schultz, R.D., Squires, R.A., Vaccination Guidelines Group of the World Small
441 Animal Veterinary, A., 2016. WSAVA Guidelines for the vaccination of dogs and cats. J Small Anim
442 Pract 57, E1-E45.
443 Decaro, N., Mari, V., Larocca, V., Losurdo, M., Lanave, G., Lucente, M.S., Corrente, M., Catella, C., Bo, S.,
444 Elia, G., Torre, G., Grandolfo, E., Martella, V., Buonavoglia, C., 2016. Molecular surveillance of
445 traditional and emerging pathogens associated with canine infectious respiratory disease. Vet
446 Microbiol 192, 21-25.
447 Ditchfield, J., Macpherson, L.W., Zbitnew, A., 1962. Association of Canine Adenovirus (Toronto A 26/61)
448 with an Outbreak of Laryngotracheitis ("Kennel Cough"): A Preliminary Report. Can Vet J 3, 238-
449 247.
450 Dundon, W.G., De Benedictis, P., Viale, E., Capua, I., 2010. Serologic evidence of pandemic (H1N1) 2009
451 infection in dogs, Italy. Emerg Infect Dis 16, 2019-2021.
452 Ellis, J.A., 2015. How well do vaccines for Bordetella bronchiseptica work in dogs? A critical review of the
453 literature 1977-2014. Vet J 204, 5-16.
454 Ellis, J.A., Krakowka, G.S., 2012. A review of canine parainfluenza virus infection in dogs. J Am Vet Med
455 Assoc 240, 273-284.
456 Ellis, J.S., Zambon, M.C., 2001. Combined PCR-heteroduplex mobility assay for detection and
457 differentiation of influenza A viruses from different animal species. J Clin Microbiol 39, 4097-
458 4102.
459 Erles, K., Dubovi, E.J., Brooks, H.W., Brownlie, J., 2004. Longitudinal study of viruses associated with
460 canine infectious respiratory disease. J Clin Microbiol 42, 4524-4529.
461 Erles, K., Toomey, C., Brooks, H.W., Brownlie, J., 2003. Detection of a group 2 coronavirus in dogs with
462 canine infectious respiratory disease. Virology 310, 216-223.
463 Falsey, A.R., 2007. Respiratory syncytial virus infection in adults. Seminars in respiratory and critical care
464 medicine 28, 171-181.
465 Glineur, S.F., Renshaw, R.W., Percopo, C.M., Dyer, K.D., Dubovi, E.J., Domachowske, J.B., Rosenberg, H.F.,
466 2013. Novel pneumoviruses (PnVs): Evolution and inflammatory pathology. Virology 443, 257-
467 264.
468 Hall, C.B., Geiman, J.M., Biggar, R., Kotok, D.I., Hogan, P.M., Douglas, G.R., Jr., 1976. Respiratory syncytial
469 virus infections within families. N Engl J Med 294, 414-419.

470 Hong, S., Kim, O., 2012. Molecular identification of Mycoplasma cynos from laboratory beagle dogs with
471 respiratory disease. Laboratory animal research 28, 61-66.
472 Kobayashi, H., Yamamoto, K., Eguchi, M., Kubo, M., Nakagami, S., Wakisaka, S., Kaizuka, M., Ishii, H.,
473 1995. Rapid detection of mycoplasma contamination in cell cultures by enzymatic detection of
474 polymerase chain reaction (PCR) products. J Vet Med Sci 57, 769-771.
475 Lin, D., Sun, S., Du, L., Ma, J., Fan, L., Pu, J., Sun, Y., Zhao, J., Sun, H., Liu, J., 2012. Natural and
476 experimental infection of dogs with pandemic H1N1/2009 influenza virus. J Gen Virol 93, 119-
477 123.
478 Mitchell, J.A., Brooks, H.W., Szladovits, B., Erles, K., Gibbons, R., Shields, S., Brownlie, J., 2013a. Tropism
479 and pathological findings associated with canine respiratory coronavirus (CRCoV). Vet Microbiol
480 162, 582-594.
481 Mitchell, J.A., Brownlie, J., 2015. The challenges in developing effective canine infectious respiratory
482 disease vaccines. The Journal of pharmacy and pharmacology 67, 372-381.
483 Mitchell, J.A., Cardwell, J.M., Renshaw, R.W., Dubovi, E.J., Brownlie, J., 2013b. Detection of canine
484 pneumovirus in dogs with canine infectious respiratory disease. J Clin Microbiol 51, 4112-4119.
485 Mochizuki, M., Yachi, A., Ohshima, T., Ohuchi, A., Ishida, T., 2008. Etiologic study of upper respiratory
486 infections of household dogs. J Vet Med Sci 70, 563-569.
487 Pratelli, A., Colao, V., 2014. A population prevalence study on influenza infection in dogs in Southern Italy.
488 New Microbiol 37, 277-283.
489 Priestnall, S.L., Brownlie, J., Dubovi, E.J., Erles, K., 2006. Serological prevalence of canine respiratory
490 coronavirus. Vet Microbiol 115, 43-53.
491 Priestnall, S.L., Mitchell, J.A., Walker, C.A., Erles, K., Brownlie, J., 2014. New and emerging pathogens in
492 canine infectious respiratory disease. Vet Pathol 51, 492-504.
493 Renshaw, R.W., Zylich, N.C., Laverack, M.A., Glaser, A.L., Dubovi, E.J., 2010. Pneumovirus in dogs with
494 acute respiratory disease. Emerg Infect Dis 16, 993-995.
495 Rosendal, S., 1972. Mycoplasmas as a possible cause of enzootic pneumonia in dogs. Acta veterinaria
496 Scandinavica 13, 137-139.
497 Rosendal, S., 1975. Canine mycoplasmas: serological studies of type and reference strains, with a
498 proposal for the new species, Mycoplasma opalescens. Acta pathologica et microbiologica
499 Scandinavica. Section B, Microbiology 83, 463-470.
500 Song, D., Kang, B., Lee, C., Jung, K., Ha, G., Kang, D., Park, S., Park, B., Oh, J., 2008. Transmission of avian
501 influenza virus (H3N2) to dogs. Emerg Infect Dis 14, 741-746.
502 Song, D., Moon, H.J., An, D.J., Jeoung, H.Y., Kim, H., Yeom, M.J., Hong, M., Nam, J.H., Park, S.J., Park, B.K.,
503 Oh, J.S., Song, M., Webster, R.G., Kim, J.K., Kang, B.K., 2012. A novel reassortant canine H3N1
504 influenza virus between pandemic H1N1 and canine H3N2 influenza viruses in Korea. J Gen Virol
505 93, 551-554.
506 Songserm, T., Amonsin, A., Jam-on, R., Sae-Heng, N., Pariyothorn, N., Payungporn, S., Theamboonlers, A.,
507 Chutinimitkul, S., Thanawongnuwech, R., Poovorawan, Y., 2006. Fatal avian influenza A H5N1 in a
508 dog. Emerg Infect Dis 12, 1744-1747.
509 Van der Poel, W.H., Kramps, J.A., Middel, W.G., Van Oirschot, J.T., Brand, A., 1993. Dynamics of bovine
510 respiratory syncytial virus infections: a longitudinal epidemiological study in dairy herds. Arch
511 Virol 133, 309-321.
512 Zeugswetter, F., Weissenbock, H., Shibly, S., Hassan, J., Spergser, J., 2007. Lethal bronchopneumonia
513 caused by Mycoplasma cynos in a litter of golden retriever puppies. Vet Rec 161, 626-627.

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520 Table 1: Study population and sample distribution

Variable					Seropositive						Pathogen detected			
					CRCoV		CnPnV		*M. cynos*		CRCoV		CnPnV	
			Dogs				(n=525)				(n=559)		(n=555)	
			n	%	n	%	n	%	n	%	n	%	n	%
	Total*		**572**	**100**	**247**	**47**	**219**	**41.7**	**236**	**45**	**43**	**7.7**	**130**	**23.4**
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	-	-	-	-	-	-	-	-	-	-
	CAdV (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	-	-	-	-	-	-	-	-	-	-

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

Variable		OR	*p*	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	*p*	95% CI	
CRCoV (N=496)	**Country**	**Italy**	*Ref*			
		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**	*Ref*			
		Positive	2.7	<0.001	1.77	4.00
	Age		1.1	0.001	1.04	1.17
CnPnV (N=525)	**Country**	**Italy**	*Ref*			
		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**	*Ref*			
		Shelter	2.8	<0.001	1.61	4.87
		Other	8.0	<0.001	3.33	19.26
	CRCoV IgG	**Negative**	*Ref*			
		Positive	2.7	<0.001	1.79	4.08
	M. cynos IgG	**Negative**	*Ref*			
		Positive	1.8	0.005	1.20	2.76
***M. cynos* (N=496)**	**Country**	**Italy**	*Ref*			
		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**	*Ref*			
		Shelter	1.8	0.024	1.08	3.15
		Other	0.5	0.126	0.18	1.23
	Clinical Group	**Clinically unaffected**	*Ref*			
		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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555 **Table 4: Final multivariable models for pathogen detection.**

Pathogen	Variable		OR	*p value*	95% CI	
CRCoV (N=517)	Country	Italy	*Ref*			
		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	*Ref*			
		2	1.9	0.155	0.78	4.51
		3	3.5	0.029	1.13	10.59
	CRCoV IgG	Negative	*Ref*			
		Positive	0.4	0.015	0.17	0.82
CnPnV (N=555)	Country	Italy	*Ref*			
		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	*Ref*			
		2	1.1	0.803	0.66	1.69
		3	2.1	0.029	1.07	4.20
	CRCoV PCR	Negative	*Ref*			
		Positive	2.03	0.040	1.03	3.98

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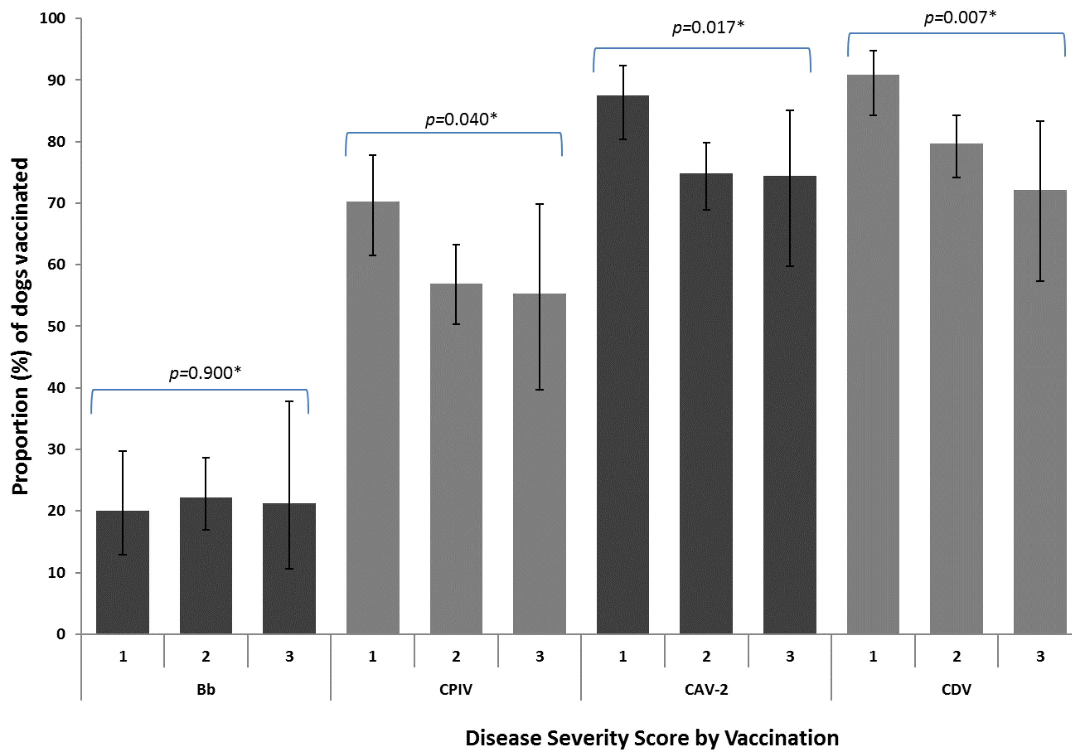

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570 **Figure 1: Proportion of vaccinated dogs with different clinical disease severity scores.**
571 Vaccinations: (Bb) Boredetella bronchiseptica, (CPIV) canine parainfluenza, (CAV-2) canine adenovirus-2,
572 (CDV) canine distemper virus. The disease 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*.

575