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Western European epidemiological survey for parvovirus and coronavirus infections in dogs

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ABSTRACT

An epidemiological survey for canine parvovirus (CPV) and canine coronavirus (CCoV) infections was conducted in Western Europe. A total of 156 faecal samples were collected from dogs with diarrhoea in Spain ($n = 47$), Italy ($n = 39$), France ($n = 26$), Germany ($n = 21$), the United Kingdom ($n = 8$), Belgium ($n = 10$), and the Netherlands ($n = 5$). Using molecular assays for virus detection and characterisation, CPV and CCoV were found to be widespread in European dog populations, either alone or in mixed infections. In agreement with previous reports, the original type CPV-2 was shown not to circulate in European dogs. The recently identified virus variant CPV-2c was predominant in Italy and Germany and present at high rates in Spain and France but was not detected in the UK or Belgium. Except for the UK, CCoV genotype I was identified in all European countries involved in the survey, albeit at a lower prevalence rates than CCoV genotype II.

Introduction

Canine parvovirus type 2 (CPV-2) and canine coronavirus (CCoV) are considered the main pathogens responsible for acute gastroenteritis in dogs (Greene and Decaro, in press). After its first emergence in the late 1970s, CPV-2 underwent a rapid evolution and the original type was replaced in the field by two antigenic variants, CPV-2a and CPV-2b, which suddenly spread to the canine population worldwide (Truyen, 2006). A third variant, CPV-2c, was identified in 2000 in Italy (Buonavoglia et al., 2001) and was subsequently found in other European countries, as well as in Asia, America and Africa (Greene and Decaro, in press).

Clinical signs of CPV infections include haemorrhagic diarrhoea, vomiting, fever, lymphopenia and sometimes death (Greene and Decaro, in press). In-house tests are available for in-practice diagnosis of CPV infection, but those methods have been shown to be poorly sensitive when compared to methods based on nucleic acid amplification (Desario et al., 2005; Schmitz et al., 2009). In addition, ELISA-based assays can detect all CPV variants without remarkable differences (Decaro et al., 2010). Increasing concerns have been expressed on the actual efficacy of vaccines based on the old CPV-2 type against field strains, chiefly against the new variant 2c (Pratelli et al., 2001a; Cavalli et al., 2008; Decaro et al., 2008), as several CPV outbreaks are being observed worldwide in dogs vaccinated regularly (Decaro et al., 2008, 2009; Calderon et al., 2009).

Whereas CPV can cause a severe, often fatal, disease, CCoV is generally recognised as an aetiological agent of mild, self-limiting enteritis followed by rapid recovery (Decaro and Buonavoglia, 2008). However, hypervirulent CCoV strains have been identified and are associated with haemorrhagic gastroenteritis and death, as well as with systemic disease under natural and experimental conditions (Decaro and Buonavoglia, 2008).

Two different genotypes of CCoV are known, namely type I (CCoV-I) and II (CCoV-II), which are often detected in mixed infections (Decaro et al., 2005b). In the genome of CCoV-I an additional open reading frame (ORF), ORF3, is present. Remnants of ORF3 have been detected in CCoV-II and in the closely related transmissible gastroenteritis virus of swine (TGEV), suggesting that CCoV-I may be an ancestor of both CCoV-II and TGEV (Lorusso et al., 2008).

In this study, an epidemiological survey for CPV and CCoV in several Western European countries was conducted and the viruses were characterised at the molecular level in order to assess the relative prevalence of the various CPV and CCoV types.

Materials and methods

Sample collection and preparation

An epidemiological survey for CPV and CCoV was conducted in Spain, Italy, France, Germany, the United Kingdom, Belgium and the Netherlands during the period January 2008–April 2009. Specimens were collected by practitioners from

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dogs with acute gastroenteritis housed in rescue and rehoming shelters and commercial breeding colonies, or presented to veterinary clinics located in different areas of the same country (Table 1). Each veterinarian participating to the survey was provided with kit boxes, shipping materials and a guidance booklet, including submission forms for collection of full anamnesis (age, breed, sex, clinical signs and vaccination status). The inclusion criteria were (1) occurrence of severe gastroenteritis especially in rescue shelters or large breeding kennels, and (2) shipping of frozen samples following the cool chain by using the provided dispensable packaging materials.

Specimens were homogenised (10% w/v) in Dulbecco's modified Eagle's medium (DMEM) and subsequently clarified by centrifuging at 2500 *g* for 10 min. Viral DNA was extracted from the supernatants of faecal homogenates by boiling for 10 min and chilling on ice. This extraction method does not appear to alter viral DNA (Desario et al., 2005). To reduce residual inhibitors of DNA polymerase activity to ineffective concentrations, the DNA extract was diluted 1:10 in distilled water (Decaro et al., 2006b). The clarified faecal suspension (140 μL) was also used for RNA extraction using the QIAamp Viral RNA Mini Kit (Qiagen), following the manufacturer's protocol and the RNA templates were stored at −70 °C until used.

Table 1
Summary of the sampling sites and their geographical location.

Country	Site no.	Town	State/region/province	Number of samples
Spain	1	Lugo	Galicia	4
	2	La Cartuja Baja	Aragòn	4
	3	Sevilla	Andalucia	6
	4	Dos Hermanas	Andalucia	4
	5	Barcelona	Catalonia	5
	6	Matarò	Catalonia	3
	7	Matarò	Catalonia	5
	8	Madrid	Madrid	2
	9	Madrid	Madrid	1
	10	Mejorada del Campo	Madrid	8
	11	Bilbao	Basque country	5
	Total			47
Italy	1	Turin	Piedmont	2
	2	Turin	Piedmont	4
	3	Genoa	Liguria	1
	4	Voghera	Lombardy	2
	5	Codogno	Lombardy	2
	6	Madignano	Lombardy	1
	7	Cordenons	Friuli Venezia-Giulia	2
	8	Casirate d'Adda	Lombardy	5
	9	Este	Veneto	2
	10	Lastra a Signa	Tuscany	4
	11	Jesi	Marche	1
	12	Macerata	Marche	2
	13	Roma	Lazio	1
	14	Follonica	Lazio	4
	15	Palma Campania	Campania	2
	16	Pomigliano d'Arco	Campania	2
	17	Bari	Apulia	2
	Total			39
France	1	Bayeux	Basse-Normandie	4
	2	Pleyben	Bretagne	3
	3	Saint-Méen-le-Grand	Bretagne	1
	4	La Mothe-Saint-Héray	Poitou–Charentes	1
	5	Le Blanc	Centre	1
	6	Saint-Jean-de-Monts	Pays de la Loire	1
	7	Flers-en-Escrebieux	Nord-Pas-de-Calais	1
	8	Aix-en-Provence	Provence-Alpes-Côte d'Azur	5
	9	Ba-Mauco	Aquitaine	6
	10	Saint-Médard-en-Jalles	Aquitaine	2
	11	Le Bourget-du-Lac	Rhône-Alpes	1
	Total			26
Germany	1	Berlin	Berlin	1
	2	Berlin	Berlin	2
	3	Berlin	Berlin	4
	4	Königs Wusterhausen	Land Brandenburg	9
	5	Erfurt	Freistaat Thüringen	1
	6	Kevelaer	Nordrhein-Westfalen	1
	7	Hannover	Land Niedersachsen	2
	8	Erfurt	Freistaat Thüringen	
	Total			21
The United Kingdom	1	Merstow Green	West Midlands	1
	2	London	Greater London	2
	3	London	Greater London	5
	Total			8
Belgium	1	Aalst	Flanders	1
	2	Varsenare	Flanders	4
	3	Koersel	Flanders	5
	Total			10
The Netherlands	1	Zeist	Utrecht	1
	2	Ermelo	Gelderland	4
	Total			5

CPV detection and characterisation

Detection of CPV DNA was obtained by real-time PCR using a conventional TaqMan probe targeting the VP2 gene (Decaro et al., 2005a). Real-time PCR was carried out in a 25 μL reaction volume containing 12.5 μL of IGTM supermix (Bio-Rad), 600 nM of primers CPV-Forward (AAACAGGAATTAACTATACTAATATATTTA) and CPV-Reverse (AAATTTGACCATTTGGATAAACT), 200 nM of probe CPV-Pb (FAM-TGGTCCTTTA-ACTGCATTAAATAATGTACC-BHQ1) and 10 μL of the extracted DNA. Serial 10-fold dilutions (representing from 10^9 to 10^2 DNA copies/10 μL of standard DNA) of a plasmid containing the nearly full length genome of CPV-2 were used to generate a standard curve. Duplicates of CPV-2 standard dilutions and sample DNA templates were analysed concomitantly by real-time PCR in the same conditions as reported.

Prediction of the virus type in CPV-positive samples was obtained by minor groove binder (MGB) real-time PCR assays specific for types 2a/2b and 2b/2c, based on single nucleotide polymorphisms in the VP2 gene of the different variants (Decaro et al., 2006b). The reactions were carried out in a total volume of 25 μL containing 10 μL of template or standard DNA, 12.5 μL of IGTM supermix (Bio-Rad), 900 nM of primers CPVa/b-Forward (AGGAAGATATCCAGAAGGAGATTGGA) and CPVa/b-Reverse (CCAATTGGATCTGTTGGTAGCAATACA) (type 2a/2b assays) or CPVb/c-Forward (GAAGATATCCAGAAGGAGATTGGATTCA) and CPVb/c-Reverse (ATGCAGTTAAAGGACCATAAGTATTAAATATATTAGTATAGTTAATTC) (type 2b/2c assays), 200 nM of probes CPVa-Pb (VIC-CTTCCTGTAACAAATGATA-MGB) and CPVb1-Pb (FAM-CTTCCTGTAACAGATGATA-MGB) (type 2a/2b assay) or CPVb2-Pb (FAM-CCTGTAACAGATGATAAT-MGB) and CPVc-Pb (VIC-CCTGTAACAGAAGATAAT-MGB) (type 2b/2c). To rule out the presence of CPV strains of vaccine origin, samples recognised as types 2/2a and 2b were subsequently tested by MGB probe assays that discriminate between vaccine and field strains of CPV (Decaro et al., 2006a,d).

CCoV detection and characterisation

All RNA extracts were subjected to a TaqMan-based real-time RT-PCR assay targeting the membrane protein gene for rapid detection of CCoV RNA (Decaro et al., 2004), with minor modifications. Briefly, a one-step method was adopted using Platinum Quantitative RT-PCR ThermoScript One-Step System (Invitrogen) and the following 50 μL mixture: 25 μL of master mix, 300 nM of primers CCoV-Forward (TTGATCGTTTTTATAACGGTTCTACAA) and CCoV-Reverse (AATGGGCCATAATAGC-CACATAAT), 200 nM of probe CCoV-Pb (FAM-ACCTCAATTTAGCTGGTTCGTGTATG-GCATT-BHQ1) and 10 μL of template RNA. Duplicates of $\log_{10}$ dilutions of standard RNA were analysed simultaneously to obtain a standard curve for absolute quantification (Decaro et al., 2004). The thermal profile consisted of reverse transcription at 50 °C for 20 min and activation of Platinum Taq DNA polymerase at 95 °C for 2 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 48 °C for 30 s and extension at 60 °C for 30 s.

The CCoV-positive samples were characterised by means of two distinct genotype-specific assays targeting the membrane protein gene (Decaro et al., 2005b). TaqMan-based real-time RT-PCRs were performed by using Platinum Quantitative RT-PCR ThermoScript One-Step System (Invitrogen) and the following oligonucleotide sets (final concentrations were 600 and 200 nM for primers and probes, respectively): primer pair CCoVI-F (CGTTAGTGCACTTGGAAGAAGCT)/CCoVI-R (AC-CAGC-CATTTTAAATCCTTCA) and probe CCoVI-Pb (FAM-CCTCTTGAAGGTACACCAA-TAMRA) for CCoV-I; primer pair CCoVII-F (TAGTGCATTAGGAAGAAGCT)/CCoVII-R (AGCAATTTTGAACCCTTC) and probe CCoVII-Pb (FAM-CCTCTTGAAGGTGTGCC-TAMRA) for CCoV-II. The thermal protocol was the same as that used for CCoV detection except for different annealing temperatures (53 °C and 48 °C for CCoV-I and CCoV-II, respectively).

Results

A total of 156 faecal samples meeting the inclusion criteria were collected in several countries, namely Spain ($n = 47$), Italy ($n = 39$), France ($n = 26$), Germany ($n = 21$), the UK ($n = 8$), Belgium ($n = 10$), and the Netherlands ($n = 5$) (Table 1). The age of the sampled dogs ranged from 4 weeks to 12 years (mean ± standard deviation (SD): 1.11 ± 2.46 years); 133 were pups aged under 3 months and 23 were dogs ⩾1 year of age. Seventy-seven dogs had either not been vaccinated or had not completed the full vaccination protocol. There were minimal differences in the vaccination protocols among the different countries. All vaccine formulations contained modified live CPV (either type 2 or 2b) strains and (when administered) killed CCoV strains. All the samples analysed were epidemiologically unrelated to each other, being representative of different disease outbreaks.

Seventy-six faecal samples (48.7%, 95% confidence interval [CI] 40.9–56.5%) tested positive for CPV, including 13/47 Spanish (27.7%, CI 14.9–40.5%), 21/39 Italian (53.8%, CI 38.2–69.4%), 16/26 French (61.5%, CI 42.8–80.2%), 15/21 German (71.4%, CI 52.1–90.7%), 7/8 British (87.5%), 4/10 Belgian (40.0%), and 0/5 Dutch (0.0%) specimens. Distribution of the CPV types varied based on the country of origin of the samples (Fig. 1a). The variant CPV-2c was predominant in Spain (9/13 CPV-positive samples) and widespread in France (7/16) and Germany (7/15), where types 2b and 2a were detected at higher frequency. In Italy, 5/21 positive samples were found to contain CPV-2c strains, with type 2a being prevalent. All the CPV strains detected in the UK ($n = 7$) were characterised as type 2b, whereas all the Belgian CPV strains ($n = 4$) were type 2a.

By TaqMan real-time RT-PCR, CCoV was detected in 60/156 tested samples (38.5%, CI 30.9–46.1%) with higher detection rates in Italy (20/39, 51.3%, CI 35.6–67.0%), Belgium (8/10, 80.0%) and the Netherlands (3/5, 60.0%) (Fig. 1b). By using genotype-specific assays, CCoV-I was detected less frequently (24/60 samples) than CCoV-II (56/60 samples). Simultaneous infections by both CCoV genotypes were identified in 20 specimens. A single sample tested positive for CCoV in the UK and was characterised as CCoV-II. CPV and CCoV were found in mixed infections in 28/156 samples (17.9%, CI 11.9–23.9%), including 4 Spanish (8.5%), 12 Italian (30.8%), 1 French (3.8%), 6 German (28.6%), 1 British, (12.5%) and 4 Belgian (40.0%) specimens.

Discussion

CPV and CCoV are the most common canine enteric pathogens worldwide and, in recent years, both viruses have given rise to new genotypes or variants. A new CPV variant, type 2c, emerged around 2000 (Buonavoglia et al., 2001) and is now circulating worldwide along with the former CPV types 2a and 2b (Greene and Decaro, in press). Likewise, CCoV-I was only detected in 2003 (Pratelli et al., 2003).

Except for a large European survey on the distribution of CPV types in 2006–2007 (Decaro et al., 2007b), only studies describing the molecular epidemiology at a national or regional level have been published in Europe (Decaro et al., 2005b; Benetka et al., 2006; Davies, 2008; Vieira et al., 2008). Based on those studies, CPV-2c appeared to be widespread in Italy, Germany (Decaro et al., 2007b), and Portugal (Decaro et al., 2007b; Vieira et al., 2008), whereas it was virtually absent in the UK and Belgium, where CPV types 2b and 2a were predominant, respectively (Decaro et al., 2007b; Davies, 2008). In the present study, CPV-2c was shown to be still common in Italy and Germany, albeit less frequent than CPV-2a, suggesting a temporal fluctuation (Decaro et al., 2007b). In contrast, the new variant CPV-2c was found to be predominant in Spain and frequent in France.

Although cross-protection between CPV-2 and its variants has been demonstrated (Spibey et al., 2008), some concerns have been raised that antigenic differences may have decreased the effectiveness of the old vaccines that are based on the original type CPV-2, a virus no longer circulating in the field (Pratelli et al., 2001a; Cavalli et al., 2008; Decaro et al., 2008). Based on these concerns, vaccines containing CPV-2b strains have been developed and licensed.

Limited data have been gathered on CCoV molecular epidemiology. Both CCoV genotypes have been found to be widespread in Italy and Austria with CCoV-I being more common (Decaro et al., 2005b; Benetka et al., 2006). In contrast, in the current survey, whereas CCoV-I was detected in most European countries, it appeared to be far less common than CCoV-II. In the UK, CCoV-I was not detected, although only a limited number of samples were available. Based on studies from the early 1990s, it may be hypothesised that the actual prevalence of CCoV in the UK is much higher (Tennant et al., 1991, 1993). Simultaneous infections by CCoV

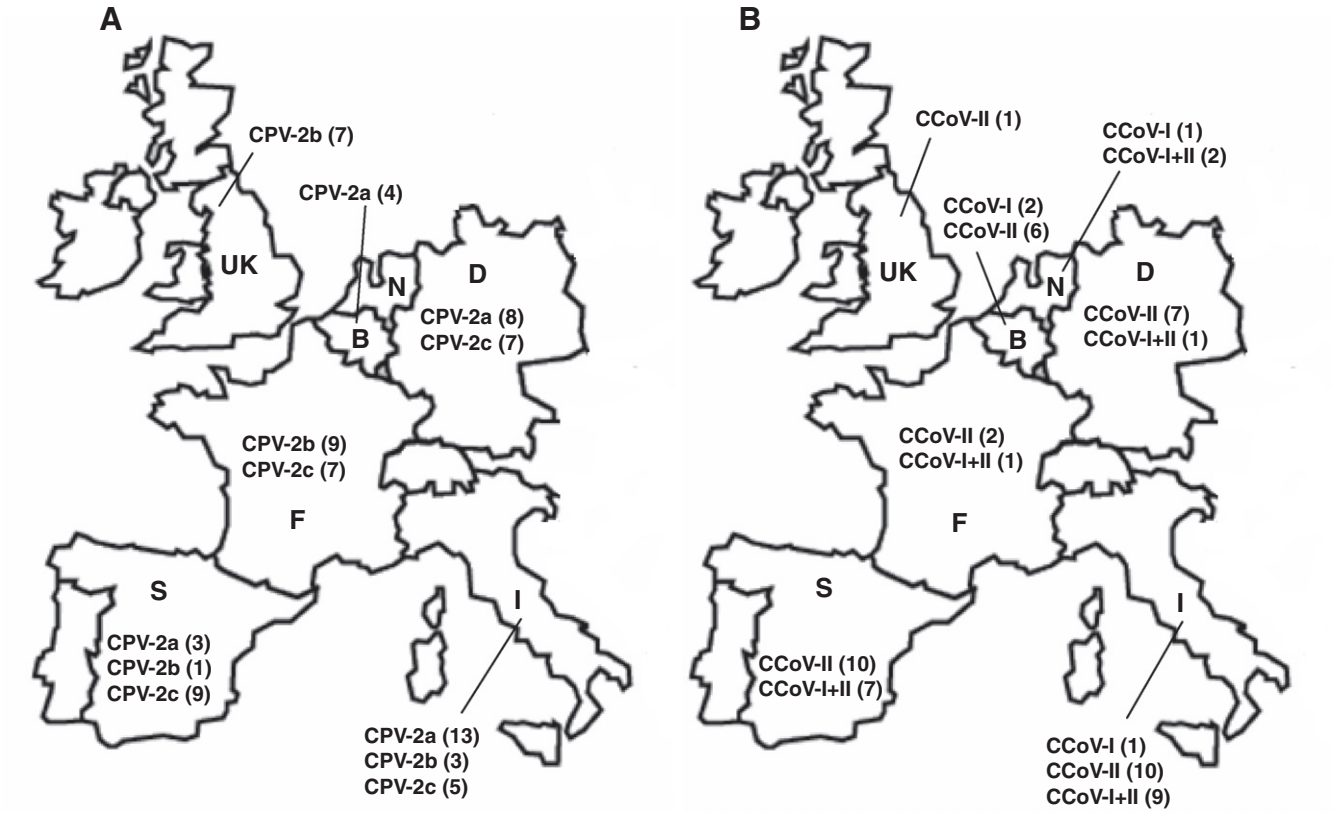

Fig. 1. Geographic distribution of the canine parvovirus (CPV) antigenic variants (A) and of the canine coronavirus (CCoV) genotypes (B) in Western Europe. Faecal specimens from different countries were analysed by molecular methods and the CPV variants or CCoV genotypes are indicated for each country by numbers in parentheses.

types I and II were detected, but at a lower frequency than in previous investigations, where up to 75% of mixed infections were documented (Decaro et al., 2005b).

An interesting finding was the presence of mixed CPV/CCoV infections in 28/156 samples. Except for the hypervirulent CCoV strains (Decaro and Buonavoglia, 2008), CCoV is usually regarded as a mild canine pathogen, since it can frequently be detected in dogs with no overt clinical signs (Schulz et al., 2008). However, CCoV may also cause severe diarrhoea and exacerbate diseases caused by other canine viruses, such as CPV (Pratelli et al., 1999; Decaro et al., 2006c) and canine adenovirus (Pratelli et al., 2001b; Decaro et al., 2007a). Although recording the complete anamnesis of diarrhoeic dogs was beyond the scope of the present study, it would have been of interest to assess whether mixed infections affected the severity and outcome of the disease. Inactivated vaccines are available in Europe and can be used to confer some protection against CCoV, thereby reducing the effects of mixed infections (Decaro and Buonavoglia, 2008). Since inactivated vaccines can protect dogs from CCoV-induced disease but not from infection, modified live virus vaccines administered oronasally have been proposed as a reliable alternative (Pratelli et al., 2004).

Our sampling procedure was planned to cover different areas of each country, but for some countries (UK, Belgium and The Netherlands), samples from only a few areas could be obtained, thus making our results not fully representative. Nevertheless, the presence of particular CPV variants and/or CCoV genotypes is reported here for the first time in some countries and this is relevant in terms of the potential efficacy of current vaccines. Since there were no remarkable differences in the vaccination protocols among the various countries, the differences in epidemiological findings are most likely related to different trading flows of the dogs imported from foreign countries rather than to vaccination protocols (Decaro et al., 2007b). For instance, it is known that there is a prosperous trade of purebred pups between Eastern and Western European countries, and this may lead to the introduction and spread in a naïve country of novel virus strains (Decaro et al., 2007a; Martella et al., 2007). However, it cannot be ruled out that some differences in the distribution of virus variants/genotypes are due to a sampling bias, at least for the countries where the number of samples was low.

Conclusions

Although the study lacks valid statistical support, it provides a snapshot of the epidemiological situation of CPV and CCoV infections in some Western European countries. This first CPV/CCoV combined epidemiological survey in Europe documents the presence and relative distribution of new viral genotypes (CCoV-I) and variants (CPV-2c) in several European countries.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisation that could inappropriately influence or bias the content of the paper.

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