Short communication

MT-PCR panel detection of canine parvovirus (CPV-2): Vaccine and wild-type CPV-2 can be difficult to differentiate in canine diagnostic fecal samples

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ABSTRACT

Canine parvovirus (CPV-2) remains an important cause of devastating enteritis in young dogs. It can be successfully prevented with live attenuated CPV-2 vaccines when given at the appropriate age and in the absence of maternal antibody interference. Rapid diagnosis of parvoviral enteritis in young dogs is essential to ensuring suitable barrier nursing protocols within veterinary hospitals. The current diagnostic trend is to use multiplexed PCR panels to detect an array of pathogens commonly responsible for diarrhea in dogs. The multiplexed PCR assays do not distinguish wild from vaccine CPV-2. They are highly sensitive and detect even a low level of virus shedding, such as those caused by the CPV-2 vaccine. The aim of this study was to identify the CPV-2 subtypes detected in diagnostic specimens and rule out occult shedding of CPV-2 vaccine strains. For a total of 21 samples that tested positive for CPV-2 in a small animal fecal pathogens diagnostic multiplexed tandem PCR (MT-PCR) panel during 2014–2016 we partially characterized the VP2 gene of CPV-2. Vaccine CPV-2 strain, wild type CPV-2a subtypes and vaccine-like CPV-2b subtypes were detected. High copy number was indicative of wild-type CPV-2a presence, but presence of vaccine-like CPV-2b had a variable copy number in fecal samples. A yardstick approach to a copy number or C_t–value to discriminate vaccine strain from a wild type virus of CPV-2 can be, in some cases, potentially misleading. Therefore, discriminating vaccine strain from a wild type subtype of CPV-2 remains ambitious.

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Canine parvovirus (CPV-2) causes a devastating enteritis and subsequent diarrhea in young and immunonaive dogs, due to viral tropism to rapidly multiplying cells, such as enterocytes in the intestinal crypts [3,11]. Successful vaccination programs using live attenuated CPV-2 vaccines exist, but controversies arise between dog owners, veterinarians and vaccine companies when diarrhea occurs within a few days of CPV-2 vaccine administration [4]. Reversion of the vaccine virus to a virulent CPV-2 virus has not been documented and is considered unlikely [3]. It has been postulated that clinical signs of canine parvovirus a few days after CPV-2 vaccination result from a different disease, or from a wild virulent CPV-2 subtypes incubating at the time of vaccination [3]. Diagnosing parvoviral enteritis in dogs is essential to ensuring suitable barrier nursing protocols within veterinary hospitals. The current diagnostic trend is to use multiplexed PCR panels to detect an array of pathogens that commonly cause diarrhea in dogs [6]. The multiplexed PCR assays do not distinguish wild from vaccine CPV-2. They are highly sensitive and detect even a low level of virus shedding, such as those caused by the CPV-2 vaccine [6]. Interpretation of test results is based on quantitation of the viral load (quantitative PCR) and clinical presentation including commonly found panleucopenia on peripheral blood. In fatal cases structural evidence can be utilized to complement qPCR and hematology to confirm parvoviral enteritis, however the aim is accurate antemortem diagnosis that rapidly differentiates vaccine associated

viral presence from wild-type parvoviral enteritis.

Canine parvovirus (CPV-2) emerged as a canine pathogen in the late 1970s. In the 1980s, two antigenic variants emerged (CPV-2a and CPV-2b) and eventually completely replaced the original CPV-2 [9]. CPV-2 is still used in many commercial vaccines with some more recently replacing this with CPV-2b. Differentiation of CPV-2 viruses is based on viral capsid protein sequence (VP2). The original CPV-2 differs in five or six amino acid residues from CPV-2a and CPV-2b [2,3], and there are only two amino acid residues differentiating CPV-2a from CPV-2b [11]. A more recently discovered strain (CPV-2c) is defined by a further single nucleotide change [3].

The aim of this study was to identify the CPV-2 subtypes detected in diagnostic specimens presented to The Veterinary Pathology Diagnostic Service at the University of Sydney. Diagnostic dilemmas for clinicians not uncommonly include the possibility of occult shedding of CPV-2 vaccine strains in diarrhea of other etiology, and the possibility of vaccine breakdown in the face of emerging CPV-2 subtypes.

A small animal fecal pathogens diagnostic multiplexed tandem PCR (MT-PCR) panel (Cat. No. 38175 Version 1 through Version 3, AusDiagnostics, Sydney, Australia) was implemented at the University of Sydney Veterinary Pathology Diagnostic Services from 2014. The MT-PCR incorporates a multiplexed initial amplification and a subsequent single-target amplification, using SYBR Green chemistry in a real-time PCR to detect DNA of parvovirus (CPV-2), as well as *Salmonella, Campylobacter, Giardia, Cryptosporidium, Dientamoeba, Blastocystis, Toxoplasma,* feline coronavirus, *Tritrichomonas,* canine distemper virus, and canine coronavirus and is used for both canine and feline fecal samples [10]. The MT-PCR panel includes amplification of host target DNA to evaluate sample adequacy, and each processed sample is spiked with proprietary DNA and tested for its presence to assess PCR inhibition in the test specimens (http://www.ausdiagnostics.com/, AusDiagnostics Pty. Ltd., Beaconsfield, NSW 2015, Australia). For each fecal sample, 250 mg of feces were first homogenized and disrupted in a FastPrep-24 Homogenisation System equipped with QuickPrep Adapter (MP Biomedicals, Australia) at a speed setting of 6.0 m/s for 40 s prior to DNA isolation. Genomic DNA was then isolated using PowerMag® Microbiome RNA/DNA Isolation Kit (magnetic separation) (Mo-Bio Laboratories; GeneWorks, Thebarton, Australia) optimised for KingFisher® Duo (Thermo-Fisher Scientific, Scoresby, Australia).

A total of 21 samples that tested positive for CPV-2 in the MT-PCR small animal fecal pathogens diagnostic panel during 2014—2016 were included in this study (Table 1; Supplementary Table 1). Using the latest Version 3 of the MT-PCR panel (see above), 76% (16/21) were CPV-2 positive (Table 1). To evaluate if the CPV-2 DNA originated from the vaccine or wild type parvovirus, we partially characterized the VP2 gene of CPV-2 from 43% (9/21) of clinical specimens (eight of which were MT-PCR V3 positive for CPV-2). Two overlapping sets of primers HFor/HRev (residues 3556—4166; **M38245**) and 555For/555Rev (residues 4003—4585; **M38245**) were used, as previously published [2]. Review of CPV-2 genome sequences available in GenBank revealed poor conservation of 555Rev primer site, therefore an alternative primer JS-2 (residues 4799—4818; **M38245**) [8] was used in combination with 555For or Hfor as an alternative combination, improving amplification success for 3 samples previously negative using 555For/ 555Rev primers (Table 2). The amplified region included the diagnostic residues 297, 300, 305, 426 and 555 for typing of CPV-2, CPV-2a, CPV-2b and CPV-2c [3]. The conventional PCR included MyTaq Red MasterMix (BioLine, Sydney, Australia) and were run on a Verity PCR cycler (Thermo Fisher Scientific, Australia). To match the obtained sequences to vaccine strains of CPV-2, we amplified VP2 from two frequently used live attenuated vaccines (CPV-2b strain, Protech C4, Boehringer Ingelheim, Australia; CPV-2, Canigen DH$_{A2}$P, Virbac, Australia). All direct DNA sequencing with the amplification primers in both directions was done by Macrogen (Seoul, Korea) using amplification primers.

Vaccine strain CPV-2 (Canigen) was detected in four samples, based on 100% match between the vaccine DNA sequence and DNA amplified from canine fecal samples (Table 2). Near identical DNA sequences to vaccine strain CPV-2b (Protech) were detected in three dog feces (Table 2, Fig. 1). The CPV-2b vaccine strain DNA had a single mismatch (nucleotide position 4710, **M38245**) in the non-coding region of the viral DNA from the canine CPV-2b DNA sequences (15—2425, 15—2378, 16—3871; Fig. 1A). This mismatch was only recognized with 555For/JS-2 primers, regions amplified with

Table 1
Small animal fecal pathogens diagnostic multiplexed tandem PCR (MT-PCR) panel during 2014—2016 testing positive for CPV-2.

ID	Breed	Age	Weight (kg)	Outcome	Spike	CPV-2	Sal	Cam	Giar
14—1644	Samoyed	2 months	3.1	Dead	14.40	11.21	24.97		
14—6993	Bullmastiff X	4 years	12.2	Recovered	14.97	24.35	25.18		
14—7931	Miniature Poodle	13 years	4.4	Recovered	15.69	25.14			
15—2378	English Cocker Spaniel	4 months	6.8	Sent home[a]	14.51	<10			19.04
15—2425	Pug X	2 months	0.4	Sent home[a]	16.73	18.87			19.86
15—2429	Daschshund X	2 months	N/A	Healthy	15.95	25.55		11.83	
15—3568	Labradoodle	2 months	N/A	Healthy	14.77	-ˆ		18.36	
15—3702	Staffordshire Bull Terrier	2 years	N/A	N/A	15.04	-ˆ			
15—4390	Greyhound	11 years	N/A	N/A	15.26	-ˆ		18.56	
15—4399	Unknown	Unknown	N/A	N/A	16.31	-ˆ			
15—4401	Staffordshire Bull Terrier	6 months	N/A	N/A	15.33	23.67	24.75	21.68	
15—5505	Unknown	Unknown	N/A	N/A	14.61			25.04	
15—6583	Unknown	Unknown	N/A	N/A	13.19	22.12		24.87	
15—6585	Unknown	Unknown	N/A	N/A	13.15	25.10	24.39	17.80	
15—6588	Unknown	Unknown	N/A	N/A	14.67	20.34			
16—0873	Miniature Pinscher X	9 months	4.0	Euthanasia	13.95	25.24			
16—3871	Golden Retriever	8 months	35.0	Recovered	14.65	17.91		24.93	24.70
16—4759	Miniature Dachshund	10 months	5.6	Recovered	12.96	23.57		19.84	
16—6027	Maltese X	11 months	6.3	Recovered	13.76	25.04			
16—7855	Kelpie X	2 months	7.4	Sent home[a]	14.66	<10			
16—9394	Staffordshire Bull Terrier X	8 months	N/A	Sent home[a]	14.25	23.72	17.14		

-ˆ Tested positive with previous version of the diagnostic kit, but was negative with Version 3.
Abbreviations: *Sal — Salmonella, Cam — Campylobacter, Giar - Giardia.*
 [a] No further information available.

Table 2
Summary of PCR amplification of CPV-2 from canine feces.

ID	Amplification primers				Outcome
	555f/555r	Hfor/Hrev	555f/JS2R	Hfor/JS2	
14−1644	Negative	Positive	Positive	n/d	wild CPV-2a
14−6993	Negative	Negative	n/d	n/d	
14−7931	Negative	Negative	n/d	n/d	
15−2378	Positive	Positive	Positive	Positive	vaccine-like/wild CPV-2b
15−2425	Negative	Positive	Positive	Negative	vaccine-like CPV-2b
15−2429	Negative	Negative	Negative	n/d	
15−3568	Positive[a]	Negative	Negative	Negative	vaccine CPV-2
15−3702	Negative	Negative	Negative	n/d	
15−4390	Negative	Negative	Negative	n/d	
15−4399	Positive	Negative	Negative	Negative	vaccine CPV-2
15−4401	Negative	Negative	Negative	n/d	
15−5505	Negative	Negative	n/d	n/d	
15−6583	Positive	Positive	Positive	n/d	vaccine CPV-2
15−6585	Positive	Positive	Positive	n/d	vaccine CPV-2
15−6588	Negative	Negative	Negative	n/d	
16−0873	Negative	Negative	Negative	n/d	
16−3871	Negative	Positive	Positive	Negative	vaccine-like CPV-2b
16−4759	Negative	Negative	Negative	n/d	
16−6027	Negative	Negative	Negative	n/d	
16−7855	Positive	Positive	Positive	n/d	wild CPV-2a
16−9394	Negative	Negative	Negative	Negative	

[a] Faint PCR product, DNA sequence confirmed; repeated amplifications negative.
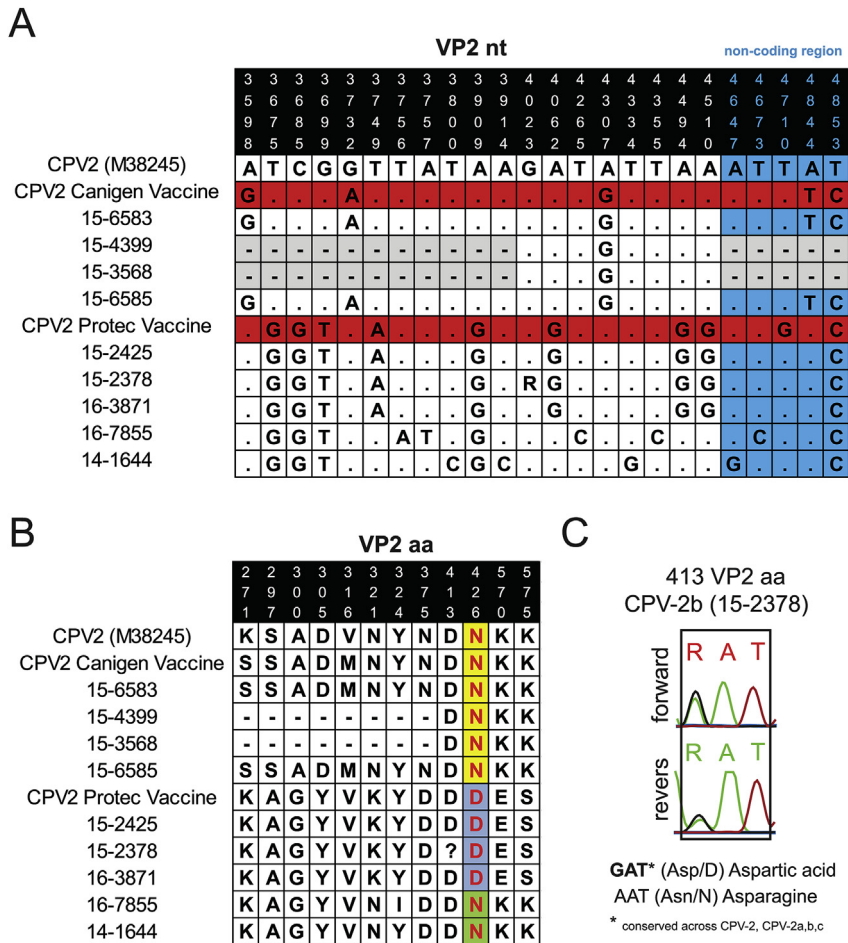

Fig. 1. Multiple sequence alignments of CPV-2 with reference strain CPV-2 (M38245). (A) Nucleotide alignment (nt) indicating VP2 gene region and non-coding region, identical residues as dots (.). (B) Translated amino acid alignment. (C) DNA sequence chromatograph indicating double peek at residue 4023 changing the amino acid from Asp > Asn.

HFor/HRev and 555Fro/555Rev were identical with CPV-2b vaccine strain DNA.

Sequence from two samples was consistent with CPV-2a sequence, considered to be a wild subtype (Fig. 1AB). Nucleotide sequence was typical for CPV-2a, including (A > G at 4062) nucleotide changes coding for amino acid changes Asn-426 > Asp differentiating type 2a from type 2b [3]. The CPV-2a subtypes were previously reported to be the most prevalent in dog populations in Australia in 2007 [8]. The small scale of the current cohort does not imply the absence of CPV-2b or CPV-2c in Australia, subtypes that are now dominant in many other countries. As high as 1450 canine parvovirus disease cases are self-reported by veterinarians in Australia annually, through Disease WatchDog, a national disease surveillance program [7,12,13]. Investigation targeting recently identified rural areas with increased prevalence of canine parvovirus by Ref. [13]; coupled with genotyping of the CPV-2 subtypes, would facilitate improved understanding of the epidemiology and evolution of individual subtypes under Australian conditions. New nucleotide sequences were deposited in GenBank under the following accession numbers: **KY242639-KY242647**.

Closer inspection of the sequence from a sample (15–2378) suspected to contain vaccine-like CPV-2b, revealed a variable nucleotide 4023 (M38245) (Fig. 1AC). The animal was vaccinated with CPV-2b strain (Boehringer Ingelheim vaccine) one week prior to the clinical presentation of vomiting and diarrhea. The nucleotide is typically a conserved guanidine in vaccine strains but, in this case, it included overlapping guanidine and adenine residues (Fig. 1C). The remaining VP2 sequence matched the CPV-2b (ex Protec vaccine), suggesting presence of vaccine strain with superinfection of a wild CPV-2b or a mutated vaccine strain. This animal (15–2378) was a 4 month old English Cocker Spaniel with concurrent giardiasis. The SNAP Canine Parvovirus Antigen Test Kit (IDEXX, Australia) returned a positive result at the time of sampling. MT-PCR returned a very low (<10) take off time (equivalent of low C_t-value) suggesting a large number of viral particles being shed in the patient's feces, unlike the low level shedding of vaccine CPV-2 strains in other dogs (Table 1, Table 2, Supplementary Table 1). The dog was released to home care and it is not known whether it represented a biosecurity issue for other dogs. The detected mutation is not reported to cause any change in virulence or strain type [1]. Further monitoring and evaluation of CPV-2 sequences is required to elucidate the frequency and origin of the observed mutation.

In the MT-PCR assay, low take-off times (<15 cycles) typically suggest presence of a wild CPV-2 subtype as these are typically shed in greater numbers than vaccine strains. In both cases of CPV-2a as well as the above mentioned case (15–2378), the take-off time was very early, providing an initial indication from MT-PCR with the confirmatory evidence of presence of wild-type canine parvovirus in the sample (via conventional PCR and DNA sequencing). Parvovirus is detected frequently in PCR panels, with 6.8% of 526 diarrheic dogs from Australia testing positive for CPV-2 using IDEXX Laboratories proprietary RealPCR™ service platform [6]. suggested that a cutoff value of 1.2 million CPV-2 gene equivalents per gram of stool discriminates vaccine strains from wild type CPV-2 infections. The load itself is a valuable outcome as it enables veterinarians to put appropriate quarantine measures in place to contain the spread of the highly resilient virus. On the other hand, higher take-off times were indicative of presence of

vaccine strain, because vaccine virus is able to replicate in the dog's intestinal mucosa and shed into the feces [4,5].

Discriminating vaccine strain from a wild type viruses of CPV-2 is ambitious in urgent diagnostics, as previously argued [4]. In this study, we have typed CPV-2 detected in canine feces by MT-PCR panel, using partial VP2 amplification and DNA sequencing. The approach has demonstrated some limitations to the commonly used assay, in particular with the application of the 555Rev primer. A yardstick approach to a copy number or C_t-value to discriminate vaccine strain from a wild type viruses of CPV-2 can be, in some cases, potentially misleading.

Acknowledgements

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mcp.2017.02.007

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