Analysis of canine parvoviruses circulating in Australia reveals predominance of variant 2b strains and identifies feline parvovirus-like mutations in the capsid proteins

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Abstract

Canine parvovirus (CPV) is a major enteric pathogen of dogs worldwide that emerged in the late 1970s from a feline parvovirus (FPV)-like ancestral virus. Shortly after its emergence, variant CPVs were generated by acquiring amino-acid (aa) mutations in key capsid residues, associated with biological and/or antigenic changes. This study aimed to identify CPV variants amongst Australian dogs, to gain insights into the evolution of CPV in Australia through phylogenetic analysis of these variants, and to investigate relationships between the disease and vaccination status of dogs from which isolates were collected. CPV VP2 sequences were amplified from 79 faecal samples collected from dogs with parvoviral enteritis at 20 veterinary practices in 5 Australian states. The median age at diagnosis was 4 months (range 1 to 96 months). Only 3.7% of dogs with vaccination histories had completed recommended vaccination schedules, while 49% were incompletely vaccinated and 47.2% were unvaccinated. For the first time, CPV-2b has emerged as the dominant antigenic CPV variant circulating in dogs with parvoviral enteritis in Australia, comprising 54.4% of strains, while CPV-2a and CPV-2 comprised 43.1% and 2.5% of strains. CPV-2c strains were not identified. Analysis of translated VP2 sequences revealed a vast repertoire of aa mutations. Several Australian CPV strains displayed signatures in the VP2 protein typical of Asian CPVs, suggestion introduction of CPV strains from Asia, and/or CPV circulation between Asia and Australia. Strains of CPV were identified containing aa residues typical of FPV at capsid (VP2) key positions, representing reverse mutations or residual mutations retained from CPV-2 during adaptation from an FPV-like ancestor, suggesting that evolutionary intermediates between CPV-2 and FPV are circulating in the field. Similarly, intermediates between CPV-2a-like viruses and CPV-2 were also identified. These findings help inform a better understanding of the evolution of CPV in dogs.

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Running title: FPV-like capsid mutations in CPVs from Australian dogs

Summary

Canine parvovirus (CPV) is a major enteric pathogen of dogs worldwide that emerged in the late 1970s from a feline parvovirus (FPV)-like ancestral virus. Shortly after its emergence, variant CPVs acquired amino-acid (aa) mutations in key capsid residues, associated with biological and/or antigenic changes. This study aimed to identify and analyse CPV variants and their capsid mutations amongst Australian dogs, to gain insights into the evolution of CPV in Australia and to investigate relationships between the disease and vaccination status of dogs from which isolates were collected.

CPV VP2 sequences were amplified from 79 faecal samples collected from dogs with parvoviral enteritis at 20 veterinary practices in 5 Australian states. The median age at diagnosis was 4 months (range 1 to 96 months). Only 3.7% of dogs with vaccination histories had completed recommended vaccination schedules, while 49% were incompletely vaccinated and 47.2% were unvaccinated. For the first time, CPV-2b has emerged as the dominant antigenic CPV variant circulating in dogs with parvoviral enteritis in Australia, comprising 54.4% of strains, while CPV-2a and CPV-2 comprised 43.1% and 2.5% of strains. CPV-2c strains were not identified. Analysis of translated VP2 sequences revealed a vast repertoire of aa mutations. Several Australian CPV strains displayed signatures in the VP2 protein typical of Asian CPVs, suggestion introduction of CPV strains from Asia, and/or CPV circulation between Asia and Australia.

Strains of CPV were identified containing aa residues typical of FPV at capsid (VP2) key positions, representing reverse mutations or residual mutations retained from CPV-2 during adaptation from an FPV-like ancestor, suggesting that evolutionary intermediates between CPV-2 and FPV are circulating in the field. Similarly, intermediates between CPV-2a-like viruses and CPV-2 were also identified. These findings help inform a better understanding of the evolution of CPV in dogs.

Keywords: Canine parvovirus; Carnivore protoparvovirus; Feline parvovirus; virus evolution

Introduction

Canine parvovirus (CPV), a *Carnivore Protoparvovirus 1* virus in the *Parvoviridae* family, is a non-enveloped, single-stranded DNA virus, that is one of the most common enteric viral pathogens of domestic dogs (Hoelzer & Parrish, 2010; D. Wang, Yuan, Davis, & Parrish, 1998). The 5.2 kb genome contains two major open reading frames, ORF1 and ORF2, which encode non-structural proteins NS1 and NS2, and viral proteins VP1 and VP2, respectively (Tsao et al., 1991).

When CPV emerged in 1978 as a new virus infecting dogs it caused a severe disease panzootic among domestic and wild dogs (*Canis familiaris*) with clinical signs including fever, vomiting and haemorrhagic diarrhoea, as well as myocarditis in young puppies (N. Decaro & Buonavoglia, 2012; Hoelzer & Parrish, 2010). The virus was named CPV-2 to distinguish it from Canine bocaparvovirus (also known as minute virus of canines or CPV-1), the first parvovirus discovered in dogs (Binn, Lazar, Eddy, & Kajima, 1970). Within a few years, CPV-2 was replaced in the field by a new antigenic variant with greater fitness in dogs called CPV-2a (426-Asp), while two other variants CPV-2b (426-Asn) and CPV-2c (426-Gly) emerged in 1984 (Parrish et al., 1991) and 1996 (Decaro et al., 2007), respectively. These three variants differ from CPV-2 at five VP2 amino acid (aa) residues (Met-87-Leu, Ile-101-Thr, Ala-300-Gly, Asp-305-Tyr and Asn-375-Asp) and from each other by a single aa substitution at position 426 (Parrish et al., 1988; Shackelton, Parrish, Truyen, Holmes, & Berns, 2005; Truyen, Evermann, Vieler, & Parrish, 1996). While the 426 substitution makes each variant antigenically distinct, phylogenetically they lack monophyletic segregation and form a clade of "CPV-2a-like" viruses distinct from the original CPV-2 virus (Voorhees et al., 2019).

The relative frequencies of CPV antigenic variants differ between countries, because, although the emergence of CPV-2a was followed by global spread, there have been no global sweeps of CPV-2b or CPV-2c (Voorhees et al., 2019). Further, the distribution of variants is not static, as illustrated by molecular surveillance in Argentina, where CPV-2c was the predominant strain circulating in 2006, but in 2010 a novel CPV-2a strain emerged and rapidly became dominant (Maya et al., 2013).

In Australia, CPV was first detected in 1978 (Colin R. Parrish et al., 1988). Molecular surveillance carried out between 1980 and 2005 showed a clear predominance of CPV-2a, which comprised 95% of isolates from dogs with parvoviral enteritis (Meers, Kyaw-Tanner, Bensink, & Zwijnenberg, 2007). Molecular analysis of isolates from 2007 to 2016, demonstrated that CPV-2b strains comprised 46.5% of isolates, while CPV-2a remained predominant (53.5%) (Clark et al., 2017). In 2017, CPV-2c was reportedly detected for the first time in Australia, from three isolates collected in 2015 from domestic dogs with parvoviral enteritis (Woolford et al., 2017).

This study aimed to identify and analyse CPV variants and their VP2 capsid mutations amongst Australian dogs, to gain insights into the evolution of CPV in Australia and to investigate relationships between the disease and vaccination status of dogs from which isolates were collected.

Materials and methods

2.1 Sample and data collection

Residual faecal samples from dogs with parvoviral enteritis diagnosed using a point-of-care (POC) faecal antigen ELISA, or qPCR performed at a commercial laboratory (VetPath, Perth), were collected. Samples were collected between March 2015 and August 2019 from five of Australia's eight States and Territories (Figure 1). The patient's age, breed, sex and postcode of origin were recorded. Postcode was used to derive region of origin defined by the Australian Government (Department of Infrastructure, Transport, Cities and Regional Development, 2018). Vaccination data obtained from medical records were used to assign cases to one of four categories: unvaccinated, incompletely vaccinated, completely vaccinated or unknown vaccination status. Completely vaccinated was defined as a dog that had received a puppy vaccination series comprising of at least two modified live virus (MLV) CPV vaccinations one month apart, with the last vaccination administered no earlier than 16-weeks of age, followed by a booster vaccine between 6 to 12 months of age, and revaccination every three years. Dogs less than 16-weeks of age that had received one or more CPV vaccinations were considered incompletely vaccinated (Day, Horzinek, Schultz, & Squires, 2016). Data obtained from patient medical records were used to classify each case according to whether treatment was given (treated or untreated), and whether the dog recovered, died or was euthanised.

2.2 DNA extraction and VP2 sequencing

Viral DNA was extracted from faeces using a commercial kit (QIAmp Fast Stool Kit, Qiagen, Germany) according to the manufacturer's instructions, or homogenisation/boiling as described previously (Decaro et al., 2006). Supernatants obtained after homogenisation/boiling were diluted 1:10 and 1:20 with nuclease-free water to abolish PCR inhibition, and stored at 4°C prior to PCR testing, which was performed within 7 days of extraction. The complete VP2 region was amplified by conventional PCR using three overlapping primer pairs as previously described, with slight modification (Table 1) (Decaro et al., 2008). Each 50 µL PCR reaction contained MyTaq HS Red reaction buffer 1X (5mM dNTPs, 15 mM $MgCl_2$), 10 mM of each forward and reverse primer, 0.5 U of MyTaq Hot Start DNA polymerase (Bioline, Meridian Life Sciences, Australia) and 10 µL of DNA template. Initial denaturation was performed at 94°C for 1 min, followed by 35-39 cycles of denaturation at 94 °C for 30 s, annealing at 50 or 55 °C for 30 s, polymerisation at 72 °C for 30 s and final extension at 72 °C for 1 min. A negative control comprising PBS subjected to the DNA extraction process was included in each run, alongside a positive control sample comprising feline parvovirus (FPV) DNA. Sanger sequencing of PCR products was performed at a commercial laboratory (Macrogen Seoul, South Korea).

2.3 *Phylogenetic analysis*

Chromatographs were analysed and VP2 sequences were edited and assembled from the three contigs obtained by PCR using CLC Main Workbench (Qiagen). Assembled sequences were deposited in GenBank under accession numbers MN258986 - MN259065, MN528597-MN528598 and MN561318 - MN561321 (FAIRsharing.org). Sequences were aligned using the ClustalW algorithm in Geneious Prime (Biomatters Ltd., Auckland, New Zealand). Sequences were translated in MEGA X (Kumar, Stecher, Li, Knyaz, & Tamura, 2018), and aa substitutions compared to the ICTV reference sequence (CPV-N GenBank accession no. M19296) were identified. To determine the evolutionary history of the CPV sequences identified in this study, a phylogenetic analysis of the VP2 region of these sequences and representative sequences of FPV and CPV taken from GenBank was performed. This resulted in a total data set of 94 sequences, 1719 nt in length. Phylogenetic analyses of these was performed using MEGA X. Evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model with 1000 bootstrap replicates (Tamura, 1992). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood value. The GTR + I model of nucleotide substitution was used. Mink enteritis virus (MEV) was used as the outgroup to root the tree.

Results

Full-length CPV VP2 sequences were amplified from 79 faecal samples collected at 20 veterinary practices in 5 Australian states (Figure 1). The median age at diagnosis was 4 months (range 1 to 96 months). Of dogs for which complete vaccination data were available, 49.1% (26/53) were incompletely vaccinated, 47.2% (25/53) were unvaccinated, and 3.77% (2/53) were completely vaccinated. The two dogs that developed parvoviral enteritis despite being completely vaccinated were a 20-month-old female Cavoodle that developed parvoviral enteritis 8 months after its first annual booster vaccination, and a 3-year-old male Staffordshire Bull Terrier that developed disease 5 months after receiving an annual booster vaccination (Table 2).

There were 44 dogs for which outcomes were known, of which 52.3% recovered (23/44), 16 (36.4%) died despite treatment and 5 (11.4%) were euthanised without treatment. CPV VP2 sequence analysis detected CPV-2b (426D) in 43/79 (54.4%) samples, CPV-2a (426N) in 34/79 (43.1%) and CPV-2 in 2/79 (2.5%). The two isolates identified as CPV-2 were from dogs with unknown vaccination histories.

On phylogenetic analysis the 79 CPV strains fell into six clades (Figure 2). The largest clade (Figure 2, Clade 1) comprised CPV-2b viruses. In addition to 426-Asp, most viruses in Clade 1 contain one novel (5-Gly) and two well-recognised (267-Tyr and 297-Ala) non-synonymous mutations compared to "classical" strains of CPV-2 (e.g. strain "N" and strain "CPV-b"), which are typically characterised by 267-Phe, 297-Phe and

5-Ala (Table 3). The majority of Clade 1 viruses originate from dogs in adjacent rural regions in central west and far west NSW (Figure 1, Table 2). Clade 1 also contains several viruses from dogs from Western Australia, all of which had a novel mutation 262-Thr compared to 262-Ala in classical CPV-2.

Clade 2, the second largest clade is also the most heterogeneous, comprising 16 CPV-2a viruses, and 5 CPV-2b viruses (Figure 1) and contains viruses from all 5 States sampled in the study. In addition to the 5-Gly, 267-Tyr and 297-Tyr aa residues described for Clade 1 viruses, unique aa mutations 564-Asn and 568-Ala were present all but one of the viruses in this clade (Table 2).

Clade 3 contains most of the viruses sequenced from Victoria in 2015. All of the viruses in this clade contain both a novel (21-Ala) and a well-recognised (324-Ile) mutation compared to "classical" CPV-2 (324-Tyr, 21-Thr). With the exception of one CPV-2b (426-Asp) virus, this clade comprises only CPV-2a viruses (426-Asn).

Viruses in Clade 4, all collected in 2019, are characterised by a novel mutation (321-Lys) compared to "classical" CPV-2 (321-Asn). Three, from dogs in Northwest NSW have identical VP2 sequences to an attenuated CPV-2b vaccine strain ("SAH"), except for residue 570-Lys, which is glutamate in the vaccine strain. The dogs were from a large breeding establishment and two had been vaccinated with the same SAH strain, while one, an unvaccinated puppy, died at 7 weeks of age after an 8-day history of lethargy, dehydration, vomiting and haemorrhagic diarrhoea. Similarly, one VP2 sequence from a dog in Western Australia differed by only one aa (413-Asn) to the SAH vaccine strain (413-Asp) (Figure 1, Table 2). The vaccination history of this dog was unknown.

Clade 5 contains CPV-2a viruses collected from dogs in metropolitan Sydney between 2016 and 2018, which, with the exception of 297-Ala, did not harbour the 5-Gly, 21-Ala and 267-Tyr mutations commonly present in other Australian CPV-2a viruses. Clade 6 contained two CPV-2 VP2 sequences from dogs with unknown vaccination history.

Discussion

This study shows that CPV-2b has emerged for the first time as the dominant antigenic CPV variant circulating in dogs with parvoviral enteritis in Australia. This finding is consistent with an increasing selective pressure for CPV-2b in Australia that was identified by analysis of time-stamped CPV isolates in a previous study in which samples were collected from all Australian States and territories (Clark et al., 2017). Since our samples were largely collected in three States (NSW, Victoria and Western Australia) we compared strain prevalence in these States between the two studies and identified that CPV-2b predominance was not the result of sampling bias; between 2008 and 2016 CPV-2b comprised 44% of strains from NSW, Victoria and Western Australia (Clark et al., 2017), whereas between 2015 and 2019 CPV-2b comprised 57%.

It is surprising that CPV-2c was not identified among the VP2 sequences in this study. CPV-2c was identified in faecal samples from three dogs hospitalised with acute gastroenteritis in 2015 in Australia (Woolford, Crocker, Bobrowski, Baker, & Hemmatzadeh, 2017). However, testing of faecal samples obtained 1-2 days earlier from the same dogs in the laboratories of two of the authors did not confirm this and the viruses were characterized as CPV-2a or CPV-2b (data not shown). A possible explanation for this discrepancy is that the dogs were co-infected with multiple CPV variants, with a change in strain predominance over time. Alternatively, the PCR and sequencing methodologies used in the three laboratories may have preferentially amplified different variants.

In the Asia Pacific region, the CPV-2c variant is not widespread although it has become the dominant strain in Taiwan (Lin et al., 2017) and Vietnam (Hoang et al., 2019). By contrast, in the most recent molecular survey of 70 CPV isolates collected in New Zealand, one of Australia's closest neighbouring countries, CPV-2c was not identified (Ohneiser, Hills, Cave, Passmore, & Dunowska, 2015).

Some of the CPV strains we identified had VP2 sequences that were intermediate between CPV and FPV (Figure 3). Although CPV and FPV share >99% nucleotide identity they have specific host ranges, antigenic and haemagglutination properties, which are controlled by the capsid VP2 gene (Chang, Sgro, & Parrish, 1992; Shackelton et al., 2005; Truyen, Agbandje, & Parrish, 1994). Subsequent to adaptations of an FPV-like ancestral virus, CPV emerged as a new virus in dogs in the late 1970s, although a specific evolutionary pathway has not been identified (Allison et al., 2016). On phylogenetic analysis, most of the viruses in Clade 2 in this study contained two FPV-defining residues, 564-Asn and 568-Ala (Figure 1 and Table 2). CPV differs from FPV at 7 VP2 residues and substitutions at three of these (564 (Asn to Ser), 568 (Ala to Gly) and 80 (Lys to Arg)) are associated with loss of replication ability of CPV-2 in cats, as determined using viruses recombinant between FPV and CPV-2 (U. Truyen et al., 1994). The 300 VP2 loop region (residues 299 to 301), structurally proximal to residues 80, 564 and 568, is a critical determinant of host range due to its interaction with the transferrin type I receptor (TfR) to mediate infection (Allison et al., 2016). Mutations in residues in close proximity to the 300 VP2 loop region can alter the efficiency of TfR binding and infection. One unique CPV-2b strain in our study, in Clade 2, contained another FPV-defining residue, 323-Asp (Table 2). VP2 aa residues 93 and 323 are critical in controlling CPV host range and a CPV-specific antigenic site on the capsid (Chang et al., 1992; Hueffer et al., 2003).

We also identified CPV-2a-like viruses that contained substitutions typical of CPV-2 in Clade 2, including four with 305-Asp and two with 87-Met (Figure 3). CPV-2a-like viruses isolated from dogs differ from CPV-2, at VP2 residues 87, 101, 300, and 305. The CPV-2a-specific residues 87-Leu and 101-Thr were likely acquired during evolution of the virus in raccoons, while the substitutions at 300 (Gly) and 305 (Tyr) were acquired when the virus transferred back to the canine host (Allison et al., 2012). Importantly, residues 87, 300, and 305 all lie within the binding footprint of the TfR, while residue 101 lies close to residue 87, just below the capsid surface (Hafenstein et al., 2007). The evolutionary trajectory between CPV-2 and CPV-2a-like viruses was likely facilitated by passage through an alternative host (Stucker et al., 2012). Another interesting finding of this study is that several Australian CPV strains displayed signatures in the VP2 protein that are typical of Asian CPVs, including 5-Gly, 267-Tyr, 324-Ile (Mira et al., 2018; Vannamahaxay et al., 2017; J. Wang et al., 2016), suggesting the introduction of CPV strains from Asia or, at least, for CPV circulation between Asia and Australia.

Our finding of intermediates between FPV and CPV-2 is unusual since intermediates have rarely been identified in molecular surveillance studies in the field. One exception was the detection of 568-Ala in a CPV-2a strain from Italy (Battilani et al., 2019), which was not accompanied by 564-Asn as seen here. The lack of identification of intermediates in other studies is likely due to the limited number of epidemiological investigations carried out before the 1990s, which restricts information on the possible geographical and temporal variations of CPV worldwide. The reversion of some VP2 aa residues to those of FPV, as identified here, could reflect alternate cycles of replication in dogs and cats. While there are no endemic wildlife *Felidae* in Australia, there are large populations of free-roaming feral cats (*Felis catus*). Alternatively, these changes could represent residues retained from CPV-2 intermediates during adaption from an FPV-like ancestor to CPV-2 rather than reverse mutations. We consider this explanation less likely since Australia does not harbor endemic wild *Felidae* or *Canidae* hosts (apart from a small population of dingoes (*Canis lupus dingo*)), thought to be required in viral evolution (Allison et al., 2012). Also, the strains with these "reverse" mutations were characterised by 297-Ala and 555-Val, both recent acquisitions among CPV-2a-like viruses (Meers et al., 2007). Finally, since recombination has been shown to play a role in the evolution of CPV (Shackelton et al., 2005), these findings could be the result of multiple evolutionary mechanisms, confounding the actual evolutionary patterns. Site-directed mutagenesis of CPV-2 virus to CPV-2b and vice versa has shown that some mutations in a given background are not well tolerated and markedly decrease virus fitness (Stucker et al., 2012). Accordingly, the discovery of intermediates with a vast repertoire of combinations of key mutations between FPV, CPV-2 and CPV-2a-like variants could inform a better understanding of the trajectory of evolution of CPV.

We identified two CPV-2 sequences, original type, from dogs without available vaccination history. These are most likely vaccine strains that were not the cause of enteritis in these dogs, since CPV-2 is no lon-

ger circulating in the field but attenuated vaccines containing this strain are used commonly in Australia. Reversion to virulence of the vaccine strains is theoretically possible but in the absence of corroborating evidence considered unlikely. Also, three dogs for which vaccination history was available, were infected with a CPV-2b strain closely related to a vaccine strain (SAH strain, accession no. FJ222822; Figure 1, Clade 4). All three dogs were from different litters from a breeding facility and had been vaccinated with the same CPV-2b SAH vaccine strain. The VP2 sequences of the virus from these dogs also had 100% nucleotide identity with two clinical isolates collected from dogs in the US in 2003 (AY742951) and 2009 (JN867605), for which accompanying vaccination data were not available (Allison et al., 2012; Shackelton et al., 2005). Viruses from these dogs differ from the SAH vaccine strain at one residue, 570-Lys, which is 570-Glu in the vaccine strain. Reversion to virulence of the vaccine strain in these cases is possible, or alternatively the CPV-2b strain in these cases may have been a vaccine mutant, but not the cause of disease. Another possible explanation is that these were wild-type viruses with a similar VP2 sequence to the vaccine virus.

Primary immunization failures (3.8%) among adult dogs that had completed a primary puppy vaccination course were rare, supporting the results of a previous Australian study where the failure rate was 3.3% (Ling, Norris, Kelman, & Ward, 2012). Although immunization failures can occur for many reasons including vaccine factors (e.g. improper storage or expiry) and various animal factors (e.g. immune-suppression due to disease or poor nutrition) (Roth & Spickler, 2010), the most likely cause of immunization failure of young adult dogs that have completed their primary vaccination course and been re-vaccinated is a genetic inability to synthesise antibodies against CPV, resulting in "non-responders" (Day et al., 2016). Some reports suggest that dogs infected with CPV-2c that have been vaccinated with a CPV-2 containing product, may be at higher risk of immunization failure compared to those receiving a CPV-2b vaccine (Decaro et al., 2008). If this is the case, then the rate of CPV immunization failures identified in Australia may not be broadly representative since only a single report exists of CPV-2c in Australia (Woolford et al., 2017). Commercial vaccines containing CPV-2c are not available. However, whether CPV-2 vaccines confer less protection *in vivo* than those containing CPV-2b, is controversial. It has been shown *in vitro* that dogs vaccinated with CPV-2 develop significantly lower antibody titres against CPV-2a-like viruses compared to antibody titres against CPV-2 (Cavalli et al., 2008). Whether the duration of immunity to CPV-2c is shorter in animals vaccinated with CPV-2 has not been investigated.

Almost half of the dogs with available vaccination histories were incompletely vaccinated. These dogs were likely permissive to infection due to interference of vaccine virus by maternally derived antibodies (MDA), since disease onset occurred before the completion of the primary vaccination series or in dogs completing the series before the age of 16 weeks. MDA in dogs generally persist until 13 to 15 weeks of age, and interfering titres of MDA have long been recognised as the most common cause of CPV immunisation failure (Decaro, Buonavoglia, & Barrs, 2020; Pollock & Carmichael, 1982).

The large proportion of dogs with parvoviral disease that were incompletely vaccinated in this study reflects the importance of final vaccination of puppies at 16-weeks of age or older to reduce immunization failure rate. However, almost half of the incompletely vaccinated dogs developed disease before completion of the primary vaccination series. Taken together, these findings indicate that strategies to overcome titres of MDA capable of interfering with vaccine protection in puppies are as important as increasing vaccination coverage overall to reduce the incidence of canine parvovirosis. Such strategies include measurement of MDA titres in puppies using haemagglutination assays to determine the optimal time for vaccination. Another tool, which has been shown to be at least partially effective in an experimental setting is the use of intranasal or oral MLV CPV vaccines (Cavalli et al., 2020; Martella et al., 2005).

In conclusion, passive surveillance between 2015 and 2019 shows that CPV-2b has emerged, for the first time, as the dominant antigenic CPV variant circulating in dogs with parvoviral enteritis in Australia. Disease was diagnosed in unvaccinated or incompletely vaccinated dogs in near equal proportions and immunization failures were uncommon. CPV-2c strains were not identified in this study. However, analysis of translated VP2 sequences revealed a vast repertoire of aa mutations. Strains of CPV that were intermediate between CPV and FPV, displayed reverse mutations, or, more likely, residual mutations retained from CPV-2 during

adaptation from an FPV-like viral ancestor. Similarly, intermediates between CPV-2a-like viruses and CPV-2 were also identified. These findings could be helpful to inform a better understanding of the evolution of CPV in dogs.

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Conflict of interest statement

No authors of this paper have financial or personal relationships with other contributors or organisations that could have inappropriately influenced the outcome of this study.

Ethics statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. No ethical approval was required as the samples used in this study were residual diagnostic samples.

Data availability statement The data that support the findings of this study are openly available in GenBank https://www.ncbi.nlm.nih.gov/genbank/ deposited under accession numbers MN258986 – MN259065, MN528597-MN528598 and MN561318-MN561321. Other data that support the findings of this study are available from the corresponding author upon reasonable request.

Table 1. Sequence, polarity and product size of PCR primers used for amplification of the VP2 region of Canine parvovirus (CPV)

Primer	Sequence 5' to 3'	Amplicon Length
CPV2679-F	AAAAAGAGACAATCTTGCACCA	853
CPV3511-R	TGAACATCATCTGGATCTGTACC	
CPV3381-F	CCATGGAAACCAACCATACC	717
CPV4116-R	AGTTAATTCCTGTTTTACCTCCAA	
555for	CAGGAAGATATCCAGAAGGA	583
555rev	GGTGCTAGTTGATATGTAATAAACA	

Table 2. Canine parvovirus antigenic variants and case data from dogs with parvoviral enteritis sampled in Australia between 2015 and 2019.

Genbank Accession no.	Sample No.	Collection Date	Age (mo)$^{++}$	Sex +	Breed	Region	Antigenic variant	Vaccination status	Treatment status
MN259015	68	04/2016	3	M	Kelpie-cross	NSW [§] – far west	CPV-2a	Unvaccinated	Treated
MN259018	72	06/2018	5	FN	Terrier-cross		CPV-2b	Incomplete	Treated

Genbank Accession no.	Sample No.	Collection Date	Age (mo)++	Sex +	Breed	Region	Antigenic variant	Vaccination status	Treatment status
MN259027	314	05/2017	2	F	Cattle Dog		CPV-2b	Incomplete	Treated
MN259029	316	05/2017	8	F	Bull Mastiff-cross		CPV-2b	Incomplete	Treated
MN259032	319	05/2018	7	M	Kelpie		CPV-2b	Incomplete	Treated
MN259035	323	07/2016	3	M	Cattle Dog		CPV-2b	Incomplete	Treated
MN258990	16	09/2015	6	M	Miniature Dachshund	NSW - central	CPV-2b	Unvaccinated	Treated
MN258995	41	12/2015	4	M	Staffordshire Bull Terrier		CPV-2b	Incomplete	Treated
MN258996	45	10/2015	6	M	Maremma-cross		CPV-2b	Unvaccinated	Treated
MN258997	46	10/2015	6	F	Great Dane-cross		CPV-2b	Unvaccinated	Treated
MN258998	47	11/2015	6	M	Maremma-cross		CPV-2b	Incomplete	Treated
MN258999	48	10/2015	3	F	Cattle Dog-cross		CPV-2a	Unvaccinated	Treated
MN259001	50	03/2015	3	M	Labrador-cross		CPV-2b	-	-
MN259007	58	02/2016	6	M	Border Collie		CPV-2b	Unvaccinated	-
MN259014	67	04/2016	4	M	Bull Arab-cross		CPV-2b	Unvaccinated	Treated
MN259019	73	06/2016	8	MN	Border Collie-cross		CPV-2b	Incomplete	Treated
MN259021	308	03/2016	4	F	Miniature Dachshund		CPV-2b	Incomplete	Treated
MN259024	311	05/2016	4	F	Staffordshire Bull Terrier-cross		CPV-2b	Incomplete	Treated
MN259025	312	05/2016	4	F	Kelpie		CPV-2a	Unvaccinated	Treated
MN259026	313	05/2017	6	M	Irish Wolfhound		CPV-2b	Unvaccinated	Treated
MN259028	315	05/2017	2	-	Cross-breed		CPV-2b	Unvaccinated	Untreated

Genbank Accession no.	Sample No.	Collection Date	Age (mo)++	Sex +	Breed	Region	Antigenic variant	Vaccination status	Treatment status
MN259030	317	01/2016	26	F	Cattle Dog		CPV-2b	Incomplete	Treated
MN259031	318	05/2017	6	M	Cattle Dog		CPV-2b	Incomplete	Treated
MN259034	322	10/2016	7	M	Cattle Dog-cross		CPV-2b	Incomplete	Treated
MN259036	324	10/2016	2	M	Bull Arab-cross		CPV-2b	Unvaccinated	Treated
MN259037	325	07/2016	2	-	Cattle Dog-cross		CPV-2a	Incomplete	Treated
MN561318	S0875	08/2019	2.5	F	Dalmatian		CPV-2a	Incomplete	Treated
MN259039	333	03/2018	3	F	Chihuahua-cross		CPV-2a	Unvaccinated	Untreated
MN528598	6064	08/2019	3.5	M	Irish Wolfhound	NSW – north west	CPV-2b	Incomplete	Treated
MN259060	Ar1	04/2019	2	F	Golden Retriever		CPV-2b	Incomplete	Treated
MN528597	Ar3841	06/2019	2	F	Cavoodle		CPV-2b	Incomplete	Treated
MN259062	Ar5	04/2019	1.75	F	Labrador-cross-Cocker Spaniel		CPV-2b	Unvaccinated	Treated
MN259000	49	03/2016	5	M	Staffordshire Bull Terrier-cross	NSW – Riverina	CPV-2a	-	-
MN259002	51	02/2016	3	F	Labrador-cross		CPV-2b	Unvaccinated	-
MN259003	52	03/2016	6	M	Labrador-cross		CPV-2b	Unvaccinated	-
MN259004	53	07/2016	3	F	Mastiff-cross		CPV-2a	Unvaccinated	-
MN259005	54	02/2016	2	F	Border Collie		CPV-2a	Unvaccinated	-
MN259011	64	04/2016	6	MN	Crossbred		CPV-2b	Incomplete	-
MN259013	66	04/2016	-	F	Cattle Dog-cross		CPV-2b	Unvaccinated	Treated
MN259016	69	04/2016	8	MN	Mastiff-cross		CPV-2b	Incomplete	Treated

Genbank Accession no.	Sample No.	Collection Date	Age (mo)++	Sex +	Breed	Region	Antigenic variant	Vaccination status	Treatment status
MN258991	18	08/2015	1	-	Crossbred	NSW – Greater Sydney	CPV-2a	Unvaccinated	Treated
MN258992	19	08/2015	1	-	Crossbred		CPV-2a	Unvaccinated	Treated
MN258993	21	08/2015	1	-	Crossbred		CPV-2b	Unvaccinated	Treated
MN259020	306	04/2016	-	M	German Shepherd		CPV-2a	-	-
MN259022	309	06/2016	-	-	-		CPV-2a	-	-
MN259023	310	04/2016	-	-	-		CPV-2a	-	-
MN259040	337	09/2018	96	FN	Labrador		CPV-2a	Incomplete	Treated
MN259041	338	08/2018	20	F	Cavoodle		CPV-2a	Complete	Treated
MN259061	Ar2	12/2017	9	M	Boxer		CPV-2a	-	-
MN259064	Ar8	12/2017	-	M	-		CPV-2a	-	-
MN259065	Ar9	01/2019	-	M	-		CPV-2a	-	-
MN561319	210	09/2017	30	M	Staffordshire Bull Terrier-cross		CPV-2b	Incomplete	Treated
MN561320	211	09/2017	1	F	Staffordshire Bull Terrier-cross		CPV-2a	Incomplete	Treated
MN258986	5	09/2015	3	M	Border Collie	Victoria	CPV-2a	-	Untreated
MN258987	11	08/2015	-	-	-		CPV-2a	-	-
MN561321	12	09/2015	3.75	M	Bull Arab		CPV-2a	Unvaccinated	
MN258988	14	09/2015	3.25	M	Great Dane-cross		CPV-2a	Unvaccinated	Untreated
MN258989	15	09/2015	5	F	Staffordshire Bull Terrier-cross		CPV-2a	Unvaccinated	Untreated
MN259008	59	08/2018	3	FN	Border Collie		CPV-2a	Incomplete	-

Genbank Accession no.	Sample No.	Collection Date	Age (mo)++	Sex +	Breed	Region	Antigenic variant	Vaccination status	Treatment status
MN259012	65	04/2016	24	FN	Crossbred		CPV-2b	Incomplete	-
MN259017	70	04/2016	3	-	Crossbred		CPV-2b	Unvaccinated	Treated
MN259033	321	02/2017	11	M	-		CPV-2a	Incomplete	-
MN259038	330	06/2018	36	M	Staffordshire Bull Terrier	South Australia	CPV-2a	Complete	Treated
MN259043	342	08/2018	7	F	Jack Russell terrier	Western Australia	CPV-2a	-	-
MN259044	343	01/2019	-	F	-		CPV-2a	-	-
MN259045	357	01/2019	6	F	Border collie		CPV-2a	-	-
MN259047	359	08/2018	7	F	Toy poodle-cross		CPV-2a	-	-
MN259048	360	01/2019	3	M	Miniature Schnauzer		CPV-2a	-	-
MN259049	361	02/2018	7	FN	Siberian Husky		CPV-2	-	-
MN259050	364	06/2018	3	M	Australian Shepherd		CPV-2b	-	-
MN259052	366	01/2019	11	MN	Crossbred		CPV-2b	-	-
MN259053	367	01/2019	-	M	-		CPV-2b	-	-
MN259054	371	01/2019	3	M	Ridgeback-cross		CPV-2b	-	-
MN259055	373	01/2019	2	F	Staffordshire Bull Terrier-cross		CPV-2b	-	-
MN259056	377	01/2019	96	FN	Pharaoh Hound		CPV-2b	-	-
MN259058	380	01/2019	1.5	M	Labradoodle		CPV-2	-	-
MN259059	383	01/2019	7	F	Doberman		CPV-2b	-	-
MN259052	384	01/2019	-	M	-		CPV-2b	-	-
MN259063	Ar6	12/2017	6	F	-	Queensland	CPV-2a	Incomplete	Treated

+ M male , F female, MN male neutered, FN female neutered

§ mo, months

++ NSW, New South Wales

Table 3. Amino acid substitutions in the VP2 region of strains of Canine parvovirus (CPV) detected in faeces from dogs with confirmed parvoviral enteritis (2015 to 2019) compared to reference strains of CPV and Feline parvovirus (FPV)

Strain	Region	Year	Clade	5	21	80	87	92	93	99	101	103	178	219	232	25
318	Central NSW	2017	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
46	Central NSW	2015	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
72	Far West NSW	2018	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
65	Victoria	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
311	Central NSW	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
322	Central NSW	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
51	Riverina	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
50	Central NSW	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
58	Central NSW	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
52	Riverina	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
317	Central NSW	2016	CPV2b	G	T	R	L	V	N	D	T	A	N	I	I	V
70	Victoria	2016	CPV2b	G	T	R	L	V	N	N	T	A	D	I	I	V
313	Central NSW	2017	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
308	Central NSW	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
324	Central NSW	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
66	Riverina	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
41	Central NSW	2015	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
21	Greater Sydney	2015	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
45	Central NSW	2015	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
47	Central NSW	2015	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
316	Far West NSW	2017	CPV2b	G	T	R	L	F	N	D	T	A	D	I	I	V
319	Far West NSW	2018	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
323	Far West NSW	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
315	Central NSW	2017	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
314	Far West NSW	2017	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
16	Central NSW	2015	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
6064	North West NSW	2019	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
373	Western Aus	2019	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
367	Western Aus	2019	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
364	Western Aus	2018	CPV2b	A	T	R	L	V	N	D	T	A	D	I	I	V
377	Western Aus	2019	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
366	Western Aus	2019	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
73	Central NSW	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
49	Riverina	2016	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
48	Central NSW	2015	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
53	Riverina	2016	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
325	Central NSW	2016	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
54	Riverina	2016	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
68	Far West NSW	2016	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
338	Greater Sydney	2018	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
64	Riverina	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
69	Riverina	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
67	Central NSW	2016	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V

Strain	Region	Year	Clade	5	21	80	87	92	93	99	101	103	178	219	232	25
330	South Aus	2018	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
59	Victoria	2018	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
383	Western Aus	2019	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
211	Greater Sydney	2017	CPV2a	A	T	R	L	V	N	D	T	A	D	I	I	V
18	Greater Sydney	2015	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
19	Greater Sydney	2015	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
359	Western Aus	2018	CPV2a	A	T	R	M	V	N	D	T	A	D	I	I	V
360	Western Aus	2019	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
343	Western Aus	2019	CPV2a	G	T	R	M	V	N	D	T	A	D	I	I	V
384	Western Aus	2019	CPV2b	G	T	R	L	V	N	D	T	A	D	I	I	V
342	Western Aus	2019	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
357	Western Aus	2019	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
321	Victoria	2018	CPV2a	G	T	R	L	V	N	D	T	A	D	I	I	V
LPZ1	China	2011	CPV2a	A	T	R	L	V	N	D	T	A	D	I	I	V
RJ7	India	2016	CPV2a	A	T	R	L	V	N	D	T	A	D	I	I	V
210	Greater Sydney	2017	CPV2b	A	T	R	L	V	N	D	T	A	D	I	I	V
5	Victoria	2015	CPV2a	G	A	R	L	V	N	D	T	A	D	I	I	V
15	Victoria	2015	CPV2a	G	A	R	L	V	N	D	T	A	D	I	I	V
12	Victoria	2015	CPV2a	G	A	R	L	V	N	D	T	A	D	I	I	V
11	Victoria	2015	CPV2a	G	A	R	L	V	N	D	T	A	D	I	I	V
14	Victoria	2015	CPV2a	G	A	R	L	V	N	D	T	A	D	I	I	V
333	Central NSW	2018	CPV2a	A	A	R	L	V	N	D	T	A	D	I	I	V
312	Central NSW	2017	CPV2a	A	A	R	L	V	N	D	T	A	D	I	I	V
S0875	Central NSW	2019	CPV2a	A	A	R	L	V	N	D	T	A	D	I	I	V
Ar6	Queensland	2017	CPV2a	A	A	R	L	V	N	D	T	A	D	I	I	I
Ar9	Greater Sydney	2017	CPV2a	A	A	R	L	V	N	D	T	A	D	I	I	V
Ar8	Greater Sydney	2017	CPV2a	A	A	R	L	V	N	D	T	A	D	I	I	V
Ar2	Greater Sydney	2017	CPV2a	A	A	R	L	V	N	D	T	A	D	I	I	V
Bel2014	Brazil	2014	CPV2b	A	T	R	L	V	N	D	T	A	D	I	I	V
PT262/14	Portugal	2014	CPV2c	A	T	R	L	V	N	D	T	A	D	I	I	V
GR09/09	Greece	2009	CPV2c	A	T	R	L	V	N	D	T	A	D	I	I	V
Cat300	Italy	2010	CPV2c	A	T	R	L	V	N	D	T	A	D	I	I	V
Ar5	North West NSW	2019	CPV2b	A	T	R	L	V	N	D	T	A	D	I	I	V
Ar3841	North West NSW	2019	CPV2b	A	T	R	L	V	N	D	T	A	D	I	I	V
Ar1	North West NSW	2019	CPV2b	A	T	R	L	V	N	D	T	A	D	I	I	V
371	Western Aus	2019	CPV2b	A	T	R	L	V	N	D	T	A	D	I	I	V
Dodge	USA	2003	CPV2b	A	T	R	L	V	N	D	T	A	D	I	I	V
9985-46	Japan	2019	CPV2b	A	T	R	L	V	K	D	T	A	D	I	I	V
333/05-4	Italy	2005	CPV2a	A	T	R	L	V	N	D	T	A	D	I	I	V
309	Greater Sydney	2016	CPV2a	A	T	R	L	V	N	D	T	A	D	I	I	V
310	Greater Sydney	2016	CPV2a	A	T	R	L	V	N	D	T	A	D	I	I	V
337	Greater Sydney	2018	CPV2a	A	T	R	L	V	N	D	T	A	D	I	I	V
306	Greater Sydney	2016	CPV2a	A	T	R	L	V	N	D	T	A	D	I	I	V
N	USA	1988	CPV2	A	T	R	M	V	N	D	I	A	D	I	I	V
CPV-b	USA	1990	CPV2	A	T	R	M	V	N	D	I	A	D	I	I	V
388/05	Italy	2005	CPV2	A	T	R	M	V	N	D	I	A	D	V	I	V
361	Western Aus	2018	CPV2	A	T	R	M	V	N	D	I	A	D	V	I	V
380	Western Aus	2019	CPV2	A	T	R	M	V	N	D	I	A	D	V	I	V
Kai.us-06	USA	2006	FPV	A	T	K	M	V	K	D	T	V	D	I	V	V
4.us 64	USA	1964	FPV	A	T	K	M	V	K	D	T	V	D	I	V	V

Strain	Region	Year	Clade	5	21	80	87	92	93	99	101	103	178	219	232	25
MEVB	China	2008	MEV	A	T	K	M	V	K	D	I	V	D	I	I	V

... well-recognized mutations compared to classical CPV2

... novel mutations compared to classical CPV2

... reversion to MEV/FPV

... reversion only to FPV

... reversion only to MEV

Figure legends

Figure 1: Geographic distribution of dogs with confirmed parvoviral enteritis, mapped according to regions defined by the Australian Government Department of Infrastructure, Transport, Cities and Regional Development.

Figure 2: Phylogenetic analysis of the VP2 region of Canine parvoviruses detected in this study, performed in MEGA X (Kumar 2018). Evolutionary history was inferred by using the Maximum Likelihood method and Tamura 3-parameter model with 1000 bootstrap replicates (Tamura, 1992). The tree with the highest likelihood (-3865.87) was selected. Bootstrap values >70% are shown on the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories [$+G$ parameter = 0.5902]). The rate variation model allowed for some sites to be evolutionarily invariable ([$+I$], 84.05% sites). Mink enteritis virus (MEV) was used as the outgroup. The scale bar indicates the number of substitutions per site.

Figure 3. Amino acid residues of the capsid protein (VP2) that differ between: (i) Feline parvovirus (FPV) (in blue) and Canine parvovirus 2 (CPV-2) (in brown), (ii) CPV-2 and CPV-2a (in green) and (iii) CPV-2a and CPV-2b or CPV-2c (green). Reverse mutations to FPV are indicated in blue font and to CPV2 in brown font. The amino acid residue mutations identified among 21 strains of CPV-2a and CPV-2b (clade 2 of this study, or FPV-like CPVs) are indicated using a font colour that matches the different viruses, FPV (blue), CPV-2 (brown) and CPV-2a, 2b and 2c (green). The frequency of mutations at each position is indicated (#1 to #21).

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