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Short communication

Typing of canine parvovirus isolates using mini-sequencing based single nucleotide polymorphism analysis

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The antigenic types of canine parvovirus (CPV) are defined based on differences in the amino acids of the major capsid protein VP2. Type specificity is conferred by a limited number of amino acid changes and in particular by few nucleotide substitutions. PCR based methods are not particularly suitable for typing circulating variants which differ in a few specific nucleotide substitutions. Assays for determining SNPs can detect efficiently nucleotide substitutions and can thus be adapted to identify CPV types. In the present study, CPV typing was performed by single nucleotide extension using the mini-sequencing technique. A mini-sequencing signature was established for all the four CPV types (CPV2, 2a, 2b and 2c) and feline panleukopenia virus. The CPV typing using the mini-sequencing reaction was performed for 13 CPV field isolates and the two vaccine strains available in our repository. All the isolates had been typed earlier by full-length sequencing of the VP2 gene. The typing results obtained from mini-sequencing matched completely with that of sequencing. Typing could be achieved with less than 100 copies of standard plasmid DNA constructs or $\leq 10^1$ $FAID_{50}$ of virus by mini-sequencing technique. The technique was also efficient for detecting multiple types in mixed infections.

Canine parvovirus (CPV) is a single stranded DNA virus that causes acute, and sometimes, fatal enteritis in dogs. The virus was identified in dogs during 1977–1978 as CPV type 2 (Appel et al., 1979; Burtonboy et al., 1979). Later, an antigenic variant, CPV2a, started appearing in the canine population (Parrish et al., 1985). In the mid-1980s, another CPV2 variant, called CPV2b appeared (Parrish et al., 1991). The CPV type 2 has since been replaced completely by the variants, type 2a and 2b throughout the world and these two variants co-exist in varying proportions in the canine population. Substitutions at amino-acid positions 426 (Asn to Asp) and 555 (Ile to Val) in the capsid protein VP2 had led to the emergence of type 2b from 2a (Parrish et al., 1991). Therefore, the differentiation of CPV2a from 2b was achieved using a PCR which used the single nucleotide polymorphisms (SNPs) arising out of substitutions at 426 (Asn to Asp) and 555 (Ile to Val) amino acid positions (Pereira et al., 2000). However, it was reported that many of the recent 2a strains do not carry the Val to Ile substitution at position 555 (Martella et al., 2006; Chinchkar et al., 2006; Wang et al., 2005). Another CPV variant (CPV 2c) with glutamic acid at position 426 has been reported from various parts of the world (Nakamura et al., 2004; Buonavoglia et al., 2001). Taken together, these mutations restrict the differences among the antigenic variants of CPV2 to just one amino acid at position 426 brought about by a single nucleotide change at either position 4062 or 4064. Therefore, the PCR based methods have become redundant for identifying the CPV2 variants (Martella et al., 2006).

Since the variant types 2a, 2b and 2c differ only by one nucleotide, molecular assays which identify SNPs may be used to type these strains. Restriction enzyme digestion of short PCR fragments of CPV-2 genome has proved to be useful for discriminating CPV-2c from other variant strains (Buonavoglia et al., 2001). Apart from this, the minor groove binder (MGB) assays which use real-time PCR were also developed for detection of SNPs present in the CPV VP2 coding sequence. Using this technique, several individual assays had to be performed at each SNP site to differentiate types 2, 2a, 2b and 2c in a sample (Decaro et al., 2006a,b).

Single nucleotide extension using the mini-sequencing technique is one of the methods used to identify SNPs (Sobrino et al., 2005). In this technique, the primers are designed in such a way that the 3′ end of the primer is one base short of the known SNP. In the mini-sequencing reaction, primers bind to a complementary template in the presence of fluorescently labeled ddNTPs and

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the polymerase extends the primer by one nucleotide. The specific nucleotide thus added, can be identified from the emitted fluorescence revealing the SNP at the said position. Various multiplex mini sequencing-based assays have been validated successfully for the analysis of mitochondrial DNA, autosomes, the Y-chromosome, Duffy and ABO blood groups, and the melanocortin 1 receptor gene (Bouakaze et al., 2007). Mini-sequencing followed by MALDI-ToF mass-spectrometry was employed for genetic typing of hepatitis B virus in a Russian population (Malakhova et al., 2009).

To the best of our knowledge, the mini-sequencing based approach using single base extension has not been reported for typing or characterizing CPV virus genome. In the present study, a multiplex mini-sequencing experiment was designed for differentiating the four CPV types and FPV (Feline Panleukopenia Virus) in a single tube.

The CPV2 strains used in vaccine manufacture by Indian Immunologicals (IIL, Hyderabad, India) were used in the study. The type 2a and 2b strains were isolated from various parts of India and were maintained in the R&D center of Indian Immunologicals (Chinchkar et al., 2006). Canine Parvovirus type 2a and 2b isolates and the type 2 vaccine strains were propagated in the A72 cell line. Total DNA from the virus infected tissue culture supernatant was extracted using DNaZol reagent (Invitrogen, NY, USA) and was used in the subsequent PCR. The PCR was performed in a reaction mixture containing, 400 μM each of dNTPs, 1 unit Taq Polymerase (Bangalore genie, Bangalore, India), 1.5 mM $MgCl_2$, 3 pmol each of primers and 50–100 ng of viral DNA. The thermal cycling was done with an initial denaturation step of 94 °C for 3 min; 40 cycles of 94 °C for 45 s, 50 °C for 45 s and 72 °C for 1 min; and final extension of 72 °C for 15 min. The primer pair, CPV Ext F and CPV Ext R, was used for the PCR amplification (Table 1). These primers were designed from the conserved regions of VP2 and were therefore 100% homologous with the VP2 DNA sequence of all the CPV types as well as FPV. Additionally, the region of CPV VP2 amplified by the PCR encompasses the SNPs which were sufficient to differentiate various CPV types. The amplified fragments were 576 bp in length and contained the sequences in between 3622 bp and 4197 bp of the CPV genome (The numbering is based on the full length CPV sequence; GenBank accession number M38245).

The PCR amplified partial VP2 coding sequences of each CPV types – type 2, 2a and 2b were cloned into pCR2.1 cloning vector of Topo-TA cloning system (Invitrogen, NY, USA) and maintained in an *E. coli* host. Plasmids extracted from these *E. coli* cultures were used as positive standards in the mini sequencing reactions. Similar positive standards for CPV type 2c and FPV were generated by introducing point mutations in CPV type 2b and CPV type 2 VP2 fragments, respectively. For creating CPV 2c standard,VP2 region of CPV 2b was amplified as two fragments with 20 bases overlap between the amplified fragments. The primers used to create the overlap contained the mutation and the mutation is incorporated in the amplified fragments (T→A at 4064). The two fragments were subjected to splicing by overlap extension (SOE) PCR to create single CPV 2c amplicon as a positive standard for CPV 2c. Similarly, the positive standard for FPV was generated from CPV2 by SOE PCR with an A→G mutation at position 3753. All the plasmid constructs were verified by sequencing.

The 576 bp PCR product (obtained from either the viral DNA samples or the plasmid clones) was added to 5 units of Shrimp Alkaline Phosphatase (SAP; New England Biolabs, Ipswich, USA) and 3 units of *Exonuclease* I (*Exo* I; New England Biolabs, Ipswich, USA). The mixture was incubated at 37 °C for 1 h to remove the unused primers and to dephosphorylate the unincorporated dNTPs. After treatment, the SAP and *Exo* I were inactivated by heating at 75 °C for 15 min. The Exo/SAP treated PCR product was used directly as template in the subsequent mini-sequencing reaction.

Five primers (CPV typing 1–5; Table 1) were used in the multiplex mini-sequencing reaction. The mini-sequencing primers were designed in such a way that the 3′ end of the respective primer is short by one base at nucleotide positions 3685, 3699, 3753, 4062 or 4064 in CPV genome (Table 1). The type-specific amino acid and the corresponding SNPs at these positions are shown in Table 1.

The mini-sequencing primers were 5′ tailed with non-homologous dGACT sequences of differing lengths to produce single base extension products ranging from 25 to 55 nucleotides. Thus, the resulting extension products differ in length from each other by at least 7 nucleotides, allowing them to be resolved sufficiently in capillary electrophoresis. When the primers are extended by the incorporation of a fluorescence labeled ddNTP in the mini-sequencing reaction, the presence of dye in the ddNTP alters the relative mobility of the extended primer products considerably. The expected relative mobilities of the primers in the capillary gel were identified by adding single ddNTP to each of the primer used in the multiplex reaction. The addition of the labeled ddNTPs was done using primer focus kit (Applied Biosystems, Foster City, USA) and the length of the primers with the fluorescently labeled ddNTP was identified in a ABI PRISM 3130xl genetic analyzer using POP-7 polymer (Applied Biosystems, Foster City, USA) in 36 cm capillary. The expected relative mobilities of the primers containing an additional labeled ddNTP are provided in Table 2. The mobility shift was more (9 to 12 bases) for shorter primers and the shift was lesser (5–7 bases) for longer primers. Three out of five primers used in the mini-sequencing reaction were anti-sense primers.

The multiplex reaction was performed in a total volume of 10 μl. The reaction mixture contained 3 μl of Exo I/SAP treated PCR product, 10 pmol each of the primers (CPV typing 1–5; Table 1) and 5 μl SNaPshot™ multiplex ready reaction mix (Applied Biosystems, Foster City, USA). The SNaPshot™ reaction mix contained AmpliTaq® DNA polymerase, reaction buffer and fluorescently labeled ddNTPs (A = dR6G, green; C = dTAMRA™, black; G = dR110, blue; T = dROX™, red). The reaction mix was subjected to 28 cycles of 96 °C for 10 s, 50 °C for 5 s and 60 °C for 30 s. During the thermal cycling all the 5 primers were extended by single ddNTP. After extension, the mix was added with SAP (1 unit) and incubated at 37 °C for 1 h to remove the 5′ phosphoryl groups of the unincorporated labeled ddNTPs. Then the SAP inactivation was carried out by heating at 75 °C for 15 min. 0.5 μl of SAP treated extension product was mixed with 9 μl of formamide and 0.5 μl of internal size standard GeneScan-120 LIZ™ (Applied Biosystems, Foster City, USA). The mix was heated at 95 °C for 5 min and snap chilled on ice to denature the product. After denaturation, the samples were run on an ABI PRISM 3130xl Genetic Analyzer using POP-7 polymer (Applied Biosystems, Foster City, USA) in 36 cm capillary. Data were collected using 3130xl (version 3) software and analyzed using the Gene-mapper v.3.7 software (Applied Biosystems, Foster City, USA). A peak with greater than 100 relative fluorescence intensity (RFU) was considered as positive.

The typical mini-sequencing signatures based on the type specific SNPs are shown in the Table 2. The five SNPs were identified in the mini-sequencing reaction by the addition of specific ddNTP to the primers. The CPV types were identified without any ambiguity in the mini-sequencing reaction (Fig. 1). Mini-sequencing pattern typical for each of these viruses (or plasmid constructs) was obtained in the experiment.

Each of the plasmid standard constructs containing part of VP2 sequences of CPV 2, 2a, 2b, 2c and FPV were diluted serially 10 fold starting with 10^7 copy numbers. 100 ng of DNA extracted from A72 cells was also mixed with each dilution of plasmid DNA. The PCR and subsequent mini-sequencing reaction was performed for each dilution of plasmid. The sensitivity was determined by determining the lowest copy number of plasmid which produced unambiguous (>100 RFU) typing result. Each reaction was performed in triplicate.

Table 1
Sequences of the primers used for PCR amplification and mini-sequencing reaction are shown. The amino acids specific for each type, the resultant SNPs and their positions are also shown. The typing primers are one base short of the respective SNP positions either in the sense strand or in the anti-sense strand as indicated.

Primer ID and primer sequence	FPV	CPV2	CPV2a	CPV2b	CPV2c	Nucleotide position in CPV genome	Amino acid position in CPV VP2
CPV Typing 1 S							
GACTGACTGTTCTTTGCCTCAA(T/G)CT GAAGGAG	C Ala	C Ala	G Gly	G Gly	G Gly	3685	300
CPV Typing 2 AS							
GACTGACTGACTGACTGTCTTTT TCTTGTTGAACTCCTATAT	G Asp	G Asp	T Tyr	T Tyr	T Tyr	3699	305
CPV Typing 3 AS							
GGTGTAACTCAAATGGGAAATACA	G Asp	A Asn	A Asn	A Asn	A Asn	3753	323
CPV Typing 4 S							
GACTGACTGACTGACTATTAACTTTAA CCTTCCTGTAACA	A Asn	A Asn	A Asn	G Asp	G Glu	4062	426
CPV Typing 5 AS							
GACTGACTGACTGACTGACTGACCAATTGGATCTGTTGGTAGCAATACATTATC	T Asn	T Asn	T Asn	T Asp	A Glu	4064	426
CPV Ext For GGCAAACAAATAGAGCATTGG **CPV Ext Rev** CCCAAATTTGACCATTTGGAT	For amplification						

Table 2
The typical mini-sequencing signature for each of the CPV types and FPV is provided below. The expected size of the primers was determined because the added labeled ddNTP varies the primer mobility in capillary gel considerably.

Nucleotide position in CPV genome ›	3685	3699	3753	4062	4064
Primer ID	Typing 1 S	Typing 2 AS	Typing 3 AS	Typing 4 S	Typing 5 AS
Primer length	32	47[a]	24[a]	40	54[a]
Expected size (with any of the four additional labeled ddNTP)	42.3–43.9	54.3–55.4	35.9–37.4	46.4–48.2	58.8–60.8
The single base added for various CPV types in mini-sequencing					
FPV	C	C	C	A	A
CPV 2	C	C	T	A	A
CPV 2a	G	A	T	A	A
CPV 2b	G	A	T	G	A
CPV 2c	G	A	T	G	T
Position of the base in capillary gel electrophoresis (depends on the primer length)[b]	2	4	1	3	5

[a] Anti-sense primers add complementary base.
[b] Position of the SNPs in the mini-sequencing was based on the size of the primer used for the particular SNP identification. Thus, the SNP arrangements in the mini-sequencing were not matching their position in the genome.

The sensitivity assay was repeated three times and the mean sensitivity was calculated. Distinct CPV typing could be attained with less than 100 copies of the plasmids per reaction. The mean sensitivity was in between 18 and 60 plasmid copies per reaction for different CPV types (Table 3).

CPV type 2, 2a and 2b virus were titrated in A72 cells using fluorescently labeled CPV specific mAb following the procedure described by Becerra and Hegland (2005) with few modifications. The titrated viruses were diluted serially 10 fold from 10^5 fluorescent antibody infectious dose 50% ($FAID_{50}$) and each of the dilutions of viruses was spiked in faeces collected from dogs free of CPV. The sensitivity of the mini-sequencing reaction was measured using the DNA extracted from the faeces samples containing different dilutions of virus. The DNA was PCR amplified and subjected to mini-sequencing reaction. The lowest dilution of virus required to obtain an explicit (>100 RFU) typing result by mini-sequencing was determined. The experiments were done in triplicate and repeated again on three different days. The mean sensitivity in terms of $FAID_{50}$ was calculated. Cycle sequencing reactions were also performed using the PCR products to determine the lowest virus titer required to obtain unambiguous sequencing results. The CPV typing by mini-sequencing was recognizable distinctly even with the DNA extracted from $\leq 10^1$ $FAID_{50}$ of virus spiked with dog feces. The mean sensitivity was in between $10^{0.62}$ and $10^{0.8}$ $FAID_{50}$ per reaction for different CPV types (Table 3). However, the cycle sequencing reaction required $>10^3$ $FAID_{50}$ virus to lead to an unambiguous sequencing result.The specificity of the mini-sequencing reaction was assessed by performing the reaction with genomic DNA samples extracted from other related canine viruses. The genomic DNA of canine adenovirus 1, canine adenovirus 2, canine distemper virus, canine caronavirus and canine parainfluenza virus were subjected to mini-sequencing reaction using CPV specific primers. Neither did the PCRs show amplification of DNA nor did it record a fluorescent signal in the capillary gel electrophoresis.The CPV typing using the mini-sequencing reaction was performed for 13 CPV field isolates and two vaccine strains (CPV 2). These isolates were typed earlier by sequencing. Of the 13 field

Table 3
Mean sensitivity of the mini-sequencing technique was determined using plasmid constructs containing CPV VP2 and dog feces samples spiked with known titer of virus. The assay was performed in triplicates and repeated on three different days. The mean ± SD ($N = 9$) for each of the types is provided in the table.

S. No.	CPV type	Virus titer ($FAID_{50}$) Log_{10}(mean ± SD)	Plasmid copies mean ± SD
1	CPV 2	0.8 ± 0.47	60 ± 47.43
2	CPV 2a	0.62 ± 0.54	18 ± 30.99
3	CPV 2b	0.69 ± 0.54	19 ± 30.52
4	CPV 2c	Not done	50 ± 47.43
5	FPV	Not done	30 ± 39.68

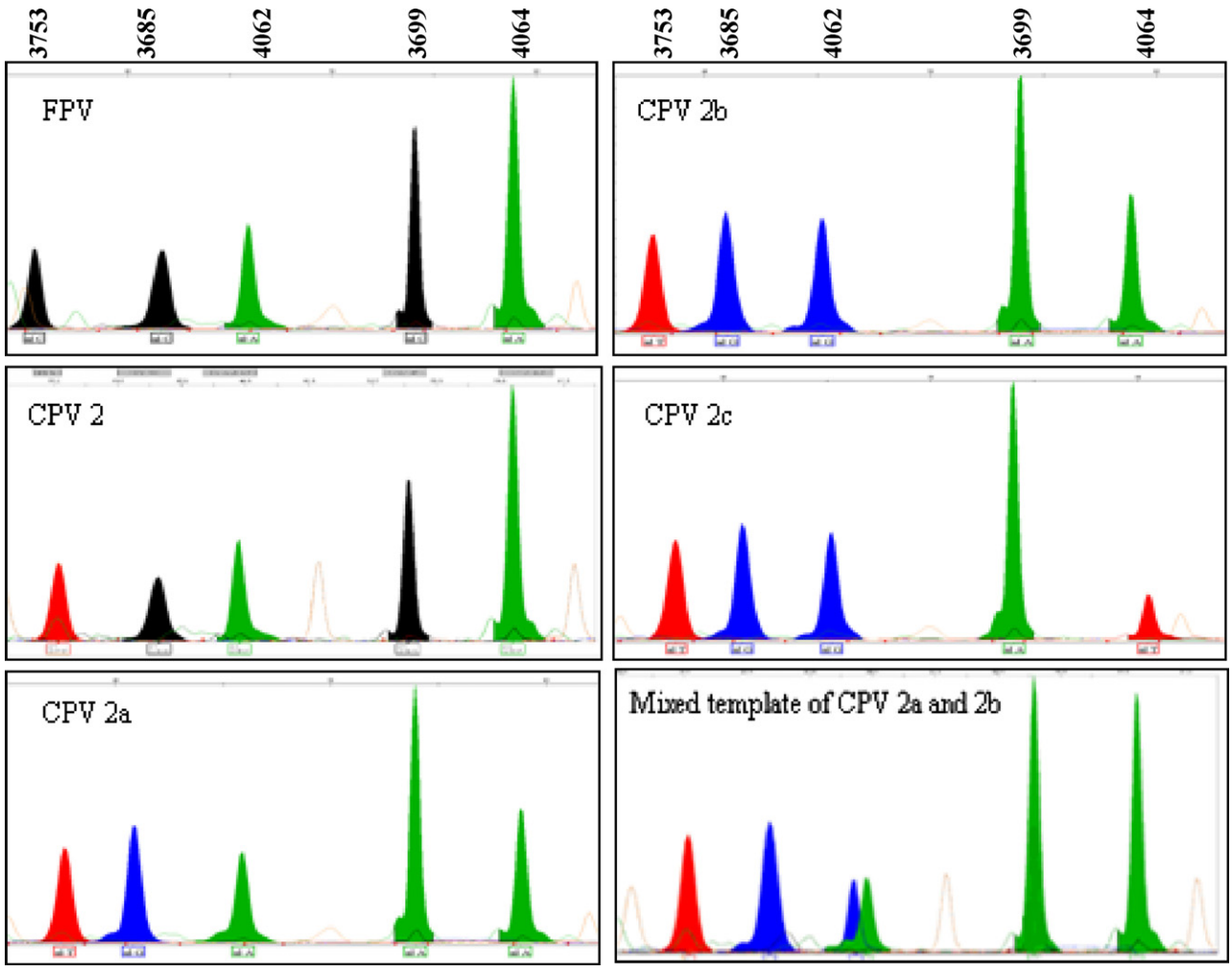

Fig. 1. The mini-sequencing peaks obtained for various CPV types after capillary gel electrophoresis. The mini-sequencing reaction mix was run in ABI PRISM 3130xl genetic analyzer. The data were collected using 3130xl version 3 software and analyzed using the Gene-mapper v.3.7 software (Applied Biosystems, Foster City, USA). The SNP position in the CPV genome is provided on the top over the corresponding peak. Mixed template of type 2a and 2b produced double peak at the relevant position (4062). A, green; C, black; G, blue; T, red. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

isolates 9 belonged to CPV2a and 4 were CPV2b. The typing results obtained from mini-sequencing matched completely with that of cycle sequencing.The mini-sequencing reaction was performed for the DNA extracted from the cell culture supernatant of mixed infection of two different CPV types. Similarly, the typing efficiency in mixed infection was evaluated by mixing varying copy numbers of the CPV plasmid constructs. Mixed infections were identified by the presence of double peaks in the specific SNP positions (Fig. 1). The presence of more than one CPV type did not significantly affect the sensitivity of the assay for identifying the individual CPV types.

The major antigenic determinants of CPV are present in the capsid protein VP2 and the changes in the amino acid sequence of capsid protein were responsible for the emergence of CPV antigenic variants. These changes in the VP2 amino acid sequences of variant types are well documented (Decaro et al., 2009; Parrish et al., 1991). The SNPs arising out of these amino acid changes have been the target in the diagnostic assays for determining the CPV type. The differentiation between the CPV2 and other variants could be achieved using a PCR with high accuracy (Pereira et al., 2000). However, the differentiation within the CPV 2 variant types (2a, 2b and 2c) could not be achieved using PCR (Martella et al., 2006; Chinchkar et al., 2006). The type 2c CPV strains harbor an *Mbo*II cleavage site at 4064 that could be detected in PCR-RFLP (restriction fragment length polymorphism; Buonavoglia et al., 2001). However, Demeter et al. (2010) reported false positive results using the PCR-RFLP method for identifying CPV type 2c. Haemagglutination (HA) using type specific monoclonal antibodies is also employed to differentiate the types. However, the sensitivity of the HA test is low and the monoclonal antibodies may not be available in every laboratory for performing the assay. Therefore, real-time PCR assays using minor groove binder (MGB) probes were reported recently to identify the SNPs present in the CPV types. Though the MGB assay is sensitive, several individual reactions have to be set up to identify and differentiate the four CPV types, the new type 2b vaccine strains and FPV (Decaro et al., 2006a,b, 2008).

In the present study, an attempt was made to identify type specific SNPs of CPV in a single reaction by the mini-sequencing technique. A 576 bp region in the CPV VP2, containing five type specific SNPs, was selected for the initial PCR amplification. The four different CPV types, and FPV, could be identified accurately on the basis of the five SNPs in the subsequent mini-sequencing reaction. The specificity of the experiment was assessed by performing the assay with DNA isolated from other canine viruses. The mini-sequencing reaction did not show any signal for the DNA from other canine viruses indicating that the specificity of the assay for detecting the CPV types.

The sensitivity of this typing technique was determined at varying plasmid copy numbers as well as virus titers. Typing could be achieved with less than 100 copies of plasmid DNA using the mini-sequencing reaction. Detection limits of the type-specific MGB probe real time-PCR assays were 10^1 and 10^2 DNA copies for types 2a and 2b, respectively (type 2a/2b assay), and 10^2 and 10^1 DNA copies for types 2b and 2c, respectively (type 2b/2c assay; Decaro et al., 2006a). Detection limit of the mini-sequencing reaction is comparable with that of MGB probe assay. Additionally, the typing by mini-sequencing could be achieved in a single tube. Since in the mini-sequencing technique a single complimentary nucleotide from among the four possible ddNTPs is incorporated, new mutations (Battilani et al., 2011) at the SNP sites would also be detected without the alteration in the assay protocol or reagents. However, in the MGB assay additional probes will have to be designed and incorporated in line with the changes in the SNPs.

The detection limit of mini-sequencing reaction was determined by spiking the dog feces samples with different dilutions of virus. Dog feces containing $\leq 10^1$ $FAID_{50}$ of CPV could be typed correctly using mini-sequencing method, whereas, the regular cycle sequencing required $>10^3$ $FAID_{50}$ of virus in recording readable peaks and detecting the CPV types with certainty. Since only specific SNPs useful in differentiating the types were involved in the mini-sequencing reaction, the analysis of result is simpler and rapid compared to the tedious sequence analysis to determine the types by cycle sequencing. Therefore, the mini-sequencing method can be used to type CPV samples in a single tube, rapidly.

The typing results of the mini-sequencing reactions for the DNA samples extracted from 15 of the isolates in this centre matched completely the cycle sequencing results indicating its reliability. Natural infection of dogs with more than one CPV types was reported earlier (Battilani et al., 2011; Vieira et al., 2008). The mini-sequencing method described above can detect mixed infection in dogs with more than one CPV types in a single reaction. Since the mini-sequencing technique can accommodate up to ten primers, there is further scope for adding additional primers in the reaction to identify the possible mutations originating from other sites of VP2 gene. The SNPs present in any site outside the 576 bp region used in the present study may also be detected by performing a multiplex PCR to amplify the relevant regions. Therefore, emerging SNP sites and type 2b vaccine strain specific SNPs can also be added in future. In conclusion, the mini-sequencing technique can be used for rapid, high-throughput and accurate initial typing of CPV isolates.

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