Research in Veterinary Science 93 (2012) 515–519

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Peptide nucleic acid-based (PNA) array for the antigenic discrimination of canine parvovirus

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ARTICLE INFO

Article history:
Received 27 April 2010
Accepted 8 June 2011

Keywords:
CPV
PNA
MGB probe

ABSTRACT

A novel peptide nucleic acid (PNA)-based array was developed for use in ante-mortem antigenic typing discrimination in dogs with canine parvovirus (CPV). Cyclic benzothiazole-2-sulfonyl PNA monomers were synthesized that recognized GTA (CPV-2) and TAT (CPV-2a, -2b and -2c) at the nt 913–915 positions, and AAT (CPV-2 and CPV-2a), GAT (CPV-2b), and GAA (CPV-2c) at the nt 1276–1278 positions of the VP2 gene. The detection limits for aa 305 and aa 426 of the VP2 proteins belonging to the four CPV antigenic types were determined optically to be 40–2000 DNA copies, and the optimal cut-off fluorescence signaling value was fixed at 5000. The PNA array described here was developed from 135 field dog fecal specimens and had 89.8% (62/69) sensitivity and 90.4% (66/73) specificity compared with a real-time PCR using the TaqMan assay, a gold standard method. This CPV PNA array could be used together with MGB probe assays as an attractive novel tool for ante-mortem antigenic typing discrimination.

1. Introduction

Canine parvovirus (CPV) is a significant pathogen of domestic dogs and various wild carnivore species. Whereas feline panleukopenia virus-induced diseases have been known since the beginning of the twentieth century (Verge and Christoforni, 1928), CPV-2 and its associated disease in dogs emerged suddenly in the late 1970's (Appel et al., 1978). Three different CPV types (CPV-2a, -2b, and -2c) were subsequently identified by their antigenic properties (Parrish et al., 1991; Buonavoglia et al., 2001).

Five amino acid substitutions in the VP2 gene differentiate CPV-2 from CPV-2a: Met87Leu, Ile101Thr, Ala300Gly, Asp305Tyr, and Val555Ile (Parrish et al., 1991). One additional substitution in the VP2 capsid protein differentiates CPV-2a from CPV-2b: Asn426Asp (Decaro et al., 2006d). An emerging CPV variant, CPV-2c, has been reported in several nations, including Italy, Vietnam, and the United States (Buonavoglia et al., 2001; Decaro et al., 2006a; Hong et al., 2007; Kapil et al., 2007; Nakamura et al., 2004). CPV-2c is distinguishable from CPV-2b by the substitution of Glu for Asp at aa 426 of VP2 (Buonavoglia et al., 2001). These amino acids are responsible for the antigenic and host-range properties of the virus (Nakamura et al., 2004; Parrish, 1991; Truyen et al., 1995).

Peptide nucleic acid (PNA), a novel oligonucleotide with the sugar phosphate backbone replaced by a pseudo-peptide skeleton, is notable for its exceptional biological and chemical stability as a nucleic acid analogue (Nielsen et al., 1991; Nielsen, 1998). The use of PNA may improve assays through improved sequence variation detection, improved detection limits, and overall higher assay sensitivity (Brandt and Hoheisel, 2004; Pellestor and Paulasova, 2004). Synthetic PNA exhibits superior hybridization characteristics, and improves chemical and enzymatic stabilities, relative to nucleic acids. Importantly, PNAs hybridize to cDNAs or cRNAs (Almarsson and Bruice, 1993; Wittung et al., 1994), with cDNA binding more strongly to PNA than to DNA because the PNA backbone is electrically neutral. Furthermore, PNA–DNA hybridization is known to be more specific than that of the corresponding DNA–DNA duplex (Weiler et al., 1997; Brandt, 2003). In this study, we sought to discriminate between the four CPV-2 antigenic types (CPV-2, -2a, -2b, and -2c) during ante-mortem diagnosis of dogs, using our newly developed PNA–DNA hybridization assay.

2. Materials and methods

2.1. Virus and samples

To evaluate the sensitivity and specificity of our assay, three plasmid clones were used, each of which included the full-length

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doi:10.1016/j.rvsc.2011.06.003

VP2 gene region of a different CPV type, as follows: CPV-2 (Vanguard®plus5 vaccine, Accession No. FJ197847), CPV-2a (K001 strain, Accession No. EU009200) and CPV-2b (DH326 strain, Accession No. EF599097). In addition, one plasmid clone, for the CPV-2b VP2 gene (DH326 strain), was changed to mimic CPV-2c through site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene Inc., CA, USA). Diarrhea specimens used to validate the developed CPV PNA were kindly supplied from three local animal hospitals (two in Seoul and one in Gyeonggi) from June 2010 to January 2011. The specimens were collected from 135 dogs, 52 of which were positive and 83 of which were negative for CPV, according to the Anigen rapid CPV Ag test kit (Bionote, Co., Korea), which functions on the same principle as immunochromatographic tests (IC).

2.2. PCR amplification and plasmid cloning

Viral DNA was extracted using the microcolumn-based QIAamp DNA FFPE tissue kit (QIAGEN, Cat. No. 56404, USA). DNA specimens were aliquoted and stored at −20 °C until use. Two biotin-labeled primer sets (CP-305f, 5′-ACAAATAGAGCATTGGGCTT-3′ and CP-305r, 5′-CTCATAATAGTAGCTTCAG-3′; and CP-426f, 5′-CAACAGGAGAAA CACCTG-3′ and CP-426r, 5′-AATATATTAGTATAGTTAATT-3′) were used for PCR amplification. Briefly, a PCR mixture [4 U *Taq* DNA polymerase (Solgent, Daejeon, Korea), 10 mM tetramethylammonium chloride (TMAC), 7.5 mM deoxynucleoside triphosphates (dNTPs), and 7 pmol of each primer in a total, final reaction volume of 50 μl] was added to 3 μl of the target DNA sample. The PCR amplification conditions included an initial denaturing step of 95 °C for 5 min, followed by 45 cycles each of 30 s at 95 °C, 60 s at 50 °C, and 60 s at 72 °C, and a final elongation step of 7 min at 72 °C. Amplifications were done in a PCR Thermal Cycler DNA Engine (BIO-RAD Laboratories, CA, USA).

2.3. Mutation of target DNAs for CPV-2c VP2 partial gene cloning

Because there are no CPV-2c infections in Korea, the CPV-2c VP2 gene used to examine the PNA array was created using a QuikChange site-directed mutagenesis kit (Stratagene Cat. No. 200519, USA). The primers used to substitute Glu for Asp at VP2 aa 426 were: 2Cmf (forward), 5′-CCTCCTGTAACAGAAGATAATGTATTGCGACCA-3′ and 2Cmr (reverse), 5′-TGGTCGCAATAC ATTATCTTCTGTTACAGGAGG-3′. The PCR amplification conditions included an initial denaturing step of 95 °C for 30 s, and 16 cycles each of 30 s at 95 °C, 60 s at 55 °C, and 60 s at 68 °C. The PCR products were cloned using the pGEM-T plasmid and identified by sequencing. The cloned mimic CPV-2c VP2 partial gene plasmid for amplification of the mimic CPV-2c was used with the biotin-labeled CP-426 primer set.

2.4. PNA probe design and synthesis

The canine parvovirus VP2 gene sequences (n = 126) had been previously obtained from the GenBank database at the National Center for Biotechnology Information and analyzed (Yoon et al., 2009). Two different amino acid regions in the VP2 protein of CPV, aa 305 (913–915 nt) and aa 426 (1276–1278 nt), were recognized by specific markers: CPV-2 (Asp305 and Asn426), CPV-2a (Tyr305 and Asn426), CPV-2b (Tyr305 and Asp426), and CPV-2c (Tyr305 and Glu426). Probes for this region were designed to discern the sequences of a one-point mutation located at the center of the 15- or 19-mer probe (Table 1). The 5′-end of each probe was modified by adding a PNA-optimized spacer and linker, to enable covalent immobilization on an epoxy-coated glass surface. PNA probes were synthesized (Panagene Inc., Daejeon, Korea) using benzothiazole-2-sulfonyl (Bts) as an amine-protecting group (Lee et al., 2007). An Agilent 1100 high performance liquid chromatographer (HPLC; Agilent Technologies, Wilmington, DE, USA) was used to purify the PNA probes. PNA probe quality was assessed by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) (AX1MA-CFR, SHIMADZ Co., Kyoto, Japan). Concentrations of the PNA probes were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.5. Fabrication of the PNA array

The PNA probes were dissolved in distilled water (DW) such that each well in the microplate contained up to 50 μM of probe in 10 μl of a PNA spotting buffer. The spotting mixture was printed onto epoxy slides using a Qarray Mini microarrayer equipped with aQu solid pins (Genetix, New Milton, UK), with the humidity in the microarrayer maintained at ~75–85%. The printed slides were incubated for >9 h in this humidified microarrayer. Next, the printed slides were blocked in succinic anhydride and dimethyl formamide (DMF) for 2 h at 40 °C. The slides were washed twice with DMF, ethanol, and DW, and then immersed in boiling water for 5 min and dried using compressed air.

2.6. Hybridization and scanning

Ten microliters of biotin-labeled target DNA was mixed with 70 μl of the PNA hybridization buffer containing Cy5-streptavidin (Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England), and then applied to the CPV PNA array and incubated for 2 h at 40 °C. After hybridization, the slides were washed twice for 5 min with PNA wash buffer and dried. Array images were obtained using a non-confocal fluorescent scanner (GenePix 4000B, Axon Instruments, Union City, USA) at a typical laser power of 100% and a photomultiplier tube gain of 700. The fluorescence signal intensities represent the hybridization signals of the probe-target duplexes.

2.7. Validation of the PNA array

To validate the developed PNA array, two real-time PCR (RT-PCR) assay based on a previously described TaqMan assay specific for all common types of CPV (Decaro et al., 2005) and based on a minor groove binder (MGB) probe specific for CPV types 2a/2b and 2b/2c (Decaro et al., 2006c) were used. Specific primers

Table 1
Sequences of specific PNA probes used for CPV antigenic typing.

Probes	Peptide nucleic acid sequence[a] (N→C)		Nt (aa) sequence position of VP2 gene	Antigenic typing
CP1	NH_2-linker-	TCCTATATCACCAAAGT	923–907 (305)	CPV-2
CP2	NH_2-linker-	CTCCTATATAACCAAAGTT	924–906 (305)	CPV-2a, -2b, 2c
CP3	NH_2-linker-	TACATTATCATTTGTTACAG	1287–1268 (426)	CPV-2 or -2a
CP4	NH_2-linker-	TACATTATCATTCGTTACAG	1287–1268 (426)	CPV-2
CP5	NH_2-linker-	AATACATTATCATCTGTTACA	1289–1269 (426)	CPV-2b
CP6	NH_2-linker-	ATTATCTTCTGTTACTGG	1284–1267 (426)	CPV-2c

[a] The underlined letters correspond to the nucleotide sequences at amino-acid positions 305 or 426.

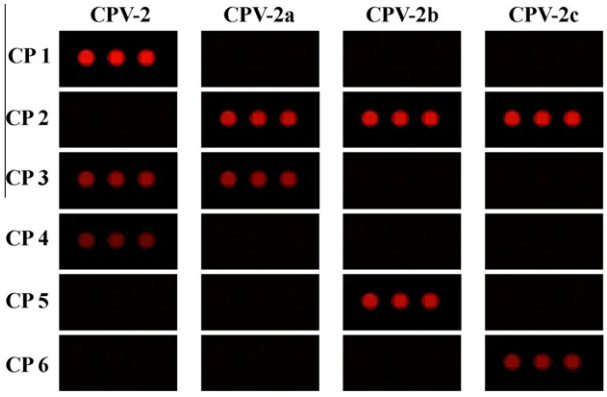

Fig. 1. Layout and scanned images of the CPV PNA array. Each PNA probe was spotted in triplicate. Scanned images were obtained through hybridization of four CPV types: CPV-2 (CP1, CP3, or CP4), CPV-2a (CP2 and CP3), CPV-2b (CP2 and CP5), and CPV-2c (CP2 and CP6).

(CPV-For and CPV-Rev) for the TaqMan assay and a specific probe (CPV-Pb) labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) at its 5′ end and with the quencher dye 6-carboxytetramethylthodamine (TAMRA) at its 3′ end were used. PCR was performed according to the following thermal cycle protocol: an initial denaturing step of 95 °C for 10 min, and 40 cycles each of 15 s at 95 °C, 15 s at 52 °C, and 60 s at 60 °C (Decaro et al., 2005). The previously described specific primers based on the MGB probe were designated CPVa/b-For and CPVa/b-Rev for the Type 2a/2b assay and CPVb/c-For and CPVb/c-Rev for the Type 2b/2c assay (Decaro et al., 2006c). PCR cycling conditions for the MGB probes designed to discriminate types 2a/2b (CPVa-Pb and CPVb1-Pb) and 2b/2c (CPVb2-Pb and CPVc-Pb) were: an initial denaturing step of 95 °C for 10 min, and 45 cycles each of 30 s at 95 °C and 60 s at 60 °C (Decaro et al., 2006c).

3. Results

3.1. Efficacy of PNA probes for CPVs

The HPLC and MALDI-TOF results showed excellent purity of the synthesized oligomer (area in HPLC, 87%). The estimated average coupling yield per base was >99% (data not shown). The CP1 and CP2 probes were designed to recognize aa 305 of VP2, and the CP3–CP6 probes were designed to recognize aa 426 of the VP2 protein (Table 1). The optimal type-specific probes (CPV-2: CP1, CP3, or CP4 probes; CPV-2a: CP2 and CP3 probes; CPV-2b: CP2 and CP5 probes; CPV-2c: CP2 and CP6 probes) were hybridized to the cloned VP2 DNAs of the four CPV types (Fig. 1). All CPV-specific probes hybridized specifically to their corresponding targets, with no cross-hybridizations with other CPVs.

3.2. Detection limit of the CPV PNA array

The detection limit of the CPV PNA array was determined by amplification and hybridization of the aa 305 and aa 426 clones. The clones of CPV-2, -2a, -2b, and -2c were diluted from 10^5 to 10^1 copies per μl to determine the optimal concentration with respect to detection limits, and were subjected to PCR with biotin-labeling using the DNA of the clones. They were then hybridized with each probe on the CPV PNA chip plate. The detection limits for the PNA array varied from 40 to 2000 copies/μl for CPV-2, 600–800 copies/μl for CPV-2a, 80–1000 copies/μl for CPV-2b, and 80–600 copies/μl for CPV-2c (Table 2). The threshold cycle (Ct) values and the inter-assay reproducibility of the PNA assay were examined using the cloned DNA fragments from the four CPV antigenic types. Each assay contained a non-template control (NTC), which never yielded fluorescence signals. Table 2 shows the coefficients of variation (CVs) of the Ct values for the various input DNA copy numbers. The CV values for CPV-2, -2a, -2b, and -2c ranged between 5.10–15.09%, 4.70–11.85%, 5.38–13.56%, and 7.14–10.73%, respectively (Table 2). A cut-off fluorescence signaling value of 5000 was determined from the detection limits of the PNA array based on the minimum number of copies of CPVs (-2, 2a, -2b, and -2c) required for detection.

3.3. Validation of the CPV PNA array using field samples

Analysis of the sensitivity and specificity of the PNA array relative to the Anigen rapid CPV Ag test kit (Bionote, South Korea) and the real-time PCR using TaqMan assay (Decaro et al., 2005) was performed on 135 field feces samples (Table 3). The positive rates according to the TaqMan assay, CPV PNA array, and Anigen rapid

Table 2
Coefficients of variations (CVs) and threshold cycle (Ct) inter-assays of the CPV PNA array.

Run	Probe	Threshold cycle (Ct)				
		10^{5}[a]	10^{4}	10^{3}	10^{2}	10^{1}
Type-2	CP1	28597 ± 2615 [b]	23683 ± 3265	19150 ± 2891	11433 ± 1315	4773 ± 430
		9.15[c]	13.79	15.09	11.50	9.01
	CP3	13170 ± 1192	8193 ± 735	9009 ± 786	2928 ± 221	890 ± 45
		9.06	8.98	8.72	7.58	5.10
	CP4	10647 ± 747	9215 ± 672	3467 ± 338	2476 ± 217	158 ± 14
		7.02	7.30	9.76	8.79	9.02
Type-2a	CP2	17091 ± 2024	15261 ± 1450	6025 ± 444	2999 ± 252	310 ± 19
		11.85	9.50	7.38	8.41	6.30
	CP3	12518 ± 1341	7517 ± 592	8353 ± 727	3496 ± 272	1992 ± 93
		10.71	7.88	8.71	7.79	4.70
Type-2b	CP2	19199 ± 1719	18092 ± 1610	16312 ± 1109	8082 ± 434	3243 ± 203
		8.96	8.90	6.80	5.38	6.28
	CP5	16096 ± 2182	14523 ± 1627	5487 ± 556	3055 ± 212	369 ± 34
		13.56	11.21	10.14	6.97	9.36
Type-2c	CP2	19734 ± 1408	18512 ± 1478	16820 ± 1612	7815 ± 695	2950 ± 243
		7.14	7.99	9.59	8.89	8.26
	CP6	14380 ± 1543	13283 ± 1202	8133 ± 730	3013 ± 221	1953 ± 165
		10.73	9.05	8.98	7.34	8.48

[a] Input copy number of control CPV DNA.
[b] Mean Ct ± S.D.
[c] CV, coefficient of variation.

Table 3
Analysis of the sensitivity and specificity of the CPV PNA array relative to those of the TaqMan assay and Anigen rapid CPV Ag test kit using feces specimens from dogs suspected of having CPV.

		TaqMan assay		Anigen rapid CPV Ag test kit		Total
		+	–	+	–	
CPV PNA array	+	62	0	50	12	62
	–	7	66	2	71	73
Total		69	66	52	83	135

The sensitivity and specificity of the CPV PNA array relative to those of the TaqMan assay were 89.8% (62/69) and 90.4% (66/73), respectively.

CPV Ag test kit were determined to be 51.1% (69/135), 45.9% (62/135), and 38.5% (52/135), respectively. The sensitivity and specificity of the CPV PNA array in comparison with the TaqMan assay was 89.8% (62/69) and 90.4% (66/73), respectively.

3.4. Antigenic typing by the CPV PNA array

The CPV PNA array and the Type 2a/2b MGB probe assay using the minor groove binding probe for discrimination of antigenic typing identified 62 positives (61 CPV-2a and 1 CPV-2b) and 69 positives (68 CPV-2a and 1 CPV-2b), respectively, in 135 field fecal samples. The linear graph of the Type 2a/2b MGB probe assay indicated a slope of $R^2 = 0.983$ for the correlation between DNA copies and threshold cycles (Fig. 2), and one CPV-2b field sample showed a significantly high value ($10^{9.97}$ copies for the 7.18 threshold cycle). Seven field samples that tested positive in the Type 2a/2b MGB probe assay but negative in the CPV PNA array showed a signal intensity below 5000 (Fig. 3) and had less than 100 copies/μl CPV according to the Type 2a/2b MGB probe assay. The signal intensities of one CPV-2b, according to the CPV PNA array, were 42,653 for the CP2 probe and 39,012 for the CP5 probe. This was the same sample detected as CPV-2b in the Type 2a/2b MGB probe assay.

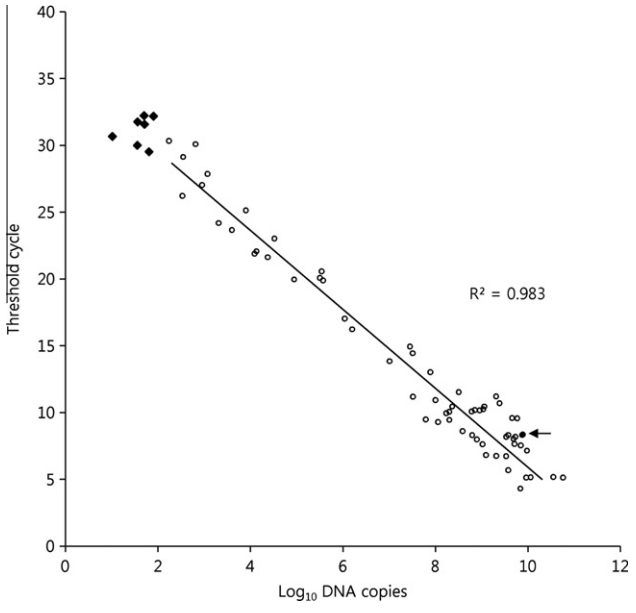

Fig. 2. One CPV-2b and 68 CPV-2a positive field samples covering the shared $\log_{10}$ dynamic ranges of the RT-PCR using the Type 2a/2b MGB probe assay. The arrow indicates the one CPV-2b positive sample. The seven diamond-shaped points represent CPV-2a positive samples below 100 copies/μl DNA concentration.

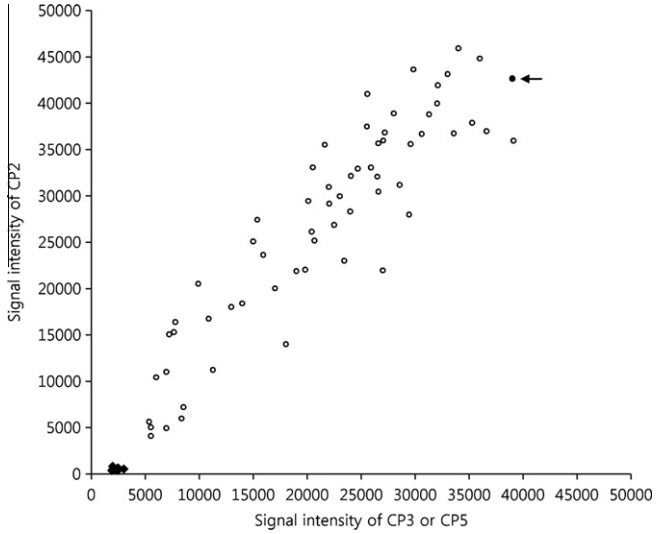

Fig. 3. One CPV-2b and 61 CPV-2a positive field samples covering the shared $\log_{10}$ dynamic ranges of the CPV PNA array. The arrow indicates the one CPV-2b positive sample. The seven diamond-shaped points represent CPV-2a negative samples below 5000 (cut-off) signaling value.

4. Discussion

Canine parvoviral enteritis is an acute, life-threatening infection that can lead to hemorrhagic diarrhea. It is therefore important that a quick and reliable method of ante-mortem diagnosis is available so that treatment can begin early (Pospischil and Yamaho, 1987). PCR and real-time PCR are reportedly more sensitive than hemagglutination, virus isolation, or immunochromatographic tests for CPV antigen detection (Desario et al., 2005; Decaro et al., 2010). Three rapid commercial CPV antigen detection tests that use immune electron microscopy and PCR have been reported to be useful for ante-mortem diagnosis (Schmitz et al., 2009), but cannot be used to rule out parvovirus infection in animals with typical clinical signs. A real-time PCR (TaqMan) assay was previously used for the discriminative detection of CPV with two MGB probes specific for CPV-2 and its antigenic variants (types -2a, -2b, and -2c) (Decaro et al., 2006c). A real-time PCR for rapid identification of vaccine and field strains of CPV-2b has also been developed via the licensing of a CPV-2b vaccine in Europe (Decaro et al., 2006b,d).

Our newly developed PNA arrays with a cut-off signaling value of 5000 had a specificity and sensitivity that were 89.8% and 90.4%, respectively, of those of the TaqMan assay. Previous studies have demonstrated that PNA oligonucleotides exhibit unprecedented thermal stability when hybridized with their DNA target molecules. PNAs increase the Tm values by ~1.0 °C per base pair for a PNA–DNA duplex, and the ΔTm values of a given PNA–DNA duplex are 2.5–8.5 °C higher than those of the corresponding DNA–DNA duplex (Choi et al., 2009).

The detection limit for the discriminative diagnosis of CPVs using a TaqMan assay with MGB probes was ~10 DNA copies (Decaro et al., 2006b), while that of the rapid immunochromatographic commercial SNAP canine parvovirus antigen test (IDEXX Laboratories) required $>10^5$ DNA copies/mg feces for non-discriminative CPV diagnosis (Schmitz et al., 2009). For human papillomavirus (HPV) types 11 and 58, the detection limits for a PNA array are as low as 10 copies/μl of the initial DNA concentration (Choi et al., 2009). However, the detection limits of the CPV PNA array with a cut-off signaling value of >5000 varied between 40 and 2000 copies/μl depending on CPV antigenic type detected by the two probes. The detection sensitivity of the CPV PNA array is some-

what lower than the sensitivity of the TaqMan assay, but an advantage of the PNA array is that it makes accurate detection possible without special nucleotide sequencing. It appears to be equivalent to the TaqMan assay with respect to its ability to detect single nucleotide polymorphisms (SNPs).

The turnaround time for our CPV antigenic-typing PNA array, including time for DNA extraction, PCR, hybridization, and scanning, was ~4.5 h, which is similar to that reported for 32 HPV typing (Choi et al., 2009). PNA arrays for CPV antigenic typing show good reproducibility, with a coefficient of variation of ⩽15.09%. When stored at room temperature, PNA arrays retain a relatively strong signaling intensity (>90%) over 13 months, whereas DNA arrays are unstable and display a relatively low signaling intensity (<10%) at 3 months (Choi et al., 2009).

The accuracy of the CPV antigenic-typing PNA array was as follows. The assay identified 62 positive samples (61 CPV-2a and 1 CPV-2b) compared to 52 positive samples for the Anigen rapid CPV Ag test kit which found that 83 samples were negative. Unfortunately, validation of the antigenic typing discrimination of CPV 2c by the PNA array was not performed because this strain does not exist in South Korea. CPV-2, a vaccine strain, was also not detected in field feces samples. Field validation should be used as a supplementary test for the detection of CPV-2 and -2c, but it is expected that CPV antigenic typing will work well with these strains as well.

In summary, the CPV PNA array described in this study could be used for fast and precise ante-mortem discriminative antigenic typing diagnosis of canine parvovirus infections.

Acknowledgment

Research on PNA probe synthesis was supported in part by Panagene Inc. (Daejeon, Korea).

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