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A minor groove binder probe real-time PCR assay for discrimination between type 2-based vaccines and field strains of canine parvovirus

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Abstract

A minor groove binder (MGB) probe assay was developed to discriminate between type 2-based vaccines and field strains of canine parvovirus (CPV). Considering that most of the CPV vaccines contain the old type 2, no longer circulating in canine population, two MGB probes specific for CPV-2 and the antigenic variants (types 2a, 2b and 2c), respectively, were labeled with different fluorophores. The MGB probe assay was able to discriminate correctly between the old type and the variants, with a detection limit of 10^1 DNA copies and a good reproducibility. Quantitation of the viral DNA loads was accurate, as demonstrated by comparing the CPV DNA titres to those calculated by means of the TaqMan assay recognising all CPV types. This assay will ensure resolution of most diagnostic problems in dogs showing CPV disease shortly after CPV vaccination, although it does not discriminate between field strains and type 2b-based vaccines, recently licensed to market in some countries.

Keywords: Dog; Parvovirus; Type 2 vaccine; Field strains; MGB probe assay

1. Introduction

Canine parvovirus type 2 (CPV-2) is responsible for acute, sometimes fatal, gastroenteritis in dogs (Carmichael and Binn, 1981). CPV-2 emerged in the late 1970s (Kelly, 1978; Appel et al., 1979; Burtonboy et al., 1979; Johnson and Spreadbrow, 1979), but it was replaced in a few years by its antigenic variants (Parrish et al., 1985, 1988, 1991). Currently, three main antigenic variants of CPV-2 are known, named type 2a, 2b and 2c and variously distributed in dog population worldwide (Mochizuki et al., 1993; De Ybanez et al., 1995; Greenwood et al., 1996; Truyen et al., 1996, 2000; Steinel et al., 1998; Sagazio et al., 1998; Buonavoglia et al., 2000; Pereira et al., 2000; Buonavoglia et al., 2001; Martella et al., 2004; Desario et al., 2005; Decaro et al., 2005b,c, 2006). CPV-2c, designated previously as CPV Glu-426 mutant, emerged in Italy in 2000 (Buonavoglia et al., 2001) and has been detected in other countries (Nakamura et al., 2004). Moreover, its pathogenicity has been investigated (Decaro et al., 2005a). The original type 2, although disappeared from the field, is still present in the CPV-2 vaccines available on the market (Parrish et al., 1988).

Traditionally, identification of the CPV-2 variants is carried out by means of time-consuming techniques, such as haemagglutination inhibition (HI) test with monoclonal antibodies (MAbs) (Parrish and Carmichael, 1983; Nakamura et al., 2004), PCR-RFLP with enzyme *Mbo*II (Buonavoglia et al., 2001), PCR-based methods (Pereira et al., 2000), or sequence analysis, often requiring the use of combined methods for the definitive prediction of antigen specificity. Recently, two real-time PCR assays using minor groove binder (MGB) probes have been developed for rapid and unambiguous characterisation of CPV-2 (Decaro et al., 2005b, 2006). The MGB probe assays are able to recognise the single nucleotide polymorphisms (SNPs) existing between types 2a/2b (A4062G) and between types 2b/2c (T4064A), which determine the presence at residue 426 of the capsid protein of amino acids Asn, Asp and Glu in types 2a, 2b and 2c, respectively (Parrish et al., 1991; Buonavoglia et al., 2001 and Table 1). Both type 2a/2b and type 2b/2c assays were found highly sensitive and specific, although the type 2a-specific probe was not able to discriminate type 2a CPVs from the orig-

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Table 1
Amino acid variations in the VP2 protein of different CPV types

	Amino acid variations at residue[a]							
	87 3045–3047[b] ATG (Met)[c] TTG (Leu)	101 3087–3089[b] ATT (Ile)[c,d] ACT (Thr)[d]	297 3675–3677[b] TCT (Ser)[c] GCT (Ala)	300 3684–3686[b] GCT (Ala)[c] GGT (Gly)	305 3699–3701[b] GAT (Asp)[c] TAT (Tyr)	375 3909–3911[b] AAT (Asn)[c] GAT (Asp)	426 4062–4064[b] AAT (Asn)[c] GAT (Asp), GAA (Glu)	555 4449–4451[b] GTA (Val)[c] ATA (Ile)
CPV-2	Met	Ile	Ser	Ala	Asp	Asn	Asn	Val
CPV-2a	Leu	Thr	Ser	Gly	Tyr	Asp	Asn	Ile
CPV-2b	Leu	Thr	Ser	Gly	Tyr	Asp	Asp	Val
New CPV-2b	Leu	Thr	Ala	Gly	Tyr	Asp	Asp	Val
New CPV-2a	Leu	Thr	Ala	Gly	Tyr	Asp	Asn	Val
Asp-300 (CPV-2a/CPV-2b)	Leu	Thr	Ala	Asp	Tyr	Asp	Asn Asp	Val
CPV-2c	Leu	Thr	Ala	Gly	Tyr	Asp	Glu	Val

[a] Positions are referred to the amino acid and nucleotide sequences of strain CPV-b (accession no. M38245).
[b] Nucleotide position.
[c] Codon observed.
[d] Codon affected by the SNP used to design the type-specific probes CPV2-Pb (type 2) and CPVv-Pb (variants).

inal type 2. In fact, both types present nucleotide A at position 4062 of the viral genome, so that the old type is characterised erroneously as type 2a using the MGB strategy (Decaro et al., 2005b, 2006). This makes the type 2a/2b MGB probe assay inadequate for discrimination between dogs vaccinated with the original type 2 and dogs infected with type 2a, although it has been noted that the vaccine virus is shed in the faeces at low titres and for a shorter time period than field strains and that usually the diagnostic tests are carried out on the faeces of sick animals (Decaro et al., 2006). Some problems may occur especially when testing faecal samples collected from dogs displaying diarrhoea few days after administration of a type 2-based vaccine, when it is crucial to be able to detect differentially the vaccine virus and the field strains of CPV-2.

In such an effort, we have developed an MGB probe assay for rapid discrimination between vaccine (old type) and field (types 2a, 2b, 2c) strains of CPV-2.

2. Materials and methods

2.1. Samples

A total of 56 samples were tested, consisting of 11 samples positive for the original type 2 and 45 samples positive for the antigenic variants (type 2a, $n = 15$; type 2b, $n = 15$; type 2c, $n = 15$). Prediction of CPV specificity was carried out by HI test with MAbs (Parrish and Carmichael, 1983; Nakamura et al., 2004), PCR-RFLP with enzyme *Mbo*II (Buonavoglia et al., 2001), or sequence analysis, as described by Desario et al. (2005). Samples containing the antigenic variants were recruited from a previous study (Desario et al., 2005). Since the old type no longer circulates in dog population, samples containing CPV-2 included only the following vaccine formulations licensed in Italy: Vanguard 7, Vanguard CPV (Pfizer Inc., NY, USA), Tetradog-CHPL, Parvodog-P, Primodog, Eurican-CHPPI2-L (Merial Italia S.p.A., Milan, Italy), Nobivac® CEPPi, Nobivac® PARVO-c, Nobivac® PUPPY CP (Intervet Italia S.r.l., Milan, Italy), Canigen CEPPi/L (Virbac s.r.l., Milan, Italy). The experimental type 2-based vaccine 17/80-ISS (Buonavoglia et al., 1983) was also tested. For each commercial vaccine, three doses from different batches were tested in separate runs.

2.2. Template preparation

Faecal samples were homogenised (10%, w/v) in phosphate buffered saline (PBS, pH 7.2) and subsequently clarified by centrifuging at $1500 \times g$ for 15 min. DNA was extracted from the supernatants by boiling the faecal homogenates for 10 min and chilling on ice (Schunck et al., 1995; Uwatoko et al., 1995). Lyophilised vaccines were resuspended in 1 ml of PBS and then processed as the faecal homogenates. To reduce residual inhibitors of DNA polymerase activity to ineffective concentrations, the DNA extracts were diluted 1:10 in distilled water (Decaro et al., 2005c, 2006).

2.3. Standard DNAs

Standard DNAs for the old type and field strains (types 2a, 2b and 2c) were obtained from vaccine Vanguard CPV (Pfizer Italia srl, Rome, Italy) and from a field faecal sample characterised as type 2a (Decaro et al., 2006), respectively. In order to increase the viral DNA titre, the vaccine strain was passaged three times on A-72 canine cell line. DNA loads for vaccine and field viruses were calculated using a real-time PCR assay able to recognise all CPV-2 strains (Decaro et al., 2005c). Ten-fold dilutions of the standard DNAs were carried out in a CPV-negative faecal suspension. Aliquots of each dilution were frozen at −70 °C and used only once.

2.4. Design of primers and MGB probes

Primers and MGB probes specific for the vaccine and wild types were designed and synthesised by Applied Biosystems (Foster City, CA) taking into account the SNP T3088C encountered in the capsid protein gene between the original type 2 and its variants (2a, 2b and 2c), which is responsible for the change

Table 2
Sequence, position and specificity of the oligonucleotides used in the study

Assay	Primer/probe	Sequence 5′ to 3′	Polarity	Specificity	Position	Amplicon size
TaqMan assay[a]	CPV-For	AAACAGGAATTAACTATACTAATATATTTA	+	All types	4104–4135[c]	93 bp
	CPV-Rev	AAATTTGACCATTTGGATAAACT	−		4176–4198[c]	
	CPV-Pb	FAM-TGGTCCTTTAACTGCATTAAATAATGTACC-TAMRA	+		4143–4172[c]	
Type 2/variants MGB probe assay[b]	CPV2/v-For	GCAGTTAACGGAAACATGGCTTTAG	+	All types	3057–3081[c] 772–796[d]	68 bp
	CPV2/v-Rev	TCAACCAATGACCAAGGTGTTACAA	−		3100–3124[c] 815–839[d]	
	CPV2-Pb	FAM-TGTGCATGAATATCAT-MGB	+	Type 2 (old type)	3082–3097[c]	
	CPVv-Pb	VIC-TTTGTGCATGAGTATCAT-MGB	+	Antigenic variants	797–814[d]	

[a] Decaro et al. (2005c).
[b] This study.
[c] Oligonucleotide positions are referred to the sequence of CPV-2 (old type) strain CPV-b (accession no. M38245).
[d] Oligonucleotide positions are referred to the sequence of CPV-2a strain CPV-15 (accession no. M24003).

Ile to Thr at residue 101 of the capsid protein (Martella et al., in press and Table 1). Probes specific for vaccine and field strains were labeled with FAM and VIC fluorophores, respectively. Specificity, sequence and position of real-time PCR primers and MGB probes are reported in Table 2.

2.5. MGB probe assay

Real-time PCR was conducted in an i-Cycler iQ™ Real-Time Detection System (Bio-Rad Laboratories Srl, Milan, Italy). The reactions (25 μl) contained 10 μl of template or standard DNA, 12.5 μl of IQ™ Supermix (Bio-Rad Laboratories Srl), 900 nM of primers CPV2/v-For and CPV2/v-Rev, 200 nM of probes CPV2-Pb and CPVv-Pb. Two different wells were used for each test sample and each dilution of standard DNA. After activation of iTaq DNA polymerase at 95 °C for 10 min, 45 cycles of two-step PCR were performed, consisting of denaturation at 95 °C for 30 s and primer annealing-extension at 60 °C for 1 min. The increase in fluorescent signal was registered during the annealing-extension step of the reaction and the data were analysed with the appropriate sequence detector software (version 3.0).

2.6. Detectability, linearity, specificity and reproducibility

Serial log 10 dilutions of each standard DNA (type 2 and type 2a) were carried out in a CPV-negative faecal suspension and used to determine the detectability and the linearity of the assay. Since vaccine virus was found to have DNA titres lower with respect to field CPVs, standard DNA concentrations ranged from 10^8 to 10^0 and from 10^9 to 10^0 DNA copies/10 μl of template for type 2 and 2a, respectively. C_T values were measured in triplicate and were plot against the log of the input DNA copy number.

The specificity of the assay was evaluated by processing high and low concentrations of DNA from the original type 2 and types 2a, 2b and 2c, as well as DNA preparations from four other unrelated DNA viruses of dogs, including minute virus of canine (Decaro et al., 2002a), canid herpesvirus 1 (Decaro et al., 2002b), canine adenovirus type 1 (Pratelli et al., 2001) and type 2 (Decaro et al., 2004), or sterile water.

The intra-assay and interassay reproducibilities were evaluated using high, intermediate and low input of the standard DNAs of the old type and CPV-2a (dilutions in a CPV-negative faecal suspension). The coefficients of variation for the absolute copy number obtained for each dilution were calculated as described previously (Decaro et al., 2005c, 2006).

2.7. Simultaneous detection of vaccine and field strains

In order to determine the ability of quantifying correctly and discriminating between vaccinal and field strains, artificially generated DNA mixtures composed of DNAs from vaccines and from field strains (2a, 2b or 2c) in various concentrations were tested by the MGB probe assay and the absolute copy numbers for each type were calculated.

2.8. Quantitation of viral DNA by TaqMan assay

All the DNA extracts were processed in parallel by a TaqMan assay able to recognise both the old type and the antigenic variants (Decaro et al., 2005c). The assay is internally controlled by using as exogenous DNA the nucleic acid extracted from ovine herpesvirus type 2 (Decaro et al., 2003). Real-time PCR was carried out in a 25 μl reaction containing 12.5 μl of master mix (Bio-Rad Laboratories Srl), 600 nM of primers CPV-For and CPV-Rev, 200 nM of probe CPV-Pb (Table 2) and 10 μl of DNA. The following thermal protocol was used: activation of iTaq DNA polymerase at 95 °C for 10 min and 40 cycles consisting of denaturation at 95 °C for 15 s, primer annealing at 52 °C for 30 s and extension at 60 °C for 1 min.

3. Results

3.1. Analytical performances

The detection limits of the MGB probe assay were shown to be 10^1 DNA copies for both types 2 and 2a. Standard curves

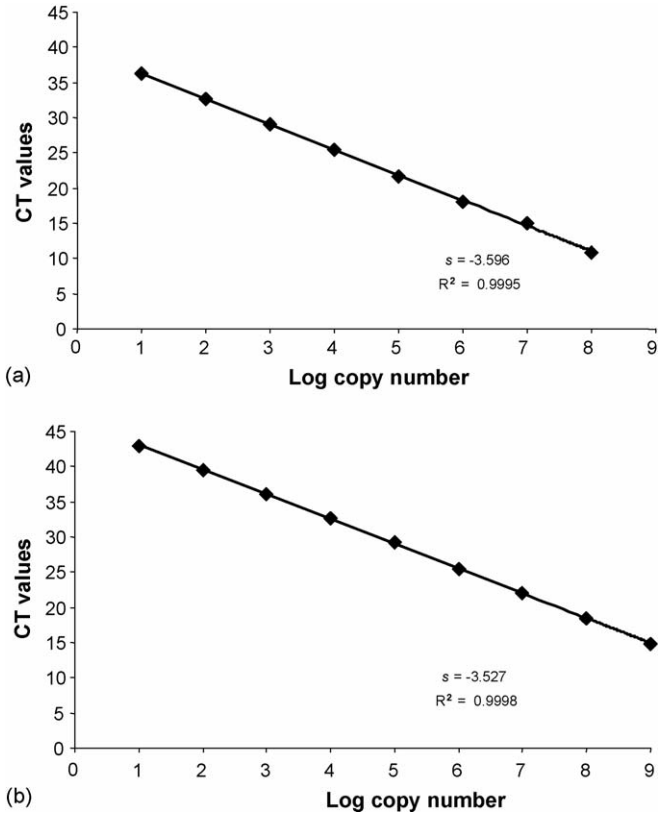

Fig. 1. Standard curves obtained for types 2 (a) and 2a (b) by the type 2/variants MGB probe assay. The dilutions of standard DNA are indicated on the *x*-axis, whereas the corresponding cycle threshold (C_T) values are presented on the *y*-axis. Each dot represents the result of duplicate amplifications of each dilution. The coefficient of determination (R^2) and the slope value (*s*) of the regression curve were calculated and are indicated.

demonstrated a strong linear correlation between 10^1 and 10^8 copies (type 2) or 10^9 copies (type 2a) (Fig. 1).

No cross-reactions were observed between the old type and the variants. FAM fluorescence signals were generated only by DNA templates from vaccines (original type 2), whereas VIC fluorescence was registered when DNA preparations from the wild types were tested. Furthermore, none of the other four canine viruses showed cross-reactivity, as well as FAM or VIC fluorescence was not obtained from sterile water.

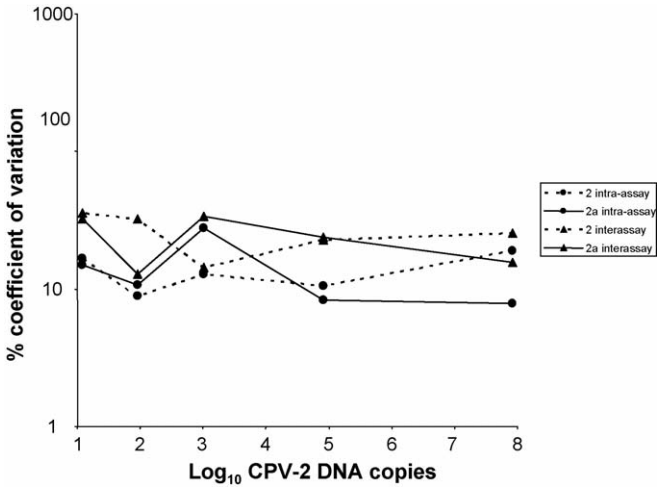

Fig. 2. Coefficients of variation (%) intra-assay and interassay over the dynamic range of the type 2/variants MGB probe assay.

CVs intra-assay ranged between 8.88 and 18.96% for the old type and between 7.80 and 27.86% for CPV-2a. CVs interassay were slightly higher, with values ranging between 14.26 and 35.44% for the original type 2 and between 12.76 and 33.54% for type 2a (Fig. 2).

3.2. Simultaneous detection of vaccine and field strains

Samples spiked with low (10^3 copies) and high (10^8 copies) concentrations of DNA from the old type and the variants showed no interference during detection and quantitation of vaccine and field strains contained in the same sample, with DNA titres calculated correctly for all CPV types (data not shown).

3.3. Quantitation of viral DNA by TaqMan and MGB probe assays

CPV DNA loads in vaccines and field samples were calculated in parallel by the developed type2/variants MGB probe

Table 3
CPV DNA titres calculated by the MGB probe assay in commercial and experimental vaccines

Vaccine	Company/reference	Specificity	Reported titre ($CCID_{50}$)/dose	Viral DNA titre/dose
Vanguard 7	Pfizer Inc.	Type 2	$\geq 10^{7}$[a]	4.43×10^{12}
Vanguard CPV	Pfizer Inc.	Type 2	$\geq 10^{7}$[a]	6.09×10^{11}
Tetradog-CHPL	Merial Italia S.p.A.	Type 2	$\geq 10^{3}$[b]	7.26×10^{8}
Parvodog-P	Merial Italia S.p.A.	Type 2	$\geq 10^{3}$[b]	5.52×10^{8}
Primodog	Merial Italia S.p.A.	Type 2	$\geq 10^{5.5}$[b]	1.90×10^{8}
Eurican-CHPPI2-L	MERIAL Italia S.p.A.	Type 2	$\geq 10^{4.9}$[b]	2.92×10^{10}
Duramune DA2LP + Pv	Fort Dodge Animal Health S.p.A.	Type 2	$>10^{4.5}$[a]	7.45×10^{7}
Nobivac® CEPPi	Intervet Italia S.r.l.	Type 2	$\geq 10^{7}$[a]	3.17×10^{10}
Nobivac® PARVO-c	Intervet Italia S.r.l.	Type 2	$\geq 10^{7}$[a]	2.08×10^{10}
Nobivac® PUPPY CP	Intervet Italia S.r.l.	Type 2	$\geq 10^{7}$[a]	2.44×10^{10}
Canigen CEPPi/L	Virbac S.r.l	Type 2	$>10^{3}$[a]	1.07×10^{10}
17/80-ISS	Buonavoglia et al. (1983)	Type 2	$10^{7.8}$	6.58×10^{10}

[a] Cell lines used for determination of viral titres are not reported.
[b] Titres calculated on feline cells.
[c] Titres calculated on canine A-72 cells.

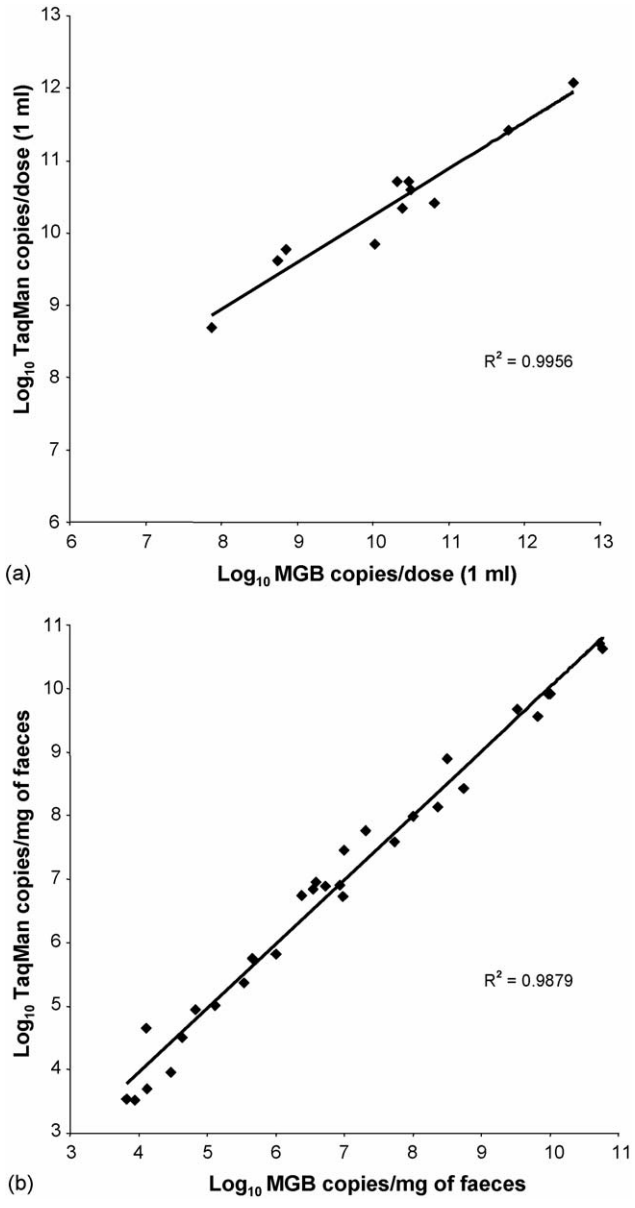

Fig. 3. CPV-2 DNA titres measured by the TaqMan assay and the type 2/variants MGB probe assay. The type 2-based vaccines marketed in Italy (a) and 30 field samples tested positive to types 2a ($n = 10$), 2b ($n = 10$) or 2c ($n = 10$) (b) and covering the shared $\log_{10}$ dynamic ranges of the assays were analysed. DNA titres are expressed as copy numbers per dose and per mg of faeces for vaccines and field samples, respectively.

assay and by the TaqMan assay established previously (Decaro et al., 2005c). As shown in Fig. 3, the two assays were found to correlate well over the shared 8 $\log_{10}$ dynamic ranges, calculating similar DNA titres for the same samples. In contrast, a poor correlation was found between the vaccine titres reported by manufacturers and expressed by $CCID_{50}$ and the DNA copy numbers calculated by the MGB probe assay (Table 3).

4. Discussion

Diagnosis of CPV infection may be ambiguous when carried out on faecal samples from dogs presenting with diarrhoea few days after vaccination. In fact, the modified-live virus contained in the vaccines is able to replicate in the intestinal mucosa of vaccinated dogs (Carmichael et al., 1984; Buonavoglia et al., 1983), despite the unnatural route of administration (intramuscular or subcutaneous instead of oronasal), and to be shed in the faeces albeit at low titres and for a shorter time period with respect to field strains, as noted in a previous work (Decaro et al., 2006). In such a circumstance, the detection of CPV-2 or its nucleic acid in the faeces of vaccinated dogs could provide false-positive results, leading to a misdiagnosis of the disease probably caused by other enteric pathogens of dogs, i.e., canine coronavirus, canine distemper virus, reoviruses, rotaviruses, *Salmonella* spp., etc. Moreover, it would be important to rule out vaccine-induced disease due to regaining of virulence of the vaccine (old type) virus. For this purpose, the characterisation of CPV using traditional techniques is often inconclusive, since a simultaneous infection by the type 2-based vaccine and wild-type virus may mislead the results of HI with MAbs, PCR-RFLP and sequence analysis.

A PCR-based approach has been proposed by Senda et al. (1995) to address this point, which takes advantage of two SNPs, A3045T and C3685G, that determine the replacement of Met by Leu at position 87 and of Ala by Gly at position 300, in old- and wild-type strains, respectively (Table 1). Two primers specific for the wild types (types 2a and 2b) were selected to have one such mutation at the very 3′ end, as nucleotide mismatches that occur at the 3′ end of a primer are highly detrimental to primer extension and strongly decrease PCR amplification. However, in our experience, such mutations were not sufficient to prevent completely amplification of the old type virus (vaccine) (V. Martella, N. Decaro and C. Buonavoglia, personal observation). Moreover, samples containing both vaccine and wild-type strains are amplified successfully, so that the PCR-based strategy will not be able to detect the simultaneous presence of the two viruses in the faeces.

The MGB probe assays developed for the rapid characterisation of the CPV strains (Decaro et al., 2005b, 2006) do not discriminate between the old type and type 2a. Thus, all samples collected from vaccinated dogs and characterised as type 2a should be tested by the novel MGB probe assay in order to assess whether they are true type 2a (field) strains or vaccine (old type) virus. A correct discrimination will help resolution of the diagnostic dilemma arising when dogs develop gastroenteric signs few days after CPV vaccination with the old virus.

Recently, type 2b vaccines have been licensed and are available on the market. Although at the moment such vaccines are not used widely, some problems may arise when testing samples collected from dogs administered a type 2b vaccine. In fact, vaccine virus shed in the faeces would be recognised by the probe specific for the CPV variants, being characterised as a field strain instead of a vaccine strain. Consequently, an additional assay should be developed which is able to discriminate the type 2b vaccines from type 2b field CPVs, in order to obtain a correct discrimination between dogs vaccinated and infected with CPV-2b. In conclusion, the test we have developed does not allow the discrimination of all vaccine viruses from field strains, but it could help a correct diagnosis when dogs display enteritis

shortly after the administration of type 2-based vaccines, that are mostly used worldwide.

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