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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.

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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., 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 MboII (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-
Table 1

Amino acid variations in the VP2 protein of different CPV types

| Amino acid variations at residuea | 87 101 297 300 305 375 426 555 |
|----------------------------------|----------------------------------|
| ATG (Met)c                        | ATT (Ile)c,d                      |
| GCT (Ala)                         | GAT (Asp)c                        |
| GTA (Val)c                        | TCT (Ser)c                        |
| GAA (Glu)                         | ATA (Ile)                         |

| CPV-2                            | Met Ile Ser Ala Asp Asn Asn Val     |
| CPV-2a                           | Leu Thr Ser Gly Tyr Asp Asp Val     |
| CPV-2b                           | Leu Thr Ser Gly Tyr Asp Asp Val     |
| New CPV-2a                       | Leu Thr Ala Gly Tyr Asp Asp Val     |
| New CPV-2b                       | Leu Thr Ala Gly Tyr Asp Asp Val     |
| Asp-300 (CPV-2a/CPV-2b)          | Leu Thr Ala Asp Tyr Asp Asp Val     |
| CPV-2c                           | Leu Thr Ala Gly Tyr Asp Asp Glu Val |

n Positions are referred to the amino acid and nucleotide sequences of virus 1 (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).

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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., 2005, 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 virus vaccine is shed in the faeces of low titre 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). Salivary problems may occur especially when testing faecal samples collected from dogs displaying diarrhoea a 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 MboII (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 formula-
2.5. MGB probe assay

MGB probes are reported in Table 2. Specificity, sequence and position of real-time PCR primers and were labeled with FAM and VIC fluorophores, respectively. Probes specific for vaccine and field strains 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.

### 2.6. Detectability, linearity, specificity and reproducibility

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 CPV2-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 15 s, primer annealing-extension at 52 °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).

### 2.7. 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.
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 (CT) values are presented on the y-axis. Each dot represents the result of duplicate amplifications of each dilution. The coefficient of determination (R²) and the slope value (s) of the regression curve were calculated and are indicated.

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 type 2/variants MGB probe assay.

### Table 3

| Vaccine Company/Reference | Specificity | Reported titre (CCID 50)/dose | Viral DNA titre/dose |
|---------------------------|-------------|-------------------------------|----------------------|
| Vanguard 7                | Type 2      | ≥10^7                         | 4.43 × 10^12         |
| Vanguard CPV              | Type 2      | ≥10^4                         | 6.09 × 10^11         |
| Tetradox-CHPL             | Type 2      | ≥10^10                        | 7.26 × 10^9          |
| Primadog-9                | Type 2      | ≥10^10                        | 5.52 × 10^9          |
| Extrac-SPP1               | Type 2      | ≥10^10                        | 1.90 × 10^9          |
| Duramine DA2LP + Prv     | Type 2      | ≥10^10                        | 2.92 × 10^9          |
| Nobivac CP                 | Type 2      | ≥10^10                        | 7.45 × 10^9          |
| Nobivac PUPPY CP          | Type 2      | ≥10^10                        | 3.17 × 10^9          |
| Canigen CEPPt             | Type 2      | ≥10^10                        | 2.08 × 10^9          |
| Canigen CEPPt             | Type 2      | ≥10^10                        | 2.44 × 10^9          |
| Canigen CEPPt             | Type 2      | ≥10^10                        | 1.07 × 10^9          |

* Cell lines used for determination of viral titres are not reported.
* Titres calculated on canine A-72 cells.
* Titres calculated on feline 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.

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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