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Comparison of three laboratorial tests for diagnosis of canine parvovirus infection

[*Comparação de três testes laboratoriais para diagnóstico da infecção pelo parvovírus canino*]

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ABSTRACT

The aim of this study was to evaluate the rapid tests currently used for canine parvovirus (CPV) diagnosis: hemagglutination test (HA), enzyme immunoassay (EIA) and polymerase chain reaction (PCR). A total of 112 fecal samples collected from diarrheic puppies up to one year of age were tested. The EIA was able to detect CPV antigen in 44 samples. By HA, 32 samples tested highly positive with titers ≥128, eight tested weakly positive (titers 32 and 64) and 72 were negative (titers ≤16). Using PCR, 57 samples were found positive including 13 EIA-negative and 19 HA-negative samples. The best correlation was observed between EIA and PCR (88.4%). These tests were able to detect all types of CPV, including CPV-2c. Considering that 23%-33% of dogs presenting enteritis did not show infection by EIA nor HA, negative results from the antigen detection tests should be confirmed through molecular methods.

Keywords: canine parvovirus, enteritis, HA, EIA, PCR

RESUMO

Avaliaram-se os métodos rápidos rotineiramente utilizados para diagnóstico da infecção por parvovírus canino (CPV): teste de hemaglutinação (HA), ensaio imunoenzimático (EIE) e reação em cadeia pela polimerase (PCR). Um total de 112 amostras fecais de cães diarreicos com até um ano de idade foi testado. O EIE foi capaz de detectar o antígeno do CPV em 44 amostras. Por HA, 32 amostras foram consideradas fortemente positivas com títulos ≥128, oito fracamente positivas (títulos 32 e 64) e 72 negativas (títulos ≤16). Por PCR, 57 amostras foram positivas incluindo 13 EIE-negativas e 19 HA-negativas. A melhor correlação foi observada entre EIE e PCR (88,4%). Os testes foram capazes de detectar todos os tipos de CPV, incluindo o CPV-2c. Considerando-se que em 23%-33% dos filhotes com enterite a infecção por CPV não foi diagnosticada pelos testes de EIE e HA, os resultados negativos nos testes de detecção de antígeno devem ser confirmados por meio de métodos moleculares.

Palavras-chave: parvovírus canino, enterite, HA, EIE, PCR

INTRODUCTION

Canine parvoviral enteritis is an acute highly contagious life-threatening infection and, therefore, laboratorial diagnosis is essential for screenning diarrheic puppies in order to prevent infection of susceptible contact animals (Goddard and Leisewitz, 2010). Soon after their appearence, the original virus (CPV-2) was subsequently replaced by the new variants, CPV-2a and CPV-2b (Parrish *et al.*, 1991). In the early 2000's, a novel CPV mutant (CPV-2c) emerged in Italy (Buonavoglia *et al.*, 2001) and is currently spreading among the canine population (Hoelzel and Parrish, 2010). These CPV variants (2a, 2b and 2c) were recently described in Rio de

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Janeiro associated with enteritis in vaccinated and unvacinnated young dogs (Castro *et al.*, 2010; 2011).

Concerns have been expressed that the continuous evolution of CPV could negatively affect the performance of diagnostic tests based on monoclonal antibodies and PCR (Buonavoglia *et al.*, 2001; Hong *et al.*, 2007).

The purpose of the present study was to evaluate the rapid laboratory tests most commonly used for CPV diagnosis: hemagglutination test (HA), enzyme immunoassay (EIA) and polymerase chain reaction (PCR). In addition, the CPV type detected in 33 fecal samples was characterized by partial VP2 sequencing.

MATERIAL AND METHODS

A total of 112 fecal samples from unvaccinated (58) and vaccinated (54) diarrheic puppies up to one year of age, collected between January 2006 and May 2009, were tested. The population comprizes both mixed (29/112) and pure bred (83/112) dogs and the median age was 3.8 months. This trial was licensed by the Ethics Committee of Animal Research-PROPPI/ UFF-CEPA/NAL under registration number 0082/09.

EIA was carried out with a commercial kit (SNAP® Parvo Antigen Test – Idexx Laboratories Inc®, USA) following the manufacturer's instructions. The tests were performed immediately after fecal sample collection. A portion of each sample was stored at -20ºC prior to HA and PCR tests.

Approximately 10% suspensions from all fecal samples were prepared in 0.01M Tris-HCl-0.0015M Ca^{2+}, pH 7.2 and tested in 96-well V-plates by hemagglutination (HA) with porcine erythrocytes (Castro *et al.*, 2007). Samples with titers up to 16 were considered positive.

Viral DNA was extracted from approximately 10% fecal suspension samples using a combination of phenol/chloroform/isoamyl-alcohol (Invitrogen®) and silica/guanidin thyocianate (Costa *et al.*, 2005). Polymerase chain reaction (PCR) was carried out using primer pair P2ab, located at nt 3025-3045 and 3685-3706 (within VP2 capsid gene) which amplifies the new CPV types circulating in dog population (CPV-2a/2b/2c) (Senda *et al.*, 1995; Costa *et al.*, 2005).

A total of 33 CPV-positive samples were randomly selected for sequencing. Primers 555For/555Rev (4003-4585) were used to amplify a 583 bp fragment of the VP2 capsid gene which encodes the two informative aminoacids (426 and 555) that allows the caracterization of CPV types (Buonavoglia *et al.*, 2001).

The amplicons were purified using QIAQuick PCR purification kit and submitted to direct sequencing using BigDye terminator v. 1.1 cycle sequencing kit (Applied Biosystems, CA, USA) (Otto *et al.*, 2008). Alignments were retrieved and analyzed by Bio-Edit sequence alignment editor v.7.0.1 and compared with sequences available at GenBank: M38245 (CPV-2), DQ340434 (CPV-2a), DQ340409 (CPV-2b) and EU797727 (CPV-2c).

The comparison of sensitivity and specificity of the HA test and EIA with the PCR was performed using Chi-square and Fisher´s exact test through StatCalc Epi Info 3.5.1. 2002. A p-value <0.05 was regarded as significant.

RESULTS

CPV antigen was detected in 44/112 samples (39.3%) by EIA and 40/112 (35.7%) by HA. By PCR, 57/112 samples (50.9%) were found positive for CPV DNA. Samples included 38 HA-positive and 19 HA-negative; they also included 44 EIA-positive and 13 EIA-negative (Table 1). Results of HA and EIA tests, when compared to PCR are both specific (96.4% and 100.0%) with low sensitivity (66.7% and 77.2% respectively) ($P<0.05$).

Of 112 samples, 89 (79.5%) gave concordant results for the three tests. Non-concordant results were found in 23 samples that tested negative (titers $\leq$16) or weakly positive (titers 32 and 64) by HA (Table 2). Among 57 positive samples, 26 were from vaccinated puppies. All CPV strains detected in the fecal samples of these puppies were confirmed as wild strains using conventional PCR (21) or nucleotide sequencing (5). By sequence analysis, most samples tested positive for CPV-2b (22), followed by CPV-2a (10) and only one as CPV-2c.

Table 1. PCR results of canine parvovirus (CPV) infection with hemagglutination test (HA) and enzyme immunoassay (EIA)

		PCR		
		(+)	(-)	Total
HA	(+)	38	2	40
	(-)	19	53	72
	Total	57	55	112

Concordance = 81.3% (91/112)
Sensitivity = 66.7% (38/57)
Specificity = 96.4% (53/55)

		PCR		
		(+)	(-)	Total
EIA	(+)	44	0	44
	(-)	13	55	68
	Total	57	55	112

Concordance = 88.4 % (99/112)
Sensitivity = 77.2% (44/57)
Specificity = 100.0% (55/55)

Table 2. Analysis of concordant and non-concordant results of 112 samples tested for canine parvovirus (CPV) diagnosis by hemagglutination (HA), enzyme immunoassay (EIA) and polymerase chain reaction (PCR)

Nº of samples	HA titre	HA	EIA	PCR	
72	≤16	(-)	(-)	(-)	53
		(-)	(-)	(+)	11
		(-)	(+)	(+)	8
8	32-64	(+)	(+)	(+)	4
		(+)	(-)	(+)	2
		(+)	(-)	(-)	2
32	≥128	(+)	(+)	(+)	32

DISCUSSION

The EIA is currently used for the confirmation of CPV infection in acute cases of enteritis in puppies presented to the veterinarians. Our results showed that EIA was more sensitive than HA although it failed to detect CPV antigen in two fecal samples which tested positive with PCR. This result might be explained by the reduced amount of free virus available for the EIA since the rapid development of an intestinal immune response to CPV results in the formation of undetectable immune complexes (Decaro *et al.*, 2005; Vieira *et al.*, 2008). These two samples tested weakly positive with HA.

HA has been used for CPV screening due to the ease of implementation and low cost. Furthermore the 96-well plates format allows the rapid processing of many samples (Carmichael *et al.*, 1980; Desario *et al.*, 2005). In this study, there was a poor correlation between HA and PCR since samples considered HA-negative were found to contain virus DNA. This discrepancy may be due to the presence of CPV strains lacking HA activity (Parrish *et al.*, 1988), or to the fact that high viral titers are required to produce HA and that specific antibodies in the intestinal lumen frequently sequestrate most of the CPV virions, thus preventing or reducing parvoviral binding to erythrocytes (Decaro *et al.*, 2005; Desario *et al.*, 2005).

It should be emphasized that non-concordant results between the tests were mostly found in those samples with low HA titers (32-64). Furthermore, the presence of nonspecific hemagglutinins in fecal samples could explain the false-positive results in two samples that tested positive only with HA (Carmichael *et al.*, 1980).

EIA, HA and PCR were able to detect all types of CPV, including the new CPV-2c, indicating that the genetic variations resulted from continuous evolution of CPV did not affect the ability of these tests based on antigen or genome detection (Decaro *et al.*, 2010). It may be concerning that about 23%-33% of dogs with suggestive clinical signs of CPV infection may not be positive for CPV with EIA or HA, so those samples should be tested by more sensitive and specific techniques such as PCR to improve the accuracy of CPV diagnosis. Similarly, nucleic acid–based tests need to be evaluated continuously to ensure that mutations have not

occurred in primer/probe binding regions (Hong *et al*., 2007).

CONCLUSIONS

CPV is the most common viral agent associated with acute enteric clinical signs in young dogs of up to 6 months-old in Rio de Janeiro. Thus a negative test result by antigen detection does not rule out parvovirosis as a differential diagnosis in a dog with hemorrhagic diarrhea and in such cases, additional tests based on DNA detection should be performed.

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