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Research paper

Comparison between Haemaglutination Test and Polymerase Chain Reaction for Diagnosis of Canine Parvovirus Infection

Akbar Ali[a], Asif Ali[a], Sajid Umar[b,*], Hajra Qadir[b], Abdul Sattar Saqib[b], Salman Ahmed Abid[a], Tamoor Azeem[a], Aqsa Mushtaq[c], Kiran Aqil[c], Habib Ullah[d], Muhammad Ali Shah[b]

[a]Department of Pathology, Faculty of Veterinary & Animals Sciences, University of Veterinary and Animal Sciences, Lahore, Pakistan
[b]Department of Pathobiology, Faculty of Veterinary & Animals Sciences, PMAS-Arid Agriculture University, Rawalpindi, Pakistan
[c] Veterinary Research Institute (VRI), Lahore, Pakistan
[d]College of Veterinary Sciences, Gomal University, Dera Ismail Khan, Pakistan

Abstract
The aim of this study was to compare the efficacy of two most commonly used diagnostic tests for canine parvovirus (CPV) diagnosis: haemagglutination test (HA), and polymerase chain reaction (PCR). A total of 50 fecal samples from dogs showing clinical signs suggestive of parvovirus enteritis were collected aseptically from different pet clinics of Lahore. Fecal samples were processed for cCPV antigen, required for HA and PCR. The HA was able to detect CPV antigen in 35 samples, 32 samples tested highly positive with titers ≥128, 3 tested weakly positive with titers ranging from 32 to 64 and 15 were negative (titers ≤16). Using PCR, 39 samples were found positive including 6 HA-negative samples. Chi square analysis showed that there was no significant difference ($P > 0.05$) between the results of HA test and PCR. Thirty percent of dogs presenting bloody diarrhea did not show infection by HA. It is concluded here that specificity and sensitivity of PCR detection is non-significantly higher ($P>0.05$) than HA. These findings have confirmed that HA test could be employed for the preliminary screening of the agent in field because of its less cost and rapid results but negative results from HA tests of suspected cases should be confirmed through molecular methods.

Keywords: Canine parvovirus, Dogs, HA, PCR.

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***Corresponding author:** Sajid Umar; **Email**: S.umar@envt.fr

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Introduction

Canine parvovirus (CPV) is a highly contagious virus mainly affecting dogs. The virus is a small, non enveloped single stranded DNA virus belonging to the *parvoviridae* family. The disease, commonly called Canine parvo viral enteritis is an acute, highly contagious life-threatening infection and is transmitted from dog to dog by direct or indirect contact by contaminated faeces [1]. Therefore, laboratorial diagnosis is essential for screening diarrheic puppies in order to prevent infection of susceptible animals [2]. Soon after their appearance, the original virus (CPV-2) was subsequently replaced by the new variants, CPV-2a and CPV-2b [3]. In the early 2000's, a novel CPV mutant (CPV-2c) emerged in Italy [4] and is currently spreading among the canine population [5]. These CPV variants (2a, 2b and 2c) were recently described associated with enteritis in vaccinated and unvaccinated young dogs [6,7]. Concerns have been expressed that the continuous evolution of CPV could negatively affect the performance of diagnostic tests based on monoclonal antibodies and polymerase chain reaction (PCR) [4,8]. Laboratory diagnosis is made through detection of CPV-2 in the feces by either an enzyme immunoassay (EIA) or a haemagglutination test, or by electron microscopy. PCR has become available to diagnose CPV-2, and can be used when potentially fewer viruses are being shed in the feces that may not be detectable by enzyme immunoassay [9]. The simplest procedure for the laboratory diagnosis of canine parvovirus infection is haemagglutination of chicken or pigs's erythrocytes (pH 6.5, 4°C) by virus present in faecal extracts [10]. The specificity of this haemagglutination is determined by titrating the fecal specimen in parallel in the presence of normal and immune dog serum. Fecal samples from dogs with acute enteritis may contain up to 20,000 haemagglutination units of virus, equivalent to about 10^9 virions per gram. Several laboratory techniques e.g. Electron microscopy, virus isolation, enzyme immunoassay, and amplification of viral DNA using the polymerase chain reaction are also used for laboratory confirmation of clinical diagnosis [11-13], but the big question has been on their accuracy, speed, cost and availability. The purpose of the present study was to compare laboratory tests most commonly used for CPV diagnosis: HA and PCR.

Materials and Methods

Faecal sample preparation

Dogs suffering from diarrhea, vomiting and suspected of CPV infection were selected for the study. Fecal samples were collected from the dogs presented to the Veterinary clinic of University of Veterinary and Animal Sciences Lahore, Pakistan and a total of 50 samples were collected, out of which 29 were collected from mixed breeds and 21 were pure bred dogs. The faecal samples were collected in the form of a rectal swab in Hank's balanced salt solution (HBBS) in a ratio of 1:9, containing streptomycin (100 mg/l) and penicillin (1 lakh IU/l)**[**9**]**. These were filtered through a disposable syringe filter (0.45 µm) (Millex, Milipore) and then centrifuged at 10 000 rpm at 4°C for 3 min in a refrigerated centrifuge for further use.

Haemagglutination (HA) test

10% fecal suspension was made with phosphate buffered saline pH 7.4 as earlier described previously [10]. They were spun along with rectal swabs suspended in VTM at 14000 rpm for 3 min. The supernatant was then collected and used for haemagglutination test. Chicken erythrocytes were collected from healthy chicken from the wing vein into an anticoagulant container. The cells were washed by adding normal saline and centrifugation at 3,000 rpm for 5 min. This was repeated two times and the cells were constituted to a final concentration of 10% from which 1% was reconstituted for the HA test. Samples with titers up to 16 were considered positive [10].

DNA extraction

Total DNA was extracted using the QIAamp® DNA Mini Kit according to manufacturer's specifications. Different procedures were adopted for DNA extraction from rectal swabs and necropsy tissues as specified by the manufacturers. A commercially available inactivated vaccine was used as a positive control of CPV and a stool sample from a healthy dog processed similarly was used as a negative control.

Amplification of DNA extracts

PCR was carried out using primer pair (within VP2 capsid gene) which amplifies the new CPV types circulating in dog population (CPV-2a/2b/2c) [10,11]. The primer set pCPV-RT (F) 5'-CAT TGG GCT TAC CAC CAT TT-3' (20-mer) and pCPV-RT (R) 5'-CCA ACC TCA GCT GGT CTC AT-3' (20-mer) from position 3136-3155 to 3276-3295 of VP1/VP2 gene of CPV-2 was custom designed and synthesized to yield an amplicon of 681 bp in PCR [14,15,16]. The PCR was performed in a thermocycler (Applied Biosystems) using a reaction volume of 50 µl which contained 5 µl of Taq DNA polymerase buffer (10×), 3 µl of MgCl2 (25mM), 200µM dNTPs, 10 pmol of each primer, 5 µl of processed sample as source of template DNA and 1 µl of Taq DNA polymerase (1 IU/µl). The thermal conditions comprised of initial denaturation at 94°C for 3 min, 30 cycles consisting of denaturation at 94°C for 30 s, annealing at 52°C for 1 min and extension at 72°C for 30 s, and a final extension at 72°C for 5 min. The PCR products were electrophoresed along with a 100 bp DNA ladder in 1% agarose gel containing 0.5 µg/ml ethidium bromide and progress of the mobility was monitored by migration of dye.

Statistical analysis

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

Results

The HA was able to detect CPV antigen in 35 samples (70%), 32 samples tested highly positive with titers ≥128, 3 tested weakly positive with titers ranging from 32 to 64 and 15 were negative (titers ≤16). Using PCR, 39/50 samples (78%) were found positive including those 6 which were HA-negative (Table 1). PCR products of 681 bp in the positive samples were visualized by gel electrophoresis (Fig.1). Analyses of the results based on breed of the examined dogs showed that the pure breed of dogs had higher positivity for the virus than the mixed breeds. Rottweilers, Labradors and German shepherd dogs all showed higher carriage capacity of the agent than the mixed breeds of dogs (data not shown).

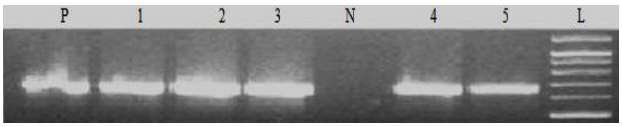

Fig. 1: Agar gel electrophoresis showing the amplicon of 681 bp of Canine parvovirus (CPV) positive samples using CPV forward and reverse primers
L= 1000 bp DNA ladder; N= negative control; P=control positive (inactivated vaccine); Lane 1-3 and 4-5: 681 bp PCR product of Canine parvovirus (CPV) positive fecal samples.

Although all PCR positive samples were also positive by HA test, not all HA positive samples were positive by PCR technique. In all, chi square analysis showed that there was no significant difference ($P > 0.05$) in

Table 1: Correlation between the HA and PCR for fecal samples defining the variables used to calculate sensitivity and specificity.

	Polymerase Chain Reaction					
	+	–	Total			
Hemaglutination Test +	A=33	B=2	A+B=35	**Sensitivity**	84.6%	(A/A+C=33/39)
–	C=6	D=9	C+D=15			
Total	A+C=39	B+D=11	A+B+C+D=50	**Specificity**	81.8%	(D/B+C=9/11)

+ = Positive samples; - = Negative samples; A=true positive; B=false positive; C=false negative; D=true negative

the number of positive samples obtained by the two diagnostic tools and found non significantly (P>0.05) specific (81.8%) with low sensitivity (84.6 %) (Table1).

Discussion

The HA is currently used for the confirmation of CPV infection in acute cases of enteritis in puppies presented to the veterinarians. Our results showed that HA was less sensitive than PCR because it failed to detect CPV antigen in six fecal samples which tested positive with PCR. This result might be explained by the reduced amount of free virus available for the HA since the rapid development of an intestinal immune response to CPV results in the formation of undetectable immune complexes [13,14].

The HA has been used for CPV screening due to the ease of implementation and low cost. Furthermore the 96-well plate's format allows the rapid processing of many samples [15,16]. 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 [17], 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 parvo virus binding to erythrocytes [13,16].Furthermore, the presence of nonspecific hemagglutinins in fecal samples could explain the false-positive results in two samples that tested positive only with HA [18]. 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 [19]. It may be concerning that about 30% of dogs with bloody diarrhea may not be positive for CPV through 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 [8].

Conclusions

CPV is the most common viral agent associated with acute enteric clinical signs in young dogs in Pakistan. However, in diagnosing CPV in clinical cases, the HA test should not be used all alone to avoid incidences of false negativity in the results. Rather, it should be run as a preliminary investigation to define a line of treatment before confirmation with a more specific technique such as PCR.

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