Full Article

A comparison between PCR and Immunochromatography assay (ICA) in diagnosis of hemorrhagic gastroenteritis caused by *Canine parvovirus*

Vakili[1], N., Mosallanejad[*2], B, Avizeh[2], R., Seyfiabad Shapouri[3], M.R., Pourmahdi[4], M.

1. Student of Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
2. Department of Clinical Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
3. Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran
4. Department of Food hygiene, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

ABSTRACT

Canine parvovirus type 2 (CPV-2) is one of the most common viruses responsible for acute hemorrhagic enteritis in dogs. A rapid and accurate diagnosis of CPV-2 infection is especially important in kennels in order to isolate infected dogs. The aim of the present study was to compare two laboratory tests i.e., Polymerase Change Reaction (PCR) and Immunochromatography assay (ICA) most commonly used for the diagnosis of *canine parvovirus* infection in companion dogs. Fecal samples were collected from fifty five dogs (50=hemorrhagic diarrheic and 5= healthy) between 2011 and 2012 in Ahvaz district, southwest of Iran. The studied dogs were divided into two age groups (<6 months, and>6 months), four different breeds (Terriers, German shepherds, Doberman pinschers and Mixed) and based on environment into two groups (open and close) also. All samples were tested by ICA and PCR methods and the results were analyzed by using Kappa test, Mc Nemar and Chi-square analysis. ICA and PCR were able to detect CPV-2 antigen or nucleic acid in 33 and 50 of the hemorrhagic diarrheic samples, respectively. Samples of healthy dogs were negative by both tests. Although sensitivity of ICA compared with PCR method was determined to be 66% (PCR more sensitive than ICA), nevertheless statistical analysis showed that the difference between two techniques were not significant (P>0.05). Kappa test was obtained 0.38 between two techniques. CBC showed that most infected dogs had leucopenia, lymphopenia and neutropenia also (82%; 41 out of 50 samples).Obtained results of this survey showed that accurate standardization of laboratory tests is required to provide veterinarian with an effective tool for a precise etiological diagnosis of hemorrhagic gastroenteritis due to CPV infection. Although Immunochromatography is a simple and quick method for screening of fecal samples of dogs suspected of CPV infection, but PCR is more sensitive and reliable than ICA. Moreover, the subtypes of the virus determined by PCR test after verifying parvovirus. In this test 48 samples were CPV-2b and another 2 samples were CPV-2a. Our results showed that CPV-2b was the predominant subtype.

Keywords: *parvovirus*, Polymerase Chain Reaction (PCR), Immunochromatography assay (ICA), dog

INTRODUCTION

Canine parvovirus type 2 (CPV-2) is a highly contagious infectious agent that can be caused acute

hemorrhagic enteritis in dogs. CPV-2 emerged in 1978 as a new dog disease agent and rapidly spread around the world in domestic and wild dogs causing high morbidity (100%) and frequent mortality (up to 10%) (Apple *et al* 1978). CPV belongs to the family of

*Author for correspondence.Email: bmosallanejad@scu.ac.ir

Parvoviridae, genus *Parvovirus*, along with the Feline Panleukopenia Virus, and possesses a negativesingle-strand DNA genome. CPVs are small, non enveloped, icosahedral virus whose single stranded DNA encodes two capsid proteins (VP1 and VP2) and two non-structural proteins (NS1 and NS2) (Decaro *et al* 2004, Hoskins 2006). CPV-2 induced disease is observed mainly in 6–12 week-old pups; whereas, younger dogs are generally protected from CPV-2 infection by maternally-derived immunity.CPV-2 spreads from infected to susceptible dogs by the fecal-oral route (Martella *et al* 2004, Decaro *et al* 2005). Virus is shed for approximately 8–12 days post-infection. Within few years, the new antigenic types, termed CPV-2a and CPV-2b, has been replaced the original type 2 completely (Hoskins, 2006). The close antigenic and genetic relationships, exist between CPV-2, Feline Panleukopenia Virus (FPV) and Mink Enteritis Virus (MEV), suggest that CPV-2 may have originated by genetic mutation in a wildlife host receptive to one of the FPV-like parvovirus that infect carnivores (Truyen *et al* 2000). The enteric form of the disease is a serious problem in breeding kennels, or where vaccination is not widely practiced (Greenwood *et al* 1996, Markovich*et al* 2012). Rapid diagnosis of CPV infection is especially important in kennels and shelters in order to isolate infected dogs and prevent secondary infections of susceptible contact animals. The clinical diagnosis of CPV-2 infection is indecisive, since several other pathogens may be caused diarrhoea in dogs, so different methods have been developed for the laboratory diagnosis of CPV-2 infection. Routinely, faeces from diarrhoeic dogs are screened using ELISA, Immunochromatographic (IC), Haemagglutination (HA), Viral Isolation (VI) or Polymerase Chain Reaction (PCR) tests (Esfandiari and Klingeborn 2000, Desario*et al* 2005, Yang*et al* 2010). VI and PCR are more sensitive (Pereira *et al* 2000, Buonavoglia *et al* 2001)., but VI is too labor-intensive and time-consuming for routine diagnostic testing. In recent years, the PCR technique has been increasingly used as a tool for the definitive diagnosis of CPV-2 DNA

(Kumar *et al* 2010). On the other hand, the immunochromatography assay is the most common rapid field diagnostic method used in clinical practice, because the test procedure is simple and rapid, and can be performed by veterinarians as well by owners (Desario*et al* 2005). Also the subtypes of the virus determined by PCR test. CPV-2b is identified as the predominant virus type (Firoozjaii *et al* 2011).The aim of the present study was to compare commercial Immunochromatography antigen detection kit and PCR for the diagnosis of CPV-2 infection in diarrheic companion dogs, in Ahvaz district, southwest of Iran.

MATERIALS AND METHODS

Sample collection and preparation. Fecal samples were collected from fifty five dogs (50= hemorrhagic diarrheic and 5= healthy) between 2011 and 2012 in Ahvaz district, capital city of the Khuzestan province, southwest of Iran. The studied dogs were divided into two age groups (<6 months, and >6 months), four different breeds (Terriers, German shepherds, Doberman pinschers and Mixed) and based on environment into two groups (open and close) also. They were young dogs from 2 to 10 months. Blood samples were collected in all of dogs to characterize CBC. Finally treatment of the affected dogs was directed at correcting the life-threatening dehydration that accompanied the diarrhea with intravenous fluids, antiemetic and broad-spectrum antibiotics to prevent secondary bacterial infection. In this study, we evaluated the detection rate of the CPV infection by using a commercially available kit compared with PCR methods.

Procedure and interpretation of the ICA test. It was carried out with a commercial rapid CPV Ag test kit (Manufactured by Anigen, Animal genetics, Inc., Korea), following the manufacturer's instructions. This kit is a chromatographic immunoassay for the qualitative detection of *parvovirus* antigen in canine feces. It can detect the pathogenic CPV subtypes CPV2a or CPV2b. Procedure of the test was as: first we provided swab from the stool and then it was inputted

and mixed into the assay diluents. Latter we left the bottle for a short time and finally add four (4) drops of supernatant from extracted sample into the sample hole. As the test begins to work, we will see purple color move across the result window in the center of the test device. Interpret test results will be at 5-10 minutes. A color band will appear in the left section of the result window to show that the test is working properly. This band is the control band. The right section of the result window indicates the test results. If another color band appears in the right section of the result window, this band is the test band. The presence of only one band within the result window indicates a negative result. The presence of two color bands (T and C) within the result window, no matter which band appears first, indicates a positive result (Figure 1). If the purple color band is not visible within the result window after performing the test, the result is considered invalid (Esfandiari & Klingeborn 2000).

PCR protocol. DNA was extracted from the fecal samples by a commercial stool DNA purification kit (Bionner, south Korea), as per the manufacturer instructions. Extracted DNAs were stored at -20 °C until be tested by PCR. PCR was performed by the primer pairs Pabs/Pabas and Pbs/Pbas, described by Senda *et al* (1995) and Pereira *et al* (2000), respectively. Firstly, the samples were tested by the Pabs/Pabas primers. The used materials for PCR consisted of 5 μl 10X PCR buffer, 1.5 μl MgCl$_2$ (50 mM), 1 μl dNTPs mix (10 mM), 1 μl (20 pmoles) of each primer, 0.5 μl (2.5 U) *Taq* DNA polymerase (Cinnagen Inc, Iran), 2 μl DMSO, 33 μl ddH20 and 5 μl of template DNA. The thermal program of PCR was 3 min incubation at 94 °C, 35 cycles of 30 second at 94 °C, 2 min at 55 °C, and 2 min at 72 °C, and a final extension step of 5 min at 72 °C. Samples which found to be positive by Pabs/Pabas primers were also tested by Pbs/Pbas primers, in order to subtype the virus. The reaction components of this PCR (except the primers) and the thermal program were as described above for the Pabs/Pabas PCR. It was expected that Pabs/Pabas

and Pbs/Pbas primers produce DNA bands of 682 and 428bp respectively.

Statistical analysis. Test results and potential association with age, sex, breed, CBC and environment situation were analyzed using SPSS 10.0 for windows and by use of Kappa test, Mc Nemar (for comparison and agreement between ICA and PCR methods) and Chi square analysis (other parameters). Differences were considered significant at p≤0.05.
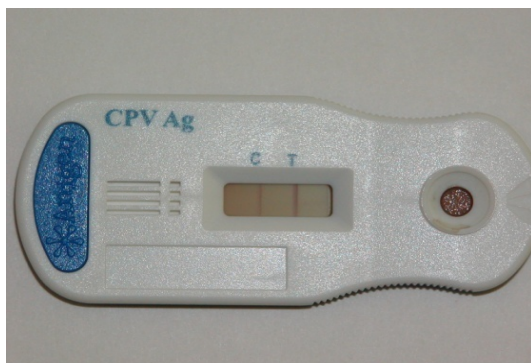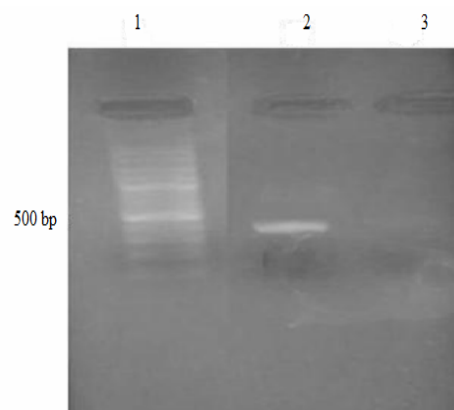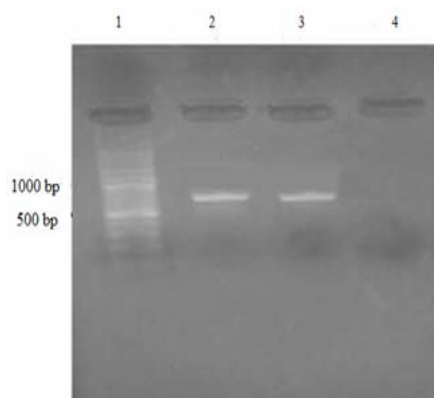
RESULTS

The results of the two tests used for the detection rate of the CPV infection in the feces of diarrheic dogs are summarized in Table 1 and 2. ICA was able, to detect CPV-2 antigen in 33 of the samples. In contrast to ICA, all the diarrheic fecal samples, including 17 samples that had tested negative by ICA were identified as positive by Pabs/Pabas PCR. All five fecal samples collected from healthy doge were negative by PCR. Statistical analysis showed that the difference between PCR and ICA techniques were not significant (P>0.05). Kappa test was obtained 0.38 between two techniques. Sensitivity and specificity of ICA compared to PCR were calculated to be 66% and 100% respectively. In order to identify the viral subtypes, DNAs extracted from all the diarrheic fecal samples were also tested by Pbs/Pbas PCR. The results showed that 48 samples were positive by this PCR and thus contained the subtype b of the virus. The two samples which were negative by Pbs/Pbas PCR were retested again negative by this PCR and finally considered as a sample containing the subtype A of the virus. Agar gel electrophoresis of some PCR products is shown in Figures 2 and 3. Prevalence was higher in male dogs (62%; 31 out of 50), age < 6 months (78%; 39 out of 50), Mixed breed (32%, 16 out of 50) and open environment (62%; 31 out of 50), but the difference was not significant between the prevalence of infection relative to host gender, breed and environment (P>0.05), but statistical analysis was significant for age (P=0.007). CBC showed that most infected dogs had

Table 1. Characteristics of the studied dogs (50) based on breed, age, environment and genus in Ahvaz district, Iran, 2011-2012.

Breed	Age			Environment		Genus	
	<3 months	3-6 months	>6 months	Open	Close	Male	Female
German shepherd	2	5	7	9	5	7	7
Doberman Pinscher	4	7	0	7	4	9	2
Terrier	3	3	1	5	2	6	1
Mixed	4	10	2	9	7	8	8
Great dane	1	0	1	1	1	1	1

Table 2: Relative performance of PCR and ICA for detection rate of CPV infection in hemorrhagic diarrheic dogs in Ahvaz district, southwest of Iran, 2011-2012.

Tests \ Results	ICA	PCR
Positive	33	50
Negative	17	0
Total	**50**	**50**

Figure 1. CPV positive Kit (ICA) (two color bands within the result window).

Figure 3. Lane 1: 100bp DNA ladder; lanes 2: samples of a dog infected with CPVb (using Pabs/Pabas primers amplifying a DNA band of 428 bp); lane 3: CPV2b negative sample.

leukopenia, lymphopenia and neutropenia (82%; 41 out of 50 samples). The difference was significant for CBC also (P<0.05).

DISCUSSION

In the present study, the results of ICA were compared with PCR method, and a high sensitivity was shown for diagnosis of CPV by PCR (100% against 66%). Although sensitivity of ICA compared with PCR method was determined to be 66% (PCR more sensitive than ICA), nevertheless statistical analysis

Figure 2. Lane 1: 100bp DNA ladder; lanes 2 and 3: samples of dogs infected with CPV2a/b (using Pabs/Pabas primers amplifying a DNA band of 682 bp); lane 4: Negative control (healthy dog).

showed that the difference between two techniques were not significant. Kappa test was obtained below 1 (0.38) between two techniques. It is indicating relatively useful to identify CPV-2 positive samples. A rapid diagnosis of CPV-2 infection is important in kennels and shelters in order to isolate infected dogs and prevent secondary infections of susceptible contact animals. Since a clinical diagnosis is not definitive, several laboratory methods have been developed to detect CPV-2 in the feces of infected dogs. The ICA test is one of the most common rapid field diagnostic methods used in clinical practice, because the test procedure is simple and fast, and can be performed by veterinarians as well by owners (Esfandiari & Klingeborn 2000). However, large amounts of viral antigen are required to produce a clearly visible band and the interpretation of results may be affected by the subjectivity of a test operator. This is especially common when amounts of virus are low. On the other hand, PCR can be carried out only in specialized laboratories. PCR-based methods have been developed to identify types 2, 2a and 2b CPVs (Pereira *et al* 2000), which take advantage of nucleotide differences between the primers restricted to one base in the end of each primer. Desario *et al* (2005) were employed five laboratory tests including IC, HA, VI, conventional and real-time PCR for diagnosis of canine parvovirus type 2 (CPV-2) infections in 89 fecal samples collected from dogs with diarrhea. IC, HA, VI and conventional or real-time PCR were able, respectively, to detect CPV-2 antigen or nucleic acid in 41, 50, 54, 68 and 73 of the samples. The best correlation was found between conventional and real-time PCR, with an overall agreement of 94.38%. In another survey by Al-Bayati *et al* (2010), the result of rapid test (ICA) showed that the ratio of positive samples was 66.6%, and this is similar to our results. In the present study, there was poor correlation between ICA and PCR. The same samples gave negative results by the ICA test, allowing us to hypothesis that fecal antibody binding CPV-2 antigen may also prevent the detection of viral antigens by this test. In contrast, PCR-based method was

demonstrated to be more sensitive. In a previous study (Decaro *et al* 2005), there was poor correlation between real-time PCR and HA titers. Those contrasting findings were attributed to the presence of high levels of CPV-2 antibodies in the feces of the infected dogs. Real time PCR is sensitive, specific, and more reproducible. It allows the detection and quantification of CPV-2 nucleic acid within few hours. Also, there is less risk of carry-over contamination (Decaro *et al* 2005). Kumar *et al* (2010) showed that PCR had more relative sensitivity, specificity and accuracy compared with ELISA. Our study showed that the prevalence of infection was more in ages between 3-6 months, though difference was no significant. We did not see any dogs that affected to parvovirus above 1 year; presumably it is due to natural resistance to the effects of parvovirus. Also, prevalence of CPV did not differ between different sexes. For unknown reasons, Doberman Pinschers, Rottweilers, Labrador retrievers, American Staffordshire terriers, German shepherds and Alaskan sled dogs seem to have an increased risk (Hoskins 2006). In our study, Mixed breeds (32%) were more involved. The molecular assays, especially PCR require expensive equipment, reagents and specialized operators; thus, their use as tests for the veterinary practice is not feasible. Nevertheless, there are efforts by several companies to adapt molecular methods to clinical practice, taking advantage of microchip technology that would reduce the cost and size of the equipment necessary for onsite testing (Desario *et al* 2005). ICA can be very useful for diagnosis of CPV-2 on fecal samples in clinics, but the PCR-based diagnosis is beyond the feasibility of diagnostic laboratories owing to its requirement of skilled manpower and costly equipments. Considering the sensitivity limits of the ICA tests that have been observed previously, negative results from the test kit should be confirmed by PCR-based methods. As previously stated, the methods used for characterization of the CPV-2 strain scan be performed only in highly specialized laboratories. A universal method that can identify all CPV-2 types is not available and more than

one method is required to identify the viral types. The ICA provides a reliable method for detection of anti-CPV antibody where laboratory support and personnel are limited. The interpretation of test results, however, is equivocal in a few cases (Oh *et al* 2006). Sequence analysis can give ample information for CPV-2 typing since the fragment amplified by conventional PCR, using primer pair 555for/555rev, as well as this primer pair allows differentiation between CPV-2, CPV-2a, CPV-2b and the Glu-426 mutant (Desario *et al* 2005). The ICA can be easily performed in the small laboratories and even at clinic level to obtain prompt, accurate and specific diagnosis of CPV-2, which can help in the effective and efficient management of the disease. Further, inhibitory substances present in the fecal samples of dogs make it necessary to extract DNA to carry out PCR and become a time-consuming process in case of large number of samples. In conclusion, accurate standardization of laboratory tests for the diagnosis of CPV infection is required to provide veterinarian with an effective tool for a precise etiological diagnosis of hemorrhagic gastroenteritis. Although ICA is a simple and quick method for screening of fecal samples of dogs suspected of CPV infection, but PCR is more specific and reliable than ICA. Further studies are needed for increasing the sensitivity and specificity.

Acknowledgment

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