DIAGNOSIS OF CANINE PARVOVIRUS USING NESTED-PCR AND COMPARISON OF BLOOD PICTURE IN AFFECTED DOGS

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Canine parvovirus (CPV) is a well known enteric pathogen of dogs throughout the world (Appel *et al.* 1979) causing hemorrhagic enteritis in young pups of 12 weeks to 6 months of age. It is characterized by vomition, diarrhea which may later turn hemorrhagic and foul smelling. There is a severe drop in the white blood cell count since virus inhibits the white blood cell division in the bone marrow (Legendre, 2000). The virus is transmitted mainly via fecal-oral route and large number of virus particles ($>10^9$ virus particles/ gm) are excreted in the feces of the affected animals (Nandil *et al.* 2007). The virus multiplies in the rapidly dividing cells of the intestinal crypts thus exhibiting its pathogenecity (Cotmore and Tattersall, 2007).

Despite widespread vaccination, CPV has remained a widespread disease of dogs because the virus forms new genetic and antigenic variants (Hoelzer and Parrish, 2010) and thus leads to vaccination failure. The clinical diagnosis of CPV infection should be confirmed with laboratory tests and PCR is employed for the diagnosis of CPV due to its higher sensitivity, specificity and rapidity and its ability to detect fewer organisms (Schunck *et al.* 1995). Nested-PCR which is a variant of PCR is a much more sensitive and specific test and has been used for the diagnosis of diseases (Hirasawa *et al.* 1994). Thus, the objective of the present study is to identify CPV infection in dogs in Ludhiana, Punjab using PCR as well as Nested-PCR, and to identify its correlation with blood parameters i.e. Hemoglobin, Total leukocyte count, etc.

Materials and Methods

Samples from canines (n=65) suspected for CPV infection were collected during September 2007 to May 2009 from the Veterinary hospital, GADVASU Ludhiana (n=40) as well as from private veterinary clinics (n=25) located at Ludhiana, Punjab. All the animals exhibited anorexia, vomition, diarrhea or hemorrhagic diarrhea along with fever. The fecal samples were collected in phosphate buffer saline (PBS pH=7.4) were transported to the Department of Veterinary Microbiology, COVS and stored at 4° C till further use. The blood samples with EDTA were also collected from all the affected dogs and were processed for the estimation of total leukocyte Count (TLC), and hemoglobin (Hb) as per the procedure of Stockham and Scott (2008a,b) at Clinical diagnostic laboratory, GADVASU, Ludhiana

Extraction of DNA from fecal samples: Extraction of DNA from the fecal samples was carried out initially by boiling 200µl of each sample for 10 minutes in a water bath. Later the contents were centrifuged at 5000g for 10 minutes and the supernatant was collected.

PCR/Nested-PCR: A PCR reaction was put with 50µl by adding 15 µl of template DNA (supernatant collected in the previous step), 1 µl of 25pm/ µl forward and reverse primers (F 5'-AGCTATGAGATCTGAGACAT-3'and R 5'-AGTATGTTAATATAATTTTCTAGGTGC-3') each, 5.0 µl of 10x PCR buffer, 3µl of 25 mM $MgCl_2$, 1.0 µl of dNTPs (10mM each), 0.5 µl of Taq polymerase (5units/ µl) and 23.5 µl of nuclease free water. Initial denaturation at 95° C for 5 minutes was done and the mixture was then subjected to 35 cycles of denaturation at 94° C for 1 minute, annealing at 55° C for 1 minute and extension at 72° C for 2.5 minutes. Later, final extension at 72° C for 10 minutes was provided to complete the reaction.

Nested-PCR: Nested-PCR was carried out with 2 µl of initial template for samples having positive PCR reaction and 5 µl for samples having negative PCR reaction. The reaction mixture was prepared by adding 1 µl of 25pm/µl forward and reverse primers (F 5'-ATACAGGAAGATATCCAGAAG-3' and R 5'-AGTATGTTAATATAATTTTCTAGGTGC-3') (Mizak and Rzezutka, 1999). each, 2.5 µl of 10x PCR buffer, 1.5 µl of 25mM $MgCl_2$, 0.5 µl of dNTPs (10mM each), 0.5 µl of Taq polymerase (5 units/ µl) and 16.0 µl of nuclease free water to make final reaction volume to 25

µl. The nested- PCR reaction was subjected to same conditions as used in PCR reaction above. The amplified DNA products of PCR and nested-PCR were photographed under ultraviolet (UV) transillumination (AlphaImager), after electrophoresing it at 5-10 volts/cm for 1 h. Gene Ruler ladder plus 100bp (MBI, Fermentas) was run for calculating the relative molecular weight.

Table No.1: Comparison of blood parameters among CPV positive samples

Blood Parameter	**Basal limit**	**No of samples positive**	**Male**	**Female**	**Age more than to 6 months**	**Age less than or equal to 6 months**
Hemoglobin (Hb) g/dl	≥ 11g/dl	9 (42.85%)	6 (66.66%)	3 (33.33%)	3 (33.33%)	6 (66.66%)
	< 11g/dl	12 (57.14%)	6 (50%)	6 (50%)	0	12 (100%)
Total Leukocyte count TLC	≥8x10^3/cu mm	8 (38.09%)	6 (75.0%)	2 (25.0%)	0	8 (100%)
	< 8x10^3 /cu mm	13 (61.90%)	6 (46.15%)	7 (53.84%)	3 (23.07%)	10 (76.92%)

Results and Discussion

Canine Parvovirus infection is one of the major causes of enteritis and subsequent death in young dogs (Kapil, 1995). In the present study, out of a total of 65 samples, 3 (4.61%) amplified 1198 bp product with PCR whereas 21 (32.3%) amplified 548bp product with Nested PCR (Figure 1). Though, viral isolation is a gold standard for the diagnosis but due to labour intensive and expensive procedure, the need for cheap, quick and reliable test is required. Since, PCR is a sensitive, specific and rapid method to detect even fewer virus particles, thus in the present study, efficacy of PCR as well as N-PCR was observed (Nandil *et al. loc.cit*).

A total of 3 (4.61%) cases were positive with PCR and 21 (32.3%) with N-PCR indicating the greater diagnostic capability of N-PCR. Higher sensitivity of N-PCR has been reported earlier by Mizak and Rzezutka, (*loc.cit*) which is in concurrent with present findings. Also, all the samples which were positive with PCR also gave positive reaction with N-PCR in the present study indicating that N PCR could detect CPV positive with PCR with 100% efficacy.

Out of 21 positive samples with N-PCR, 9 (42.85%) were females and 12 (57.24%) were males indicating no significant ($p<0.05$) effect of sex on CPV infection in animals. The above observation was similar with that of Yang Dong- Kun *et al.* (2010) and Kalli *et al.* (2010), who also reported no variation in the disease status when compared with the sex of the animal. In contrast Khan *et al.* (2006) and Houston *et al.* (1996) reported the prevalence of CPV infection higher in males when compared with females.

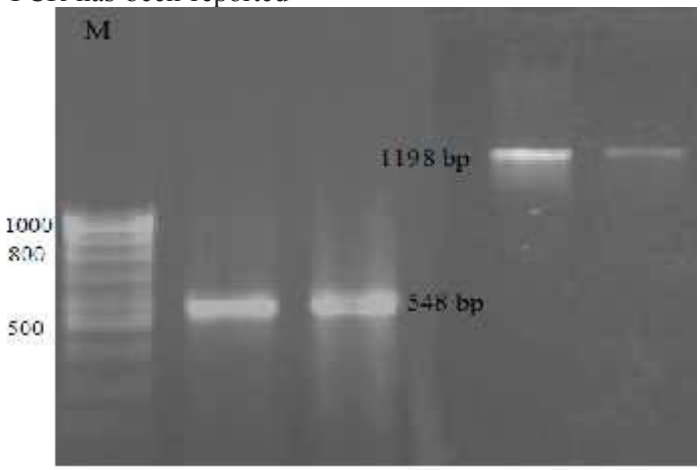

Figure 1 PCR and Nested PCR of CPV

Out of all the 65 samples, 40 samples were from animals below or equal to 6 months of age. Among these 18 (45%) were positive by N-PCR whereas out of the rest 25 samples from animals having age more than 6 months 3 (12%) were positive by N-PCR. Thus, out of a total 21 positive samples with N-PCR, 18 (86%) were below or equal to 6 months of age indicating that the infection caused by CPV is more severe in young animals. Similar findings have been observed by various earlier workers too (McCaw and Hoskins, 2006).

Month-wise variation revealed that the incidence was more during March and October (28.5%) followed by April (14.28%) and November (9.52%) whereas 1 (4.76%) case was positive during January, August, September and December months. The above findings indicate that with the change of season (i.e. approaching summers and winters) puppies are rendered more susceptible towards CPV infection. The possible reason for this may be stress due to changing weather, though earlier studies have indicated that during warm and humid conditions the number of CPV positive cases increases (Horner, 1983, Houston *et al. loc.cit*, Rewerts and Cohn, 2000 and Khan *et al. loc.cit*) which is in contrast to our findings. However, Stann *et al*. (1984) reported no difference between the warm and cold period of the year regarding the occurrence of CPV enteritis.

The hemoglobin per cent when compared revealed 9 (42.8%) of the animals were having Hb more than or equal to the basal limit 11g/dl (Benjamin, 1985) and 12 (57.4%) animals were having Hb less than11g/dl (Table 1) indicating that the effect of Hb is not correlating with CPV infection as has been reported earlier too (Grigonis *et al.* 2002). Since the animal suffering from CPV undergoes profuse diarrhea, thus leading to hemoconcentration which should have been the normal observed course but it was not found in the present study. It may be either due to the prompt therapy provided to the animals because all the animals/cases collected were from veterinary hospital or private clinics where initial treatment was have administered rapidly.

On the basis of TLC count, 13 (61.90%) animals were having TLC less than $8x10^3$ /cu mm and 8 (38.09%) animals were having TLC count greater than or equal to basal point (Benjamin, *loc.cit*). Leucopenia associated with CPV infection has been reported earlier by Grigonis *et al.* (*loc.cit*) however, Potgieter et al. (1981) reported that leukocyte count does not change during the course of infection. This may be because the virus inhibits the white blood cell division in the bone marrow (Legendre, *loc.cit*) and thus leading to leukopenia.

Summary

Canine Parvovirus (CPV) infection is an important viral disease mainly affecting young pups of 12 weeks to 6 months of age and is characterized by vomition and diarrhea. Despite widespread vaccination, CPV has remained a widespread disease of dogs mainly due to the formation of new genetic and antigenic variants. A total of 65 samples from affected dogs collected during September 2007 to May 2009, when subjected to PCR as well as N-PCR yielded 3(4.61%) and 21(32.3%) positive reactions respectively. Out of 21 positive cases with N-PCR, 9 (42.85%) were females and 12 (57.24%) were males. Month-wise variation revealed that 6 (28.5%) were positive during March and October indicating that these months are more prone for the infection in dogs in the Punjab region. Total leukocyte count (TLC) identified 13 (61.90%) animals having TLC count less than the basal point, whereas 8 (38.09%) animals were having TLC greater than the basal point. The study concluded that CPV infection is predominant in young pups and it infects both male and female equally and does not differentiate between sex.

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