Prevalence of Canine Parvovirus Infection at Different Pet Clinics in Lahore, Pakistan

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Abstract.- Prevalence of canine parvovirus (CPV) infection was studied in the dog population of Lahore. A total of 198 fecal samples were taken aseptically from dogs clinically suspected for parvovirus infection from different pet clinics of Lahore during 2010-2011. Most animals had a history of hemorrhagic diarrhoea, vomiting and a few had yellow diarrhea with mucus. Cases were categorized and recorded on the basis of sex, age and breed. Fecal samples were processed for haemagglutination test (HAT) and slide agglutination test (SAT) for CPV antigen. The overall prevalence of CPV was 22.7 % (45/198). It was observed that CPV was more prevalent in the 0-2 month age group (44.5%), dogs of the German shepherd breed were more susceptible (40%), and female dogs were more at risk (58.5%). The cardiac form of the disease was noted in young puppies while the enteric form of the disease was noted in both puppies and in young dogs. Tissue samples were collected in 10% formalin for histological study of heart and intestinal tissue. In the cardiac form there was a severe to mild loss of myofibres, sarcolemmal proliferation, and intranuclear inclusion bodies while in the enteric form a complete loss of intestinal villi, compaction of the lamina propria with dilated crypts and a lack of leucocytes were observed. The results showed that SAT is a cheap and rapid screening test for the diagnosis of CPV infection. It is suggested that dogs should be vaccinated against CPV in order to eradicate this life threatening disease of dogs from Pakistan.

Key words: Canine parvovirus, pet clinic, slide agglutination test**,** histopathology.

INTRODUCTION

Canine parvovirus (CPV) is the number one viral cause of puppy enteritis and mortality (Kapel, 1995; Shabbir *et al*., 2009). Unique properties of CPV make it an emerging and re-emerging pathogen of dogs worldwide (Buonavoglia, 2006). Parvoviruses have a single-stranded DNA genome of 5,000 bases with a hairpin structure (Cotmore and Tattersall, 2007). Parvoviruses have exceptional evolutionary abilities (Chicnchkar *et al*., 2006; Lopez-Bueno *et al*., 2006; Truyen, 2006). Both genotypes of CPV type 2 [CPV-2 and CPV-2b], are prevalent in Pakistan (Tawakal *et al*., 2010). Parvoviruses are extremely stable in the environment and relatively resistant to disinfectants because they are non-enveloped viruses (Saknimit *et al.*, 1988). CPV multiplies in the rapidly dividing cells of the crypts of the intestine, leading to diarrhea and dehydration (Cotmore and Tattersall, 2007; Manzoor and Jamil, 2013).

In the kennel environment, a large number of susceptible puppies, environmental stress, and the unique properties of CPV form an ideal scenario for the rapid spread of CPV. Effective commercial modified live virus vaccines containing genotype CPV-2 or CPV-2b are available. There is currently no commercial vaccine available containing CPV-2c. However, induction of active immunity in puppies is blocked by maternal immunity (Pollock and Carmichael, 1982). The stability of CPV in the kennel environment and excretion of large amounts of CPV by sick puppies can expose susceptible puppies to massive infectious doses of CPV. This CPV susceptibility window coincides with weaning of puppies in the age of about 6 to 8 weeks. Eight weeks is therefore the age when most puppies succumb to CPV. Moreover, there are variations in the amount of antibodies and induction of active

immunity after vaccination due to the genetics of the puppies (Buonavoglia *et al*., 2001).

It would be clinically useful if there were diagnostic tests available that could detect the amount of CPV in a sample, the genotype of the virus, and quantify the amount of antibodies against different CPV subtypes quickly in the kennel environment. Thus, we have developed and validated instant CPV antigen test *i.e*. the slide agglutination test (SAT). These tests are fast, sensitive, quantitative, and generic for all CPV types. We are confident that these safe, economical, and rapid tests will encourage timely use of the vaccines and will help to manage outbreaks of CPV in kennels requiring only minimum training of personnel. There are a few tests that have been used for rapid detection of CPV in fecal samples and CPV antibodies. These tests include an immunochromatography (Oh *et al*., 2006), latex agglutination (Sanekata *et al*., 1996), and coagglutination (Singh *et al*., 1998). The present study was designed to determine the prevalence of CPV in dog populations of Lahore by using economical and rapid in house tests (slide agglutination tests).

MATERIALS AND METHODS

Clinical samples

A total of 198 fecal samples were taken aseptically from dogs clinically suspected for parvovirus infection from different pet clinics of Lahore during 2010-2011. All samples were collected from dogs that had a history of vomiting and diarrhoea and processed at the University Diagnostic Laboratory (UDL). Most animals had a history of hemorrhagic diarrhoea, and had yellow diarrhoea with mucus. Fecal samples and intestinal contents were processed as 10% (Wt/Vol) suspensions in phosphate-buffered saline (PBS) (pH 7.2) for this study. The CPV status of the samples was confirmed by conventional assays such as haemagglutination (HA) test. Hyper immune serum against the canine parvo virus was raised in rabbits using commercial vaccine (Primadog; Merial, France). Haemagglutination agents were processed for haemagglutination inhibition test using known serum against vaccinal strain of canine parvovirus

for its sero confirmation (Shashidhara *et al*., 2009).

Haemagglutination test (HAT)

HAT was performed as described by Carmichael *et al*. (1980). The samples were serially diluted twofold in PBS (0.2 M) in V-bottom plates. First, 50 µl of PBS was added to each well of the plate. In the first column, 50 µl of sample (fecal suspension) was added. The sample was mixed five times, and 50 µl was transferred to the next well. Each sample was diluted from 1:2 through 1:4,096. Then, 50µl PBS was added to each well. The HAT was performed using chicken erythrocytes (0.5%). The plate was shaken for 30 seconds. The plates were covered with lids and incubated at 4 to 7°C for 2 to 4 h. Positive agglutination was indicated by mat formation, and a button indicated lack of agglutination. The titer was calculated as the reciprocal of the last well showing agglutination. Control negative wells (PBS+ chicken RBCs) and control positive wells (Known CPV-2 + RBCs+ PBS) showed a button formation and mat formation respectively. Red blood cells control was also run for auto-agglutination along with other controls in each plate.

Slide agglutination test (SAT)

Slide agglutination is a modified form of HAT being more economical and less time consuming. For the SAT, the conditions of the test were standardized to obtain agglutination results in 30 to 60 s of mixing the reaction mixture components. The buffer was the same as routinely used for the HAT, *i.e*. PBS (0.2 M PBS, pH 7.2). Glass slides were kept in the freezer compartment of the refrigerator, cleaned, and were ready to use. For the assay, each glass slide was wiped with a paper towel to remove moisture. Twenty microliters of unknown sample separation was added as a drop on the slide and then 20 µl of chicken erythrocytes (0.5%) were added as a separate drop. The total volume was made up to 50 µl with 10 µl of 0.2 M PBS. The drops were mixed with a wooden toothpick in a circular motion for 30s. CPV-positive samples produced agglutination within 1 min. Negative samples were homogeneous and showed no agglutination. However, all samples were further incubated in the refrigerator for an additional 5 min

before the results were recorded and confirmed microscopically. CPV-positive samples showed large clumps of agglutination and CPV-negative samples showed single erythrocytes homogenously spread in the well. Partial agglutination was microscopically confirmed with smaller clumps of chicken erythrocytes. Weak CPV-positive samples can take up to 3 min to agglutinate in the refrigerator. Using the SAT procedure, the results were recorded as agglutination, no agglutination, and partial agglutination. For determination of the amount of the virus, the CPV-positive samples were diluted two fold in a U-bottom well plate (Linbro/Titertek) (96-well U-well plate) (ICN Biologicals, Inc., Aurora, Ohio). Using the SAT procedure, the results were recorded as agglutination, no agglutination, and partial agglutination. The dilution of the sample that showed partial agglutination was recorded as 1 hemagglutinating unit (HAU). This convention and calculation were adopted from the hemagglutination inhibition assays for CPV. The dilution that contained 1 HAU was divided by 8 to calculate the dilution containing 8 HAU of CPV (Shashidhara *et al.*, 2009)

Slide inhibition test (SIT)

Hyperimmune serum sample (10 µl) was diluted two fold in the wells on a U-bottom plate with PBS (0.2 M PBS, pH 7.2) for slide inhibition test. The serum was diluted up to 1:4.096. CPV isolates at 8 HAU (CPV-2, CPV-2b) were used. CPV isolate (20 µl) was added to the serum dilutions, and the plate was incubated for 1 min at 37°C in an incubator. Thirty microliters containing CPV and serum dilution mixtures were added to the cool glass slides. Twenty microliters of chicken erythrocytes (0.5%) was added and immediately mixed with a toothpick. The reaction mixture volume was 50 µl. The presence of CPV antibody in the serum was indicated by the lack of agglutination due to inhibition/block of the agglutination. The antibody titer was recorded as the inverse of the highest dilution that produced complete inhibition of erythrocyte agglutination.

Histopathological study

Necropsy was performed on those dogs that tested positive for CPV with SAT and died. Gross pathological lesions were recorded on heart and small intestine, and tissue samples of heart and small intestine (duodenum, jejunum and ileum) were collected and preserved in 10% buffered formalin immediately for histological investigation. The histopathological examination was carried out on all samples according to standard procedure described by Bancroft and Gamble (2002). Fixed tissues were processed by the routine method of dehydration and paraffin embedding. Sections of 4–5 µm thickness were cut and stained with hematoxylin and eosin dyes (Bancroft and Gamble, 2002). Histopathological changes in tissue were observed under bright field compound microscope (Olympia, USA) using 10X and 40X objectives and photographed. Two by two tables were constructed to determine the correlation between the HAT and SAT for fecal samples showing the variables used to calculate sensitivity, specificity, and accuracy of both tests. Data was entered and analyzed using SPSS version 16.0. The comparison of sensitivity and specificity of the HAT and SAT 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. The overall prevalence of canine parvovirus infection was calculated from the data. For the analysis of certain additional variables (age, sex, season, sensitivity, specificity) percentage calculations were also calculated. The differences were evaluated at P value of 0.05.

RESULTS

The present study highlighted that CPV infection is more common in puppies aged up to two months. It is also observed that German shepherded dogs are most often affected (40%), than Labrador 31.8%, pointer 13.6%, Rottweiler 8%, boxer 1.5% or crossbreed 5.5% (Table I). This study also showed that the prevalence of the CPV infection is higher in females. Interestingly, more cases of CPV infection are observed in summer season (62%) than in winter (38%) (Table I). The main clinical signs of canine parvovirus infection were vomiting, bloody foul smelling diarrhoea, dehydration, sunken eyes, depression and weakness. Clots of blood and broken intestinal epithelium were most often observed in

Table I.-   **Percentage of risk factors.**

Age	0-2 months	88 (44.5%)
	3-6 months	67 (34%)
	7-9 months	34 (17%)
	>10 months	9 (4.5%)
Breed	German shepherd dogs	79 (40%)
	Labrador	63 (31.8%)
	Pointer	27 (13.6%)
	Rottweiler	16 (8%)
	Boxer	3 (1.5%)
	Cross breed	10 (5.5%)
Season	Winter	75 (38%)
	Summer	123 (62%)
Sex	Male	83 (41.5%)
	Female	115 (58.5%)

the faeces. On gross examination the myocardium was seen as mottled with pale areas. The intestine was found filled with blood and showed severe congestion and enteritis. The histopathological study showed the complete or partial loss of intestinal villi, leaving a compacted lamina propria almost completely devoid of epithelial cells, distension of the crypts lumen which contained erythrocytes, necrosis of epithelial cells, dilatation of crypts, and depletion of Payer's patches (Figs. 1A-C). Histologically, sacrolemmal proliferation, loss of myofibrils, and necrosis of cardiac muscle fibers, inclusion bodies in muscle nuclei in the intestinal epithelium and few inflammatory areas were observed (Fig. 1D).

DISCUSSION

Canine parvovirus can be detected in faecal material by using different techniques including direct microscopy, haemagglutination test (HAT) and polymerase chain reaction (Pereira *et al*., 2000; Muzaffar *et al*., 2006). Similarly, HAT was performed for the demonstration of canine parvovirus in faeces by using avian and mammalian erythrocytes (Martella *et al.,* 2005; Silva *et al*., 2013). In the present study, slide agglutination test (a modified form of the HA test) was used because it is more economical and rapid (Desario *et al*., 2005; Shashidhara *et al*., 2009; Silva *et al*., 2013).

The disease was found to be more common in puppies from 0-2 months of age (*p<0.05*). The main reason of this high incidence seems to be caused by lack of maternal immunity and poor efficiency of the immune system. Similar results have been described by some other authors (Lopez-Bueno *et al.,* 2006; Muzaffar *et al.,* 2006), who made similar observation in dogs between weaning and six month of age. It has been documented earlier that CPV is dependent on the mitotic activity of myocardial and intestinal cells which is at its maximum in younger puppies (Muzaffar *et al.,* 2006; Shashidhara *et al*., 2009). Moreover in the present study, similar clinical signs (vomiting, bloody foul smelling diarrhoea, dehydration, sunken eyes, depression, weakness etc.) have been found like in previous studies (Pollock and Carmichael, 1982; Shashidhara *et al*., 2009; Manzoor and Jamil, 2013). In all positive cases severe diarrhea was observed because the virus multiplies mainly in the gastrointestinal epithelium as described previously (Martella *et al*., 2004; Shashidhara *et al*., 2009).

The present study highlighted that German shepherd dog is the most affected breed (40%), where as Labradors (31.5%), pointers (13.5%), Rottweilers (8%), boxers (1.5%) and crossbreeds (5.5%) are less affected. These findings disagree with findings of previous studies (Martella *et al*., 2005; Muzaffar *et al.,* 2006) that recorded the highest incidence of the disease in Rottweilers. In Pakistan, people keep German shepherd dogs usually as pet animals and this could be the major possible reason for the high incidence of the disease in this particular breed. Secondly, the number of Rottweilers kept as pet animals in Pakistan is decreasing due to the aggressive nature of the Rottweiler. Interestingly, more cases of canine parvovirus infection are observed in the summer season. This agrees with the findings of recent publication (Muzaffar *et al.,* 2006).

This study also showed that the prevalence of the disease is higher in females than in males because Pakistani mostly keeps females for breeding purposes. Our results totally disagree with previous observations (Muzaffar *et al*., 2006). This could be due to the fact that female breeding places have contamination of parvovirus due to lack of hygiene and biosecurity measures and have become a
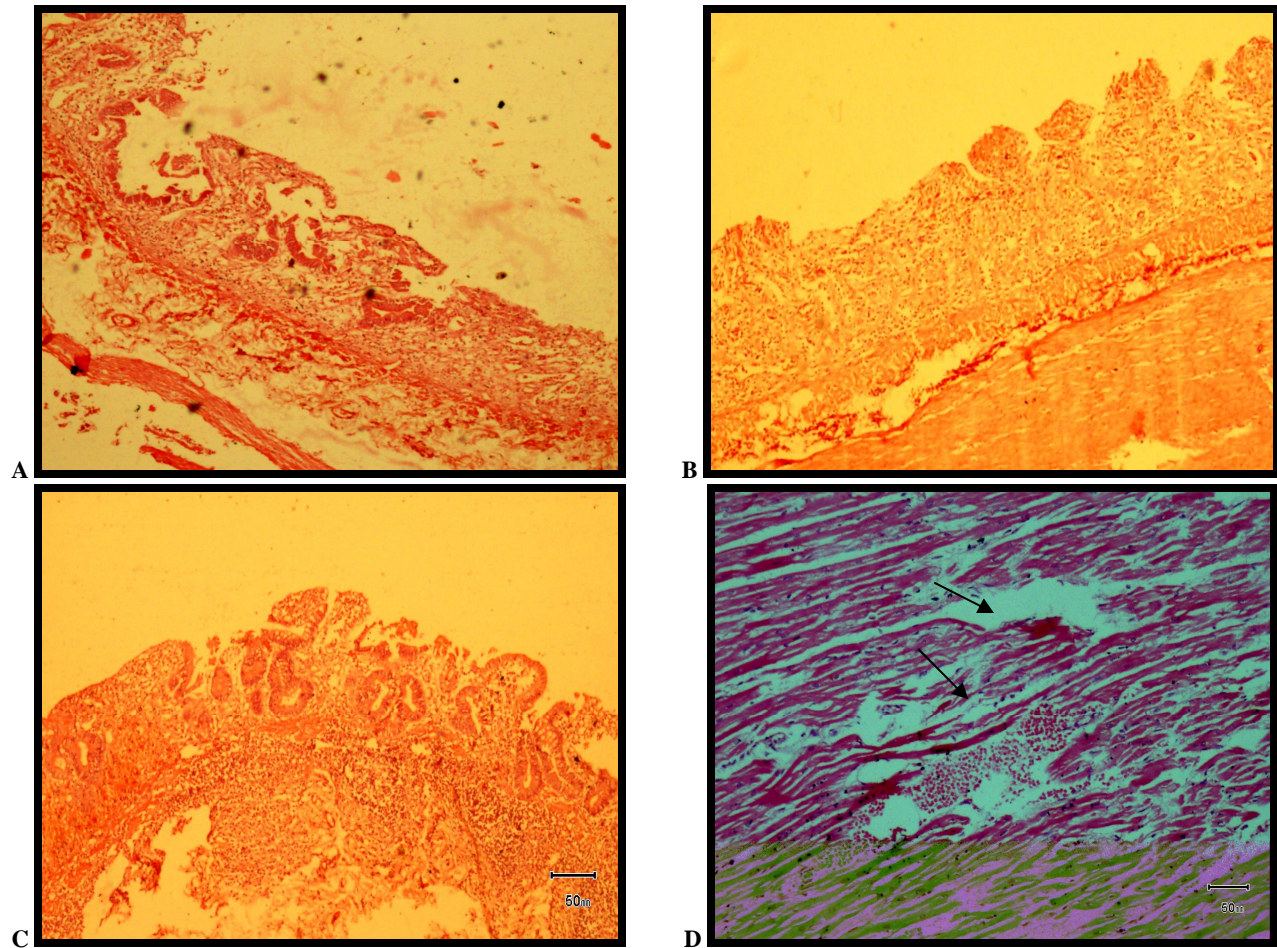
Fig. 1. Histological structure of (A) Jejunum of a 6.5 month old German shepherd dog showing severe villus atrophy and degeneration; (B) Ileum of a 7.5 month old Labrador dog with villus atrophy; (C) Duodenum of a 5.5 month old German shepherd dog showing severe atrophy of villi (D) The heart of a 2.5 month old pointer puppy showing necrosis of cardiac muscle fibers infiltration of mononuclear cells and neutrophils (arrow head). Stain: haematoxylin & Eosin. Magnification, 40X.

permanent source of infection. The histopathological findings of this study are in line with findings of Haligur *et al*. (2009). In young puppies the cardiac form of disease was more severe. On gross examination the myocardium was seen mottled with pale areas. Histologically, sacrolemmal proliferation, loss of myofibrils, and necrosis of cardiac muscle fibers, inclusion bodies in muscle nuclei in the intestinal epithelium and few inflammatory lesions were observed. Similar observations were made by (Buonavoglia *et al*., 2006; Muzaffar *et al*., 2006). The intestine was found filled with blood and showed severe hemorrhagic enteritis. This is the prominent feature of the intestinal form of canine parvo infection (Decaro *et al*., 2006; Heligur *et al.*, 2009).

We found these twin assays, HAT and SAT, to be very useful for field applications for the management of CPV outbreaks in kennels. The only potential limitation of our assay is the need to bleed a chicken to obtain erythrocytes. We recommend to properly fix chicken erythrocyte to provide a longer shelf life at room temperature.

CONCLUSION

It is concluded that CPV is more prevalent in puppies of age between 0-2 months ($p<0.05$). HAT

and SAT are cheap and rapid screening tests for the diagnosis of CPV infection especially in outbreak situations in poor developed countries like Pakistan. It is recommended that a prevalence study should be done all over Pakistan. Proper attention should be given to CPV vaccination in order to eradicate this disease from the dog population of Pakistan.

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Conflict of interest

The authors declare that they have no competing interests.

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