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Comparative efficacy of conventional and molecular assays for detection of canine parvovirus

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Canine parvovirus type 2 (CPV-2) emerged in 1978 as the cause of enteric disease in dogs throughout the world (Truyen *et al.* 1996). Diarrhoea is a common manifestation caused by parvovirus infection in pups causing high morbidity and mortality. The infected domestic dogs may not necessarily exhibit clinical manifestations but they may shed the virus in faeces during the acute phase of enteric fever and show significant rise in the serum antibody titres (Stann *et al.*1984). The stability of CPV in the kennel environment and excretion of large amounts of virus by sick puppies can expose susceptible puppies to massive doses of virus (Marulappa and Kapil 2009). As clinical diagnosis is sometimes not possible due to difficult differential diagnosis with other diseases rapid and specific diagnosis of parvovirus infection is important. Haemagglutination test (Senda 1986) though sensitive and relatively inexpensive to perform, has its limitation in the requirement of continuous source of reactive RBCs and the need to monitor specificity of low-titred reaction with a haemagglutination inhibition assay. Virus isolation and detection being laborious and time consuming were replaced by polymerase chain reaction analysis (Uwatoko *et al*. 1995) because of rapidity, sensitivity and specificity (Costa *et al.* 2005). Keeping in view the importance of the disease, conventionally used assays are compared with molecular detection assays for rapid diagnosis.

Faecal samples: Faecal samples/rectal swabs were collected in sterile 2 ml Sorensen phosphate buffer (SPB) from 25 different dogs (irrespective of breed, age and sex) showing clinical signs of anorexia, vomition, dehydration, haemorrhagic diarrhoea and fever presented to Veterinary Clinics of the University.

Processing of samples: The rectal swabs were squeezed and agitated vigorously for 1 min and centrifuged at 2000 rpm for 15 min. Supernatant was collected and 1 ml of chloroform was added to it. The mixture was again shaken vigorously and allowed to stand for 10 min at 4°C. It was again centrifuged at 2000 rpm for 15 min and supernatant collected as faecal antigen for use in HA and virus isolation assays (Carmichael 1980).

Haemagglutination (HA) and haemagglutination inhibition (HI) assays: HA activity was assayed as per OIE (2001) using 1% pig RBC suspension. Alpha (α) method of haemagglutination inhibition (varying virus and constant serum) was done to identify the haemagglutinating virus as CPV using hyperimmune serum raised in rabbits (Carmichael 1980).

Virus isolation: Faecal samples were simultaneously used for virus isolation using Madin Darby canine kidney (MDCK) cells grown in 25 cm^2 cell culture flasks containing DMEM with 10% fetal calf serum. The infected cultures were examined daily for the appearance of cytopathic effects (CPE). Each sample was considered negative for virus if, after 3 passages, no cytopathic effect (CPE) was observed. At the end of incubation period, the cells were frozen and thawed 3 times and then harvested along with the culture fluid, which was tested for the presence of CPV by alpha method of VNT and by PCR/nested PCR.

PCR/Nested PCR: The viral DNA was extracted from cell culture fluid using QIAmp DNA extraction columns. However, DNA from faecal samples was extracted by boiling 200 µl of each sample at 100°C for 10 min followed by collection of supernatant after centrifugation at 12000 rpm for 10 min. DNA extracted from a commercial CPV vaccine was used as a positive and negative control consisted of DNA extracted from faecal sample of healthy pup and cell culture harvest which did not show any CPE. PCR and nested PCR for detection of CPV was carried out as Mizak and Rzezutka (1999), using DNA amplification kit. The PCR and nested

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PCR products were separated by agar gel electrophoresis in 1% agarose gel with 0.5 mg/ml of ethidium bromide at 80 V for 1 h and visualized by ultraviolet (UV) transillumination.

Evaluation of the tests for detection of canine parvovirus

$$\text{Sensitivity (\%)} = \frac{\text{Sample positive by both tests}}{\text{Sample positive by reference test (PCR)}} \times 100$$

$$\text{Specificity (\%)} = \frac{\text{Sample positive by both tests}}{\text{Sample positive by reference test (PCR)}} \times 100$$

The suspensions prepared from rectal swabs collected from 25 different field cases were passaged in MDCK cells to isolate the virus. The faecal antigens were subjected to HA before virus isolation. Out of 25 samples, 15 showed haemagglutinating activities with HA titre ranging from 16 – 8192. The samples, positive for HA were subjected to a-method of HI to identify the haemagglutatinating virus as CPV. The titres of HI confirmed the presence of CPV in all these 15 samples. HA and HI could detect the presence of virus in 60% (15/25) of the samples suspected for CPV infection. Several workers have used HA/HI for detection of canine parvovirus with a success rate varying from 40–80% (Kim *et al.* 1997, Oke *et al.* 2000).

The isolates were serially passaged in MDCK cells until clear cut CPE were produced. The CPE observed was rounding and clumping of cells as early as 24–36 h post-inoculation followed by detachment of cells from monolayer, which was completed by 96 h post- inoculation. Different isolates showed CPE at different passage levels after cell culture. Eleven out of 25 samples showed characteristic extensive CPE on third passage onwards. The presence of virus in cell culture was confirmed by alpha method of VNT. CPV could be isolated from only 11 samples using MDCK cells. Isolation and adaptation of CPV in CRFK and MDCK cell culture at various passage levels was reported by Mochizuki *et al.* (1989) who obtained 21 CPV isolates from 96 suspected samples.

PCR and nested PCR was used for detection of CPV from MDCK cell culture passaged material and faecal samples directly. Out of 25 cell culture passaged samples, 11 (44%) samples showed presence of 1198 base pair and 548 base pair product in PCR and nested PCR, respectively (Figs 1, 2). No amplification was detected in uninfected cell

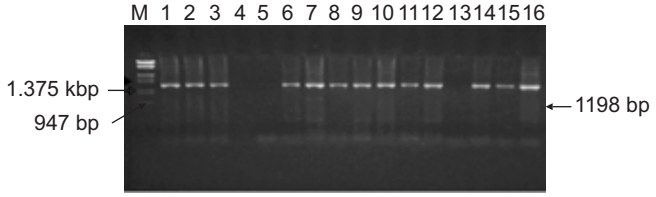

Fig. 1 PCR amplification of CPV from faecal samples of dogs

Lane M : DNA molecular weight marker (l DNA/ECO RI and Hind III, 564-21226 bp); lane 1 : positive control; lanes 1–3, 6–12, 14–16: positive samples; lanes 4,5,13, negative samples.

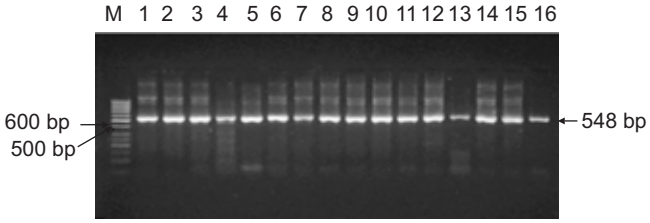

Fig. 2 Nested PCR amplification of CPV from faecal samples of dogs

Lane M, DNA molecular weight marker (80–1031 bp); lane 1, positive control; lanes 2 to 16, positive samples.

culture isolates (not shown). PCR and nested-PCR was also used for detection of CPV directly from faecal samples. Nineteen faecal samples (76%) showed presence of 1198 base pair product in PCR (Fig.1) and 22 faecal samples (88%) showed presence of 548 base pair product in nested PCR (Fig. 2). PCR could detect the presence of CPV in 76% of faecal samples while nested PCR in 88% of faecal samples. PCR and nested PCR were used for detection of CPV with a success rate of 64 and 87%, respectively (Kim *et al.*1997).

In comparison to PCR the sensitivity and specificity of HA-HI were 68.18 and 100.0%, respectively, while that of VI was 50 and 100%, respectively. A marked difference was observed between the proportion of PCR/nested PCR positive, HA/HI negative and VI negative samples indicating nested PCR to be more sensitive than HA/HI and VI in CPV diagnosis.

Less number of samples detected positive by HA/HI than PCR/nested PCR shows a lower sensitivity of HA test in the present study. There are several reports of lower sensitivity of HA test for detecting CPV due to presence of antibodies in the intestinal lumen of the infected dogs, which may bind virions and prevent agglutination (Desario *et al.* 2005). Moreover canine parvovirus can be detected by HA only for few days post infection (Decaro *et al.* 2005 and Desario *et al.* 2005). Several CPV-2 strains lacking HA activity had also been reported by Parrish *et al.* (1988) and Cavalli *et al*. (2001). In addition, erythrocytes from stress and disease free healthy donor pigs only can ensure a clear reading of HA (Desario *et al.* 2005). CPV could not be isolated from 4 HA/HI positive samples as these showed cytotoxic effect on inoculation to MDCK cells. The detection limits of viral isolation is less probably due to the low number of virus particles in the faeces at the time of sampling, or may be attributable to the pH, or to the presence of ions and colloids in faeces which might inactivate the virus (Tennant *et al.* 1994).

PCR/nested PCR could detect presence of CPV in more number of samples because these have the added advantage that they detect the virus nucleic acid even if the virus gets inactivated during transportation, storage, processing or it fails to grow *in vitro* (Mizak and Rzezutka1999, Costa *et al.* 2005). The nested PCR in general is sensitive than even PCR

for the detection of CPV in faecal samples as Hirsawa *et al.* (1994) have reported that single PCR could detect 10^9–10^{11}/g of genome copies, however 10^{11}–10^{13}/g copies could be detected by the nested PCR.

SUMMARY

The HA/HI and virus isolation could detect CPV from 15/25 and 11/25 cases respectively, while PCR/nested PCR when applied directly on faecal samples could detect CPV from 22/25 cases. In comparison to PCR/nested PCR the sensitivity and specificity of HA/HI was 68.18% and 100% respectively whereas sensitivity and specificity of virus isolation was 50% and 100% respectively. A marked difference observed between proportion of nested PCR positive and HA/HI, VI negative samples suggested nested PCR to be a more sensitive method for detection of CPV.

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