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POLYMERASE CHAIN REACTION FOR THE DETECTION OF CANINE PARVOVIRUS IN FAECES OF DOGS

JOSEMI MATHEW

Thesis submitted in partial fulfillment of the requirement for the degree of

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Department of Microbiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR - 680 651
KERALA, INDIA

DECLARATION

I hereby declare that the thesis entitled **"POLYMERASE CHAIN REACTION FOR THE DETECTION OF CANINE PARVOVIRUS IN FAECES OF DOGS"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

27-11-2004
Mannuthy

JOSEMI MATHEW

CERTIFICATE

Certified that the thesis entitled **"POLYMERASE CHAIN REACTION FOR THE DETECTION OF CANINE PARVOVIRUS IN FAECES OF DOGS"** is a record of research work done independently by **Dr. Josemi Mathew**, under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

Dr. M. Mini
(Chairperson, Advisory Committee)
Assistant Professor (S.S.)
Department of Microbiology
College of Veterinary and
Animal Sciences, Mannuthy

27-11-04
Mannuthy

CERTIFICATE

We, the undersigned members of the Advisory Committee of **Dr. Josemi Mathew,** a candidate for the degree of Master of Veterinary Science in Microbiology, agree that the thesis entitled **"POLYMERASE CHAIN REACTION FOR THE DETECTION OF CANINE PARVOVIRUS IN FAECES OF DOGS"** may be submitted by Dr. Josemi Mathew, in partial fulfilment of the requirement for the degree.

Dr. M. Mini
(Chairperson, Advisory Committee)
Assistant Professor (S.S.)
Department of Microbiology
College of Veterinary and Animal Sciences, Mannuthy, Thrissur

Dr. V. Jayaprakasan
Associate Professor and Head
Department of Microbiology
College of Veterinary and
Animal Sciences, Mannuthy
(Member)

Dr. G. Krishnan Nair
Associate Professor
Department of Microbiology
College of Veterinary and
Animal Sciences, Mannuthy
(Member)

Dr. S. Ajithkumar
Assistant Professor
Department of Clinical Medicine
College of Veterinary and
Animal Sciences, Mannuthy
(Member)

External Examiner

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Introduction

1. INTRODUCTION

Dogs have been part of human society for longer period than any other domestic species. Now dogs are engaged for an array of purposes like sporting, tracking, policing and guiding, in addition to their role as a coveted pet and guard. Over the years, because of its tremendous multi-faceted values, dog rearing has increased tremendously, especially in urban and semi-urban areas. Dog breeding has become a most lucrative enterprise, offering good self-employment opportunities and substantial financial gains. Therefore, health cover to dogs has assumed great importance

These animals are susceptible to various infectious diseases, which have always been a cause of great concern for pet owners, veterinarians and scientists all over the world. As in most fields of science today, knowledge has been accumulating about viral diseases of dogs.

Canine parvovirus type 2 (CPV-2) has emerged as a new virus of dogs in the late 1970s, possibly derived as a variant of the related virus, feline panleukopenia virus (FPV) or other FPV-like viruses, by natural genetic mutation (Koptopoulos *et al.*, 1986 and Horiuchi *et al.*, 1998). The pandemicity of this disease is so great that since its first report in 1978, it has spread to about 50 countries in the world by the end of 1983. In India the disease was first recorded in Tamil Nadu in 1981 (Balu and Thangaraj, 1981). In Kerala, outbreak of canine parvovirus (CPV) enteritis was reported by Sulochana *et al.* (1987).

This disease is probably one of the most common infectious disorders of dogs. Three distinct age related canine parvovirus disease syndromes have been recognized in dogs (Murphy *et al.*, 1999). They are generalized neonatal syndrome, myocarditis syndrome and leucopaenia/enteritis syndrome, which affect age groups of 2 to 12 days, three to eight weeks and 2 to 12 months respectively.

Although clinical symptoms such as vomiting and haemorrhagic enteritis are considered characteristic of CPV infection, it could be misleading (Hoskins, 1998). Hence, this disease must be differentiated from other causes of acute onset of vomiting, diarrhoea and leucopenia, such as salmonellosis, campylobacteriosis, canine distemper, infectious canine hepatitis, canine corona viral enteritis, poisonings and small intestinal obstruction. In addition, this disease is highly contagious, spreading from host to host via faecal-oral transmission and causes even life threatening illness in susceptible canine population. Canine parvovirus, like other parvoviruses, is extremely resistant to adverse environmental conditions for long periods. Moreover, it is difficult to inactivate CPV with disinfectants. Therefore, it is important to isolate infected dogs from uninfected animals promptly. Thus, it becomes evident that an early and accurate diagnosis is absolutely essential for the proper treatment and control of the disease.

Different methods for diagnosis of CPV include virus isolation (VI) in cell culture, haemagglutination (HA) test, electronmicroscopy (EM), enzyme linked immunosorbent assay (ELISA) and agar gel precipitation test (AGPT) (Mochizuki *et al.*, 1984 and Teramoto *et al.*, 1984). Among these, HA test seems to be the most acceptable in the routine diagnosis because the test is relatively simple, rapid and inexpensive, but it is relatively insensitive, less specific and also requires continuous supply of porcine erythrocytes. Virus isolation and EM, although highly specific, are too time consuming and expensive.

Serological tests also fail to diagnose an early infection and often are less helpful in confirming the diagnosis of parvoviral gastro-enteritis, because vaccinated dogs or puppies with residual passive antibody obtained from colostrum will have elevated parvoviral antibody titres.

Since these conventional methods are time consuming and less sensitive, the search for alternative methods of early diagnosis of CPV infection have been focussed on DNA based techniques, like DNA hybridization and polymerase chain reaction (PCR), taking the advantage of the recent developments in molecular biology. Polymerase chain reaction can detect fewer particles of CPV

than ELISA and it is 10 to 100 fold more sensitive than EM (Schunck *et al.*, 1995).

Even though the use of effective vaccines has reduced the incidence of CPV infection among domestic dogs, outbreaks of canine parvoviral enteritis among vaccinated dogs have been reported occasionally.

Soon after its first appearance, CPV-2 was replaced during the next three years by antigenically variant viruses (CPV-2a and CPV-2b) by genetic alteration, which now co-exist in dog population world-wide (Murphy *et al.*, 1999). Molecular biological techniques such as restriction endonuclease analysis (REA) has enabled the study on the characteristic of the virus at molecular levels, to detect differences among closely related genomes and also strain variation.

Canine parvovirus has been isolated from faecal samples of CPV infected dogs, in various cell cultures like primary and secondary feline kidney and canine kidney cells, feline and canine lung, raccoon salivary gland and bovine foetal spleen cells and also in several cell lines such as MDCK, CRFK, NLFK feline cell line and A-72, a canine fibroma derived cell line (Appel *et al.*, 1979; Mochizuki *et al.*, 1993a; Mizak and Utka, 1997 and Joshi *et al.*, 1998).

Keeping these in view, the present study was undertaken with the following objectives.

1. Detection of canine parvovirus (CPV) in faecal samples by HA, PCR and seminested PCR.
2. Comparison of HA, PCR and seminested PCR for the diagnosis of CPV infection.
3. Characterization of CPV vaccine strain as well as field strain by REA of PCR amplified product of CPV capsid protein VP1/ VP2 gene.
4. Attempts for isolation of CPV using cell lines (MDCK/CRFK/NLFK).
5. To study the influence of age, breed, season and vaccination on the occurrence of CPV infection.

Review of Literature

2. REVIEW OF LITERATURE

2.1 HISTORICAL BACKGROUND OF THE DISEASE

Minute virus of canines (MVC) is an autonomous parvovirus of dogs that was first discovered in the faeces of normal dogs in 1967 (Binn *et al*., 1970) and was known as the only canine parvovirus(CPV) until a second CPV emerged about 10 years later.

As early as 1977, in United States, Eugster and Nairn (1977) demonstrated parvovirus-like particles in the faeces of puppies showing symptoms of diarrhoea, by electronmicroscopy (EM), but the virus they detected was not successfully propagated in cell culture and comparisons with MVC could not be made.

A newly recognized highly contagious and infectious disease of dogs characterized by acute gastro-enteritis with diarrhoea and myocarditis had emerged as an epizootic in 1978 and its etiology was identified to be canine parvovirus 2 (CPV-2). (Appel *et al*., 1979; Gagnon and Povey, 1979 and Johnson and Spradbrow, 1979).

Retrospective serological studies showed that no anti CPV-2 antibodies were detected in the sera of domestic dogs or wild canine population until the mid-1970s. This indicated that CPV was comparatively a new pathogen for dogs (Koptopoulos *et al*., 1986).

Although the conclusive origin of CPV was unknown, the most widely accepted hypothesis for its emergence was that, it was derived from feline panleukopenia virus (FPV) or other FPV-like viruses by natural genetic mutations. (Horiuchi *et al*., 1998)

The stability of the virus, its efficient faecal-oral transmission and the near universal susceptibility of dog population of the world accounted for the epizootic in 1978. Retrospective serologic studies had indicated that the apparent immediate ancestor of the virus began infecting dogs in Europe during early or mid 1970s. (Murphy *et al*., 1999).

2.2 INCIDENCE

2.2.1 Global

Subsequent to identification of CPV in mid 1978, the disease was reported from several countries such as USA (Appel *et al*., 1979), Canada (Gagnon and Povey, 1979), New Zealand (Gumbrell, 1979), United Kingdom (Hitchcock and Scarnell, 1979), Australia (Johnson and Spradbrow, 1979), France (Moraillon *et al.,* 1979), Israel (Perl *et al*., 1980), Japan (Azetaka *et al*., 1981), India (Ramadass and Khader, 1982) and China (Cui *et al*., 1984).

2.2.2 India

Disease resembling CPV infection occurred since 1980 and proved fatal in many parts of India. Balu and Thangaraj (1981) reported the devastating gastro-enteritis outbreaks in canines at Madras that closely resembled CPV. However, Ramadass and Khader (1982) reported the first confirmation of etiological agent in India in 1982 from Madras.

Sherikar and Paranjape (1985) recorded the outbreak of parvoviral enteritis in dogs in and around Bombay city in August 1981. High mortality (63.7 per cent) was observed among unvaccinated dogs.

Narasimhaswamy (1988) demonstrated CPV haemagglutinating antigen in 34.7 per cent of faecal samples from dogs with clinical signs of gastro-enteritis in Bangalore.

Mohan *et al*. (1992) using haemagglutination inhibition (HI) test detected 33 per cent seropositive samples for CPV infection in Punjab.

2.2.3 Kerala

Sulochana *et al*. (1987) reported an outbreak of canine parvoviral enteritis among dogs in Kerala, particularly in Trichur and Ernakulam districts, during June to August 1986 and mortality was about 26 per cent. Out of the apparently healthy animals, nine had titres ranging from 64 to 1024. Since vaccination against this disease was not being practiced at that time, it might be due to previous exposure to this virus.

A high incidence of sero positiveness (71.3 per cent) to CPV was recorded by Dot-ELISA (Deepa *et al*., 2000).

2.3 ETIOLOGY

2.3.1 Taxonomy

Canine parvovirus 2 belongs to the family *Parvoviridae*, subfamily *Parvovirinae* and genus *Parvovirus*. This virus was found to be genetically and taxonomically distinct from a previously described parvovirus of dogs, MVC or canine parvovirus-1 (CPV-1) (Siegl, 1985 and Murphy *et al.,* 1999).

2.3.2 Virion Properties

2.3.2.1 Genetic and Capsid Structure

The CPV is a nonenveloped, icosahedral particle of 21 ± 3 nm in diameter (Gagnon and Povey, 1979 and Murphy *et al*., 1999).

The genome of CPV is a single stranded (ss) negative sense DNA of about 5,200 nucleotides in length, which has two promoters which result in the expression of two structural proteins (VP1 and VP2) and two nonstructural proteins (NS1 and NS2). (Reed *et al*., 1988).

Parvovirus has a diameter of about 255 A°, a molecular mass of between 5.5×10^3 and 6.2×10^3 k da and a ss DNA of 5000 bases, with three viral proteins, namely, VP1, VP2 and VP3 with approximate molecular weights of 82,500, 67,000 and 63,000 daltons respectively (Tsao *et al.,* 1991).

Canine parvovirus, the smallest autonomous parvovirus, morphologically and biochemically consists of a ss DNA genome and is surrounded by a protein coat (Turiso *et al*., 1992).

Parrish (1999) demonstrated the surface features of CPV capsid by X-ray crystallography, which included a raised area (spike) surrounding the three-fold axis of symmetry, a depression (dimple) spanning the two-fold axis of symmetry and a further depressed area (canyon) surrounding the five-fold axis of symmetry. A non coding region near the right hand end of the CPV genome contains a variable number of direct repeat sequences and there are palindromic hairpins at either end of the genome that are used in the replication of the viral DNA.

2.3.2.2 Physico-chemical Properties

Johnson and Spradbrow (1979) indicated that CPV could resist heating to 60°C for at least one hour and was stable at pH 3.0.

Canine parvovirus had been recovered from dog faeces after more than three months at room temperature (McCandlish *et al.,* 1981).

Canine parvovirus was extremely stable to environmental conditions (extremes of heat and pH) and was noted for its resistance to inactivation by detergents and disinfectants. However, sodium hypochlorite (one part in 30 parts of water) was effective in inactivation of CPV. (Hoskins,1998 and Murphy *et al.,* 1999)

2.3.2.3 Antigenic Relationship

Canine parvovirus was closely related antigenically to mink enteritis virus (MEV) and FPV and these viruses were different from MVC. (Appel *et al.,* 1979 and Carmichael *et al*., 1980).

Osterhans *et al*. (1980) reported a close antigenic relationship between CPV and FPV by using agar gel immunodiffusion and fluorescent antibody test.

Chang *et al*. (1992) observed that CPV, FPV, MEV and raccoon parvovirus (RPV) had greater than 98 per cent similarity in DNA sequence within the capsid protein gene and studies with monoclonal antibodies indicated that these viruses were very similar antigenically.

2.3.2.4 Antigenic Variation and Evolution of CPV

Many workers (Parrish *et al*., 1988; Parrish *et al*.,1991; Murphy *et al*., 1999; Parrish, 1999 and Truyen, 1999) recorded that CPV, FPV and MEV were closely related and the mink and canine viruses were assumed to have arisen as host range mutants of the feline virus. Since its emergence in 1978, CPV-2 had undergone further mutations affecting its antigenic and genetic properties and these changes were recognized as subtypes 2a and 2b.The first antigenic variant CPV-2a had emerged between 1979 and 1981 that differed in three coding changes in the capsid protein gene where as the second antigenic variant CPV-2b was appeared by around 1984 and it differed from CPV-2a by only one aminoacid substitution. These variants could replicate and spread more effectively in susceptible dogs.

The close antigenic and genetic relationship and also approximately 98 per cent homology that exist between CPV-2, FPV, MEV and raccoon parvovirus (Truyen *et al.,*1994) suggest that CPV-2 might have originated by genetic mutation in a wild life host receptive to one of the FPV-like parvoviruses that infect carnivores (Truyen *et al*.,1995).

Greenwood *et al*.(1996) and Truyen *et al*.(1996) antigenically typed CPV isolates from clinical cases of gastro-enteritis into CPV-2, CPV-2a and CPV-2b, by restriction enzyme analysis and HI assay using monoclonal antibodies.

The most widely accepted hypothesis on the ancestor of CPV-2 is the emergence from a variant of FPV or of a closely related virus infecting another carnivore such as mink or fox. (Truyen *et al.,* 1998).

2.4 EPIDEMIOLOGY

2.4.1 Natural Host Range

The natural host range of FPV, MEV and CPV were by definition, the cat, mink and dog, respectively (Siegl *et al*., 1985).

The determinants of the host range differences between feline and canine parvoviruses had been attributed to the spike present in the capsid where there were two aminoacid differences between the two viruses (Hoskins, 1998).

Murphy *et al*. (1999) reported that all members of the family *Canidae* (dogs, wolves and coyotes) were known to be susceptible to natural infection by CPV.

Ikeda *et al*. (2000) indicated that, the antigenic variants CPV-2a and 2b infected cats efficiently, unlike CPV-2 and these two variants are further evolving in cats.

2.4.2 Age, Breed, Sex and Seasonal Factors Influencing CPV Enteritis

2.4.2.1 Age

Eugster *et al.* (1978) observed that dogs of all ages, any breed and sex could be affected with CPV enteritis.

Rogers (1987) emphasized that young unvaccinated dogs between 12 and 16 week-old were commonly affected with CPV infection.

Sulochana *et al.* (1987) noticed majority of CPV infection in pups below eight months of age, even though dogs of all ages were affected.

Ernst *et al.* (1988) reported that the risk for CPV infection in dogs younger than six months was significantly greater than other age groups. However, Sherikar *et al*. (1989) reported that adults seemed to be more affected than pups.

Canine parvovirus affected all age groups of dogs with high morbidity and mortality in young puppies (Joshi *et al.*, 1998).

Murphy *et al*. (1999)) opined that canine parvoviral enteritis was seen most commonly in pups at two to four months of age.

Garcia *et al*. (2002) observed that 76 per cent of CPV-2 infection involved two to four months old puppies.

2.4.2.2 Breed

Ramadass and Khader (1982) indicated that all breeds were equally susceptible to CPV infection.

Rogers (1987) reported an increased incidence of CPV infection in Rottweilers, Doberman pinchers and German shepherd dogs.

Gunaseelan (1993) observed higher percentage of incidence in non-descript dogs, compared to purebred dogs.

Mizak and Mizak (1994) and Deepa (1999) observed highest distribution of CPV infection in German shepherd dogs compared to other breeds.

2.4.2.3 Sex

Fluckiger (1980) observed more occurrence of CPV infection among males than in females.

Both sexes were equally susceptible to CPV infection (Ramadass and Khader, 1982 and Rogers, 1987).

2.4.2.4 Season

Kelly (1978) in Australia reported an outbreak of CPV in the month of August, September, October and March through May.

Horner (1983) reported the peak incidence of CPV infection over spring and summer months from October to March in New Zealand. This probably reflected breeding cycles and also greater movement of animals to shows and boarding kennels.

Sherikar and Paranjape (1985) observed that the incidence of CPV infection varied from month to month and the seasonal influence on the occurrence of CPV was not significant.

According to Ernst *et al*. (1992), dogs were at higher risk of developing CPV infection during January, February, March, May and October.

Gunaseelan (1993) noticed that the incidence of CPV infection were higher during April, June and July in Madras.

Houston (1996) observed seasonal fluctuation with an excess of cases during July, August and September compared to rest of the year in Canada.

The inherent stability of CPV and extreme seasonal temperature variation might be contributing factors to the seasonal distribution of CPV enteritis (Sohini, 1997).

The possible explanation for time peaks in incidence of CPV enteritis would be a corresponding increase in susceptible population through whelping and weaned puppies (Greene, 1998) or due to onset of rainy season. (Deepa *et al.,* 2000)

2.5 VACCINE FAILURE

Janseen *et al*. (1982) reported the occurrence of CPV infection in 10 of 17 vaccinated juvenile bush dogs between 5 and 19 weeks old, suggesting that maternal antibodies might have interfered with immunization.

Serological analysis by haemagglutination inhibition (HI) test revealed that five per cent of dogs which had received modified live parvovirus vaccination and five per cent of animals which had received inactivated virus vaccination did not respond to the vaccine (Narasimhaswamy, 1988).

Dahlgaard (1989) investigated antibody level for CPV in vaccinated dogs and found that 25 per cent were negative, which explained why parvovirus could be the cause of gastro-enteritis in such dogs.

Greene (1998) suggested that the vaccination failure might be attributed to inherent host factors, difficulties with the vaccine or errors made in the process of administration. Usually for puppies in household environments, a complete series of vaccination with doses given every three to four weeks was recommended until pups are 16 weeks old. In contrast, pups born to bitches with high antibody titres or certain breeds of pups would not respond always to conventional CPV-2 vaccines until upto 20 weeks of age.

Vaccine failure due to CPV strain variation could be observed as a CPV independent enteritis in puppies immediately after CPV-2 vaccination (Sagazio *et al*., 1998).

Canine parvoviral enteritis was also reported in dogs vaccinated with killed as well as modified live vaccine by Deepa (1999).

2.6 DIAGNOSIS OF CPV INFECTION

According to the study conducted by Sabine *et al.* (1982) 31 per cent of practicing veterinarians in Australia and New Zealand used clinical signs, as the sole method for the diagnosis of CPV and during severe outbreaks, the information obtained was reasonably accurate.

2.6.1 Clinical Signs

Eugster *et al.* (1978) suggested that the main clinical signs in CPV infection was vomiting, diarrhoea and dehydration. The vomitus was usually greyish white and watery. The faeces was first greyish or yellowish and then contained various amounts of unclotted or partially clotted blood, one or two days later.

Infection with CPV may be manifested by myocarditis or gastro-enteritis in susceptible dogs of any breed, sex and age group (Black *et al.*, 1979).

Mohan *et al.* (1993) described anorexia, depression and dehydration as the common initial signs of CPV infection, followed by vomiting and diarrhoea within 6-36 hours. In 92 per cent of the cases faeces contained blood. High body temperature was observed in 26.7 per cent cases, convulsions in two cases and oral mucosal lesions in one case.

2.6.2 Laboratory Diagnosis

Even though CPV infected dogs excreted infectious virus in their faeces for upto 10 days after the onset of disease, faecal samples within the first three days of clinical illness was frequently necessary to be of value in confirming a diagnosis (Carmichael and Binn, 1981 and McCandlish *et al.*, 1981). Studdert *et al.* (1983) suggested that clinical signs supported the diagnosis of CPV enteritis in approximately 30 per cent of cases. However, the clinical symptoms, although considered characteristic of CPV infection, could be misleading. Hence, other enteropathogenic viral and bacterial infections should also be considered.

(Hoskins, 1998). Therefore, virus detection or isolation was the most specific means of confirming parvoviral infection.

2.6.2.1 Agar Gel Immunodiffusion (AGID) Test

Flower *et al.* (1980) demonstrated the antigenic variation between CPV and FPV using AGID. A precipitation line of partial identity was produced between anti-CPV serum and the CPV and FPV antigens.

Ramadass and Khader (1982) suggested that AGID was as sensitive as immunofluorescence and could be used for the routine diagnosis of CPV Infection.

Saseendranath *et al.* (1992) reported that 28 per cent of faecal samples from puppies aged 6 to12 weeks, with gastro-enteritis were positive to CPV infection by AGID.

Gunaseelan *et al.* (1993) used concentrated anti-CPV hyperimmune serum for the detection of CPV in faeces by AGID. Though there was 100 per cent correlation with the test using unconcentrated anti-CPV serum, the time taken was very short (three to four hours), when concentrated serum was used.

Out of 52 faecal samples collected from dogs with haemorrhagic enteritis, 15.7 per cent were found to be positive to CPV infection by AGID (Deepa, 1999).

2.6.2.2 Counter Immunoelectrophoresis (CIEP)

Saseendranath *et al.* (1992) indicated that 28 per cent of faecal samples from puppies aged between 6 to 12 weeks, with gastro-enteritis were positive to CPV infection by using CIEP.

Deepa (1999) observed that haemagglutination inhibition (HI) test and Dot-ELISA were more sensitive than AGID and CIEP for detecting CPV antibodies in serum samples of both vaccinated and non-vaccinated dogs.

Joshi *et al.* (2000) detected CPV antigen in 23.02 per cent of feacal specimens by CIEP.

2.6.2.3 Haemagglutination (HA) Test

Canine parvovirus agglutinated pig and rhesus monkey RBC at 4°C and 25°C, but not at 37°C (Appel *et al.*, 1979).

Carmichael *et al.* (1980) employed HA test in estimating the amounts of CPV haemagglutinin in faecal samples and suggested that a titre of 64 and above could be taken as positive. They also reported that CPV strongly agglutinated erythrocytes of pig, rhesus macaque, horse and to a lesser extent, the cat, under restricted pH and temperature conditions. Cow, chicken, human-O, goat, sheep, dog, rat, mouse, guinea pig and hamster erythrocytes were not agglutinated by CPV. They observed that faecal HA titres of CPV exceeded 3,27,680 per gram of faeces, but commonly ranged from 320 to 10,2400 between four and seven days post infection or when signs of enteritis commenced and HA ceased generally between seven and nine days post infection. Since low amounts of non-specific haemagglutinins were often present in faeces, the specificity of HA could be determined by simultaneous testing of samples by HI test with a CPV reference antiserum.

Sabine *et al*. (1982) diagnosed 30 per cent of clinically suspected cases as positive to CPV infection by HA test.

Mathys *et al*. (1983a) found that formalin treated rhesus macaque erythrocytes could be used for HA test, without influencing sensitivity or specificity of HA test. Further, it extended the useful life of the stored erythrocytes.

Mathys *et al.* (1983b) revealed that chloroform treatment of faecal samples, which was known to result in partial purification of CPV, had no

influence on HA titres above 32, and because titres of 32 and less are considered negative, anyway, it can probably be omitted.

The most simple, sensitive and rapid method of detecting CPV virus was HA, which was twice sensitive than virus isolation and eight times sensitive than electronmicroscopy. (Studdert *et al.*, 1983).

Sherikar and Paranjape (1985) investigated an outbreak of parvoviral enteritis in dogs and found that 71 per cent and 72 per cent faecal samples were positive by HA and HI test respectively.

Senda *et al.* (1986) modified the HA test using two buffers, one alkaline (borate buffered saline) and one acid (virus adjusting diluent) and opined that CPV strains agglutinated erythrocytes from different species both at 4°C and 37°C. Highest HA titre was obtained at pH 6.0. They claimed the procedure as inconvenient and premixing of these two buffers resulted in lowering the CPV titres to the extent of eight fold.

Sulochana *et al.* (1987) showed that in an outbreak of CPV infection in dogs, 75.5 per cent were positive by HA test, with HA titres ranging from 40 to 5020.

Komolafe (1988) suggested the existence of carrier status in dogs recovered from CPV infection, which can act as a potential source of infection to other dogs.

Narasimhaswamy (1988) detected CPV in 34.8 per cent of cases with gastro-enteritis by HA test.

Senda *et al.* (1988) divided the CPV strains into two types, namely, earlier strains and new type strains. Earlier strains showed similar antigenicity to FPV and MEV and were temperature dependent. The new type strains showed different antigenicity from FPV and MEV and had temperature independent HA activity.

Sherikar *et al.* (1989) suggested that faecal HA and HI test were more rapid and economic than serum HI test, electronmicroscopy and fluorescent antibody technique.

According to Drane *et al.* (1994) HA was considered unsuitable for rapid diagnosis because of the need to perform a confirmatory HI test.

Gunaseelan (1993) reported that 18.1 per cent faecal samples were positive to CPV infection by HA test with titres ranging from 64 to 2048 and also observed that chloroform treatment of faecal samples did not significantly alter the HA titres of samples, signifying the absence of non-specific agglutinins.

Mohan *et al.* (1993) confirmed the specificity of haemagglutinating activity of CPV by HI test using specific CPV antiserum.

Rai *et al.* (1994) found that majority of the HA activity of 8 $\log_2$ to14 $\log_2$ was obtained from faecal samples collected three to six days after the onset of clinical signs.

Sohini (1997) indicated that 25.7 per cent of dogs with clinical gastro-enteritis were positive to CPV infection by HA test, with titres ranging from 64 to 32,768 and the specificity of HA test was confirmed by HI test.

Udupa and Sastry (1997) found that 60.5 per cent of dogs with clinical gastro-enteritis were positive to CPV infection by serum HI test and of these 59.7 per cent of dogs had positive faecal HA titres.

In comparing HA, AGID, CIEP, EM and immunoelectron microscopy (IEM) for detection of CPV in faeces, Deepa (1999) showed that HA test gave best results. Out of 57 faecal samples screened, 61.4 per cent of samples could be diagnosed as positive to CPV infection by HA test.

The simplest procedure for the diagnosis of CPV infection was HA of pig or rhesus monkey red blood cells (pH 6.5, 4°C) by virus present in faecal extracts

(Murphy *et al.*, 1999). Haemagglutination test could not be used for the detection of CPV if less than 5 x 10^4 virions were present per gram of faecal materials.

The excretion of CPV in faeces of vaccinated dogs is at a lower level when compared to natural infection (Hoskins, 2000).

Garcia *et al.* (2002) reported that among 92 CPV positive dogs by HA test, 28 had been previously vaccinated and in11 of these animals, the positive HA titre might have been as a result of vaccination.

Faecal excretion of CPV could only be detected from 5 to 15 days after recent modified live virus (MLV) vaccination by faecal parvoviral antigen testing (www.antechdiagnostics.com).

Kumar *et al.* (2003a) analyzed the effect of erythrocytes of different species, different incubation temperatures and pH on HA activity by CPV and found that porcine erythrocytes at 4°C towards acidic pH in the range of four to six gave best results. They also studied the influence of different buffers [PBS, PBS-BSA, phosphate buffered saline salt solution (PBSS)] on HA activity of CPV and suggested that these buffers were comparable and could be effectively used for HA test.

2.6.2.4 Haemagglutination inhibition (HI) Test

Pollock and Carmichael (1979) observed significant amounts of CPV antibodies in the serum of affected dogs, even five days following infection and recommended HI test for routine serological diagnosis of CPV.

Canine parvovirus antibodies in sera were reported to persist at high levels for at least one year and a HI titre of more than 320 was considered as positive to CPV infection (Carmichael *et al.*, 1980).They also opined that absorption of sera with 25 per cent kaolin was not found essential as post infection titres were high and non-specific inhibitors of HI were rare and did not exceed tires of 1:80.

According to Walker *et al.* (1980) a HI titre of 256 or more should be regarded as positive.

An outbreak of CPV infection among laboratory Beagles showed that 78 per cent of the affected dogs and 83 per cent of apparently healthy dogs were positive to CPV antibodies (Binn *et al.*, 1981).

Mohri *et al.* (1982) found that 16.7 per cent serum samples from stray dogs were positive to CPV infection by HI test.

In an experimental infection, MaCartney (1984) detected circulating antibodies by HI test at day five after inoculation and the level increased rapidly in all sera collected on or after day seven.

Olson *et al.* (1988) opined that there was no significant difference in HI titres between vaccinated and non-vaccinated dogs, regardless of the time after vaccination.

Mohan *et al.* (1992) reported that 87.6 per cent of serum samples showed positive reaction to CPV by HI test.

Gorski *et al.* (1993) observed that non-specific thermostable inhibitors of haemgglutination of CPV could be efficiently removed from serum samples by absorption with 25 per cent kaolin suspension, with which the sensitivity of HI could be increased.

Gunaseelan (1993) noticed the seropositivity to CPV infection among vaccinated (82.1 per cent) was significantly higher when compared to non-vaccinated (65.3 per cent).

Rai *et al.* (1994) reported that 91.6 per cent serum samples from clinical cases of gastro-enteritis, between 4 to 10 days, were positive to CPV infection by HI test.

Udupa and Sastry (1996) suggested that a HI titre of 80 or more could be considered as positive to CPV infection.

Udupa and Sastry (1997) employed serum HI test and faecal HA test for diagnosis of CPV infection from clinical cases of gastro-enteritis and proposed that serum HI test was more sensitive than faecal HA test.

2.6.2.5 Latex agglutination Test (LAT)

Sonekata *et al.* (1996) proposed that the sensitivity of LAT was similar to that of HA test. The agglutination on a glass slide was observed macroscopically within two minutes.

Subhashini *et al.* (1997) compared HA and LAT for diagnosis of CPV in faecal samples of clinically suspected dogs and on statistical analysis, HA test was found to be significantly superior in sensitivity to LAT.

2.6.2.6 Enzyme Linked Immunosorbant Assay (ELISA)

2.6.2.6a Detection of Antigen

Teramoto *et al.* (1984) evaluated ELISA, DNA hybridization, HA and EM for the detection of CPV in faeces and observed highest correlation between ELISA and HA. The study indicated ELISA to be a sensitive and specific diagnostic assay for CPV infection.

Drane *et al.* (1994) compared ELISA and HA test for the detection of CPV antigen and found that ELISA had a sensitivity of 87 per cent and specificity of 100 per cent, when compared with 87 and 63 per cent respectively for HA test.

The faecal samples from dogs with gastro-enteritis were tested by rapid Dot- ELISA and observed that 26.3 per cent were positive for CPV (Sohini, 1997).

Pokorova *et al*. (2000) analyzed whole blood and serum samples of suspected as well as vaccinated dogs using ELISA to evaluate diagnostic value of whole blood examination in detecting anti-CPV antibodies.The difference between serum and whole blood titres were found to be insignificant.

Sen *et al*. (2000) compared the efficacy of ELISA in detecting CPV antigen from faeces of suspected dogs and HI test in detecting anti-CPV antibodies in the serum of same dogs and suggested that both the tests were equally valuable as diagnostic tools.

Joshi *et al.* (2001) described a rapid Dot immunobinding assay (DIA) for the diagnosis of parvoviral enteritis in dogs and noticed a 100 per cent correlation between DIA and HA.

2.6.2.6b Detection of Antibody

Rice *et al.* (1982) compared systemic and local immunity in dogs with canine parvoviral enteritis and found that dogs with high levels of CPV copro-antibodies and high serum antibody levels measured, by HI and ELISA, showed low viral HA titres in faeces. Dogs with no coproantibody had high viral titres in the faeces.

Florent (1986) noticed that 28 dogs with positive HI titre for CPV antibodies were strongly positive for CPV IgG by ELISA, but negative for CPV antibodies of the IgM class, thereby indicating that the HI test could be conveniently used for the serological assay for CPV antibodies.

Hara *et al.* (1994) on comparing indirect ELISA and HI test demonstrated the higher sensitivity of the ELISA with its titres sharing a good correlation with the HI test.

2.6.2.7 Electronmicroscopy (EM)

Examination of faeces, intestinal contents and affected tissues by EM had been widely used to detect CPV particles (Eugster *et al.*, 1978).

Williams (1980) detected astro virus-like, corona virus-like and parvovirus- like particles by EM of diarrhoeic faeces from pups and suggested that corona virus and parvo virus were the recognized agents of canine viral enteritis, even though astro virus had not been previously reported in dogs.

Valicek *et al.* (1981) examined 39 samples from diarrhoeic dogs by EM and demonstrated parvo virus in six samples, rota virus in eight samples and both parvo and rota viruses in two samples.

An EM examination of faecal and intestinal contents from dogs with gastro-enteritis showed that CPV was the major virus identified (48 per cent). In addition to CPV, the other viruses detected were corona virus, rota-like and astro-like viruses (Hammond and Timoney, 1983). They also proposed that immunoelectron microscopy (IEM) was more sensitive than EM for identification of CPV.

Mochizuki *et al.* (1984) confirmed CPV infection in 16.9 per cent of suspected dogs by IEM and HA test.

2.6.2.8 Fluorescent Antibody Technique (FAT)

The immunofluorescent assay by direct or indirect method was employed to examine infected tissue culture and faecal samples for CPV using virus specific antibody preparation (Black *et al.*, 1979).

Rivera and Karlsson (1987) developed a solid phase fluorescent immunoassay using antibody coated polyacrylamide beads (immunobeads) for detection of CPV in faecal samples of infected dogs and suggested that

immunobead assay (IBA) was as sensitive as ELISA, but more sensitive than HA test and immunofluorescene test using tissue cultures.

Dohse and Rudolph (1988) were able to demonstrate CPV in adrenal cortex specimens from infected dogs, by FAT.

Matsui *et al.* (1993) detected CPV in tongue epithelium, using immunofluorescent method.

2.6.2.9 Immunochromatography

A one step immunochromatographic test, based on the use of monoclonal antibodies (Mab), was developed by Esfandiari and Klingeborn (2000) for the detection of CPV in dog faeces and they concluded that the one step test was a rapid, simple, reproducible and sensitive diagnostic tool for detecting parvovirus in faecal samples.

Yamaguchi *et al.* (2000) employed a new test kit to identify CPV antigen in faeces using immunochromatographic method with Mab and considerd this kit as a simple diagnostic tool for detecting CPV.

Javdenic *et al*. (2001) revealed that HA test and FAT were more sensitive than immunochromatographic method for demonstration of CPV antigen in faecal specimens.

2.6.2.10 Molecular Diagnostic Methods

2.6.2.10a Polymerase Chain Reaction (PCR)

Polymerase chain reaction assay enabled the specific amplication of DNA of CPV from faeces after a fast and simple boiling pre-treatment or infected Crandell Feline Kidney (CRFK) cells (Parrish and Carmichael, 1986).

Widjojutmodjo *et al.* (1992) reported the inhibitory effect of bile acids on PCR amplification and suspected the acids as the inhibitory substances in faeces.

They also opined that this inhibitory effect of bile acids was heat stable since the inhibition was not diminished by heat treatment.

Mochizuki *et al.* (1993a) observed that PCR could be applied to spoiled or contaminated faecal specimen in which viruses had been inactivated.They proposed that PCR was as sensitive as virus isolation (VI) assay using Madin Darby canine kidney (MDCK) cells and more sensitive than VI assay using CRFK cells or HA assay.But they opined that false negative result could occur when only the PCR assay was used for diagnosis of CPV, which might be due to inhibitory substances present in faecal sample.

Schunck *et al.* (1995) compared the efficiency of extraction of CPV DNA from faecal material, using different pre-treatment methods such as phenol chloroform extraction after proteinase K digestion, boiling and treatment with Chelex (R) and revealed that boiling protocol gave best results in amplification of virion DNA by PCR.They also suggested that the sensitivity of PCR was as high as 10 infectious particles per reaction which corresponded to a titre of about 10^3 infectious particles per gram of unprocessed faeces. This rendered the PCR about 10 to 100 fold more sensitive than EM.

Senda *et al.* (1995) described PCR as a method for detecting wild-type CPV strains, which contaminated vaccines for dogs and found that the sensitivity of PCR was 100 to 10,000 fold higher than VI assay using CRFK cells. They indicated that in case of of combined vaccines, sensitivity was somewhat lower than those for monovalent vaccines, perhaps because of some inhibitory substance for PCR in those vaccines.

Uwatoko *et al.* (1995) opined that PCR assay could detect fewer particles of CPV than ELISA and VI assay. They also indicated that heat treatment can inactivate certain PCR inhibiting substances like DNAase and other proteins present in faeces. They reported the existence both heat-stable and heat-labile

inhibitory materials in faeces, the proportion of which might vary from dog to dog.

Meerarani *et al.* (1996) employed HA and PCR assay for early detection of CPV from faecal samples. Haemagglutination test detected 61.1 per cent of cases and PCR assay detected 72.7 per cent of cases, which indicated the higher sensitivity of PCR assay over HA test.

To detect pathogenic viruses in animal faecal specimens by PCR assay, it was important to remove or inactivate PCR inhibitory substances from faeces (Uwatoko *et al.*, 1996b). They opined that cationic surfuctant Catrimox – 14 TM more effectively removed PCR inhibiting substances in canine faecal specimens than gel filtration or boiling.

Mizak and Utka (1997) described PCR as a rapid, sensitive and specific method for early demonstration of CPV in faecal samples.

Sohini (1997) demonstrated CPV in faeces of dogs with gastro-enteritis by PCR, HA, Dot-ELISA and slot blot hydridization in 49.5 per cent, 25.7 per cent, 26.3 per cent and 39.13 per cent of cases respectively and proposed PCR not only as a very sensitive and specific, but also as a fast, simple and reliable technique for detection of parvovirus in faeces.

Subhashini *et al.* (1997) proved PCR as a sensitive and specific diagnostic method over LAT and HA test for detection of CPV infection in dogs.

Mingjeng *et al.*(2000) reported that the sensitivity of PCR was 10^2 to 10^4 fold higher than that of culture method and HA test for detecting CPV in faecal samples.

Kumar *et al.* (2003b) applied PCR assay successfully to detect CPV in cell culture supernatant as well as in faecal samples after heat treatment and suggested that PCR could be used as a viable alternative in routine diagnosis of CPV infection.

Recent modified parvovirus vaccination within 14 days may cause false positive results (www.antechdiagnostics.com).

2.6.2.10b Nested PCR

Mochizuki *et al.* (1993a) employed seminested PCR to confirm the specificity of the PCR product obtained in detecting CPV from faecal samples of dogs.

Hirasawa *et al.* (1994) developed nested PCR to increase the sensitivity as well as specificity of PCR assay for the detection of CPV in faecal samples and demonstrated that nested PCR was 100 times more sensitive than the single PCR. The number of the genome copy in positive samples was estimated as 10^{9}-10^{11} by simple PCR and 10^{11}-10^{13}/g by nested PCR. They also suggested that nested PCR seemed to be a sensitive, specific and practical method for the detection of CPV in faecal samples.

The sensitivity of detection of CPV in stool specimens by nested PCR was increased 60 per cent in comparison to single PCR method (Mizak and Utka, 1999). They also reported a variation in the intensity of band while amplifying the DNA of CPV from faecal samples, indicating that the final concentration of the products was not equal in each reaction.

2.6.2.10c Nucleic Acid Hybridization

Remond *et al.* (1992) proposed that nucleic acid hybridization might be an alternative diagnostic method to ascertain the presence of CPV, especially in frozen tissue samples.

Waldvogel *et al.* (1992) demonstrated the exact cellular localization of parvo virus in formalin fixed and paraffin wax embedded tissue section by *in situ* hybridization, which was proved to be a valuable specific tool for detecting CPV.

Sohini (1997) indicated that slot blot hybridization was equally as sensitive as PCR and no significant difference could be obtained on statistical analysis.

Meerarani *et al.* (1998) developed a sensitive and specific DNA probe for identification of CPV in faecal samples and demonstrated CPV in 64.4 per cent of cases by slot blot hybridization.

2.7 MOLECULAR CHARACTERIZATION OF CPV

2.7.1 Protein Profile of CPV by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE)

Structurally and biochemically CPV was very similar to other autonomous parvoviruses and the proteins comprising the capsid structure when analyzed by SDS-PAGE revealed three capsid proteins, which were characteristic of the autonomous parvoviruses (Parasido *et al.*, 1982).

Carman and Povey (1983) reported that the number and distribution of the viral proteins and the equivalent protein molecular weights were similar for CPV-2, MEV and FPV in SDS-PAGE. Four viral proteins were identified and their molecular weights were determined. ProteinA (77,500 to 79,500 daltons),protein B (63,000-63,500 daltons), protein C (61,500-63,000 daltons) and protein D (50,000-55,000 daltons).

Mengeling *et al.* (1986) determined the viral proteins (VP) of CPV, FPV, porcine parvovirus (PPV), MEV and bovine parvovirus (BPV) by SDS-PAGE and found that the mature virions of these viruses were composed of three VPs designated as VP1, VP2 and VP3 and the corresponding proteins of each virus were similar in molecular weights, 79,000 to 82,500 daltons (VP1), 65,000 to 66,000 daltons (VP2) and 62,000 to 62,500 daltons (VP3).

Gunaseelan (1993) obtained three major bands of molecular weights 85,000, 66,000 and 55,000 daltons from the partially purified virus samples by SDS-PAGE.

2.7.2 Restriction Enzyme Analysis (REA)

The DNA analysis by restriction endonucleases is a powerful technique to establish relationship between viruses at the genome level and to characterize different viral strains. McMaster *et al.* (1981) compared the genomes of CPV and MEV by REA and found 86 per cent homology for both types of DNA, indicating that CPV and MEV were closely related viruses.

Parrish and Carmichael (1986) reported differences after digesting the replicative form (RF) of DNA with restriction enzymes *Hinf I, Hae III, Dde I* and *Rsa1*. They concluded that the differences were probably due to mutations of restriction enzyme recognition sites, while others might have been due to rearrangements in the genome of the viruses.

Canine parvovirus isolates could be differentiated into three groups, viz., CPV-2, CPV-2a and CPV-2b by REA. The three groups were readily identified by their *Hph I* restriction profile. CPV-2a and CPV-2b had a characteristic 360 bp *Hph I* fragment that was absent from CPV-2. The restriction enzyme analysis also indicated that the CPV-2b viruses were about 60bp smaller than the CPV-2a and CPV-2 virus types (Greenwood *et al.*, 1995).

2.7.3 Polymerase Chain Reaction

Pereira *et al.* (2000) designed a PCR assay to type CPV strains in faecal samples collected from symptomatic dogs and proved the emergence of CPV-2, followed by replacement by the variants CPV-2a and CPV-2b.

2.7.4 PCR-based Restriction Fragment Length Polymorphism (PCR-RFLP)

Genomic variabilities between various canine and feline isolates and also the reference CPV and FPV strains were evidenced by the restriction analysis of the PCR amplified genes encoding the capsid protein (Mochizuki *et al.*, 1993b).

Hirasawa *et al.* (1995) employed PCR and REA for the differentiation of wild and vaccine type CPV.Their study with 21 isolates showed no differences in RFLP patterns as compared to the vaccine strain.

Polymerase chain reaction based REA is probably the most sensitive method for comparing closely related genomes, since a single base pair change in a recognition sequence, undetectable by other techniques, leads to a change in restriction endonuclease fragment pattern (Mochizuki *et al.*, 1996).

Sagazio *et al.* (1998) amplified the fragments of representative strains of CPV-2, CPV-2a and CPV-2b by PCR and the products were digested by restriction enzymes *Rsa I, Hpa II, Hind III* and *Pva II.* The *Rsa I* enzyme allowed the differentiation of CPV-2 from CPV-2a and CPV-2b.

Sohini *et al.* (1999) analyzed the genomic properties of CPV by restriction cleavage patterns of amplified DNA fragments encoding the capsid protein VP1 and VP2. All the isolates showed similar fragment length patterns with restriction enzymes *Hae III, Hpa II* and *Hind III,* whereas the isolates were readily differentiated by digestion with *Alu I.*

2.7.5 DNA Sequencing

The genome of a CPV isolate (CPV-N) was cloned and the DNA sequence was determined (Reed *et al.*, 1988). The entire genome including ends, was 5,323 nucleotides in length. They indicated that very few nucleotide or amino acid changes differentiated the antigenic and host range specificity of FPV and CPV.

Parrish *et al.* (1991) revealed that, by DNA sequence analysis, the CPV-2b strains differed by only two amino acids in the VP-1/VP2 genes from the CPV-2a strains, which differed in only five or six amino acids from CPV-2 isolates.

The cloning and sequencing of an *EcoRI- Pst I* fragment derived from the RF DNA of CPV vaccine strain was reported by Remond *et al.* (1992). The variability of the 5'end of NSI protein gene in the genome was confirmed by comparison with previously determined DNA sequences. A 15 nucleotide deletion was observed in the vaccine strain.

Serological, sequence and *in vitro* host range analysis of FPV isolates in Vietnam and Taiwan revealed that more than 80 per cent of the isolates from cats were of CPV type rather than FPV (Ikeda *et al.*, 2000).

Sequence analysis of the capsid protein encoding gene of CPV by Buonavoglia *et al.* (2001) revealed two amino acid changes. One of the changes affected position 426 (Asp to Glu) in a major antigenic site of the viral capsid, determining the replacement of the residue unique to CPV type 2b.

Battilani *et al.* (2002) sequenced the VP2 genes of Italian CPV type 2 strains isolated from dogs and wolves and detected mutation, one at residue 297 and the other at residue 265. These results demonstrated that the same strain of CPV could circulate among domestic and wild canids.

2.8 ISOLATION AND IDENTIFICATION OF CPV

2.8.1 Isolation of CPV

Appel *et al*. (1979) reported the isolation of a newly recognized canine parvo-like virus from faeces of dogs with haemorrhagic enteritis. Cell cultures from several species such as primary and secondary feline kidney and canine kidney cells, mink lung (CCL-64), Madin-Darby Canine Kidney (MDCK), feline

lung, raccoon salivary gland and bovine foetal spleen cells were all found susceptible to CPV. Also, virus could be serially propagated in these cells.

Hitchcock and Scarnell (1979) recovered CPV from the mesenteric lymph node of a puppy with haemorrhagic enteritis. The virus was grown in both primary canine kidney cell culture and in a feline cell line.

Johnson and Spradbrow (1979) tried the isolation of CPV in various cell cultures including low passage canine foetal kidney, a canine melanoma cell line, primary canine foetal kidney, heart, lung and liver, a feline kidney cell line and Vero cells and detected virus growth in primary canine foetal lung cells and in feline kidney cell line. The virus grew poorly or failed to grow in the other cells tested.

McCandlish *et al*. (1979) isolated CPV from dogs with gastro-enteritis, in dog kidney cell line (GH) and a feline embryonic cell line (FEA) and observed that virus appeared to grow better in the FEA cell line.

Canine parvovirus appeared to grow better in feline embryonic cell culture and was isolated in primary feline kidney and CRFK cell culture (Azetaka *et al*., 1981).

A new cell line, A-72, was established from a tumor, surgically removed from a female, eight years old Golden Retriever dog and was found supporting the growth of canine viruses and the cytopathic effect could be observed in initial culture or after one laboratory passage (Carmichael *et al*., 1981).

Mochizuki and Hashimoto (1986) stated that feline cells were more sensitive than canine cells for the propagation of CPV. They used three strains of MDCK cell line (A, B and C) for propagation of CPV and found that the strain A and B supported the replication of CPV, but no viral replication was recorded by HA test in the strain C cells. However, a few inclusion bodies were found in

cells of strain C. This indicated that there was some qualitative difference in susceptibility of each MDCK cell strain to CPV.

A mutant of CPV was derived from culture of a highly passaged isolate of CPV in the NLFK feline cell line (Parrish and Carmichael, 1986).

Narasimhaswamy (1988) used MDCK and Vero cell lines for the propagation of CPV isolates and revealed that Vero cells did not support the viral replication. Attempts to revive the virus from experimentally infected pups in primary and secondary canine kidney cultures were unsuccessful, for which the exact reason could not be established.

Mochizuki *et al*. (1993a) suggested that isolation of CPV using feline CRFK cells was less sensitive than that using canine MDCK cells, which were almost as sensitive as the PCR assay and they reported this finding was unexpected when compared to the previous study conducted by Mochizuki and Hashimoto (1986).

Mochizuki *et al.* (1996) reported the isolation of CPV from a cat manifesting clinical signs of feline panleukopenia in CRFK cell line.

Both permissive cell lines (CRFK, A-72 and MDCK) and non-permissive cell lines (Vero, ESK and L929) bound CPV and possessed multiple CPV binding protein in cellular membrane fraction (Uwatoko *et al*., 1996a).

A new continuous feline kidney cell line (FK-91) was developed by Durymanov and Shestopalov (1999) and the growth of parvovirus in this cell line was found to be comparable with that of primary canine kidney cell culture and CRFK cell line.

Mizak and Utka (1999) isolated CPV from faecal samples of dogs in feline NLFK cell line.

2.8.2 Identification of CPV in Cell Culture

2.8.2.1 Cytopathic Effect (CPE)

Production of intranuclear inclusion bodies in infected cell culture was described as a characteristic CPE of CPV by many workers (Appel *et al*., 1979; McCandlish *et al*., 1979 and Mochizuki and Hashimoto,1986).

Many workers are of the opinion that most of the isolates of CPV produce subtle cytopathic effects in cell culture inspite of the rapid replication (Appel *et al.,* 1979, McCandlish *et al*., 1979; Narasimhaswamy, 1988 and Gunaseelan *et al*., 1997).

Johnson and Spradbrow (1979) detected wide spread intranuclear inclusions in primary canine foetal lung cells and in the feline kidney cell line at 8 to15 days post infection.

Azetaka *et al.* (1981) reported that intranuclear inclusions were prominent in feline kidney cells after inoculation of freshly seeded cells, but not when monolayers had formed before inoculation

Hirasawa *et al*. (1985) observed that the number of infected CRFK cells bearing intranuclear inclusion bodies were much larger in those cells synchronized with thymidine treatment than the asynchronized cells, at 72 h post inoculation (PI) even though the inclusion bodies were first observed in both type of cells at 10 h PI.

Gunaseelan *et al*. (1997) indicated that haematoxylin and eosin stained infected coverslips were a good indicator of cytopathic changes showing multinucleated giant cells with intranuclear inclusions, which were irregular in shape. They also noticed some variable morphological changes such as rounding and floating of cells and opined that these changes were not characteristic of viral growth.

Sohini (1997) reported syncytia formation with cell vacuolation and intracytoplasmic inclusions as CPE produced by CPV at five days PI. Initial changes like rounding and clumping of infected cells appeared after two passages.

The infected CRFK cells at their third passage revealed CPE characterized by rounding and clumping of cells as early as 24 to 36 h PI and detachment of cells at 72 h, which was completed by 96 h PI (Joshi *et al.,* 1998). They also demonstrated characteristic intranuclear inclusion bodies with a clear halo at 48 h PI, which were darkly stained at 72 h PI on May-Grunwald Giemsa staining.

2.8.2.2 Fluorescent Antibody Technique (FAT)

The presence of parvovirus in cell culture was confirmed by the detection of specific intranuclear fluorescence in virus infected cells (Appel *et al*., 1979; McCandlish et al., 1979 and Hirasawa *et al*., 1985).

By indirect FAT, Basak and Compans (1989) were able to show that CPV entered only from basolateral surface of infected MDCK cells.

Indirect FAT of CPV infected cells showed bright apple green, yellow intranuclear fluorescence between 48 to72 h PI at third passage (Joshi *et al.,* 1998).

Cavalli *et al*. (2001) opined that *in vitro* cultivation of CPV was difficult and after a few passages on canine and feline cells, the presence of virus was detectable only by immunoflourescent assay on the feline cells, since HA activity had disappeared.

2.8.2.3 Immunoperoxidase Test

Hirasawa *et al*. (1985) employed immunoperoxidase test to detect CPV in cell culture and observed the viral antigen as brown coloured products.

2.8.2.4 Haemagglutination (HA) and Haemagglutination Inhibition (HI) Test

The supernatant fluids from CPV infected cell cultures agglutinated porcine and rhesus monkey erythrocytes at 4°C and 25°C to titres over 1024 (Johnson and Spradbrow, 1979).

Narasimhaswamy (1988) noticed a residual HA activity of eight at first passage and maximum HA activity was obtained at fourth passage level. The HA titres was appeared 36 h PI and reached a peak of 4096 at 120 h and steadied thereafter.

Gunaseelan *et al*. (1997) opined that the progressive viral replication in cell culture could be monitored by HA test.

The cell culture fluid after five days of growth at third passage when assayed by HA test showed the presence of CPV haemagglutination with high reciprocal HA titre of 2048. The specificity of HA was further confirmed by HI test (Sohini, 1997).

The cell culture fluid after five days of growth at third passage when assayed by HA test showed the presence of CPV haemagglutination with high reciprocal HA titre of 2048 (Joshi *et al.,* 1998).

2.8.2.5 Agar Gel Immunodiffusion (AGID) Test

Narasimhaswamy (1988) and Sohini (1997) demonstrated CPV antigen in infected tissue culture fluid by AGID using anti-CPV hyperimmune serum.

2.8.2.6 Polymerase Chain Reaction (PCR)

The PCR, HA-HI assay and AGID were used for detecting CPV growth in cell culture after each passage (Mochizuki and Hashimoto, 1986; Parrish and Carmichael, 1986 and Drane *et al*., 1994).

Sohini (1997) confirmed the presence of CPV in tissue culture fluid by PCR at second passage.

2.8.2.7 Acridine Orange Staining

The presence of CPV in infected cell culture could be revealed by acridine orange staining as bright greenish yellow intranuclear fluorescence (Gunaseelan *et al*., 1997).

Materials and Methods

3. MATERIALS AND METHODS

Molecular grade chemicals procured from Genei, Bangalore and analytical grade chemicals purchased from Sisco Research Laboratory (SRL) and Hi-Media, Mumbai were used, wherever the source is not mentioned. Glassware of Borosil brand and Tarsons brand plasticware were used in this study.

3.1 COLLECTION AND PROCESSING OF FAECAL SAMPLES

Faecal samples were collected from dogs of various breeds and of both sexes, suspected for canine parvoviral infection, and also from normal healthy non-vaccinated as well as vaccinated dogs, that were brought to Veterinary Hospitals attached to Kerala Agricultural University (KAU). Detailed case history with particular reference to age, breed, vaccination and clinical symptoms was also gathered. Collection of samples was made using sterile rectal swabs and these were immersed in sterile phosphate buffered saline (PBS, pH 7.2). The samples were then clarified by centrifuging at 9500 x g for 10 min in a cooling centrifuge (Remi C-24). The supernatant of samples were collected and tested for canine parvovirus (CPV) by haemagglutination (HA) test, polymerase chain reaction (PCR) and seminested PCR. Those samples showing high HA titres as well as positive by PCR were subjected for isolation trials using cell line.

3.2 HAEMAGGLUTINATION (HA) TEST

The supernatant of faecal samples for HA test were subjected to two different reactions, one after treating the supernatant with chloroform and another without treatment with chloroform. In the case of HA test without chloroform treatment, the supernatant of faecal samples were subjected to HA for studying the effect of different diluents [PBS (pH 7.2) and PBS-BSA] and also different diluents having different pH (PBS with pH 7.2, 6.0, 5.0 and 4.0) on HA activity

of CPV. Phosphate buffered saline with pH 7.2 was taken as the standard diluent of HA test for comparing the HA titres.

3.2.1 Preparation of One Per Cent Pig Red Blood Cells (PRBC) Suspension

3.2.1.1 Materials

(i) Alsever's solution

Dextrose	10.25 g
Trisodium citrate	4.0 g
Sodium chloride	2.10 g
Citric acid	0.275 g
Triple distilled water	500 ml

The pH of the solution was adjusted to 7.2 and autoclaved at 10 lbs pressure and 110°C for 20 min.

(ii) Phosphate Buffered Saline (PBS) (0.15 M, pH 7.2)

Sodium chloride	8.0 g
Potassium chloride	0.2 g
Potassium dihydrogen phosphate	0.02 g
Disodium hydrogen phosphate	1.15 g
Triple distilled water	1000 ml

Sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure.

3.2.1.2 Method

Blood was collected in Alsever's solution (1:1 or 2:1) from pigs maintained at Centre for Pig Production and Research, KAU. Erythrocytes were sedimented by centrifugation at 1500 x g for 10 min and washed three times with the ice cold PBS (0.15 M, pH 7.2) and finally one per cent suspension was prepared in ice cold PBS.

3.2.2 Different Diluents for HA Test

(i) PBS (0.15 M, pH 7.2) [3.2.1.1(ii)]

(ii) PBS (0.15 M, pH 6)

Composition was similar to PBS (pH 7.2), but the pH of solution was adjusted to six with 1N HCl and then autoclaved.

(iii) PBS (0.15 M, pH 5) [3.2.1.1(ii)].

The pH of the solution was adjusted to 5.0.

(iv) PBS (0.15 M, pH 4) [3.2.1.1(ii)]

Using 1N HCl pH was adjusted to 4.0.

(v) PBS-BSA

Bovine serum albumin, fraction V (Merck)	0.1 g
PBS (0.15 M, pH 7.2)	100 ml

Diluents were stored at 4°C till use.

3.2.3 Protocol for HA Test

Haemagglutination test was carried out as per the method of Carmichael *et al*. (1980), with few modifications.

3.2.3.1 Materials

(i) One per cent PRBC suspension

(ii) PBS (pH 7.2) [3.2.1.1(ii)]

(iii) Clarified faecal suspension

(iv) Canine parvovirus (CPV) positive sample

Live attenuated parvovirus vaccine (Fort Dodge, US) and inactivated parvovirus vaccine supplied by M/S Indian Immunologicals were used.

(v) U bottom microtitre plate

3.2.3.2 Method

Two fold serial dilutions of the test faecal sample (50µl) were prepared in ice cold PBS (pH 7.2) to provide dilutions ranging from 1:2 to 1:4096, in U bottom microtitre plate. Fifty microlitre of cold one per cent PRBC was added to all the wells and the plate was incubated at 4°C for four to six hours. Positive CPV sample control and RBC controls were included in separate rows of the microtitre plate. The results were read when buttons were formed in the control wells and the HA titre was expressed on the basis of $\log_2$.

The same protocol was repeated with other diluents [3.2.2 (ii)] to [3.2.2.(v)] and the HA titres were compared.

3.2.4 Protocol for HA Test with Chloroform Treatment

3.2.4.1 Materials

(i) One per cent PRBC suspension

(ii) PBS (pH 7.2) [3.2.1.1(ii)]

(iii) Chloroform treated faecal sample

The supernatants of the samples were treated with 1/10 volume of chloroform. After vigorous shaking the samples were kept for 10 min undisturbed, then centrifuged at 1500 rpm for 15 min and the supernatant was collected.

(iv) Canine parvovirus (CPV) positive sample [3.2.3.1 (iv)]

(v) U bottom microtitre plate

3.2.4.2 Method

The supernatant of chloroform treated faecal suspension was subjected to HA as described earlier. (3.2.3.2) and the HA titre was analyzed.

3.3 PREPARATION OF ANTI-CANINE PARVOVIRUS (ANTI-CPV) HYPERIMMUNE SERUM

Anti-canine parvovirus hyperimmune serum was produced in rabbits as per the method described by Ramadass and Khader (1982).

3.3.1 Materials

(i) Inactivated canine parvovirus vaccine supplied by M/S Indian Immunologicals

(ii) Freund's complete and incomplete adjuvant (Sigma)

(iii) Two healthy rabbits of Newzealand White breed, aged six months and weighing 2.5 kg, procured from Small Animal Breeding Station, KAU.

3.3.2 Method

Each rabbit was injected intramuscularly with one millilitre of vaccine emulsified in equal quantity of Freund's adjuvant. Totally four injections were given at an interval of 10 days. Freund's complete adjuvant (FCA) was used for the first injection and Freund's incomplete adjuvant (FICA) was used for subsequent injections. Ten days after the last injection, the rabbits were test bled

from ear vein and serum samples were tested by agar gel immunodiffusion (AGID) and haemagglutination inhibition (HI) tests for the presence of antibody. When the results were found satisfactory, the rabbits were bled from the heart and the serum was separated, inactivated at 56°C for 30 min and stored in aliquots at –20°C.

3.4 AGAR GEL IMMUNODIFFUSION (AGID) TEST

3.4.1 Materials

(i)	Agarose	0.8 g
(ii)	Sodium azide	0.01 g
(iii)	Normal saline	100 ml
	Sodium chloride	0.85 g
	Distilled water	100 ml

3.4.2 Method

Clean microscopic slides were dipped in one per cent melted agar and dried in air by keeping the slides horizontally. The dried slides were stored at room temperature until use.

Agarose (0.8 g) in 100 ml of normal saline solution was melted, cooled to 60°C and 0.01 per cent sodium azide was added. Four millilitres of melted agarose was poured onto pre-coated slides and allowed to solidify and then kept at 4°C till use.

Wells of five millimetre diameter were punched out on the solidified agar over the slide at a distance of three to four millimetre. Separate wells were charged with two to three drops of test rabbit serum and positive CPV control.

The slides were incubated in a moist chamber at room temperature for about 24 to 48 h.

3.5 HAEMAGGLUTINATION INHIBITION (HI) TEST

This test was conducted according to the method described by Carmichael *et al*. (1980), with few modifications.

3.5.1 Materials

(i) One per cent PRBC suspension(3.2.1).

(ii) Fifty per cent PRBC suspension. This was prepared in the same manner as described earlier (3.2.1).

(iii) PBS (0.15 M, pH 7.2) [3.2.1.1(ii)]

(iv) Positive CPV sample [3.2.3.1 (iv)]

(v) U bottom microtitre plate

3.5.2 Method

The test rabbit serum was inactivated at 56°C for 30 min and 1:10 dilution was made in PBS (pH 7.2). This was treated with 0.1 ml of 50 per cent PRBC to remove non-specific inhibitors of HA and allowed to stand overnight at 4°C. The serum sample was then centrifuged at 1500 rpm for 15 min to remove PRBC. The supernatant was used for HI test.

A two fold serial dilution (25 µl) of the test rabbit serum which was inactivated and PRBC treated was made in ice cold PBS (pH 7.2), starting with a dilution of 1:20 to 1:40960, in U bottom microtitre plate. Then, 25 µl of positive CPV sample containing four HA units was added to the serum dilutions and kept at room temperature for one hour. Fifty microlitre of one per cent PRBC was

added to all the wells and the plate was incubated at 4°C. The HI end point was determined after an overnight incubation.

The controls consisted of PRBC, virus control and serum control. The HI titre was expressed as the reciprocal of highest serum dilution with complete inhibition of HA.

3.6 POLYMERASE CHAIN REACTION (PCR)

3.6.1 Materials

3.6.1.1 Reagents for PCR

(i) Primers

A set of 19-mer primers VPF and VPR, which were chosen from conserved region in the CPV capsid protein VPI/VP2 gene, were used in the present study. The primers were selected from published reports (Mochizuki *et al*., 1993a). The sequences of the primers were as follows

VPF, 5'-ATGGCACCTCCGGCAAAGA-3' (identical to nucleotides 2285 to 2303, numbered according to Reed *et al*., 1988).

VPR, 5'-TTTCTAGGTGCTAGTTGAG-3' (complementary to nucleotides 4512 to 4530).

(ii) PCR Reaction Buffer (10x)

This contained 500 mM KCl, 100 mM, Tris hydrochloride (pH 9), 15 mM Magnesium chloride and 0.01 per cent gelatin.

(iii) *Taq* DNA polymerase with a concentration of 3 u/µl.

(iv) Deoxy nucleotide triphosphates (dNTP mix) 10 mM (2.5 mM of each dGTP/dCTP/dATP/dTTP).

3.6.1.2 Vaccine Strain of CPV

Puppy DP vaccine (Intervet) which contained live attenuated CPV was used as positive control for PCR. The vaccine was diluted to 1:10 in sterile distilled water before being subjected to PCR.

3.6.1.3 Clarified Faecal Suspension

Two hundred microlitre of the clarified faecal suspension was boiled for 10 min and cooled to 4°C for 15 min. Then the sample was centrifuged at 3000 x g for 10 min in a cooling centrifuge (Remi C-24). The supernatant was collected and diluted to 1:10 with sterile distilled water, which was then used as template DNA for PCR reaction.

3.6.2 Reconstitution and Dilution of Primers

The primers obtained in lyophilized form were reconstituted in sterile triple glass distilled water to a concentration of 100 pM/µl. The tubes with primers were kept at room temperature with occassional shaking for one hour. They were spun briefly to pellet down the insoluble particles, if any, and the stock solution was distributed into 10 µl aliquots and stored at –20°C. At the time of use, the aliquots were thawed and further diluted to 10 fold to obtain a concentration of 10 pM/µl before using for PCR.

3.6.3 Setting up of PCR (Test Proper)

The PCR reaction was carried out as per the method elaborated by Mochizuki *et al*. (1993a) with some modifications.

Polymerase chain reaction was performed in a total volume of 50 µl reaction mixture. A master mix was prepared before setting up the PCR reaction by combining the following reagents in 45 µl volume.

PCR reaction buffer	50 mM KCl, 10 mM Tris HCl (pH 9)
	1.5 mM Mg Cl_2
Primers	10 pM of each primer
dNTPs	200 µM of each dNTP
Taq DNA polymerse	one unit

Preparation of 450 µl of master mix for 10reactions was as follows

Reagents	Quantity
PCR reaction buffer (10x)	50 µl
Forward primer	10 µl
Reverse primer	10 µl
dNTP mix	20 µl
Taq polymerase	3.3 µl
Sterile triple distilled water to make	450 µl

To each PCR tube, 45 µl of master mix and five microlitre of diluted faecal suspension were added and mixed. One negative control without template DNA was included to monitor contamination, if any. The tubes were placed in the thermal cycler (Eppendorf master cycle gradient). After the reaction mixture had been incubated at 94°C for five minutes, it was subjected to 30 cycles of amplification.

The programme of amplification was as follows.

Denaturation	94°C for 30 seconds
Annealing	55°C for two minutes
Extension	72°C for two minutes
Number of cycles	30
Final Extension	72°C for five minutes

3.7 SEMINESTED PCR

3.7.1 Reagents for Seminested PCR

(i) Primers

The forward primer (VPF) was same as that of the simple PCR. The reverse primer was an internal primer designated as VPi which was chosen from the middle part of the target VP1/VP2 gene in the first round of amplification [Mochizuki *et al*. (1993a)].

The sequence of the internal primer was as follows

VP_i 5^1 – CATCTGGATCTGTACCATGG - 3^1

[complementary to nucleotides 3484 to 3503 and numbered according to Reed *et al*. (1988)].

(ii) PCR Reaction Buffer (10x) [3.6.1.1 (ii)]

(iii) *Taq* DNA polymerase [3.6.1.1 (iii)]

(iv) Deoxy Nucleotide Triphosphates [3.6.1.1(iv)]

3.7.2 Template DNA

Both positive and negative PCR products of first round of amplification was used as template DNA for second round of amplification after diluting 1:4 in sterile distilled water.

3.7.3 Setting up of Seminested PCR

The seminested PCR reaction was conducted as per the method described by Mochizuki *et al*. (1993a), with some modifications.

The preparation of mastermix was same as that of simple PCR. After distributing 45µ1 of master mix in every PCR tube, five microlitre of first amplified product diluted to 1:4 was added to each tube. Both positive and negative controls were also included in each reaction. The amplification was carried out in thermal cycler (Eppendorf master cycle gradient) and the programme of amplification was similar to simple PCR.

3.8 DETECTION OF AMPLIFIED PRODUCTS

3.8.1 Submarine Agarose Gel Electrophoresis

3.8.1.1 Materials

(i) Agarose

(ii) EDTA stock solution (0.5 M) pH 8.0

Sodium EDTA. $2H_2O$	186.1 g
Distilled water	800 ml

The pH was adjusted to 8.0 with 1N NaOH. Distilled water was added to make up the volume to one litre. The solution was sterilized by autoclaving at 121°C for 15 min at 15 lbs pressure.

(iii) Tris Borate EDTA Buffer (TBE) pH 8.2

Stock solution (10x)

Tris base 108.0g

Boric acid 55.0 g

EDTA (0.5 M, pH 8.0) 40 ml

Triple distilled water to make 1 litre

(iv) Tris Borate EDTA Buffer (TBE) 1x

TBE stock solution 10 ml

Triple distilled water to make 90 ml

(v) Ethidium Bromide Stock Solution

Ethidium bromide 10 mg

Triple distilled water 1 ml

The solution was mixed well and stored in amber coloured bottles at 4°C.

(vi) Gel Loading Buffer (6x)

Bromophenol blue 0.25 per cent

Xylene cyanol 0.25 per cent

Sucrose 40 per cent (w/v) in water

(vii) DNA Molecular Size Marker (500 µg/ml)

The Low Range DNA Ruler consisting of 9 double stranded DNA fragments of 100, 200, 300, 600, 1000, 1500, 2000, 2500 and 3000 base pairs was used as molecular size marker.

(viii) Amplified PCR products

3.8.1.2 Method

The PCR products were detected by electrophoresis in a two per cent agarose gel in TBE buffer (Ix). Agarose was dissolved in TBE buffer (Ix) by heating and cooled to 50°C. To this, ethidium bromide was added to a final concentration of 0.5 µg/ml.

The clean, dry, gel platform was sealed with adhesive tape and the comb was kept in proper position before pouring agarose. Once the gel was solidified, the adhesive tape was removed gently and placed the gel tray in the buffer tank. Poured TBE buffer (Ix) till it covered the top of the gel.

Ten microlitre of PCR product was mixed with one microlitre of 6x gel loading buffer and the samples were loaded into the respective wells carefully. The Low Range DNA Ruler (one microgram) was also loaded in one of the wells as DNA molecular size marker.

Electrophoresis was carried out at 5 v/cm for 2 h (or) until the bromophenol blue migrated the full length of the gel.

3.8.2 Recording of the Results

The gel was visualized under UV transilluminator (Hoefer, USA) and the results were documented in a gel documentation system (Bio-Rad laboratories, USA).

3.9 RESTRICTION ENZYME ANALYSIS OF PCR PRODUCTS

3.9.1 Materials

(i) Amplified PCR products

(ii) Restriction Enzymes (RE)

RE	Concentration	Recognition sequence	Procured from
Hinf I	10 U/µl	5^1G$\downarrow$ANTC 3^1 3^1CTNA$\uparrow$G 5^1	Genei, Bangalore
Rsa I	20 U/µl	3^1GT$\downarrow$AC 5^1 5^1CA$\uparrow$TG 3^1	Gene Craft, Germany
Sau 961	10 U/µl	3^1G$\downarrow$G NCC 5^1 5^1 CCNG$\uparrow$G 3^1	Genei, Bangalore

(iii) RE buffer (10x)

It was supplied by the manufacturer along with RE. Restriction enzyme (*Rsa1*) was selected from published reports (Sagazio *et al*., 1998) and the other two enzymes were chosen using web cutter programme.

3.9.2 Method

The PCR products were digested with restriction enzymes according to the manufacture's instructions. Digestion of PCR products by RE was carried out in a total volume of 20 µl. The following components were added to sterile microfuge tubes.

PCR product	15 µl
RE buffer (10x)	2 µl
RE	1 µl (10U)
Sterile distilled water to make	20 µl

The digestion mixture was incubated at 37°C for two hours. The RE, *Rsa I* was inactivated by heating at 65°C for 20 min, where as the other two enzymes were inactivated at 82°C for 20 min. The digestion as well as inactivation was carried out in Eppendorf master cycler (Germany).

3.9.3 Separation of Restriction Fragments

3.9.3.1 Polyacrylamide Gel Electrophoresis (PAGE)

The PCR products digested separately by *Sau 961, Hinf I* and *Rsa 1* were separated by PAGE.

3.9.3.1a Materials

(i) Preparation of eight per cent Acrylamide gel

Acrylamide: Bisacrylamide 30:8	3.72 ml
TBE (5x)	2.8 ml
Distilled water	7.80 ml
10 per cent Ammonium per sulphate	50 µl
TEMED	3 µl

(ii) TBE 1x [3.8.1.1 (iv)]

(iii) Restriction enzyme digested PCR products

(iv) Ethidium bromide stock solution [3.8.1.1 (v)]

(v) Gel loading buffer (6x) [3.8.1.1(vi)]

(vi) DNA Molecular Size Marker

The Low Range DNA Ruler[3.8.1.1(vii)] or *pUC 18/Sau 3A1* (200 µg/ml) which consits of DNA fragments of size 1444, 943, 754, 585, 458, 341, 258, 153 and 77/75 was used as DNA molecular size marker.

3.9.3.1b Method

Eight per cent acrylamide gel was prepared and poured between two glass plates. Then a comb was inserted in the top and the solution was allowed to polymerize. The comb was removed after complete polymerization. The glassplates containing polyacrylamide gel was transferred to the vertical slab gel electrophoresis system (Hoefer, USA). The wells were washed with TBE 1x to remove unpolymerised particles and then the wells were half filled with TBE 1x. Five micro litres of the digested PCR product was mixed with one microlitre of 6x gel loading buffer and carefully layered under the buffer column in the wells. Undigested amplified PCR product and DNA molecular size marker [3.9.3.1.1(vi)] were also loaded in separate wells. The upper and lower buffer tanks were filled with TBE 1x and electrophoresis was carried out at 70V till the bromophenol blue dye reached the bottom of the gel.

When the electrophoresis was over, glass slab containing the gel was dismantled and the gel was separated out and stained with ethidium bromide as in (3.8.1.2.).

3.9.4 Recording of Results

The gels were viewed in a transilluminator and photographed using a gel documentation system.

3.10 ISOLATION OF CANINE PARVOVIRUS

Madin-Darby Canine Kidney (MDCK) cell line obtained from National Centre for Cell Sciences (NCCS), Pune, was employed for the purpose.

3.10.1 Subculturing and Maintenance of Cell Line

3.10.1.1 Materials

(i) Eagle's minimum essential medium (MEM)

Dehydrated powder was reconstituted as per manufacturer's instruction and filtered using membrane filter (0.2 µm).

(ii) 7.5 per cent sodium bicarbonate in triple distilled water, sterilized by filtration.

(iii) Calcium – Magnesium Free Phosphate Buffered Saline (CMF – PBS) (0.15 M, pH 7.2)

Dehydrated powder was reconstituted as per manufacturer's instruction and sterilized by autoclaving at 15 lbs pressure at 121°C for 15 min.

(iv) Trypsin-Versene Glucose (TVG) solution (working solution)

Trypsin (1:250)	0.25 g
EDTA	0.02 g
Glucose	0.05 g
CMF-PBS (0.15 M, pH 7.2)	100 ml

Sterilized by filtration through membrane filter (0.2 µm), distributed in small quantities and stored at –20°C and prewarmed before use.

(v) Neonatal calf serum – collected aseptically from colostrum deprived male calves maintained in University Livestock Farm, Mannuthy.

(vi) Cell culture growth medium: Eagle's MEM containing 10 per cent foetal calf serum and 1 mM sodium pyruvate was prepared and pH was adjusted to 7.2 with 7.5 per cent sodium bicarbonate.

(vii) Cell culture maintenance medium: Differed from growth medium in having two per cent foetal calf serum.

(viii) Antibiotics

Benzyl penicillin	10 lac units
Streptomycin sulphate	1 g
Sterile triple distilled water	40 ml

Sterilized by filtration through membrane filter (0.2 µm), distributed in aliquots and stored at –20°C. It was used both in growth and maintenance media at a final concentration of 100 IU of penicillin and 100 µg of streptomycin per millilitre of the medium.

(ix) Tissue culture bottle of 50ml capacity

3.10.1.2 Method

The maintenance medium was poured off from tissue culture bottle containing confluent monolayer of healthy growing cells. The cell sheet was washed twice with CMF-PBS. Two millilitres of prewarmed TVG solution was added to bottle containing monolayer and then shaken gently for one minute. Then, the TVG solution was discarded and the bottle was incubated at 37°C for five minutes. When the cells started dislodging from the monolayers, added a small quantity of freshly prepared growth medium and the cells were detached from the surface by mechanical disruption using sterile pipette attached with bulb. A split ratio of 1:2 was employed for seeding into new tissue culture bottles and the cells were also seeded into test tubes containing coverslips. Enriched the tissue culture bottles and test tubes containing coverslip with growth medium at the rate of eight millilitres and two millilitres respectively. The bottles and tubes were incubated at 37°C. They were observed daily for the formation of monolayer. When monolayer was formed (usually within three to four days), it was used for further infection with CPV.

3.10.2 Inoculation of Cell Lines

3.10.2.1 Materials

(i) Fresh monolayer or partly formed monolayer of MDCK cells in tissue culture bottles and coverslips

(ii) Faecal samples processed for inoculation.

Faecal sample positive for CPV, with high HA titre was either filtered through Millipore syringe filter(0.2 µm) or treated with antibiotics at the rate of 500 µg of Streptomycin sulphate and 500 IU of Benzyl penicillin per millilitre of faecal suspension and incubated at 37°C for one hour and was used as inoculum for infecting cell line.

3.10.2.2 Method

Tissue culture bottle with fresh monolayer or partly formed monolayer was selected, the growth medium was poured off and washed with CMF-PBS. The monolayer was inoculated with 0.5 millilitres of inoculum and incubated at 37°C for one hour to facilitate adsorption of virus. The inoculum was removed and the monolayer was washed with CMF-PBS. Then eight millilitres of maintenance medium and eight millilitres of growth media were added to tissue culture bottle containing fresh monolayer and partly formed monolayer respectively. Control culture bottles were prepared simultaneously in which CMF-PBS was used as inoculum instead of clinical samples.

All the tissue culture bottles were incubated at 37°C and were examined at 24 h interval for a period of five to six days, under an inverted microscope for evidence of any cytopathic effect (CPE).

For infecting coverslip cultures, 0.2 millilitres of inoculum and two millilitres of maintenance medium were used. Control tubes were also treated in the same manner in which CMF-PBS was used as inoculum. Infected coverslip

cultures were collected at 24 h interval for a period of five days, for studying the CPE by May-Grunwald Giemsa staining and also for detecting presence of virus in cell culture by indirect immunofluorescence technique. The control cover slips were also stained and studied.

3.10.3 Passaging of Virus

3.10.3.1 Materials

(i) Inoculated monolayer in tissue culture bottles

(ii) Fresh monolayer or partly formed monolayer of MDCK cells in tissue culture bottles and coverslips

3.10.3.2 Method

Five days post inoculation, the inoculated monolayes were freeze thawed three times and centrifuged at 2000 x g for 15 min in a cooling centrifuge (Remi C-24), to sediment cell debris. The supernatant was used as inoculum for the next passage. The inoculation was performed as mentioned earlier (3.10.2.2). After every passage, the supernatant was tested for presence of CPV by PCR and HA test. If the presence of CPV was not detected even after third passage, the cell culture supernatant after initial clarification was subjected to ultracentrifugation at 100000 x g for two hours in a SW-type rotor to pellet the virus ,if present. Then the pellet was reconstituted in 200µl of PBS (pH 7.2), and subjected to PCR. Passaging of virus was stopped when PCR was found to be negative.

3.10.4 Staining of Coverslip Cultures

3.10.4.1 May-Grunwald Giemsa Staining

3.10.4.1a Materials

(i) Cover slip cultures

(ii) May-Grunwald stain

Prepared by dissolving 2.5 g of May-Grunwald stain powder in 100 ml of absolute methanol and allowed to age for one month.

(iii) Giemsa stain

Prepared by dissolving one gram of stain powder in 66 ml of glycerol and kept at 60°C till the stain powder got dissolved completely. Then added 66 ml of absolute methanol and kept for a day.

(iv) Methanol

(v) Acetone

(vi) Xylene

(vii) DPX mountant

3.10.4.1b Method

The coverslip cultures were fixed overnight in methanol. They were stained for 10 min, in May-Grunwald stain and for 20 min in 1 in 10 diluted Giemsa stain. The cover slips were rinsed rapidly in two changes of acetone and then in two parts of acetone and one part of xylene for five seconds. They were then placed in one part of acetone and two parts of xylene for one minute, cleared in two changes of xylene, two minutes each, dried and mounted with DPX on a clean grease-free glass slide and examined for CPE and /or inclusion bodies under microscope. The uninfected coverslips were also fixed as described above and studied in detail.

3.10.4.2 Indirect Fluorescent Antibody Technique (IFAT)

3.10.4.2a Materials

(i) Coverslip cultures

(ii) Anti-CPV hyperimmune serum raised in rabbits (3.3).

(iii) Fluorescein isothiocyanate (FITC) labeled goat antirabbit IgG (Genei, Bangalore).

(iv) PBS (0.15M, pH 7.2) [3.2.1.1(ii)]

(v) Ice cold acetone

(vi) Glycerol saline

Glycerol - 50 ml

Physiological saline (0.85 percent) - 50 ml

Mixed and sterilized by autoclaving at 15 lbs pressure and 115°C for 15 min.

3.10.4.2b Method

The infected and uninfected coverslip cultures were fixed in cold acetone for five minutes and washed with PBS. Anti-CPV hyperimmune serum developed in rabbit was diluted to 1:10 and added to each monolayer at the rate of 0.1 milliliter and incubated at 37°C for one hour in a moist chamber. Monolayer were then thoroughly washed with PBS and triple distilled water thrice respectively. Coverslips were allowed to react with 0.1 millilitre of FITC conjugated goat antirabbit IgG at a dilution of 1:50 in PBS and incubated at 37°C for one hour. They were again rinsed well in PBS and triple distilled water. The coverslips were mounted in glycerol saline and examined under a microscope with U.V. light source.

Results

4. RESULTS

In the present study, faecal samples were collected from 126 dogs clinically suspected for canine parvovirus (CPV) infection. Majority of the dogs had a history of vomiting, pyrexia, inappetence, dehydration and haemorrhagic enteritis.

A total of 29 samples from healthy vaccinated dogs after 15 days of vaccination and 11 samples from healthy non-vaccinated dogs were also collected.

All the faecal samples were screened for the presence of CPV by haemagglutination (HA) test, polymerase chain reaction (PCR) and seminested PCR. Attempts were made to isolate CPV in MDCK cell line, from those samples with high HA titre.

4.1 HAEMAGGLUTINATION (HA) TEST

Phosphate buffered saline (PBS, pH 7.2) was taken as the standard diluent of HA test for comparing HA titres. The HA titres were expressed on the base of $\log_2$. Titres of 6 $\log_2$ and above were taken as positive to CPV infection as suggested by Carmichael *et al.* (1980). The HA titre was analyzed using two different diluents (PBS, pH 7.2 and PBS-BSA).

Among 126 clinically suspected dogs, 41 (32.54 per cent) were positive to CPV infection by HA test using PBS (pH7.2) as diluent. The HA titres of the samples ranged between 0 and 15 and six of the positive samples were having HA titres of 12 and above. Among 41 positive cases, eight (6.35 per cent), seven (5.56 per cent), nine (7.14 per cent), four (3.17 per cent), two (1.59 per cent), five (3.97 per cent), two (1.59 per cent), one (0.79 per cent), two (1.59 per cent) and one (0.79 per cent) cases were having HA titres of 6, 7, 8, 9, 10, 11, 12, 13,14 and 15 respectively. Haemagglutination by CPV in faecal samples is shown in Fig.1.

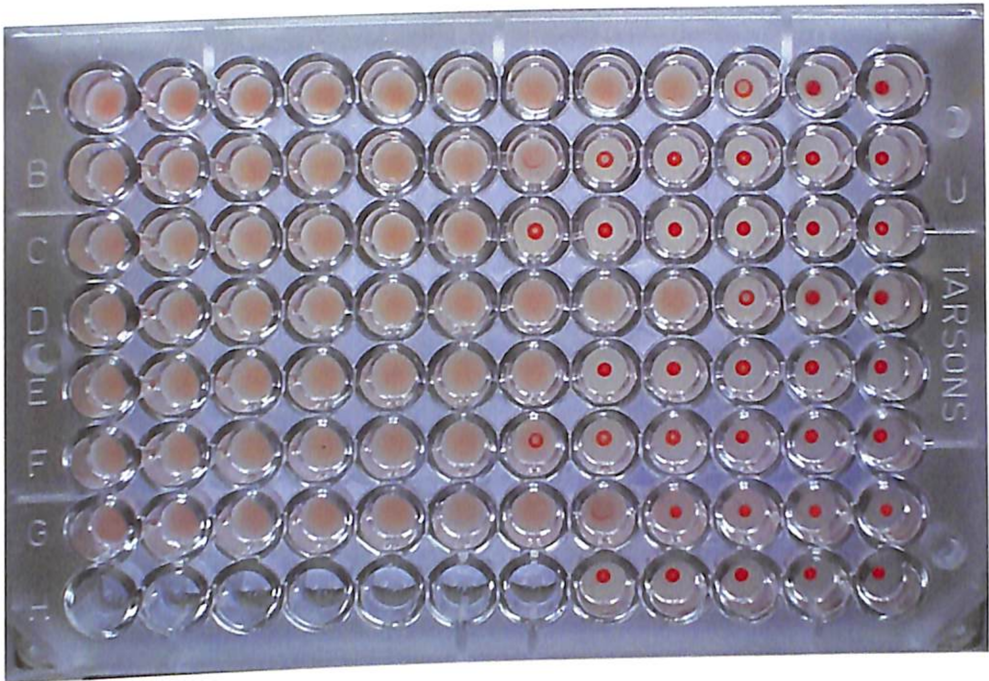

Fig. 1. Haemagglutination test for CPV in faecal samples

Rows A-F : Positive faecal samples
Row G : Positive control
Row H : RBC control

Using PBS-BSA, out of 126 samples from clinically suspected dogs, 39 samples (30.95 per cent) were found positive by HA test and the tires ranged from 0 to 14. Out of 39 positive cases, six (4.76 per cent), nine (7.14 per cent), eight (6.35 per cent), five (3.97 per cent), two (1.59 per cent), four (3.17 per cent), two (1.59 per cent), two (1.59 per cent) and one (0.79 per cent) cases were having HA titres of 6, 7, 8, 9, 10, 11, 12, 13 and 14 respectively. The frequency of the samples with their corresponding HA titre using different diluents are shown in Table 1.

Table 1. Canine parvovirus haemagglutination titres in faecal samples using PBS (pH 7.2) and PBS-BSA as diluents for HA test.

HA titre (log_2)	Number of samples		Per cent of total samples	
	PBS (pH 7.2)	PBS-BSA)	PBS (pH 7.2)	PBS-BSA)
0	45	46	35.71	36.51
1	12	10	9.52	7.93
2	8	9	6.35	7.14
3	11	9	8.73	7.14
4	4	7	3.17	5.56
5	5	6	3.97	4.76
6	8	6	6.35	4.76
7	7	9	5.56	7.14
8	9	8	7.14	6.35
9	4	5	3.17	3.97
10	2	2	1.59	1.59
11	5	4	3.97	3.17
12	2	2	1.59	1.59
13	1	2	0.79	1.59
14	2	1	1.59	0.79
15	1	-	0.79	-

None of the samples collected from normal healthy vaccinated as well as non-vaccinated dogs had positive HA titres.

4.1.1 Haemagglutination (HA) Test Using PBS with Different pH

The effect of pH on haemagglutination of CPV was studied using PBS with different pH and the HA titres were compared.

All the samples showing HA titre of 6 log_2 and above when PBS (pH 7.2) was used as diluent, revealed a progressive increase in HA titre when the pH of the diluent varied from 6.0 to 4.0.

Faecal samples from 40 suspected cases were having HA titre ranging from 1 log_2 to 5 log_2 while using PBS (pH 7.2) as diluent for HA test and among these, six cases, 12 cases and 14 cases were having a positive HA titre of 6 log_2 and above at pH 6.0, 5.0 and 4.0 respectively. When PBS with pH 4.0 was used as diluent 14 more cases could be detected as positive by HA test when compared to HA test using PBS (pH 7.2) as diluent. These 14 samples were also found positive by PCR. Out of 126 samples tested, 55 samples (43.65 per cent) were found positive to CPV infection by HA test using PBS (pH 4.0). Some of the samples showing very low HA titre while using PBS (pH 7.2), could be diagnosed as positive by HA test when PBS (pH 4.0) was used as diluent.

The frequency of the samples with their corresponding HA titre using PBS at different pH is shown in Table 2 and in Fig.2. The progressive increase in HA titre in some of the samples are presented in Table 3.

Table 2. Canine parvovirus haemagglutination titres in faecal samples using PBS at different pH.

HA titre (log_2)	Number of samples			
	PBS (pH 7.2)	PBS (pH 6.0)	PBS (pH 5.0)	PBS (pH 4.0)
0	45	44	41	39
1	12	11	10	7
2	8	8	7	7
3	11	7	6	5
4	4	5	4	6
5	5	4	5	7
6	8	4	6	4
7	7	3	5	3
8	9	7	9	4
9	4	11	7	7
10	2	6	8	11
11	5	6	5	9
12	2	3	4	5
13	1	2	3	4
14	2	1	2	4
15	1	2	1	1
16	-	1	1	1
17	-	-	1	1
18	-	1	1	-
19	-	-	-	1

Fig.2 Frequency of samples tested positive and negative by HA test using PBS at different pH

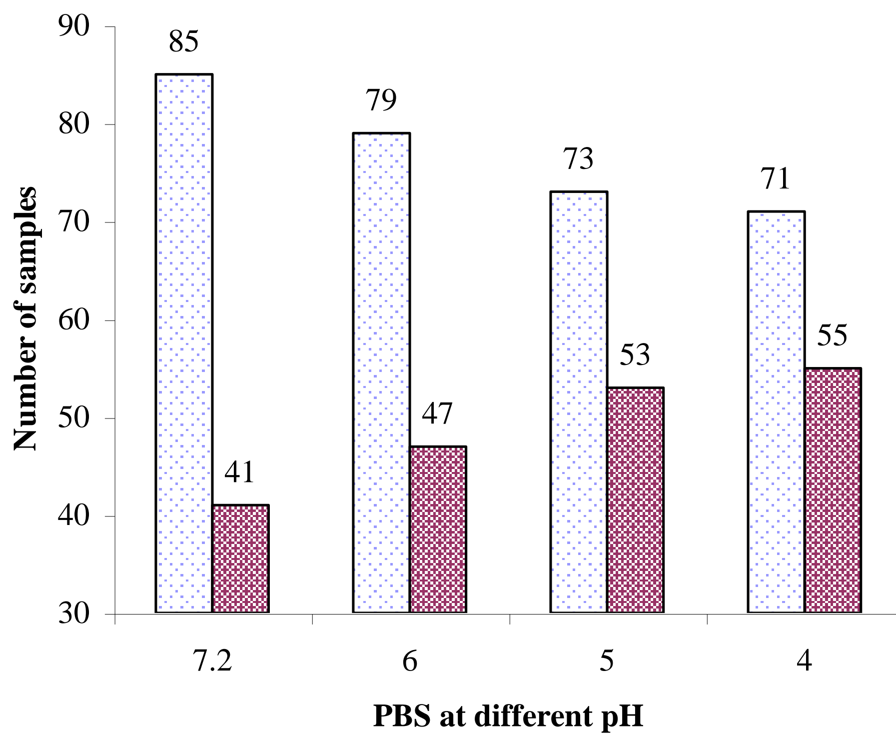

Table 3. Progressive increase in HA titre at different pH in terms of log_2

PBS (pH7.2)	PBS (pH6.0)		PBS (pH5.0)		PBS (pH4.0)	
	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum
1	2	4	2	5	3	8
2	2	5	3	7	4	9
3	3	6	4	7	5	9
4	4	6	5	8	5	9
5	6	8	6	10	7	10
6	6	9	7	10	8	10
7	7	9	8	10	8	11
8	9	9	9	11	10	11
9	9	11	10	12	10	12
10	10	12	11	13	11	13
11	11	12	11	13	12	14
12	12	13	13	14	13	14
13	14	14	15	15	15	15
14	14	15	15	17	16	17
15	18	18	18	18	19	19

All the faecal samples with no HA activity using PBS (pH 7.2) were also found negative to CPV infection by HA test, when diluents at different pH were used.

4.2 HAEMAGGLUTINATION (HA) TEST WITH CHLOROFORM TREATMENT

Chloroform treatment of faecal samples had no influence on HA titres above 5 log_2. Out of 126 samples, 41 (32.54 per cent) were tested positive by HA test both with and without chloroform.

4.3 POLYMERASE CHAIN REACTION

A PCR product of approximately 2.2 Kb was generated with DNA templates of CPV vaccine strain (Fig.3). Presence of canine parvoviral DNA in faecal samples, after boiling pre-treatment, was observed by the amplification of approximately 2.2 Kb fragment (Fig.4). The negative control kept along with the samples during each reaction had not produced any amplification.

Among 126 faecal samples tested for CPV by PCR, 77 samples (61.11 per cent) were found positive.

All the faecal samples collected from healthy vaccinated as well as non-vaccinated dogs were found negative by PCR.

4.4 SEMINESTED PCR

All the PCR products of both positive and negative faecal samples, after first round of amplification, was subjected to seminested PCR.

A DNA fragment of approximately 1.2 Kb was obtained with PCR product of CPV vaccine strain by seminested PCR. All the PCR products of the faecal samples positive by simple PCR also generated a DNA fragment of same size in seminested PCR. (Fig.5). The negative control had not produced any amplification during each reaction.

All the faecal samples which were found positive by PCR, were also tested positive by seminested PCR. Out of 126 samples screened, 93 samples (73.81 per cent) could be diagnosed as positive to CPV infection by seminested PCR.

A one and half year old dog that had taken annual booster vaccination one week back showed clinical signs such as haemorrhagic enteritis, vomiting and inappetence and was tested positive by only seminested PCR.

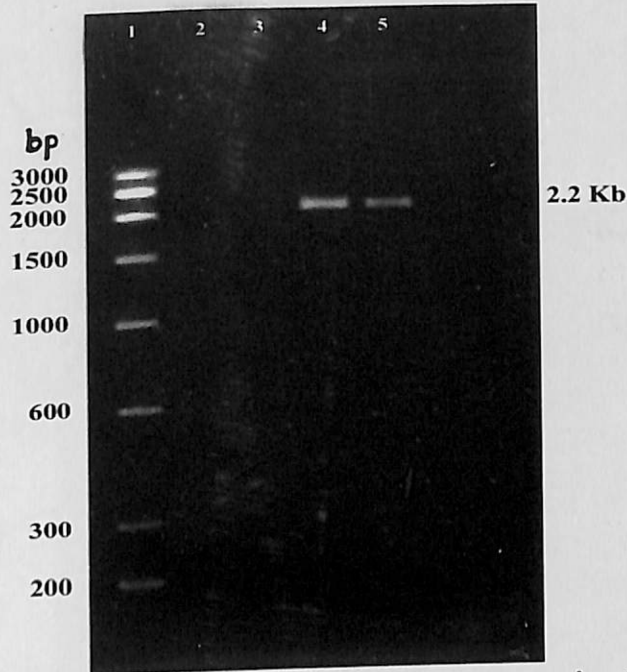

Fig. 3. Detection of CPV vaccine strain by PCR

Lane 1 - Low Range DNA Ruler
Lane 2 - Negative control
Lane 3 - Undiluted vaccine
Lane 4 - I:10 diluted vaccine
Lane 5 - I:10 diluted vaccine after boiling pre-treatment

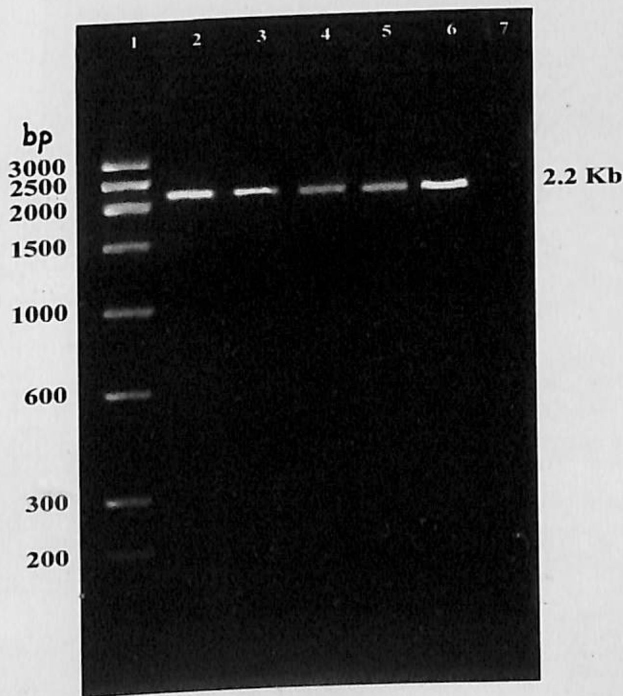

Fig. 4. Detection of CPV in faecal samples by PCR

Lane 1 - Low Range DNA Ruler
Lane 2 to 5 - Faecal samples
Lane 6 - Positive control
Lane 7 - Negative control

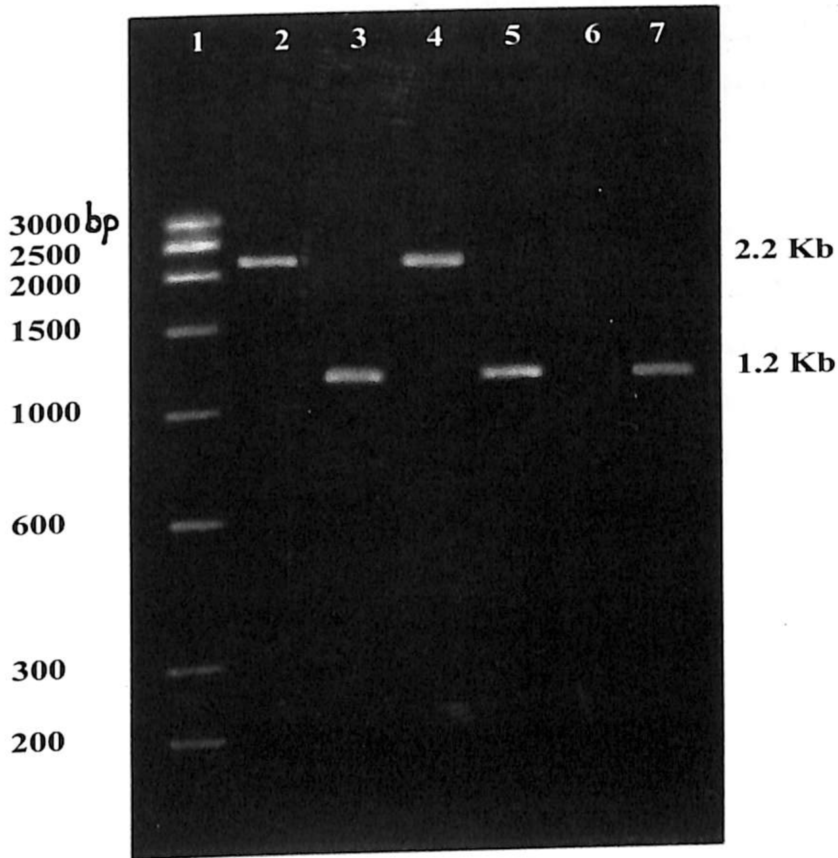

Fig. 5. Detection of CPV in faecal samples by seminested PCR

Lane 1 : Low Range DNA Ruler

Lane 2 : Positive control(first amplification)

Lane 3 : Positive control(second amplification)

Lane 4 : Positive faecal sample(first amplification)

Lane 5 : Faecal sample (second amplification)

Lane 6 : Negative faecal sample (first amplification)

Lane 7 : Faecal sample (second amplification)

All the faecal samples from healthy vaccinated as well as nonvaccinated dogs were tested negative by seminested PCR.

4.5 COMPARISON OF HA, PCR AND SEMINESTED PCR

A total of 126 samples were tested by HA, PCR and seminested PCR. Of these, 55 (43.65 per cent), 77 (61.11 per cent) and 93 (73.81 per cent) samples were found positive by HA, PCR and seminested PCR respectively.

When the results of HA, PCR and seminested PCR were compared, PCR detected CPV in 23 samples and seminested PCR demonstrated CPV in 39 samples that were tested negative by HA test. Among 49 samples which were tested negative by simple PCR, 16 more samples could be found positive by seminested PCR.

One faecal sample which was tested negative by both PCR and seminested PCR, was showing HA titre of 8 $\log_2$. The specificity of HA was confirmed by HI test using anti-CPV hyperimmune serum and the HI titre was found to be 6 $\log_2$. All other samples having HA titre above 6 $\log_2$ were tested positive by both PCR and seminested PCR.

The results of HA, PCR and seminested PCR are compared in Table 4 and 5.

Table 4. Results of HA, PCR and seminested PCR

HA titre (log2)	Number of samples	Number positive by PCR	Number positive by seminested PCR
Below 6 (HA negative)	71/126	23/71	39/71
6 and above (HA positive)	55/126	54/55	54/55
Total positive	55/126	77/126	93/126

Table 5. Comparison of HA, PCR and seminested PCR

Test	Number of samples tested	Number of positive samples	% positive
HA	126	55	43.65
PCR	126	77	61.11
Seminested PCR	126	93	73.81

4.6 RESTRICTION ENZYME ANALYSIS OF PCR PRODUCT

The PCR product of 2.2 Kb size from 26 field cases as well as the CPV vaccine strain were digested with *Hinf I*, *Rsa I* and *Sau 961*. The fragment patterns were studied by polyacrylamide gel electrophoresis.

4.6.1 Restriction Enzyme Pattern of *Hinf I*

Canine parvovirus PCR amplicon of 2.2 Kb when digested with *Hinf I* produced four fragments of sizes approximately 111, 393, 778 and 963 bp (Fig.6).

4.6.2 Restriction Enzyme Pattern of *Rsa I*

Seven fragments of sizes around 50, 61, 76, 242, 449, 633 and 718 bp were generated when PCR products were digested with *Rsa I*. (Fig.7)

4.6.3 Restriction Enzyme Pattern of *Sau 961*

When the PCR products were digested with *Sau 961*, three fragments of sizes around 331, 448 and 1466 bp were observed (Fig.8).

4.7 ISOLATION OF CPV IN MDCK CELL LINE

Attempts were made to isolate CPV in MDCK cell line from eight faecal samples with HA titre of $log_2 11$ and above. Out of these eight samples, four

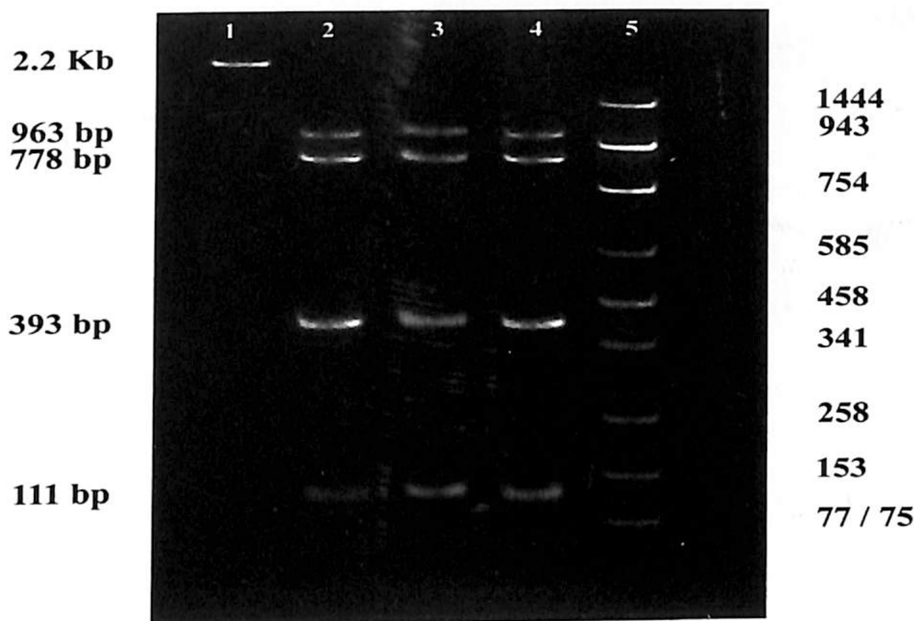

Fig. 6. Polyacrylamide gel electrophoresis of *Hinf I* digested PCR product

Lane 1 - Undigested amplified product
Lane 2 - Restriction fragments of CPV vaccine strain
Lane 3,4 - Restriction fragments of CPV field strain
Lane 5 - *pUC 18/Sau 3A1* digest

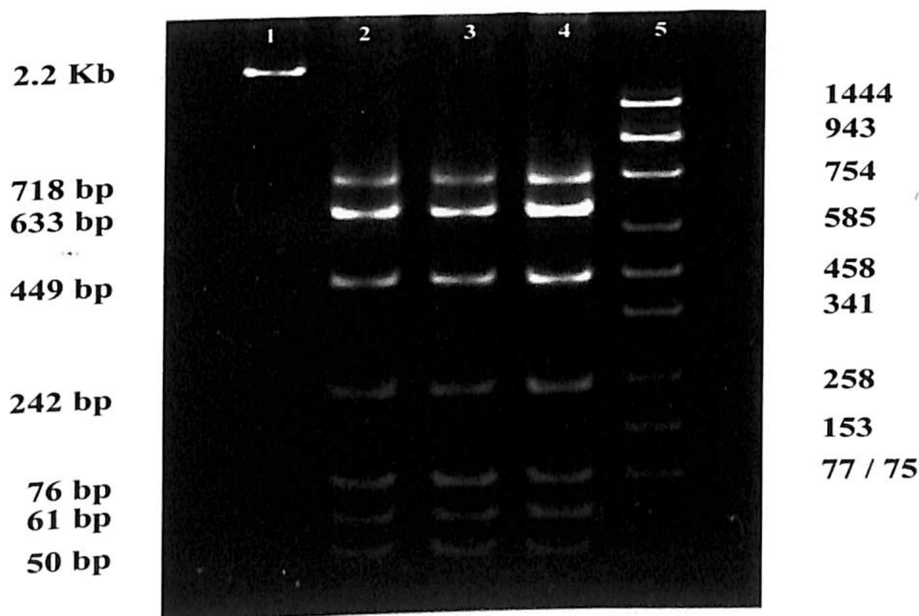

Fig. 7. Polyacrylamide gel electrophoresis of *Rsa I* digested PCR product

Lane 1 - Undigested amplified product
Lane 2 - Restriction fragments of CPV vaccine strain
Lane 3,4 - Restriction fragments of CPV field strain
Lane 5 - *pUC 18/Sau 3A1* digest

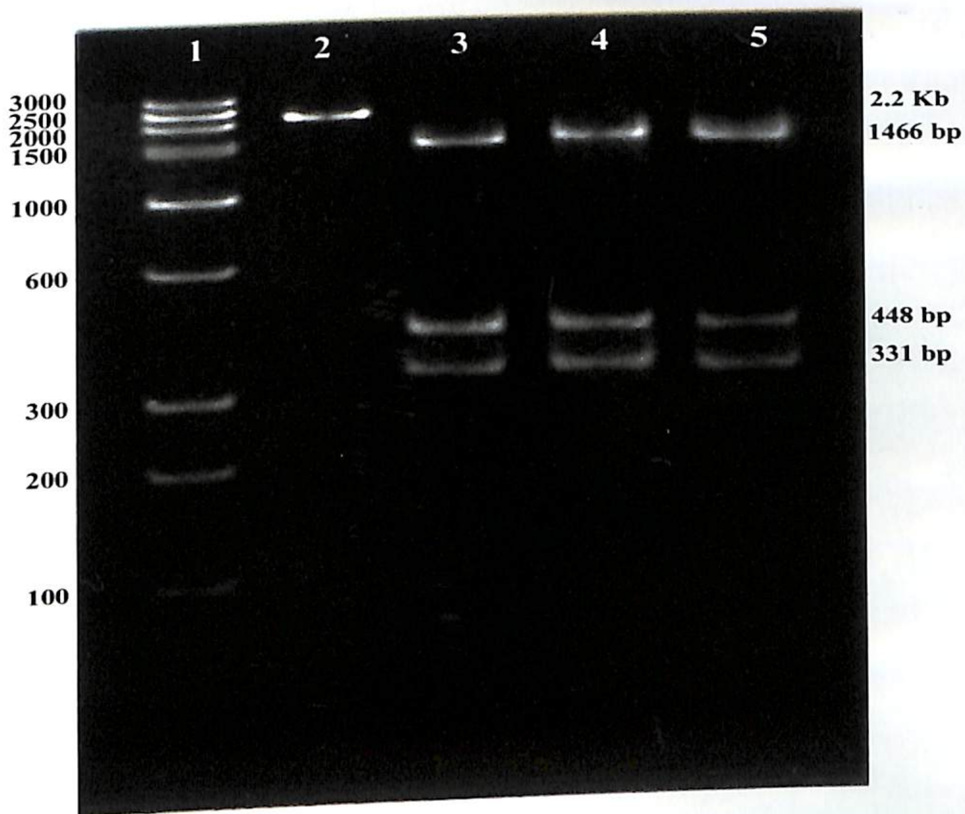

Fig. 8. Polyacrylamide gel electrophoresis of *Sau 961* digested PCR product

Lane 1 - Low Range DNA Ruler

Lane 2 - Undigested amplified product

Lane 3 - Restriction fragments of CPV vaccine strain

Lane 4,5 - Restriction fragments of CPV field strain

samples were inoculated after filtration and four samples after antibiotic treatment. Among four samples which were antibiotic treated, two samples showed contamination within 24 h after primary inoculation of cell line. None of the filtered samples showed contamination after primary inoculation. All the six samples without contamination after primary inoculation were passaged three times to detect the presence of CPV.Out of these six samples, four samples were passaged in fresh monolayer of MDCK cells and two samples in partly formed monolayer.

4.8 IDENTIFICATION OF CPV IN MDCK CELL LINE

4.8.1 Cytopathic Effect (CPE)

Both infected as well as control coverslips were stained with May-Grunwald Giemsa stain.

No evident CPE could be appreciated in any of the samples at all passage levels. Eventhough multinucleated giant cells could be observed in the infected coverslips, the same change was detected in controls also. Partial detachment and floating of cells were detected within 24 h in one of the samples after initial inoculation.

4.8.2 Indirect Fluorescent Antibody Technique (IFAT)

Both infected and control coverslips, were examined by IFAT for the presence of virus replication in cell. No fluorescence could be detected at all passage levels in all the six samples.

4.8.3 Haemagglutination (HA) Test

The cell culture fluid after initial inoculation of the sample showed a residual HA titre of 2 $\log_2$ in one of the samples and on subsequent passages no progressive increase in the HA titre could be appreciated in any of the samples.

4.8.4 Polymerase Chain Reaction

After every passage the cell culture fluid was subjected to PCR to detect CPV. The presence of CPV in cell culture fluid was detected in three samples five days after initial inoculation. But on subsequent passages, presence of CPV DNA could not be detected by PCR.

After third passage, ultracentrifugation was carried out to pellet CPV, if present, and the pellet was processed for PCR. But, no specific amplification could be obtained by PCR assay in any of the six samples tested even after third passage.

4.9 OCCURRENCE OF CPV INFECTION AMONG VACCINATED AND NON-VACCINATED DOGS

The results of occurrence of CPV infection among vaccinated as well as non-vaccinated dogs are presented in Table 6 and 7.

Table 6. Distribution of canine parvovirus infection among vaccinated dogs

Category	Details of vaccination	Number tested	Number tested positive	% positive
I	Taken vaccination as per the vaccination schedule	8	3	37.50
II	Get disease before taking booster vaccination	7	3	42.86
III	Not taken booster	7	6	85.7
	Total	22	12	54.55

Table 7. Distribution of canine parvovirus infection among non-vaccinated dogs

Number of non-vaccinated dogs tested	Number of positive	Number of negative	% positive
104	81	23	77.88

Out of 126 faecal samples taken from suspected dogs, 22 samples were from vaccinated dogs and the rest of the samples (104) were from non-vaccinated dogs. Among 22 vaccinated dogs only eight dogs received vaccination as per the vaccination schedule, seven other dogs developed enteritis before the date of taking booster vaccination and another seven dogs did not receive booster vaccination at all. All vaccinated dogs were immunized with multicomponent vaccine containing modified live CPV-2 virus.

Among the regularly vaccinated dogs in category I, three dogs were tested positive for CPV. Out of these three dogs, two dogs aged five months developed characteristic clinical signs one month after booster vaccination and were tested positive by HA, PCR and seminested PCR, where as the other dog which had received annual booster vaccination one week back was tested positive by seminested PCR only.

In category II, out of three dogs which were found positive for CPV before taking booster vaccination, two developed signs four days after taking first dose of vaccination and one developed disease four days before annual booster vaccination.

In category III, six dogs were tested positive for CPV enteritis and the time period after vaccination was ranging from 1 month to 2 years.

Out of 104 non-vaccinated dogs 81 dogs (77.88 per cent) were found to be positive for CPV enteritis.

4.10 AGE-WISE DISTRIBUTION OF CPV INFECTION

The results of occurrence of CPV infection among different age groups are presented in table 8.

Table 8. Age-wise distribution of CPV infection

Age in months	Number of samples	Number positive	Per cent of total positive
0-4	74	63	67.74
5-12	33	24	25.81
>12	19	6	6.45
Total	126	93	

The distribution of CPV infection was highest (67.74 per cent) among dogs of 0-4 months age, followed by dogs of 5-12 months (25.81 per cent) and dogs above 12 months of age (6.45 per cent).

4.11 BREED-WISE DISTRIBUTION OF CPV INFECTION

The results of breed-wise distribution of CPV infection in clinically suspected dogs are presented in table 9.

Table 9. Breed-wise distribution of CPV infection

Breed	Number of positive	Per cent
German shepherd	37	39.78
Dachshund	14	15.05
Doberman pinscher	12	12.90
Rottweiler	12	12.90
Other breeds and crossbreds	13	13.98
Non-descript	5	5.38
Total	93	

The highest distribution of CPV infection was noted among the German shepherd dogs (39.78 per cent), followed by Dachshund (15.05 per cent), Doberman pinscher (12.9 per cent), Rottweiler (12.9 per cent), other breeds and crossbreds (13.98 per cent) and non-descripts (5.38 per cent).

4.12 MONTH-WISE DISTRIBUTION OF CPV INFECTION

The results of month-wise occurrence of CPV infection are presented in Table 10.

Table 10. Month-wise distribution of CPV infection

Year	Month	Number of positive	Per cent
2003	February	16	17.2
	March	1	1.08
	June	1	1.08
	July	9	9.68
	August	4	4.3
	December	1	1.08
2004	January	3	3.23
	February	3	3.23
	March	11	11.83
	April	10	10.75
	May	23	24.73
	June	11	11.83

In the year 2003, most number of cases of CPV infection was noted during February (17.2 per cent) and July (9.67 per cent) where as in the year 2004, majority of CPV positive cases were observed during March to June, with highest positivity (24.73 per cent) during May.

Discussion

5. DISCUSSION

Canine parvovirus type 2 (CPV-2) emerged as a new virus of dogs in the late 1970's and is a highly contagious infection of dogs, characterized by sudden onset of vomiting, diarrhoea, dysentery, anorexia, pyrexia, severe dehydration, leucopaenia and death.

It was first recognized in North America in 1978 and shortly afterwards it was reported in Europe, Australia and Asia. In India, the disease was recorded in Tamil Nadu in 1981. Subsequently the disease has been recorded in various states of the country including Kerala.

Canine parvovirus affects all age groups of dogs with high morbidity and mortality in young puppies (Joshi *et al.*, 1998). The sudden onset of foul smelling bloody diarrhoea in a young dog less than two years is often considered indicative of CPV-2 infection. However, all dogs with bloody diarrhoea, with or without vomiting, are not necessarily infected with CPV-2. Other enteropathogenic viral and bacterial infections should also be considered (Hoskins, 1998). All clinical signs characteristic of CPV-2 infection are seldom present at any one time. The diagnosis of fulminant cases of CPV is relatively easy, but less severe cases, which are more common, pose a diagnostic dilemma. Therefore, demonstration of virus specific antigen is an important factor for confirmation of CPV infection.

Dogs infected with CPV excrete infectious virus in their faeces for upto 10 days after the onset of disease (Carmichael and Binn, 1981). This virus is extremely stable and resistant to adverse environmental conditions for long periods. Canine parvovirus type 2 is highly contagious and most infections occur as a result of exposure to contaminated faeces. In addition, humans, instruments, insects and rodents serve as vectors. Hence, a rapid, sensitive and specific

diagnosis of CPV infection is necessary so that effective treatment as well as control measures can be adopted to check further spread of disease.

Previously, confirmatory diagnosis of CPV infection was mostly based on the detection of CPV antigen or antibody by conventional methods. Since conventional methods are time consuming and less sensitive, they are now being replaced by modern molecular biology techniques which allow rapid and presumptive identification of organisms directly from clinical samples. With the introduction of PCR, rapid detection of fewer particles of CPV in faecal samples has become practical, by specific amplification of DNA of CPV (Uwatoko *et al.*, 1995).

In the present study, the ability of HA, PCR and seminested PCR to detect CPV in faecal samples was compared for the diagnosis of CPV infection.

Since its emergence in 1978, CPV-2 has undergone genetic alterations developing new virus strains, namely, CPV-2a and CPV-2b. In the United States CPV-2b has largely replaced previously isolated strains, whereas in the Far East and in Europe, both type 2a and 2b predominate. In the present study, DNA of CPV amplified by PCR was further analyzed by REA using *Rsa I, Hinf I* and *Sau 961* to detect CPV strain variation as well as the differences in genotype of field and vaccine viruses.

Isolation of CPV from faecal samples of dogs infected with CPV was also attempted in MDCK cell line in this study.

5.1 HAEMAGGLUTINATION (HA) TEST

The HA activity of CPV was utilized diagnostically in detecting the presence of virus in faecal samples by many workers (Appel *et al.*, 1979; Johnson *et al.*, 1979 and Carmichael *et al.*, 1980). Titres of 6 $\log_2$ and above were taken as positive to CPV infection as suggested by Carmichael *et al.* (1980).

Phosphate buffered saline (PBS, pH 7.2) was taken as the diluent for performing HA test by many workers (Sherikar and Paranjape, 1985; Sohini, 1997 and Kumar *et al.*, 2003a).

One per cent pig erythrocyte suspension at 4°C was used to perform HA test in the present work. Carmichael *et al.* (1980) and Murphy *et al.* (1999) reported that CPV strongly agglutinated the erythrocytes of porcine and rhesus macaque at 4°C. With regard to the incubation temperature of HA test, Kumar *et al.* (2003a) found that the best result was obtained at 4°C, followed by incubation temperature of 23°C to 26°C and the least HA titer was obtained at 37°C.

In the present study, among 126 faecal samples collected from dogs suspected for CPV infection, 41 (32.54 per cent) samples were positive to CPV infection by HA test when PBS (pH 7.2) was used as diluent.

Sabine *et al.* (1982), Studdert *et al.* (1983), Narasimhaswamy (1988) and Sohini (1997) observed that 30 per cent, 30 per cent, 34.8 per cent and 25.7 per cent respectively of clinically suspected dogs were found positive to CPV infection. The results of the present study are in agreement with the results of the above studies.

Gunaseelan (1993) and Subhashini *et al.* (1997) reported that 18.1 per cent and 20.32 per cent respectively of the faecal samples from dogs with characteristic clinical signs were positive to CPV infection by HA test.

However, many workers (Sherikar and Paranjape, 1985; Sulochana *et al.*, 1987 and Deepa, 1999) detected CPV infection in 71 per cent, 75.5 per cent and 61.4 per cent of faecal samples respectively by HA.

In the present work, the HA titres of CPV in faecal sample was ranging from 6 $\log_2$ to 15 $\log_2$. Sohini (1997) and Subhashini *et al.* (1997) also reported similar findings.

The high HA titre in the present study may be due to collection of faecal samples between one to four days after the onset of clinical illness. Rai *et al.* (1994) found that majority of the HA activity of 8 log_2 to14 log_2 was obtained from faecal samples collected three to six days after the onset of clinical signs.

All the samples collected from normal healthy vaccinated as well as non-vaccinated dogs after 15 days of vaccination were negative by HA test. Faecal excretion of CPV could only be detected from 5 to 15 days after recent modified live virus (MLV) vaccination by faecal parvoviral antigen testing (www.antechdiagnostics.com).

5.1.1 Haemagglutination Test Using Different Diluents

In the present study, no significant difference in HA titre as well as in per cent of positive cases could be appreciated when two different diluents (PBS, pH 7.2 and PBS-BSA) were used to perform HA test. Similar finding was reported by Kumar *et al.* (2003a) who suggested that these buffers were comparable and could be effectively used for HA test.

5.1.2 Effect of pH on Haemagglutination of CPV

The effect of pH on haemagglutination of CPV was studied using PBS with pH ranging from 7.2 to 4.0 and a progressive increase in the HA titre could be appreciated in a pH range of six to four.

Kumar *et al.* (2003a) also analyzed the effect of different buffers at different pH on HA and it was found that best results were obtained with PBS, PBS with 0.1 per cent BSA and PBSS in a pH range of six to four.

The simplest procedure for the diagnosis of CPV infection was HA of pig or rhesus monkey erythrocytes (pH 6.5, 4°C) by virus present in faecal extracts (Murphy *et al.*, 1999).

Carmichael *et al.* (1980) suggested that the optimum value for HA by CPV, FPV and MEV was pH 5.8 to 6.0 and they also indicated that CPV agglutinated porcine and monkey RBC over a wide pH range of 5.8 to 7.4.

In the present study, when PBS with pH 4.0 was used as diluent, 14 more cases could be detected as positive by HA test, when compared to HA test using PBS (pH 7.2) as diluent. These 14 samples were found positive by PCR. All the faecal samples, which showed progressive increase in HA titre at different pH, were also found positive to CPV by PCR. These findings indicated the reliability of haemagglutination by CPV at acidic pH.

On the basis of observation made by Carmichael *et al.* (1980), Murphy *et al.* (1999) and Kumar *et al.* (2003a), and the results obtained in the present study, it could be concluded that slightly acidic pH in the range of four to six is favourable for haemagglutination reaction by CPV and it could be effectively utilized in the diagnosis of CPV infection by HA test.

5.2 HAEMAGGLUTINATION TEST WITH CHLOROFORM TREATMENT

Treatment of faecal samples with chloroform as suggested by Carmichael *et al.* (1980) to remove non-specific haemagglutinins was adopted in this study.

The numbers of positive cases of HA test with and without chloroform was absolutely the same and also no significant difference in HA titre could be appreciated in either cases. But in some of the cases with HA titre below 5 $\log_2$ without chloroform treatment, it was found that the titre was reduced or absent after chloroform treatment, indicating the presence of non-specific agglutinins in lower dilutions. But chloroform treatment of faecal samples had no influence on HA titre above 5 $\log_2$ in the present study. This finding is in accordance with that of Mathys *et al.* (1983b) who revealed that chloroform treatment of faecal samples, which was known to result in partial purification of CPV, had no influence on HA titres above 5 $\log_2$, and because titres of 5 $\log_2$ and less are considered negative anyway, it can probably be omitted. Carmichael *et al.* (1980)

and Mathys *et al*. (1983a) indicated that non-specific HA activity could be observed at lower dilutions in some samples while performing HA test.

Gunaseelan (1993) and Deepa (1999) also reported that chloroform treatment of faecal samples did not significantly alter the HA titre of samples, signifying the absence of non-specific agglutinins.

5.3 POLYMERASE CHAIN REACTION

Amplification of DNA templates of CPV vaccine strain as well as the DNA of CPV in faecal samples generated a PCR product of approximately 2.2 Kb size and non-specific amplification was not produced during any cycle of amplification. None of the negative control samples tested by this assay showed amplification, which indicates this test to be highly specific. These results were in accordance with those of Mochizuki *et al.* (1993a); Subhashini *et al.* (1997); Sagazio *et al.* (1998) and Battilani *et al.* (2002).

A combined vaccine was used as source of DNA of CPV in the present study. No amplification was obtained when undiluted, unprocessed vaccine was used as template. This inhibitory effect on PCR may be either due to high template DNA concentration or some inhibitory substance present in the vaccine itself. But good amplification could be appreciated when diluted unprocessed vaccine as well as diluted processed vaccine was used. This indicates that, the concentration of template DNA and (or) PCR inhibitory substance present in vaccine get diluted, leading to good amplification. Senda *et al.* (1995) and Sagazio *et al.* (1998) amplified DNA of CPV using monovalent as well as combined vaccines. Senda *et al.* (1995) suggested that amplification of CPV vaccine strains by PCR was extremely sensitive as well as rapid, after dilution in either PBS or vaccine diluent. They also indicated that in the case of combined vaccines, sensitivity was somewhat lower than those for monovalent vaccines, perhaps because of some inhibitory substance for PCR in those vaccines.

In this study, simple pre-treatment of faecal sample by boiling enabled the specific amplification of DNA of CPV from faeces by PCR, making this assay a very rapid and simple one. Similar finding had been reported by many workers (Parrish and Carmichael, 1986; Senda *et al.*, 1995; Sohini *et al.*, 1997 and Kumar *et al.*, 2003b) who opined that heat treatment could inactivate PCR inhibitory substances like DNAase and other proteins present in faeces.

In the present study, among 126 samples tested, 61.11 per cent were found positive by PCR. Meerarani *et al.* (1996); Sohini (1997) and Subhashini (1997) demonstrated CPV in 72.7 per cent, 49.5 per cent and 56.67 per cent of samples respectively, by PCR.

When DNA of CPV was amplified from faecal sample, an increase in the intensity of bands could be appreciated with corresponding increase in the HA titre. This difference in intensity is due to variation in the final concentration of PCR product, which directly depends on the initial template DNA concentration. Mizak and Utka (1997) also reported a variation in the intensity of band while amplifying the DNA of CPV from faecal samples, indicating that the final concentration of the products was not equal in each reaction.

None of the faecal samples collected from healthy vaccinated as well as non-vaccinated dogs were found positive by PCR, in this study. All the faecal samples from vaccinated dogs were collected 15 days after vaccination. Recent modified parvovirus vaccination within 14 days may cause false positive results (www.antechdiagnostics.com). On the basis of above finding it can be concluded that both normal healthy non-vaccinated dogs and vaccinated dogs after 15 days of vaccination are not excreting detectable level of CPV in their faeces.

5.4 SEMINESTED PCR

Mochizuki *et al.* (1993a) employed seminested PCR to confirm the specificity of the PCR product obtained in detecting CPV from faecal samples of dogs.

Hirasawa *et al.* (1994) and Mizak and Utka (1999) suggested nested PCR as a more sensitive and specific method over simple PCR for detection of CPV in faecal samples.

In the present work, the PCR products of positive faecal samples and CPV vaccine strain by simple PCR generated a DNA fragment of 1.2 Kb size in seminested PCR. The negative control had not produced any amplification during each reaction. This indicates that seminested PCR is a highly specific test to confirm the specificity of PCR product in detecting CPV from faecal samples. Similar finding has been reported by Mochizuki *et al.* (1993a)

A dog, which received annual booster vaccination one week back, showed characteristic clinical signs of CPV infection and was tested positive by seminested PCR. This case could be considered as 'false positive' since even simple PCR can give false positive result within 14 days after modified live CPV vaccination (www.antechdiagnostics.com). Sagazio *et al.* (1998) and Garcia *et al.* (2002) also reported that recent MLV vaccination could lead to false positive result since vaccine virus is excreted in faeces. This indicates that we have to look into the vaccination history before giving a positive diagnostic result in case of CPV infection.

In this study, it was also observed that healthy non-vaccinated dogs and healthy vaccinated dogs after 15 days of vaccination are not carriers of CPV as indicated by seminested PCR. However, Komolafe (1988) suggested the existence of carrier status in dogs recovered from CPV infection, which can act as a potential source of infection to other dogs.

5.5 COMPARISON OF HA, PCR AND SEMINESTED PCR

Mochizuki *et al.* (1993a) proposed that PCR was as sensitive as VI assay using MDCK cells and more sensitive than VI assay using CRFK cells or HA assay.

Schunk *et al.* (1995) indicated that the sensitivity of PCR was as high as 10 infectious particles per reaction, which corresponded to a titre of about 10^3 infectious particles per gram of unprocessed faeces.

Meerarani *et al.* (1996) and Sohini (1997) indicated the higher sensitivity of PCR assay over HA test.

Mingjeng *et al.* (2000) reported that the sensitivity of PCR was 10^2 to 10^4 fold higher than that of culture method and HA test, for detecting CPV in faecal samples.

Hirasawa *et al.* (1994) developed nested PCR to increase the sensitivity as well as specificity of PCR assay for the detection of CPV in faecal samples and demonstrated that nested PCR was 100 times more sensitive than the simple PCR.

Improved sensitivity of nested PCR assay for enhanced detection of CPV in faecal samples had been reported by Mizak and Utka (1999) who observed 60 per cent increase in sensitivity of nested PCR in comparison to simple PCR. They indicated that nested PCR seemed to be a sensitive, specific and practical method for detection of CPV in faecal samples.

In this study, out of 71 samples that were tested negative by HA test, 23 samples and 39 samples were tested positive by PCR and seminested PCR respectively, thus indicating the higher sensitivity of both PCR and seminested PCR over HA test in detecting CPV from faecal samples. Many workers have reported similar finding (Mochizuki *et al.*, 1993a; Meerarani *et al.*, 1996; Sohini, 1999 and Mingjeng *et al.*, 2000).

Sixteen samples that were found negative by simple PCR were also found positive by seminested PCR. Hence, the present study reveals that seminested PCR is more sensitive than PCR for detection of CPV in faecal samples. This

finding is in agreement with the observations of Hirasawa *et al.* (1994) and Mizak and Utka (1999).

In the present study, both PCR and seminested PCR were found to be negative in only one faecal sample, which was CPV positive by both HA and HI test. Although the sample was repeatedly tested at different dilutions (1:10, 1:50 and 1:100) in the PCR assay, it was still negative. It may be assumed as some substance, or substances, in the faeces might have interfered with the PCR assay. Mochizuki *et al.* (1993a) also reported false negative result (1.7 per cent) by PCR assay, which might be due to inhibitory substances in the faeces. Widjojutmodjo *et al.* (1992) reported the inhibitory effect of bile acids on PCR amplification and suspected the acids as the inhibitory substances in faeces. They also opined that this inhibitory effect of bile acids was heat stable since the inhibition was not diminished by heat treatment. Uwatoko *et al.* (1995) indicated the existence of both heat-stable and heat-labile inhibitory materials in faeces, the composition of which might vary from dog to dog.

In the present study, even though one false negative result (0.79 per cent) was obtained by PCR, this assay was able to detect CPV in 61.11 per cent of faecal samples tested. Hence PCR could be considered not only as a sensitive and specific, but also a rapid and simple technique for routine diagnosis of CPV infection in dogs. Since seminested PCR was specific and also could detect more positive cases of CPV infection than PCR, it can be recommended as a valuable diagnostic tool for confirming CPV infection in laboratories. Though routine use of seminested PCR for diagnosis is expensive and complicated, its higher sensitivity appears to be useful to detect low level of virus shedding during early stages of infection so that effective treatment as well as control measures can be adopted to prevent further spread of infection.

5.6 RESTRICTION ENZYME ANALYSIS OF PCR PRODUCTS

Polymerase chain reaction based REA is probably the most sensitive method for comparing closely related genomes, since a single base pair change in a recognition sequence, undetectable by other techniques, leads to a change in restriction endonuclease fragement pattern (Mochizuki *et al.*, 1996).

Canine parvovirus and FPV isolates, as well as vaccine and wild type strains, could be differentiated on the basis of differences in the restriction cleavage patterns of the PCR amplified DNA fragment (Hirasawa *et al.*, 1993).

5.6.1 Restriction Enzyme Patterns of *Hinf I*

Upon REA of PCR product using *Hinf I*, all the digested products of field strain generated same pattern as that of the CPV vaccine strain.

5.6.2 Restriction Enzyme Pattern of *Rsa I*

Sagazio *et al.* (1998) amplified a DNA fragment of CPV VPI/VP2 capsid protein gene (2.2 kb size) by PCR and the products were digested by restriction enzymes *Rsa I, Hpa II, Hind III* and *Pva II*. The *Rsa I* enzyme allowed the differentiation of CPV-2 from CPV-2a and CPV-2b.

In the present study, REA revealed that both field and vaccine strains of CPV were having similar patterns of digested products. The fragment pattern obtained in this study was matching with that of CPV-2 strain shown by Sagazio *et al.* (1998). No CPV-2 strain variation could be observed in this study.

Reed *et al.* (1988) reported that, on sequencing and REA of VP1 gene and some portions of the CPV genome, most of the isolates on digestion revealed same number and sized fragments.

5.6.3 Restriction Enzyme Pattern of *Sau 961*

There was no difference in fragment pattern of both field strain as well as vaccine strain of CPV by REA using *Sau 961*.

Hirasawa *et al.* (1995) employed PCR and REA for the differentiation of wild and vaccine type CPV. Their study with 21 isolates showed no differences in RFLP patterns as compared to the vaccine strain.

Sohini *et al.* (1999) analyzed the genomic properties of CPV by REA and all the isolates showed similar fragment length patterns with restriction enzymes *Hae III*, *Hpa II and Hind III*, whereas the isolates were readily differentiated by digestion with *Alu I.*

In the present study, REA using *Hinf I*, *Rsa I* and *Sau 961* could not detect any mutagenic variants of CPV. However, a detailed study employing more number of samples and different restriction enzymes is required to get concrete evidence about the existence of different types of CPV in Thrissur.

5.7 ISOLATION OF CPV IN MDCK CELL LINE

Attempts were made to isolate CPV in MDCK cell line from faecal samples in the present study. Canine parvovirus in faeces of diarrhoeic dogs has been isolated in MDCK cell line by Appel *et al.* (1979); Mochizuki and Hashimoto (1986) and Mochizuki *et al.* (1993a). Mochizuki *et al.* (1993a) compared VI assay using CRFK cells and MDCK cells and found that VI assay using feline CRFK cells was less sensitive than that using canine MDCK cells which was almost as sensitive as the PCR assay. They also opined that this finding was unexpected as in a previous study conducted by Mochizuki and Hashimoto (1986) feline cells were found more sensitive than canine cells for propagation of CPV. Hence MDCK was chosen as the cell line for isolation of CPV in this study.

According to Appel *et al.* (1979), primary and secondary feline kidney and canine kidney cells, mink lung (CLL-64), MDCK, feline lung, raccoon salivary gland and bovine foetal spleen cells were all found susceptible to CPV and also virus could be serially propagated in these cells.

However, McCandlish *et al.* (1979) and Azetaka *et al.* (1981) mentioned that CPV appeared to grow better in primary feline kidney (PFK) and CRFK cell cultures.

In the present study, faecal samples were subjected to two types of processing (filtration and antibiotic treatment) before inoculation into cell culture. Out of four samples, which were treated with antibiotic, two samples showed contamination within 24 h after primary inoculation of cell line, whereas none of the filtered samples showed contamination after primary inoculation. Since faecal sample contains lots of contaminant bacteria, it is better to go for filtration rather than antibiotic treatment before inoculation to avoid bacterial contamination of cell culture. Many workers (Appel *et al.,* 1979; McCandlish *et al.,* 1979 and Mochizuki *et al.,* 1993a) preferred filtration of faecal material over antibiotic treatment before inoculation into cell culture.

In the present work, while propagating the virus, care was taken to see that infection of the cells was performed in actively growing stage, since resting cells and aged cultures did not support CPV replication very well. Though isolation was attempted in fresh monolayer as well as partly formed monolayer of MDCK cells, CPV could not be detected in either case, even after third passage. Johnson and Spradbrow (1979) isolated CPV in partly formed monolayer of primary canine foetal lung cells and feline kidney cell line. Appel *et al.* (1979) and Azetaka *et al.* (1981) have reported that intranuclear inclusions and intranuclear fluorescence were prominent in feline kidney cells after inoculation of freshly seeded cells, but not when monolayers had formed before inoculation. These changes were less distinct in other cells tested. However, Gunaseelan *et*

al. (1997) and Joshi *et al.* (1998) successfully isolated CPV in monolayers of CRFK cells.

5.8 IDENTIFICATION OF CPV IN MDCK CELL LINE

5.8.1 Cytopathic Effect (CPE)

Many workers are of the opinion (Appel *et al.,* 1979; Johnson and Spradbrow, 1979 and Gunaseelan *et al.,* 1997) that despite rapid replication, most CPV isolates produce subtle cytopathic effects and hence appearance of CPE is not a consistent feature of viral replication in case of CPV.

In this study, no CPE could be appreciated in any of the samples at all passage levels. Similar finding was also reported by Narasimhaswamy (1988). No CPE was observed upto fifth passage by Sohini (1997).

Gunaseelan *et al.* (1997) showed multi-nucleated giant cells with intranuclear inclusions in CPV infected CRFK cells by haematoxylin and eosin staining. In the present study, eventhough multinucleated giant cells could be observed in the infected coverslips, the same change was detected in controls also and hence this change could not be considered as virus induced.

Partial detachment and floating of cells were detected in one of the samples after 24 h of initial inoculation. Joshi *et al.* (1998) also observed detachment of CPV infected CRFK cells at 72 h PI, which was completed by 96 h PI and they demonstrated intranuclear inclusion bodies also. Gunaseelan *et al.* (1997) noticed some variable morphological changes such as rounding and floating of cells and opined that these changes were not characteristic of viral growth. On the basis of observations of Gunaseelan *et al.* (1997) and the present study, it could be concluded that the partial detachment of infected cells within 24 h of initial inoculation could not be considered as a virus induced change.

5.8.2 Indirect Fluorescent Antibody Technique (IFAT)

In this study, no fluorescence could be detected at all passage levels in all the six samples, indicating the absence of virus replication in infected cells.

Indirect FAT of infected cells showed bright apple green intranuclear fluorescence between 48-72 h PI at third passage (Joshi *et al.,* 1998).

Demonstration of intranuclear fluorescence by IFAT was used as an indicator of virus replication in infected cells by Appel *et al.* (1979) and Hirasawa *et al.* (1984).

5.8.3 Haemagglutination (HA) Test

The progressive viral replication in cell culture on subsequent passages could be monitored by HA and HI test (Appel *et al.,* 1979; Narasimhaswamy, 1988 and Gunaseelan *et al.,* 1997).

Although a residual HA titre of 2 $\log_2$ was observed in one of the samples after primary inoculation, no progression increase in HA titre could be appreciated in any of the samples on subsequent passages, which reveals the absence of progressive viral replication.

The cell culture fluid after five days of growth at third passage when assayed by HA test showed the presence of CPV haemagglutination with high reciprocal HA titre of 2048 (Joshi *et al.,* 1998). The HA titres were observed around 32 h and reached a peak of 4096 at 120 h PI and steadied thereafter (Narasimhaswamy, 1988).

5.8.4 Polymerase Chain Reaction

Polymerase chain reaction has been used as a sensitive and specific method for detection of DNA of CPV in infected cell cultures by Mochizuki *et al.* (1993a) and Sohini (1997).

Sohini (1997) confirmed the presence of CPV in tissue culture fluid by PCR in the second passage.

In the present study, though CPV could be detected in cell culture fluid after primary inoculation, PCR failed to detect CPV on subsequent passages, indicating the absence of virus replication in infected cell cultures. Since faecal sample with high HA titre was used as inoculum and also because of the extreme sensitivity of PCR, this assay was able to detect CPV in cell culture fluid after primary inoculation.

In the present study, after third passage, ultracentrifugation was carried out to pellet CPV, if present and the pellet was subjected to PCR. But, no specific amplification could be obtained in any of the six samples tested, which indicates that CPV was not successfully propagated in MDCK cell line.

Narasimhaswamy (1988) employed Vero cells and MDCK cells for propagation of CPV isolates and found that Vero cells did not support the viral replication. The cell line, MDCK, on the other hand supported the virus only after three silent passages. Attempts were also made to revive the virus collected from experimentally infected pups in primary and secondary canine kidney cultures and were found unsuccessful.

Mochizuki and Hashimoto (1986) used three strains of MDCK cell line (A, B and C) for propagation of CPV and found that the strain A and B supported the replication of CPV, but no viral replication was recorded by HA test in the strain C cells. However, a few inclusion bodies were found in cells of strain C. This indicates that there is some qualitative difference in susceptibility of each MDCK cell strain to CPV. The strain of MDCK cell line used in this study was unknown.

Cavalli *et al*. (2001) opined that *in vitro* cultivation of CPV was difficult and after a few passages on canine and feline cells, the presence of virus was

detectable only by immunoflourescent assay on the feline cells, since HA activity had disappeared.

On the basis of observations made by McCandlish *et al.* (1979), Azetaka *et al*. (1981), Mochizuki and Hashimoto (1986), Narasimhaswamy (1988), Gunaseelan *et al*. (1997), Sohini (1997) and Joshi *et al.* (1998), it could be concluded that feline cells are more sensitive than canine cells for isolation of CPV from clinical samples, though both feline and canine cells can be used for propagation of CPV isolates.

5.9 OCCURRENCE OF CPV INFECTION AMONG VACCINATED AND NON-VACCINATED DOGS

Among the regularly vaccinated dogs, two dogs aged five months developed characteristic haemorrhagic enteritis one month after booster vaccination with MLV vaccine and were tested positive by HA, PCR and seminested PCR. The reason for the failure of immune response to vaccination could be either due to vaccine factor or host factors (Greene, 1998). In this particular case, the primary cause of failure of MLV vaccine may be due to either interfering levels of maternal antibody or due to lack of sufficient seroconversion to the CPV-2 vaccine administered, which is in agreement with Greene, 1998. According to Greene (1998), usually for puppies in household environments, recommendations are to give a complete series of vaccinations, with doses given every three to four weeks until pups are 16 weeks old. In contrast, pups born to bitches with high antibody titres or certain breeds of pups may not respond to conventional CPV-2 vaccines until upto 20 weeks of age.

Janseen *et al.* (1982) suggested that maternal antibody may interfere with immunization and may cause vaccine failures.

Serological analysis by HI test revealed that five per cent of dogs which had received modified live parvovirus vaccination and five per cent of animals

which had received inactivated virus vaccination did not respond to the vaccine (Narasimhaswamy, 1988).

Dahlgaard (1989) investigated antibody level for CPV in vaccinated dogs and found that 25 per cent were negative, which explained why parvovirus could be the cause of gastro-enteritis in such dogs.

Deepa (1999) also reported vaccine failure in dogs after vaccination with killed as well as modified live CPV vaccines.

In the case of regularly vaccinated dogs, one dog which received annual booster vaccination one week back was tested positive for CPV and it can be considered as a false positive result which is already discussed earlier (5.4).

In the present study, two pups which received first dose of MLV vaccination four days back, showed characteristic signs of CPV infection and were tested positive by all the three assays, with a HA titre of 8 log_2. Their companion dog also died of CPV infection one week back. Faecal parvoviral antigen testing can give false positive result from 5 to 15 days after recent MLV vaccination (www.antechdiagnostics.com). Also the excretion of CPV in faeces of vaccinated dogs is at a lower level when compared to natural infection (Hoskins, 2000). On the basis of the above observations, it can be concluded that, the positive HA titre in this case is due to CPV infection, which indicates that the dogs might already have been in the incubation period at the time of vaccination.

Another dog developed CPV infection four days before annual booster vaccination, which indicate that the protective anti-CPV antibody level was not maintained till the due date of annual booster vaccination. This is in accordance with the observations of Dahlgaard (1989) and Greene (1998).

In category III, six dogs developed CPV infection, which clearly indicate that the protective anti-CPV antibody titre was reduced in these dogs since they did not take booster vaccination.

In the present study, the occurrence of CPV infection among non-vaccinated dogs was higher when compared to vaccinated dogs. Similar finding was also reported by Sherikar and Paranjape (1985) and Rogers (1987).

On the basis of above observation in the present study, it can be concluded that the high prevalence of CPV infection in the area under study is due to absence of regular vaccination of dogs.

5.10 AGE-WISE DISTRIBUTION OF CPV INFECTION

In the present study, the distribution of CPV infection was highest among dogs below four months of age and lowest among dogs above 12 months of age.

Many workers (Rogers, 1987; Murphy *et al.*, 1999 and Garcia *et al.*, 2002) have emphasized that the CPV infection was seen most commonly in pups at two to four months of age than other age groups. This observation is in accordance with the present study.

Ernst *et al.* (1988) and Deepa (1997) noticed majority of CPV infection in pups below six months of age.

However, Eugster *et al.* (1978) observed that dogs of all ages, any breed and sex could be affected with CPV enteritis. Sherikar *et al.* (1989) reported that adults seemed to be more affected than pups.

On the basis of observations made by Rogers (1987), Murphy *et al.* (1999) and Garcia *et al.* (2002), and the results obtained in the present study, it could be concluded that young unvaccinated dogs between two to four months of age are at higher risk for getting CPV infection than other age groups.

5.11 BREED-WISE DISTRIBUTION OF CPV INFECTION

The result from this study showed highest distribution of CPV infection in German shepherd dogs (39.78 per cent), compared to other breeds. Similar finding was also reported by Mizak and Mizak (1994) and Deepa (1999).

Rogers (1987) and Houston *et al.* (1996) reported an increased incidence of CPV infection in Rottweilers, Dobermann pinchers and German shepherd dogs.

However, Gunaseelan (1993) and Sohini (1997) observed higher percentage of incidence in non-descript dogs, compared to purebred dogs.

Ramadass and Khader (1982) indicated that all breeds were equally susceptible to CPV infection.

In the present study, it was noticed that there were more number of German shepherd dogs which were brought to hospital in Thrissur with symptoms of CPV infection and most of these dogs were found to be unvaccinated, which may be considered as the reason for increased susceptibility to CPV infection.

5.12 MONTH-WISE DISTRIBUTION OF CPV INFECTION

In this study, higher numbers of CPV positive cases were noticed during February (17.2 per cent) and July (9.68 per cent) in the year 2003 and during March to June with highest positivity (24.73 per cent) during May in the year 2004.

According to Ernst *et al.* (1992), dogs were at higher risk of developing CPV infection during January, February, March, May and October.

Horner (1983) reported the peak incidence of CPV infection over spring and summer months from October to March in New Zealand. This probably

reflects breeding cycles and also greater movement of animals to shows and boarding kennels.

Many workers (Kelly, 1978; Gunaseelan, 1993 and Houston *et al.*, 1996) have reported the highest incidence of CPV infection during different periods.

Sherikar and Paranjape (1985) observed that the incidence of CPV infection varied from month to month and the seasonal influence on the occurrence of CPV was not significant.

The inherent stability of CPV and extreme seasonal temperature variation may be contributing factors to the seasonal distribution of CPV enteritis (Sohini, 1997).

In the present study, the possible explanation for time peaks in occurrence of CPV enteritis would be corresponding increase in susceptible dog population through whelping and weaned puppies and also due to onset of rainy season. This observation is in agreement with that of Greene (1998) and Deepa *et al.* (2000).

In the present study, REA using *Hinf I*, *Rsa I* and *Sau 961* could not detect any mutagenic variants of CPV. However, a detailed study employing more number of samples and different restriction enzymes is required to get a concrete evidence about the existence of different types of CPV in Thrissur.

Summary

6. SUMMARY

Canine parvovirus type 2 (CPV-2), a relatively new virus in the late 1970s has ravaged the canine population in the recent past. Canine parvoviral enteritis has been reported from many countries, world-wide, and also from different states of India, including Kerala. It affects all age groups of dogs, with high morbidity and mortality in young puppies. This disease has to be differentially diagnosed from other enteropathogenic viral and bacterial infections and also since this virus is highly contagious and extremely resistant, a rapid, sensitive and specific diagnosis is absolutely necessary for the proper treatment and control of disease. The conventional methods employed for the detection of CPV are considered to be relatively less sensitive, laborious and time consuming, and do not contribute to an early diagnosis. Recently, DNA based techniques like PCR has been introduced for the detection of various infectious organisms directly from clinical samples. Hence, the present study was undertaken to compare the ability of HA, PCR and seminested PCR for the diagnosis of CPV infection by detecting the virus from faecal samples. Restriction enzyme analysis has been employed in this study to characterize CPV vaccine strain and field strain. Attempts for isolation of CPV from faecal samples were also performed in MDCK cell line.

Faecal samples were collected from 126 dogs clinically suspected for CPV infection, that were brought to veterinary hospitals of KAU. Forty faecal samples were also collected from normal healthy non-vaccinated as well as vaccinated dogs after 15 days of vaccination. All these samples were tested for the presence of CPV by HA, PCR and seminested PCR.

Haemagglutination titre was compared using two different diluents (PBS, pH 7.2 and PBS-BSA) and no significant difference could be appreciated suggesting that these two buffers can be effectively utilized to perform HA test.

The effect of pH on haemagglutination of CPV was studied using PBS with pH ranging from 7.2 to 4.0. Forty one samples (32.54 per cent) could be diagnosed as positive to CPV infection when PBS (pH 7.2) was used as diluent. But 14 more cases (43.65 per cent) were found positive to CPV infection using PBS (pH 4.0) as diluent for HA test. Thus, a slightly acidic pH in the range of 4.0 to 6.0 was found favourable for haemagglutination reaction by CPV and it could be effectively utilized in the diagnosis of CPV infection by HA test.

Chloroform treatment of faecal samples had no influence on HA titres above 5 $log_{2,}$ signifying the absence of nonspecific agglutinins. However, nonspecific HA activity could be observed at lower dilution in some of the samples and because titres of 5 log_2 and less are considered negative anyway, it can probably be omitted.

Among 126 faecal samples examined, 43.65 per cent, 61.11 per cent and 73.81 per cent were tested positive for CPV infection by HA, PCR and seminested PCR respectively, indicating the higher sensitivity of both PCR and seminested PCR over HA test in detecting CPV from faecal samples. Therefore, PCR can be considered not only as a sensitive and specific, but also as a rapid and simple technique for routine diagnosis of CPV infection in dogs. Though seminested PCR is expensive and complicated for routine diagnostic purpose, because of its extreme sensitivity and specificity, it can be recommended as a valuable alternative for early diagnosis of CPV infection, which is absolutely essential for proper treatment and control of the disease.

Both PCR and seminested PCR were found to be negative in only one sample which was tested positive by both HA and HI test, which indicate that some substance in the faeces might have interfered with the PCR assay. Both PCR and nested PCR can give false positive results immediately after MLV vaccination.

All the faecal samples collected from healthy non-vaccinated dogs and vaccinated dogs after 15 days of vaccination were tested negative for CPV by HA, PCR and seminested PCR, which reveals that they are not excreting CPV in the faeces.

Characterization of CPV vaccine strain and field strain was done by REA using *Hinf I, Rsa I* and *Sau 961* and no differences in the fragment length patterns could be observed.

Since faecal sample contained lots of contaminant bacteria, filtration was found to be a better processing method rather than antibiotic treatment, before inoculation, to avoid bacterial contamination of cell culture. Attempts to isolate CPV from faecal samples in MDCK cell line were found unsuccessful.

The occurrence of CPV infection was higher among non-vaccinated dogs when compared to vaccinated dogs. The high prevalence of CPV infection in this area is due to lack of regular vaccination of dogs, thus providing a susceptible canine population to CPV. Vaccine failure was also observed after vaccination with modified live CPV.

The distribution of CPV infection was highest among dogs between two to four months of age and lowest among dogs above 12 months of age. Breed-wise distribution of CPV infection showed highest distribution in German shepherd dogs when compared to other breeds. Most number of cases of CPV infection was noticed during February (17.2 per cent) and July (9.67 per cent) in the year 2003 and during March to June, with highest positivity (24.73 per cent) during May in the year 2004. The time peaks in occurrence of CPV enteritis would be due to corresponding increase in susceptible dog population through whelping and weaned puppies and also due to onset of rainy reason.

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POLYMERASE CHAIN REACTION FOR THE DETECTION OF CANINE PARVOVIRUS IN FAECES OF DOGS

JOSEMI MATHEW

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Kerala Agricultural University, Thrissur

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Department of Microbiology
COLLEGE OF VETERINARY AND ANIMAL SCIENCES
MANNUTHY, THRISSUR - 680 651
KERALA, INDIA

ABSTRACT

A study was undertaken to compare the ability of haemagglutination (HA), polymerase chain reaction (PCR) and seminested PCR for the diagnosis of canine parvovirus (CPV) infection by detecting CPV from faecal samples of clinically suspected dogs. Characterization of vaccine strain and field strains of CPV was performed by restriction enzyme analysis (REA) in this study. Attempts were made to isolate CPV from faecal samples in MDCK cell line.

One hundred and twentysix faecal samples were collected from dogs suspected for CPV infection and 40 faecal samples were also collected from normal healthy non-vaccinated as well as vaccinated dogs after 15 days of vaccination, that were brought to veterinary hospitals attached to KAU. All the samples were screened by HA, PCR and seminested PCR to detect CPV.

No significant difference in HA titre could be appreciated on comparing the titre using PBS, pH 7.2 and PBS – BSA as diluents. The haemagglutination reaction by CPV was found to be favoured by a slightly acidic pH in the range of 4.0 to 6.0. Chloroform treatment of faecal samples had no influence on HA titres above 5 $\log_2$.

Among 126 faecal samples screened, 43.65 per cent, 61.11 per cent and 73.81 per cent were tested positive for CPV infection by HA, PCR and seminested PCR respectively. Therefore, seminested PCR was found to be a more sensitive and specific method over HA and PCR for the early diagnosis of CPV infection.

All the faecal samples from healthy non-vaccinated and vaccinated dogs after 15 days of vaccination were tested negative by HA, PCR and seminested PCR.

Restriction enzyme analysis using *HinfI, Rsa I* and *Sau 961* revealed no difference in the fragment length patterns between CPV vaccine strain and field strain.

Attempts to isolate CPV from faecal samples in MDCK cell line were found unsuccessful.

The occurrence of CPV infection among non-vaccinated dogs was found to be higher than that of vaccinated dogs. Vaccine failure was also observed after MLV vaccination.

The distribution of CPV infection was highest among dogs between two to four months of age. Breed-wise distribution of CPV infection showed highest distribution in German shepherd dogs when compared to other breeds. Most of the cases of CPV were noticed during February and July in the year 2003 and during March to June in the year 2004.