Canine parvovirus: Identification and evaluation of vaccines

by

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ABBREVIATIONS

BPV	bovine parvovirus
CPV	canine parvovirus
CRFK	Crandell feline kidney
EPK	embryonic porcine kidney
FA	fluorescent antibody
FPV	feline panleukopenia virus
HA	hemagglutination
HI	hemagglutination inhibition
KV	killed virus
MDCK	Madin-Darby canine kidney
MEV	mink enteritis virus
MLV	modified-live virus
pBL	primary bovine lung
PBS	phosphate buffered saline
pCK	primary canine kidney
PPV	porcine parvovirus
SN	serum neutralization
$TCID_{50}$	tissue culture infective dose$_{50}$

INTRODUCTION

The increased use of tissue culture and electron microscopy as diagnostic tools in small animal medicine has recently led to the addition of canine coronavirus (CCV), canine parvovirus (CPV), and rotavirus to the list of viruses associated with enteritis in the dog. In contrast to CCV which was apparently present in the canine population for some time before it was associated with enteritis, CPV appears to be a new pathogen of dogs.

Soon after CPV was recognized, it was shown to be serologically indistinguishable from feline panleukopenia virus (FPV). This serological relationship led to the use of FPV vaccines in dogs in an effort to prevent CPV infection. But, their efficacy in dogs had not been established.

The present study deals with the relationship of CPV to FPV and to mink enteritis virus (MEV), another serologically related parvovirus. The study was undertaken to discover markers that could distinguish individual isolates of CPV, FPV, and MEV and to evaluate the antigenic relationship of CPV and FPV by determining if FPV vaccines could, in fact, be used to protect dogs against CPV enteritis. To accomplish this latter objective, a vaccination-challenge procedure had to be established.

LITERATURE REVIEW

Enteric Viruses of the Dog

Viral enteritis in the dog manifested by vomiting and diarrhea has long been associated with systemic infections of canine distemper virus and infectious canine hepatitis virus (Severin, 1968). In neither case, are the symptoms confined to the gastrointestinal system.

In 1971, Binn et al. (1975) isolated a coronavirus from military dogs in Germany that were undergoing an epizootic of diarrheal disease. The virus was shown by serum neutralization studies to be antigenically related to transmissible gastroenteritis virus (TGE), a coronavirus of swine, but swine anti-TGE sera neutralized the canine coronavirus virus at one-sixteenth the level it neutralized TGE virus. Infectivity studies conducted in neonatal and 2.5-month-old seronegative pigs indicated that a tissue culture preparation of the canine origin virus was not pathogenic for or able to cause seroconversion in swine. In dogs, diarrhea could be experimentally produced in 2 litters of 3- to 5-day-old pups, but the dams remained asymptomatic. All the dogs seroconverted. The passage of the virus in tissue culture may have affected its virulence for adult dogs.

In 1972, an outbreak of acute diarrhea, vomiting, and anorexia occurring in dogs at a breeding kennel was reported

from England (Cartwright and Lucas, 1972). Although the younger pups in the kennel appeared to show more severe signs, all the dogs were infected and one of the 40 dogs died, a 3-week-old pup. No pathogenic bacteria were isolated and attempts to isolate virus in a canine kidney cell line and primary porcine kidney cells failed. Sera collected at intervals following the outbreak showed a rise in antibody titer to TGE virus although none of the dogs had access to pigs. The serological evidence would indicate that CCV was involved in this outbreak.

The presence of anti-TGE antibody in the dog population was discovered prior to the isolation of CCV by Binn et al. (1975). Haelterman (1962) reported that dogs and foxes fed TGE virus infected pig gut would shed the virus for at least 7 days and show seroconversion by a constant serum-varying virus neutralization test. No clinical symptoms were observed in any of the animals. This led Norman et al. (1970) to evaluate the incidence of TGE antibody in dogs from the midwest United States. Serum samples from 116 dogs with different histories were tested. Only dogs from a hysterectomy-derived closed colony were consistently free of antibody. Adult dogs of mixed breeding, adult beagles from a closed colony, and 6- to 10-week-old beagle pups from 3 different commercial suppliers of laboratory animals had antibody to TGE. In all, 72 dogs which had no contact with

swine were positive indicating the antibody was to a viral agent that could perpetuate itself in dogs. Higher antibody titers were seen in the older animals which the authors attributed to either persistent or recurrent infections. The possibility of low level maternal antibodies in the pups was not considered.

Additional studies conducted by these same investigators (McClurkin et al., 1970) dealt with the pathogenicity of TGE virus for dogs. Eleven dogs were fed 0.45 micron filtered TGE infected gut material twice at 21 day intervals. No clinical signs were observed and viral isolation attempts from fecal swabs using swine testicle cells failed on all but one sample taken at 2 days after the first feeding. Composites of rectal swab material from all 11 dogs taken 7 and 14 days after the first feeding did produce disease typical of TGE in 2-day-old piglets. Of the 11 dogs, only 9 showed seroconversion, 7 at only 1:5, and the antibody levels obtained were generally lower than those seen in their previous survey (Norman et al., 1970). The work indicated, as dogs that of Haelterman (1962), that TGE can replicate in dogs and dogs can transmit TGE to pigs. However, the low incidence of virus in the feces and the fact that only 9 of 11 dogs serologically converted after two oral doses of virus leaves doubt that TGE virus perpetuates itself in dogs. This would suggest that the antibody titers

seen in the earlier study (Norman et al., 1970) were due to CCV and not TGE virus. The CCV may have been subclinical since no enteritis associated with the dogs had been mentioned.

In 1977, Eugster and Nairn (1977) reported a case of diarrhea in a litter of 9-day-old pups. The feces were described as being thin, watery, and light tan in color but returned to normal by 11 days. The diarrhea was not considered severe and no other clinical signs were observed. Parasitological and bacteriological examinations of the feces were negative but a cytopathic effect (CPE) with total cell destruction in 6 days was observed when a fecal preparation was inoculated onto Madin-Darby canine kidney (MDCK) cells. By the third tissue culture passage, no CPE was observed and no CPE was ever detected with primary canine cells. Electron microscopic studies of the fecal material and the first two MDCK passages revealed 18-20 nm diameter particles which, because of their morphology, were considered to be parvoviruses. The fact that the virus could not be maintained in tissue culture made it impossible to experimentally determine if the parvovirus was the causative agent of the diarrhea seen in the pups. It should be noted that Binn et al. (1970) previously recovered a parvovirus, designated the minute virus of canines (MVC), from apparently normal dogs. Of 23 cell types tested, MVC would only grow in a canine cell line

derived from a subdermoid cyst, but the susceptibility of MDCK cells was not determined. No pathogenicity has been associated with MCV but serological studies have indicated it is widespread (70 percent) in the dog population (Siegl, 1976). This author suggests that the virus isolated by Eugster and Nairn (1977) may have been MVC.

In July 1978, an epizootic of contagious and sometimes fatal gastroenteritis in show dogs was reported with the initial case being observed in mid-March (Carmichael, 1978). There were vomiting and diarrhea with variable amounts of mucus and blood in the feces. The feces, which was often "orangish", was exceptionally foul smelling. Animals generally recovered in 7 to 10 days, however, there were some deaths reported, particularly in young pups. A coronavirus was recovered in cell culture from 5 widely separated outbreaks, and the isolates were shown to be serologically related to the CCV previously isolated by Binn et al. (1975) (Appel et al., 1978). Experimental CCV infection resulted in mild symptoms of diarrhea or soft feces which persisted for 1 to 2 weeks in some dogs while others remained asymptomatic. The reason for the sudden occurrence of an enteric syndrome associated with CCV infection is not clear since studies indicated it was prevalent in the United States prior to 1978 (Appel et al., 1978; Norman et al., 1970). The number of cases being reported decreased dramatically after a few months.

In the summer of 1978, Eugster et al. (1978a, 1978b) noted cases of enteritis in dogs in which massive numbers of parvoviruses could be observed in intestinal contents by electron microscopy. The clinical syndrome was reportedly different than that observed earlier with parvovirus associated diarrhea (Eugster and Nairn, 1977) and resembled feline panleukopenia both clinically and histologically. The morbidity approached 100 percent and the mortality ranged from 10 to 50 percent with the higher rates being seen in the younger animals. The clinical syndrome assumed epidemic proportions in certain areas of Texas.

Similar outbreaks of canine enteritis resembling feline panleukopenia were occurring simultaneously in Canada (Thomson and Gagnon, 1978), Australia (Kelly, 1978), and England (Jefferies and Blakemore, 1979). A definite relationship between the canine parvovirus (CPV) and FPV was established when it was shown that intestinal epithelium from cases of enteritis in which CPV was isolated, fluoresced when stained with FPV conjugate (Black et al., 1979). These same authors demonstrated a high level of cross-reactivity between the two viruses with a serum neutralization (SN) test. Cross-reactivity was also observed with hemagglutination inhibition (HI) tests by which CPV and FPV could not be differentiated (Johnson and Spradbrow, 1979). However, the fact that CPV would hemagglutinate

porcine and monkey erythrocytes at 4 and 25°C was said to differentiate it from FPV which reportedly only agglutinates porcine erythrocytes and then only at 4°C (Johnson and Cruikshank, 1966).

At approximately the same time as the canine enteritis outbreaks, a sudden death syndrome was occurring in young puppies in which the primary lesion was myocarditis. A viral etiology was assumed because intranuclear inclusion bodies were noted in myocardial cells (Kelly and Atwell, 1979; Huxtable et al., 1979; Thompson et al., 1979), and on ultrastructural examination, these inclusion bodies contained particles which closely resembled parvoviruses (Hayes et al., 1979b). Subsequently, the fluorescence of myocardial inclusion bodies stained with canine origin conjugate specific for the canine enteritis parvovirus (Hayes et al., 1979a) and the presence of both myocarditis and enteritis in the same animal (Jefferies and Blackmore, 1979) suggested that the two syndromes were caused by the same virus; canine parvovirus.

In 1979, Eugster and Sidwa (1979) reported the presence of a rotavirus and an apparent picornavirus in the feces of a dog with diarrhea. The pup was from a pet store in which dogs were known to be infected with CPV and Giardia. No isolation and subsequent pathogenicity studies were conducted so the role of the virus in causing the diarrhea could not be

established.

Rotaviruses have also been reported in feces and intestinal contents of dogs with diarrhea submitted for electron microscopic examination in the United States (Pollock and Carmichael, 1979) and in England (McNulty et al., 1980). A serological survey indicated that 49 of 62 dogs from the Belfast area had antibodies to rotavirus (McNulty et al., 1978). The actual role of rotaviruses in canine enteritis is not clear since human rotaviruses will infect pups without causing clinical diarrhea (Tzipori and Makin, 1978).

England and Poston (1980) reported the isolation of a rotavirus from a 3-day-old pup with fatal diarrhea in which the virus was propagated in MDCK cells through at least 11 subpassages. Intestinal contents from the pup were orally inoculated into two healthy 6-month-old beagle dogs but the dogs remained asymptomatic and virus was not observed in the feces. It was suggested by the authors that the failure to induce disease may have been due to the animals age but this does not explain the lack of virus in the feces since rotavirus infections, some subclinical, can occur in other animals at all ages (Woode and Crouch, 1978). Serological studies were not reported and the failure to achieve infection may have been due to the immunity of the dogs by prior exposure to a rotavirus.

Canine Parvovirus

As previously discussed, outbreaks in dogs of enteritis and myocarditis of apparent parvoviral etiology occurred on several continents during the summer of 1978. Retrospective serological studies have been conducted by several authors to determine if the associated agent, CPV, epidemiologically resembled canine coronavirus which was widespread in the dog population prior to being associated with a disease syndrome, or if CPV was a primary pathogen that had just entered a naive dog population thereby accounting for the rapid spread of the virus.

The earliest canine sera in which CPV antibody could be detected were from Europe. Three of 56 sera collected from dogs in Belgium between June 1976 and June 1977 were found to be positive by an HI test (Schwers, et al., 1979). Osterhaus et al. (1980b) reported positive serological results from one serum collected at the end of 1977 in the Netherlands. They also reported that both the enteric and cardiac forms of CPV infection were occurring at that time.

In Australia, Johnson and Spradbrow (1979) reported that no HI antibody could be detected in 22 serum samples collected in Townsville, Queensland, in 1976 while sera collected in the latter part of 1978, after clinical cases of CPV were observed, were positive for CPV antibody. No testing of 1977 serum samples was reported.

A larger serological study (428 samples) was conducted in New South Wales, Australia, by Walker et al. (1980). Of the 150 serum samples collected in 1978 from dogs of unknown history, 19 had HI antibody titers ≥1:256 with the first positive serum being collected in May. None of the 74 sera collected from 1969 to 1977 were positive. The authors reported that 35 of 45 sera from clinically normal dogs in breeding colonies were positive and attributed these findings to widespread subclinical infection. Prior vaccination with FPV vaccine was considered not to be a factor since none of the 20 sera tested from FPV vaccinated cats had antibodies to CPV ≥1:256 by the HI test. This is in disagreement with vaccination trials reported by Appel et al. (1979) where HI titers of ≥1:256 were commonly seen in dogs vaccinated with FPV vaccines. In addition, Carmichael et al. (1980) reported HI titers to CPV of ≥1:2,500 in cats given live FPV.

In the United States, 95 sera from Tennessee dogs with undisclosed histories were tested for CPV antibody using an indirect fluorescent antibody test (Black et al. 1979). Seven of 30 sera collected in 1978 were positive while 27 sera from 1977 and 38 sera from 1976 were negative. Carmichael et al. (1980) tested 757 sera from across the United States submitted primarily for canine brucellosis testing and 303 serum samples submitted from June 1978 to June 1979 from dogs with suspected viral enteritis. None

of the 139 serum samples received from 1971 to 1975 or the 177 sera received in 1977 had HI titers $\geq$1:320, the level the authors felt was a definite indication of CPV infection. Of 171 sera submitted from January to June 1978, three received in late June for canine brucellosis testing were positive; one from Pennsylvania and two from California. An additional serum from a dog recently imported from Hong Kong and submitted for brucellosis testing was positive in early July 1978. The percent of positive sera rose to approximately 20% by August and September of 1978 but this rate was biased since most of the sera from this period were being submitted for diagnosis of enteric illness. The sera received prior to June 1978 were from purebred dogs and commercial breeding kennels located in many regions of the United States. Because of the high degree of mobility and interaction with breeding and show dogs, it is felt by this author that CPV would appear in these dogs very soon after its introduction into the United States and then spread rapidly among them. The rapid increase in the number of positive sera collected from widely distributed areas of the United States would tend to support this hypothesis. It is therefore felt that CPV did not exist in the dog population of the United States much before the summer of 1978, and it was imported from some other continent such as Europe where it had been detected at least a year earlier.

The actual source of CPV may never be determined but the close serological relationship with FPV and MEV suggests that it resulted from a mutation in one of these viruses that broadened its species specificity to include dogs. A similar phenomenon is believed to have occurred with MEV. Feline panleukopenia was recognized as a clinical entity in the latter half of the last century but was thought to be a bacterial infection (Croghan, 1968). In 1928, filtration studies indicated that it was probably caused by a virus (Verge and Christoforoni, 1928), and by 1934 a formalized vaccine had been developed (Leasure et al., 1934). Mink enteritis, on the other hand, was not recognized until 1947 when it caused an outbreak of enteritis on a mink ranch in the Fort Williams area of Ontario, Canada (Schofield, 1949). The virus slowly spread throughout North America and was world-wide by 1960 (Burger and Gorham, 1970). It is felt that MEV is a mutant of FPV that has become adapted to mink.

Clinico-Pathological Features

The effect of age

Canine parvovirus has been associated with two distinct clinical syndromes, myocarditis and enteritis, with the age of the animal being an influential factor in determining which clinical manifestation is seen. Myocarditis is usually a disease of young pups 4 to 8 weeks of age (Jezyk

et al., 1979; Mulvey et al., 1980) although deaths due to myocardial scarring have been reported in dogs up to 5 months of age (Robinson et al., 1980a; Carpenter et al., 1980). Clinical signs of the enteric form of CPV infection are more severe in pups 6 weeks to 6 months of age but, unlike the myocardial form, older adult dogs can also be infected (Fritz, 1979; Harcourt et al., 1980). It has been possible to experimentally produce the enteric form of CPV with virus isolated from cases of myocarditis (Hayes et al., 1979a; Robinson et al., 1980b) but myocarditis has not been experimentally produced except by in utero inoculation of virus of myocardial origin (Lenghaus et al., 1980).

Parvoviruses require dividing cells in order to replicate (Rhode, 1973; Siegl, 1976) and this dependency on cells that are mitotically active may account for the age differences seen with the two forms of CPV infection. Myocardial cell division is maximal in pups during the first 3 weeks of life (Bishop, 1972) while the mitotic index of intestinal epithelial cell in the neonate compared to older animals is relatively low. By comparing migration rates of tritiated thymidine labeled intestinal epithelial cells in rats of various ages, Koldovsky et al. (1966) showed that the rate of cell proliferation in suckling rats was one-fourth of that seen in weanling or adult rats. A similar study conducted in pigs (Moon, 1971) indicated that the rate of

cell division in the 1-day-old pig was one-third that seen in the 3-week-old pig. It is hypothesized by this author that a similar situation occurs in the intestinal epithelial cells in the dog. Therefore, if a pup is exposed to parvovirus in the first 4 to 6 weeks of age, the virus replicates in the mitotically active myocardial cells producing myocarditis. The virus manages to produce only a low level infection in the gut with limited pathogenesis because of the low number of dividing cells. However, if the pup is exposed to parvovirus after weaning, the rate of myocardial cell division is reduced and myocardites does not develop but the increased mitotic actively in the gut allows for extensive virus replication and cell destruction resulting in enteritis.

The study by Csiza et al., (1971) supports this hypothesis. They examined the pathogenesis of FPV in newborn kittens and observed severe enteric lesions in only 3 of 21 cats although it is a consistent finding in older animals. The reason for the relative lack of enteric involvement in newborn cats was not given but may have been due to a decreased rate of intestinal cell proliferation. In studies conducted with FPV in germfree and specific pathogen free (SPF) cats (Rohovsky and Griesemer, 1967; Carlson et al., 1977), it was observed that, as in the neonate, the enteric lesions in the germfree cats were considerably less severe than those seen in SPF cats. However, differences in degree

of thymic involution or leukopenia, both signs of FPV infection, did not occur between the two types of cats. Carlson and Scott (1977) determined that mucosal crypt length, cells per crypt, mitotic index and villous length were greater in SPF cats than in germfree cats and suggested that either bacteria or their metabolic by-products present in the SPF cats were responsible for the differences. They felt the lower mitotic index and lower number of cells per crypt in the germfree cat decreased the chance of FPV replication in the gut and thus the severity of the lesions produced.

Myocarditis has not been associated with FPV infection in cats nor has cerebellar hypoplasia, an FPV induced lesion in neonatal kittens, been seen in pups. However, when neonatal kittens were infected with FPV (Csiza et al., 1971), intranuclear inclusions and extensive fluorescence were observed in myocardial cells 5 to 13 days postinfection. In another study conducted on 8- to 9-week-old cats (Carlson et al., 1977), virus could be recovered from the heart but no inclusion bodies were observed. The virus may have been present in the heart in these individuals because of a viremia following replication of the virus in endothelial cells (Csiza et al., 1971) rather than myocardial cells. These two studies suggest that in the cat at least, myocardial cells of the neonate are more susceptible to

parvovirus infection than myocardial cells of older animals.

Myocardial syndrome

The clinical picture seen with the myocardial syndrome is one of sudden death with signs of acute respiratory distress occurring as a result of cardiac failure. The pups are usually reported to be in good health prior to the episode which usually occurs after feeding (Thompson et al., 1979; Jezyk et al., 1979). On gross examination, pulmonary congestion and edema are common and the myocardium, especially the left ventricle, will appear pale (Kelly, 1978; Thompson et al., 1979). Lymph nodes may be enlarged and edematous (Hayes et al., 1979a; Thompson et al., 1979).

Histological examination of the heart shows non-suppurative myocarditis with interstitial fibrosis and infiltration of plasma cells, lymphocytes, and some macrophages (Kelly, 1978; Jezyk et al., 1979). The left side is usually more involved. In older animals, focal myocardial necrosis and mineralization have been observed (Jezyk et al., 1979). Basophilic homogeneous intranuclear inclusion bodies in myocardial cells may be seen scattered throughout the myocardium (Kelly, 1978; Thompson et al., 1979).

The cause of death in the myocardial form of CPV infection depends on whether the infection is acute or chronic. In some pups, death occurs in the acute phase by

conduction failure as determined by electrocardiograms (Carpenter, et al., 1980). Despite the observations by Carpenter et al. (1980), electrocardiogram findings have not correlated well with the severity of the myocardial lesions seen postmortum and pups have died of myocarditis within 24 hours of a normal electrocardiogram (Lenghaus et al., 1980). If the dogs survive the acute infection, they may still die as the result of extensive myocardial fibrosis which, by contraction of the musculature, causes valvular insufficiencies and congestive heart failure (Robinson et al., 1980a; Lenghaus et al., 1980).

Enteric syndrome

The clinical signs of enteric CPV infection have been well-summarized by Appel et al. (1978). They include vomiting, diarrhea, rapid dehydration, and anorexia. The feces is light gray in the early stage of diarrhea but may become hemorrhagic. Copious amounts of mucus have also been reported with the diarrhea (Gagnon and Povey, 1979). Temperatures from 40 to 41°C have been reported (Appel et al., 1978), but it is not a consistent finding, especially in older dogs. A leukopenia has been observed accompanying the fever with total counts of 500/mm^3 to 2000/mm^3 being reported (Appel et al., 1978), but an absolute lymphopenia of <2,000/mm^3 is a more consistent finding (Fritz, 1979; Appel et al., 1979). All ages of animals are susceptible

although the mortality rate appears to be higher in the younger animals. Most deaths occur within 24 to 48 hours of the first sign of disease which is usually vomition (Appel et al., 1978; Fritz, 1979). The average mortality rate in litters has been estimated at 20% (Johnson and Spradbrow, 1979).

Gross pathological lesions are confined to the intestinal tract. The gut is often distended with gas and the contents in the lower and middle small intestine are watery and may vary from light colored with a large amount of mucus to dark and hemorrhagic (Walker et al., 1979; Thomson and Gagnon, 1978). Shallow erosions with scant fibrinous exudate have also been reported (Thomson and Gagnon, 1978).

The lesions seen on histopathological examination resemble those seen with FPV infection in cats (Kahn, 1978). There is severe loss of intestinal epithelium in the crypts of the small intestine with shortening of the villi. There is also a depletion and necrosis of lymphoid tissue evident in Peyer's patches as well as lymph nodes, spleen, and thymus (Cooper et al., 1979; Nelson et al., 1979). Bone marrow sections show a marked depletion of mature elements of both red and white cell series (Kelly, 1978).

Properties of the Virus

The characteristics of the family Parvoviridae have recently been reported by the Study Group on Parvoviridae, Coordinating Subcommittee, International Committee on Taxonomy of Viruses (Bachmann et al., 1979). Members of the family contain linear, single-stranded DNA with a molecular weight of between 1.5 and 2.2 X 10^6 daltons. The virions are isometric, nonenveloped particles, 18-26 nm in diameter with icosahedral symmetry. The virion probably contains 32 capsomers, 3-4 nm in diameter, but a configuration with 12 capsomers has also been suggested (Croghan, 1968; Kongsvik et al., 1974). The infectious particles have a buoyant density in CsCl gradients of between 1.39 and 1.42 g/cm^3. The viruses are ether- and chloroform-resistant, and heat- and acid-stable (56°C, pH 3 for 60 minutes). Most members possess a hemagglutinin on the virion for at least one species of red blood cells. The viruses multiply in the nucleus and replication is dependent upon certain functions of the host cell or helper functions provided by other viruses.

The family Parvoviridae contains three genera. They are Parvovirus which contains viruses that can replicate in susceptible cell cultures without a helper virus, produce intranuclear inclusion bodies, and contain only plus strands of DNA; Adeno-associated virus which contains viruses that

are dependent on helper viruses (adenoviruses and to some extent herpesviruses), produce no detectable cytopathology, and contain either plus or minus strands of DNA; and Densovirus which contains parvoviruses of arthropods that can replicate without helper viruses, cause hypertrophy of the nucleus with formation of intranuclear masses, but have either plus or minus strands of DNA in the mature virus particles. It had been previously determined by Johnson et al., (1974) that based on size, buoyant density, and DNA characteristics, FPV and MEV met the then existent classification requirements for Parvovirus Subgenus A set by the International Committee on Virus Nomenclature (Wildy, 1971) and are now considered members of the genus Parvovirus and strains of the species Feline Parvovirus (Bachmann et al., 1979).

Canine parvovirus has not yet been formally included in the genus Parvovirus by the International Committee on Taxonomy of Viruses but it has been shown to possess the characteristics of that group. Basophilic intranuclear inclusion bodies are a common observation in myocardial cells of dogs with myocarditis (Kelly and Atwell, 1979; Huxtable et al., 1979; Thompson et al., 1979) and both eosinophilic and basophilic intranuclear inclusion bodies have been observed, though not consistently, in crypt epithelial cells of dogs with enteritis (Kelly, 1978;

Fritz, 1979; Jefferies and Blakemore, 1979). The basophilic inclusion bodies seen in myocardial cells and some enterocytes may be indicative of older lesions since in cell cultures, parvovirus inclusions are at first lightly eosinophilic and then become basophilic with age (Cheville, 1975). The particles observed by electron microscopy in the inclusion bodies associated with CPV infection (Huxtable et al., 1979; Jefferies and Blakemore, 1979; Hayes et al., 1979b) and the feces of dogs with enteritis (Eugster et al., 1978b; Appel et al., 1978, Black et al., 1979) were 18-22 nm in diameter and resembled a parvovirus in morphology. Burtonboy et al. (1979) described the intact particles as 24 nm in diameter with an icosahedral shape and a buoyant density of 1.43 g/cm^3 in CsCl. Canine parvovirus isolates from cases of enteritis were grown in primary canine fetal lung cells and a feline kidney cell line (Johnson and Spradbrow, 1979). Subsequent testing indicated that CPV possessed a hemagglutinin for red blood cells, resisted heating at 60°C for one hour, resisted a pH of 3 and ether treatment, and behaved as a DNA virus in the presence of 2 bromodeoxyuridine; all characteristics of a parvovirus. More recently, McMaster et al., (1981) have shown by restriction enzyme analysis that a high degree of homogenicity exist in the DNA from CPV and MEV. Sixty-eight of 79 mapped sites were common and most of the 11 restriction sites which were

different were in regions coding for viral capsid proteins which may account for slight serological differences and for the difference in host cell range observed for the two viruses.

Relationships with Other Viruses

Serological cross-reactivity

Canine parvovirus has been found to be indistinguishable from FPV and MEV by HI or SN tests (Carmichael et al., 1980; Black et al., 1979; Johnson and Spradbrow, 1979) which is in agreement with earlier studies that indicated no serological differences between FPV and MEV (Johnson, 1967). Lenghaus and Studdert (1980) reported that CPV was significantly different from FPV by SN but not HI. There was, however, only a 4-fold increase in antibody titer when CPV was neutralized by the homologous antiserum over the titer obtained with the same serum and FPV. This author feels that the difference was too small to be significant. An apparent cross-reaction between CPV and fluorescent antibody (FA) conjugate to porcine parvovirus has been reported (Black et al., 1979; Eugster, 1980). The extent of this cross-reactivity and its occurrence with FPV and MEV has not been examined. There appears to be no relationship between CPV, FPV, or MEV and MVC (Carmichael et al., 1980; Siegl, 1976).

Hemagglutination reactions

The difference in hemagglutination (HA) reactions for CPV, FPV, and MEV may serve as a means of differentiating the three viruses. Johnson and Cruikshank (1966) indicated MEV or ultrasonicated FPV would hemagglutinate pig erythrocytes at 4°C but not room temperature. Further studies by one of the authors (Johnson, 1971) indicated 6 locally isolated MEV strains agglutinated pig cells while 10 local isolates of FPV did not. Because the 16 viruses tested were really different isolations and not necessarily different strains, the uniformity of the agglutination of pig erythrocytes by MEV but not FPV was not truly established.

Johnson and Spradbrow (1979) indicated that CPV from cases of enteritis would agglutinate pig and monkey (cynamologous and crab-eating Macaque) erthrocytes at 4° and 25°C, with the reaction at 25°C differentiating it from FPV and MEV. The authors cite one author's previous study (Johnson and Cruikshank, 1966) to state that FPV (and presumably MEV) will not agglutinate monkey erythrocytes, but the article cited does not specify the species of cells with which negative hemagglutination reactions occurred. In another article by this same author (Johnson, 1971), rhesus monkey, horse, and cat erythrocytes were among those tested that would not agglutinate.

The hemagglutination reaction of fecal origin CPV with pig and monkey (rhesus) erythrocytes at 4°C was confirmed by Burtonboy et al. (1979). They did not incubate the test at 25°C but obtained negative results at 37°C. Gagnon and Povey (1979) and Osterhaus et al. (1980b) reported hemagglutination at 4°C with cat as well as pig and rhesus monkey erythrocytes and an enteric isolate of CPV, and Robinson et al. (1979) indicated that myocardial isolates of CPV agglutinated pig erythrocytes at room temperature.

Konishi et al. (1975) demonstrated that the ability of FPV to agglutinate pig erythrocytes was related to the pH at which the test was conducted. A viral preparation of FPV had an HA titer of 1:8 at pH 7.4; 1:64 at pH 7.0, and 1:1,024 at pH 6.6 when incubated at 4°C. Seven isolates of FPV acquired at Tokyo University had similar HA patterns. No agglutination was seen with calf, horse, sheep, cat, guinea pig, chicken, or goose erythrocytes at pH 6.0-8.0. The failure of Johnson (1971) to note hemagglutination with FPV may have been due to the pH used in his testing but this is conjecture since the actual pH used was not specified.

Carmichael et al. (1980) studied the effect of pH on the agglutination of various species of erythrocytes at 4°C by CPV, FPV, and MEV. Canine parvovirus agglutinated pig, rhesus monkey, horse, and cat erythrocytes over a wide pH range (6.0, 6.8, and 7.2 except for pig cells that

spontaneously agglutinated at 6.0) with the highest titers occurring with pig erythrocytes. Feline panleukopenia virus and MEV agglutinated rhesus monkey and horse cells at pH 6.0 only and did not agglutinate cat cells. The results obtained with pig erythrocytes indicated a higher titer with FPV than with MEV. Although this is in contrast to earlier studies (Johnson and Cruikshank, 1966; Johnson, 1971), no apparent attempt was made in either study to standardize the CPV, FPV, and MEV preparations so that viral titers were similar. The agglutination of horse erythrocytes by CPV is inconsistent with the negative results seen by Gagnon and Povey (1979) and Walker et al. (1979). A similar inconsistency exists with FPV since Konishi et al. (1975) reported no hemagglutination with horse erythrocytes at pH 6.0.

Moraillon et al. (1980) reported no differences in the effect of pH on the hemagglutination reactions seen with CPV and MEV using cat and rhesus monkey erythrocytes but the strain of MEV used was from an outbreak of enteritis in mink occurring in October, 1979. Since this is after the appearance of CPV in Europe, and the pathogenicity of CPV for mink has not been determined, the identification of the isolate as MEV is questioned by this author.

In summary, it appears that at the proper pH, CPV, FPV, and MEV will all agglutinate monkey, pig, horse, and possibly cat erythrocytes. Differences in the agglutination

titers seen with erythrocytes from various species may be an important tool for differentiating the three viruses but these test should be standardized as were those by Toolan (1967) and Hallauer et al. (1972) where different agglutination patterns for rodent parvoviruses were determined after the viruses were diluted to contain an equal HA titer with one species of erythrocytes.

The use of the hemagglutination test on fecal preparations with either pig or rhesus monkey erythrocytes has been shown to be a sensitive diagnostic aid when used in combination with an HI test to eliminate nonspecific hemagglutination (Carmichael et al., 1980). Pig erythrocytes are preferred over monkey erythrocytes since the latter will also agglutinate in the presence of MCV (Binn et al., 1970).

Species susceptibility

In vitro Johnson and Cruikshank (1966) indicated that of a wide range of primary and line cells, FPV and MEV would only cause CPE in cat, tiger, mink, or ferret tissue cultures; but they did not specify the negative cells. In an earlier publication (Johnson, 1964), bovine, dog, monkey, human epidermoid cancer cells, or HeLa cells were not affected by FPV. Lee et al. (1969) observed intranuclear inclusion bodies in feline and lion cells infected with FPV but not dolphin, raccoon, mink, pig, rabbit, bovine, whale,

or bat cell lines or primary dog kidney cells. Scott et al. (1970) confirmed these findings.

Intranuclear inclusions caused by CPV have been observed in both feline and canine cells (Johnson and Spradbrow, 1979; McCandlish et al., 1979) with feline cells appearing to be more susceptible (Black et al., 1979; McCandlish et al., 1979). Intranuclear inclusions were either not seen or were considered indefinite when CPV was inoculated onto Vero cells (Johnson and Spradbrow, 1979) or Madin-Darby bovine kidney cells, primary human embryo kidney cells, primary rhesus monkey kidney cells, and HeLa cells (Burtonboy et al., 1979). However, when a fluorescent antibody test was used as an indicator system (Appel et al., 1979), Vero cells, raccoon salivary gland cells, and bovine fetal spleen cells in addition to canine, feline, and mink cells were found to be susceptible. Intranuclear inclusions were prominent only in feline kidney cells.

Canine parvovirus appears to have a wider host range in vitro than FPV or MEV. However, the studies conducted with FPV utilized the presence of CPE rather than specific fluorescence as an indicator system. Very little work has been done with MEV to establish its in vitro host range.

In vivo Feline panleukopenia virus has been isolated from domestic cats, leopards, tigers, lions, and panthers, and most probably all members of the family Felidae are

naturally susceptible (Siegl, 1976). The pathogenicity of FPV for mink (family Mustelidae) is questionable. Gorham et al. (1966) and Farrell et al. (1972) could not produce disease in mink with an FPV isolate that was highly pathogenic for cats. Burger (1961) experienced similar problems in producing enteritis in mink but could get disease symptoms if he gave the mink hydrocortisone or exposed them to 300 r of whole body radiation. Continuous passage in mink or alternate passages in mink and cats could not increase the virulence of FPV for mink. MacPherson (1956) on the other hand, did produce enteritis in mink by giving them portions of spleen, liver, and bowel from the carcass of a cat which had died with typical symptoms and clinical signs of FPV. Whether the cat could have been infected with MEV and not FPV was not made clear. Feline panleukopenia virus has been shown not to be an enteric pathogen of ferrets, another member of the family Mustelidae (Burger, 1961), but will cause cerebellar hypoplasia in neonatal ferrets (Johnson et al., 1967; Duenwald et al., 1971). The isolation of a virus with characteristics of either FPV or MEV from coati-mundi which were in close contact with cats (Johnson and Halliwell, 1968) and an outbreak of enteritis in raccoons in 1939 caused by an agent that produced disease typical of FPV when inoculated into cats (Waller, 1940) would tend to indicate that members of the family

Procyanidae are also susceptible to enteric infections with FPV. The susceptibility of the raccoon to FPV was experimentally confirmed by Burger (1961). Although replication of FPV in dogs has been reported (Appel et al., 1980a), no clinical signs have ever been observed (Appel et al., 1980a; Hindle and Findley, 1932; Urbain, 1933).

Mink enteritis virus is, of course, pathogenic for one member of the family Mustelidae, mink. The pathogenicity for ferrets has not been definitely established and Schofield (1949) and MacPherson (1956) indicated they were resistant to infection. Cats are susceptible to MEV but to a much lesser degree than mink. Wills (1952) reported that "under strictly controlled conditions, tissues from infected mink produced leucopenia in kittens." No experimental details were given. MacPherson (1956) produced a marked diarrhea 3 and 4 days postchallenge and a temperature of 40°C 7 days postchallenge in 1 of 24 cats fed mink enteritis material. The leukocyte count remained normal which suggests that the diarrhea may not have been of viral etiology. The susceptibility of the 24 cats for MEV was questioned by the author since he was also unable to produce clinical disease in 14 cats given material from a "typical case of feline enteritis." Burger (1961) had results similar to those of Wills (1952) in that he could produce a leukopenia but no signs in susceptible cats given MEV. When MEV

was given to raccoons (Burger, 1961), 8 of 9 animals remained well while the other developed a diarrhea on day 9.

Canine parvovirus has been associated with cases of enteritis in the dog as well as other members of the family Canidae including the coyote, bush dog, maned wolf and crab-eating fox (Evermann et al., 1980; Fletcher et al., 1979; Mann et al., 1980). Canine parvovirus has also been suggested as the cause of an outbreak of severe enteritis in raccoons (Nettles et al., 1980) but the lack of high antibody titers in sera from the raccoons and negative serological results against FPV causes this author to doubt the diagnosis. Similar outbreaks of enteritis had been occurring in raccoons since October 1976 and this was long before there was any serological evidence of CPV in the United States.

The pathogenicity of CPV for members of the families Mustilidae and Felidae have not been established as of this date. Moraillon et al., (1980) reported that the virus from a naturally occurring outbreak of enteritis in mink would produce disease identical to CPV when fed to young pups. Although hemagglutination studies are not definitive, the virus appeared to be closely related to CPV. This and the fact that the isolation from mink was made after CPV was present in the country suggests that CPV caused the enteritis in mink instead of MEV being pathogenic for dogs.

Osterhaus et al., (1980b) reported a slight temperature rise and a gradual fall in total leucocyte counts, but remaining within the normal range, in one FPV susceptible cat given CPV. Although no response to challenge was seen in another cat that was immune to FPV, the lack of a significant number of animals in the experiment limits the value of any conclusions that can be drawn from it.

An outbreak of gastrointestinal disease in laboratory personnel occurring simultaneously with an outbreak of CPV at a laboratory colony has been reported (Fritz, 1979) indicating that the host range of CPV might include man, but a relationship with CPV could not be established. Further, sera from humans who had diarrhea after handling CPV infected dogs were negative for CPV antibody (Appel et al., 1980b).

Vaccination

When mink enteritis spread through the fur industry during the early 1950s resulting in great economic losses, heterologous FPV vaccine manufactured for use in cats was employed as a means of protection and was shown to be efficacious in mink (Wills and Belcher, 1956). Since that time, the ability of FPV and MEV to immunize against one another has been well-established (Gorham et al., 1965; Gorham et al., 1966; King and Gutekunst, 1970). When CPV

emerged in 1978 as an important pathogen of dogs, the close serological relationship between FPV and CPV led to the recommendation that commercially available FPV vaccines be used once again for the prevention of a heterologous disease, CPV enteritis (Appel, 1979; Johnson and Spradbrow, 1979; Royal College of Veterinary Surgeons, 1979). Because the pathogenicity of modified-live FPV in dogs had not been established, the use of only inactivated vaccines was recommended.

Experimental confirmation of the efficacy of FPV vaccine in preventing CPV caused disease was first presented by Appel et al., (1979). Although serological conversion was obtained in all vaccinated dogs, and dogs vaccinated twice with inactivated or once with modified-live FPV vaccine did not develop elevated body temperature or lymphopenia; pyrexia and lymphopenia were the only signs of infection produced in the controls by the challenge preparation used in the experiment. Therefore, the efficacy of the vaccines in preventing CPV enteritis had not been established.

In another vaccination-challenge experiment (Chapek et al., 1980), inactivated FPV vaccine was shown to prevent viral shed but the method employed, viral isolation from rectal swabs, is considered by this author to be less sensitive than the method employed by Carmichael et al.

(1980) in which 10% fecal preparations were tested by hemagglutination. Isolations were made on day 4 to 7 by rectal swabs as compared to days 3 to 9 by hemagglutination. Chapek et al. (1980) also stated that inactivated FPV vaccine protected dogs from leukopenia which was present in the controls. The severity of leukopenia produced and the percentage of controls in which it was observed were not given.

As stated earlier, the safety of live FPV vaccines in dogs had to be established prior to their being recommended for use in dogs. Although an early report (Lloyd-Evans, 1980) indicated vaccine virus could not be recovered from, or identified by immunofluorescence in internal organs of newborn pups given FPV vaccine, Appel et al. (1980a) reported that small amounts of FPV were isolated from certain tissues of dogs vaccinated 2 to 3 days earlier. The authors suggested that the virus recovered was the result of replication in the dog and not just residual vaccine virus. The authors also suggested that the replication of FPV in dogs accounts for the high and more persistent antibody levels seen following vaccination with modified-live as compared to inactivated FPV vaccines (Appel et al., 1979; Moraillon, 1980; Appel et al., 1980a).

Feline panleukopenia virus apparently does not replicate in all vaccinated dogs. In field trials in over

100 dogs, only 58% of the dogs developed hemagglutination inhibition antibody levels of $\geq$1:80 (Carmichael and Pollock, 1981). Although comparable antibody titers are seen following the administration of a single dose of inactivated FPV vaccine (Appel et al., 1979), it was the view of the authors that in the majority of the dogs in which titers of $\geq$1:80 were generated, they were generated because of viral replication. The authors also presented data that indicated 8 of 8 dogs given an experimental vaccine containing $10^{7.5}$ tissue culture infective dose$_{50}$ ($TCID_{50}$) of virus developed antibody titers of at least 1:80, while only 5 of 8 dogs given $10^{5.5}$ $TCID_{50}$ of virus developed antibody levels of this magnitude. The authors concluded that the higher percentage of responding animals seen with the higher titered vaccine was due to viral replication and by increasing the virus titer, the proportion of dogs in which the virus replicated, also increased. In actuality, if viral replication was a prominent feature of vaccination with modified-live FPV vaccine, there should not be as great a difference in the immunity produced by a vaccine with $10^{5.5}$ $TCID_{50}$ of virus and one with $10^{7.5}$ $TCID_{50}$ of virus as replication of the virus in the host would soon equate any initial differences. An increase in total antigenic mass in the higher titered vaccine with little or no increase in the proportion of vaccinated dogs in which replication occurred

seem a more logical explanation of the observed response differences.

Vaccines produced from homologous CPV have also been developed. Appel et al., (1979) demonstrated high post-vaccinal serology in dogs given an inactivated CPV vaccine. The dogs were protected from challenge but, as mentioned earlier, the challenge was not capable of producing more than a lymphopenia and pyrexia in control dogs. Studies conduced by other investigators (Smith et al., 1980; Eugster, 1980) using more virulent challenges have established the efficacy of inactivated CPV vaccines. The immunity generated by the inactivated CPV vaccine appears, however, to be short-lived (Appel et al., 1980a; Carmichael and Pollock, 1981).

An isolate of live CPV was reported to be attenuated for pups after 80 serial passages in dog kidney cells (Appel et al., 1980a; Carmichael et al., 1981) and capable of producing a high initial antibody response and "long-lived immunity" (Carmichael and Pollock, 1981). Although no evidence of virulence has been observed with this attenuated strain of CPV when given alone to dogs, its use in a vaccine would most probably be in combination with other modified-live viruses. Potgieter et al. (1980) reported the occurrence of enteritis and neutropenia in 3 of 3 dogs given CPV one week after being vaccinated with canine distemper

and infectious canine hepatitis vaccine; one dog died. Dogs not previously vaccinated remained clinically normal following exposure to the CPV preparation used. It has also been reported (Jezyk, 1980) that dogs given distemper vaccine while ill with presumed CPV enteritis have subsequently died with confirmed distemper. In light of these findings, the safety of modified-live CPV vaccine used in combination with other modified-live virus vaccines, should be established.

PART I. IDENTIFICATION OF CANINE PARVOVIRUS

INTRODUCTION

Serologically CPV, FPV, and MEV appear to be indistinguishable from one another making identification of individual isolates difficult. This study examined several parameters; antigenic relationship to porcine parvovirus (PPV), differences in hemagglutination activity, and in vitro species susceptibility; to establish a simple method of differentiating the 3 viruses.

Specific fluorescence with PPV conjugate and CPV has been reported, but the presence of a similar cross-reaction with PPV conjugate and FPV or MEV has not been established. Hemagglutination inhibition tests have, however, indicated there is no serological relationship between the latter 2 viruses and PPV. In this study, the extent of the cross-reactivity of PPV antiserum with CPV, FPV, and MEV was examined by FA and SN tests to determine if it was a distinct characteristic of CPV. Bovine parvovirus (BPV) was included in the FA test to further determine the extent of the cross-fluorescence reported with PPV conjugate.

Although differences in the abilities of CPV, FPV, and MEV to agglutinate erythrocytes of various species have been reported, these differences appear to be influenced by the pH at which the tests were conducted. An attempt was made to determine if the pH associated differences in HA titers seen with the most reactive cell, porcine erythrocytes

(Carmichael et al., 1980), were reproducible among isolates of each virus.

A number of reports suggest that, based on the presence of intranuclear inclusion bodies, there are differences in in vitro species susceptibility of CPV, FPV, and MEV. The FA technique was, however, shown to be more sensitive than inclusion body observations in detecting CPV in cells from various species. In this study, the presence of specific fluorescence was employed to determine differences in in vitro species susceptibility for several isolates of all 3 viruses.

MATERIALS AND METHODS

Tissue Culture Procedures

The medium used for all tissue culture procedures was Eagle's minimum essential medium with Earle's salts, L-glutamine and nonessential amino acids[1]. To this, 5% fetal calf serum and gentamycin,[2] at a final concentration of 50 mcg/ml, were added. The same medium, without fetal calf serum, was used as virus and serum diluent in all procedures except hemagglutination.

The fluorescent antibody technique was the indicator system used to determine tissue culture infectivity. Eight chamber slides[3] were seeded with 0.4 ml/well of suspended cells, 200,000 cells/ml. Wells were inoculated with 0.1 ml of virus or serum-virus mixture within 2 hours of seeding and incubated at 37°C in an atmosphere of 5% CO_2 until fixed in acetone and stained with fluorescein tagged conjugate. Cells were examined for typical fluorescence using an Orthoplan[4] microscope equipped with a 200 watt mercury light source and an incident-light illuminator.

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[1]F-15, Grand Island Biological Company, Grand Island, New York.

[2]Garamycin, Schering Corporation, Kenilworth, New Jersey.

[3]Lab-Tek Products, Naperville, Illinois.

[4]E. Leitz Inc., Rockleigh, New Jersey.

Virus titrations were stained at 5 days postinoculation while SN tests were stained at 3 days.

Cross-Reactivity with PPV

Fluorescent cross-reactivity

Cells Crandell feline kidney (CRFK) cells were used to propagate CPV, FPV, and MEV; PPV was grown in embryonic porcine kidney (EPK) cells. Cross-reactivity with BPV was included in the test design and the virus was propagated in primary bovine lung (pBL) cells.

Viruses The CPV used was the KB strain obtained from a dog in Ames, Iowa that was showing typical signs of canine parvoviral enteritis. The virus had been passaged twice in CRFK cells. The FPV was the ICK-33 challenge strain that had been passaged 5 times in CRFK cells. The MEV, here designated MEV-2, was a tissue culture adapted strain obtained from a commercial producer of MEV vaccine. It was passaged once in CRFK cells after receipt. Even though this virus may not have been used in the production of a vaccine, the actual source of this virus or any other virus obtained from a biologics manufacturer will not be given because of the possibility of disclosing proprietary information. The PPV used in this study was lot TC-3 of a virus that had been isolated from porcine trypsin (Croghan et al., 1973) and passaged 3 times in EPK cells. The HADEN strain of BPV

(Abinanti and Warfield, 1961) adapted to bovine lung cells was used.

Conjugates The FPV conjugate was prepared from rabbits hyperimmunized with the ICK-33 virus strain, the PPV conjugate was prepared from a sow vaccinated and then challenged with PPV, and the BPV conjugate was prepared from a calf hyperimmunized with the HADEN virus strain.

Test design Between 100 and 1000 $TCID_{50}$ of each virus were inoculated onto the applicable cell culture. Three days postinoculation, the infected cell cultures were fixed, stained with each conjugate, and examined for typical parvoviral fluorescence. Uninoculated control cultures were treated in a similar manner.

Serum neutralization cross-reactivity

The cells and virus strains used in this study were identical to those used in the fluorescent cross-reactivity test except that BPV was excluded from the study. Any cross-reactivity between BPV and PPV would not be a factor in differentiating CPV from FPV and MEV, and viral neutralization of CPV, FPV, or MEV should not occur unless cross-fluorescence was first present. A constant serum-varying virus neutralization test was conduced with a porcine origin PPV antiserum with an SN titer of 1:6912 and a porcine serum free of SN antibody to PPV. Each were diluted 1:5 and mixed with an equal volume of 10-fold

dilutions of virus; PPV, CPV, FPV, and MEV. After incubation at room temperature for 30 minutes, the virus-serum mixtures were inoculated, 4 wells/dilution, into 8-chamber slides containing freshly planted EPK cells (PPV) or CRFK cells (CPV, FPV, and MEV). Three days postinoculation, the slides were fixed and stained with PPV or FPV conjugate. Each well that contained at least one fluorescing cell was considered positive and virus titers were determined by the Spearman-Karber method as refined by Finney (1971).

The antibody titer of the PPV antiserum was determined against each virus. Two-fold dilutions of serum were mixed with each virus preparation diluted to contain 200 $TCID_{50}$/ 0.1 ml. The virus-serum mixtures were then incubated and inoculated onto cell cultures in the same manner described for the constant serum-varying virus neutralization test. After staining, each well that was free of fluorescing cells was considered positive and the antibody titers were calculated by the Spearman-Karber method.

Hemagglutination Reactions

The effect of pH on the HA titers observed with several strains of CPV, FPV, and MEV was determined as was the relationship between HA titer and infectivity titer.

Viruses

The CPV strains utilized were FLF1-2, a challenge strain that had been passaged twice in embryonic feline fibrobast cells after being isolated from a dog in Ames, Iowa that died of canine parvoviral enteritis; KB strain previously described; A78-22 strain[1] passaged once in CRFK cells; and the TN-233 strain[2] that had been passaged approximately 40 times in CRFK cells prior to receipt. The MEV strains utilized were MEV-2, previously described; MEV-1, a mink tissue origin challenge strain; and MEV-3, another tissue culture adapted strain. The FPV strains included in this study were the ICK-33 strain, previously described; 2 isolates of the Johnson strain, here designated FPV-J1 and FPV-J2; the Crawley strain, FPV-C1; and the Gorham strain, FPV-G1. The isolates of MEV and FPV were passaged once in CRFK cells after receipt. Virus infectivity titers for all strains were determined by the fluorescent antibody technique described previously.

Porcine erythrocytes

Ten ml of porcine blood were collected in an equal volume of Alsever's solution. The cells were placed in a

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[1]Obtained from M.J.G. Appel, Ithaca, New York.

[2]Obtained from J.W. Black, Nashville, Tennessee.

polypropylene centrifuge tube and the volume raised to 50 ml by the addition of Dulbecco's phosphate buffered saline (PBS) free of Ca^{++} and Mg^{++}. The cells were then centrifuged at 500 X g for 10 minutes, decanted from the supernatant, and resuspended in Dulbecco's PBS. This washing procedure was repeated twice but the cells were not resuspended after the last centrifugation step. The packed cells were stored at 4°C for not more than 8 hours before being used.

Diluent

Dulbecco's PBS with 0.5% bovine serum albumin added was used as diluent for viruses and erythrocytes. The PBS was divided into 3 lots. One lot was maintained at the initial pH of 7.4, one lot was lowered to 7.0, and the other lowered to 6.6 by the addition of 1N HCl.

Test design

The 12 virus strains were titered for HA activity at 3 different pH levels. The tests were conducted in 96-well U bottom microtiter plates.[1] A variable volume multichannel pipette[2] was used to deliver 0.05 ml of diluent into each well. A 0.05 ml pipette[3] was used to dispense that volume

[1]Dynatech Laboratories Inc., Alexandria, Virginia.

[2]Titertek, Flow Laboratories Inc., Rockville, Maryland.

[3]Medical Laboratory Automation Inc., Mount Vernon, NY.

of each virus into the first well. The multichannel pipette was then used to make 23, 2-fold dilutions, after which 0.025 ml of a 1% suspension of packed porcine erythrocytes in the appropriate pH diluent was added to each well. The plates were incubated at 4°C overnight and the HA titer recorded as the highest dilution of virus that would agglutinate cells.

In Vitro Species Susceptibility

Cells

The host-cell ranges of CPV, FPV, and MEV were evaluated in CRFK, EPK, and pBL cells previously described. Primary canine kidney (pCK) cells, a mink lung cell line (MV1-Lu), a ferret lung cell line (FeL-MA139) a dog fibroblastic cell line (A-72), MDCK cell line, and an African green monkey cell line (Vero) were also included in the study.

Viruses

The 12 viruses listed for the HA reaction study were included in this study. Two additional FPV isolates were included. They were another Crawley isolate, FPV-C2, and a ferret tissue culture adapted isolate, FPV-F1.

Test design

Preliminary studies in which 0.1 ml of undiluted MEV-2, ICK-33, FPV-J1, and FPV-C1 were inoculated onto pDK cells, indicated that 1-10 fluorescing cells/100,000 cells would be detected with all 4 viruses when high infectivity titers were used ($10^{3.5}$ $TCID_{50}$ for MEV-2 to $10^{5.1}$ $TCID_{50}$ for ICK-33). In an effort to standardize this study, each of the 14 virus preparations used were diluted to contain approximately 1000 $TCID_{50}$/0.1 ml. The dilutions were based on previous titrations conducted in CRFK cells. Eight chamber slides planted with each cell type were inoculated with 0.1 ml/well, 4 wells/virus when the cells were approximately 70% confluent. Twenty-four hours postinoculation, the cells were washed twice with tissue culture medium, the chambers refilled with medium, and incubated for an additional 3 days. At that time, the slides were stained with FPV conjugate and the number of fluorescing cells were determined.

RESULTS AND DISCUSSION

Cross-Reactivity with PPV

Fluorescent cross-reactivity

The presence of typical parvoviral fluorescence in infected cells stained with PPV, FPV, and BPV conjugates is recorded in Table 1. The cross-fluorescence observed with PPV conjugate and CPV, FPV, and MEV was considerably less brilliant than that observed with the 3 viruses and FPV conjugate or with the homologous PPV system (Figure 1). The cross-reactivity appeared to be one-way since no fluorescence was noted between PPV and the conjugate for FPV. None of the parvoviruses appeared to cross-react with BPV. There was no discernible difference in intensity of fluorescence observed between CPV, FPV, and MEV with either PPV or FPV conjugates. No specific fluorescence was observed in the uninoculated control cells.

Serum neutralization cross-reactivity

Although the antibody titers obtained for PPV antiserum against FPV and MEV were <1:2 in the varying serum-constant virus neutralization test (Table 2), the numbers of fluorescing cells detected at the 1:2 dilution were greatly reduced from the number observed in the 1:2 dilution of the normal serum and virus. The results of the constant serum-varying virus neutralization test also indicated low level

Figure 1. Fluorescent cross-reactivity with PPV conjugate: (a) FPV stained with FPV conjugate diluted 1:20, x 130; (b) PPV stained with PPV conjugate diluted 1:20, x 130; (c) FPV stained with PPV conjugate diluted 1:20, x 130; (d) FPV stained with PPV conjugate diluted 1:4, x 250

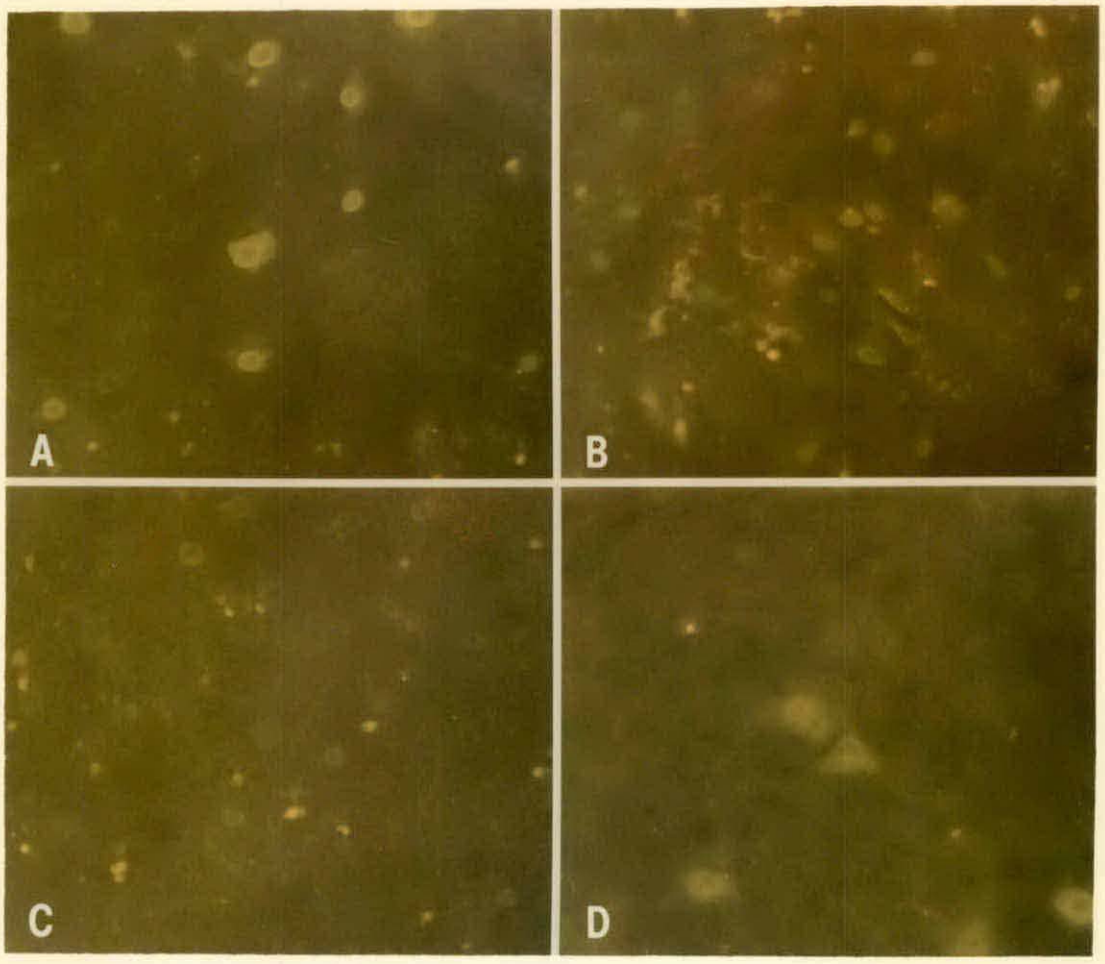
A
B
C
D

Table 1. Fluorescent cross-reactivity between parvoviruses

Virus	PPV conjugate	FPV conjugate	BPV conjugate
PPV	+	-	-
CPV	+	+	-
FPV	+	+	-
MEV	+	+	-
BPV	-	-	+

Table 2. Serum neutralization cross-reactivity between parvoviruses; PPV antiserum

Virus	Virus titer[a]		Log_{10} neutralized	Antibody titer[b]
	NS	AS		
PPV	6.5	3.0	3.5	6912
CPV	5.3	3.9	1.4	4
FPV	6.1	4.5	1.6	<2
MEV	4.5	3.5	1.0	<2

[a]Virus titer expressed in log_{10} $TCID_{50}$/ml obtained with normal serum (NS) and PPV antiserum (AS).

[b]Recripocal of serum dilution of PPV antiserum that would neutralize approximately 100 TCID of virus.

reduction with PPV antiserum. The titers obtained for CPV and FPV after correcting for the 1:2 serum-virus dilution were significantly below ($p < .05$ and $p < .01$ respectively) the expected results based on previous titrations. The reduction seen with MEV was not significant but this may have been due to the small number of previous titrations performed rather than a difference in the neutralization characteristics of MEV. The mean and standard deviation of the infectivity titer for CPV was $10^{5.4 \pm .2}$ ($n = 5$), for FPV it was $10^{6.7 \pm .2}$ ($n = 11$), and for MEV it was $10^{4.8 \pm .3}$ ($n = 3$).

Although the reductions in infectivity titer of CPV and FPV seen with the PPV antiserum were statistically significant, they were less than $10^{2.0}$, the minimum level of reduction commonly considered significant for specific antibody neutralization. The results obtained from both test systems indicate serum neutralization cross-reactivity with PPV antiserum does not occur at a great enough level to be used as a method of differentiating CPV, FPV, and MEV from one another.

Hemagglutination Reactions

The variation in hemagglutination reactions appeared as great among individual isolates of a virus as it did between viruses. This is true in both the relationship of

infectivity titer to HA titer and in the effect of pH on HA titer (Table 3).

The HA titer/infectivity titer ratio appeared to decrease as the number of tissue culture passages of an isolate increased. This was true regardless of whether the virus was CPV, FPV, or MEV. The lowest ratios for the MEV isolates were seen with the tissue culture adapted strains, MEV-2 and MEV-3. Likewise, the TN-233 isolate of CPV with approximately 40 tissue culture passages had a lower HA titer/infectivity titer ratio than the other CPV strains; FLF1-2, KB, and A78-22; which had been passaged less than 3 times. With the FPV isolates, the ICK-33 strain with only 5 tissue culture passages had the second highest ratio while the 2 Johnson vaccine strains, FPV-J1 and FPV-J2 had the lowest.

The HA titers of the CPV isolates were affected less by changes in pH than the FPV and MEV isolates; but again, the responses were not consistent among isolates. The HA titer of the tissue culture adapted TN-233 isolate increased by 4-fold as the pH increased while the titer of the other isolates either remained unchanged or decreased.

While the HA titers of all the FPV and MEV isolates decreased as the pH increased, the reduction ranged from a 4-fold decrease to >32-fold decrease. The 2 Johnson strains of FPV and the MEV-3 isolate of MEV showed the largest drops.

Table 3. Effects of pH on hemagglutination titers of CPV, FPV, and MEV

Virus	Strain	Infectivity titer	pH 6.6	pH 7.0	pH 7.4
CPV	FLF1-2	5.0[a]	1024[b]	1024	512
CPV	KB	5.5	16,384	16,384	16,384
CPV	A78-22	5.4	2048	1024	2048
CPV	TN-233	6.7	256	512	1024
FPV	FPV-J1	5.7	32	<2	<2
FPV	FPV-J2	6.5	512	16	16
FPV	ICK-33	6.1	2048	128	256
FPV	FPV-C1	5.7	2048	512	256
FPV	FPV-G1	4.5	512	256	128
MEV	MEV-1	5.0	16,384	4096	4096
MEV	MEV-2	4.5	256	64	64
MEV	MEV-3	4.5	64	8	<2

[a]Infectivity titer expressed in $\log_{10}$ $TCID_{50}$/ml.

[b]Hemagglutination titer.

In this test system, changes of less than an 8-fold were not considered significant, thus 1 FPV isolate, FPV-G1, and 2 MEV isolates, MEV-1 and MEV-2, reacted similarly to the CPV isolates. Hemagglutination reactions were, therefore, not reliable in differentiating CPV, FPV, and MEV.

In Vitro Species Susceptibility

All of the viruses tested were able to replicate in CRFK cells as evidenced by >1000 infected cells/well and a reduction in cell numbers due to the cytopathogenicity of the viruses. No specific fluorescence was detected in EPK cells or pBL cells with any of the 14 viruses used in the study.

Although the CPV isolates tested were able to induce the formation of specific fluorescence in all 3 canine cell types, the A-72 cells appeared to be the most susceptible to CPV (Table 4). While none of the FPV isolates showed evidence of viral replication in canine cells, the challenge strain of MEV (MEV-1) was able to induce fluorescence, but only in A-72 cells. The lack of fluorescence with MEV-2 and MEV-3 may have been due to either their adaptation to CRFK cells, or to the use of lower titered inoculums for these isolates. In spite of the low amount of fluorescence seen with MEV-1 in A-72 cells, the data obtained indicate that the ability of CPV to replicate in A-72 cells to approximately the same degree as it does in CRFK cells is a

Table 4. Specific parvoviral fluorescence in canine cells

Virus	Strain	Titer	Cells		
			MDCK	pDK	A-72
CPV	FLF1-2	2.5[a]	<5[b]	<5	>1000
CPV	KB	3.5	27 ± 14	5-10	>1000
CPV	A78-22	3.5	5-10	<5	>1000
CPV	TN-233	2.8	<5	<5	>1000
FPV	FPV-J1	3.5	NSF[c]	NSF	NSF
FPV	FPV-J2	3.0	NSF	NSF	NSF
FPV	FPV-C1	3.3	NSF	NSF	NSF
FPV	FPV-C2	3.8	NSF	NSF	NSF
FPV	FPV-F1	3.5	NSF	NSF	NSF
FPV	FPV-G1	3.5	NSF	NSF	NSF
FPV	ICK-33	2.8	NSF	NSF	NSF
MEV	MEV-1	3.8	NSF	NSF	5-10
MEV	MEV-2	2.5	NSF	NSF	NSF
MEV	MEV-3	2.5	NSF	NSF	NSF

[a]Infectivity titer of inoculum expressed in $\log_{10}$ $TCID_{50}$/0.1 ml. Titer was determined in CRFK cells.

[b]Average number of fluorescing cells/well. When the mean was between 11 and 1000, the mean ± 1 standard deviation is given.

[c]No specific fluorescence.

characteristic that can be used to distinguish CPV from FPV and MEV.

Although it appears CPV replicates better than FPV or MEV in mink lung, ferret lung, and Vero cells (Table 5), there is too much variation among the isolates to make any definite conclusions on the usefulness of these cells for virus identification. One reason is that the amount of fluorescence observed with MEV-3 in mink lung cells was more indicative of an FPV isolate than MEV. However, when the history of MEV-3 was investigated, it was discovered that the virus was obtained from R. H. Johnson. The similarities of the HA reactions (low HA titer/infectivity titer ratio) of MEV-3 and the Johnson isolates of FPV (Table 3) suggest that MEV-3 may have been misidentified at some time and that it is really the Johnson strain of FPV. If this is true, then the ability to replicate in mink lung cells to a greater extent than ferret lung or A-72 cells would be a characteristic of MEV that would differentiate it from CPV and FPV.

The ferret tissue culture adapted FPV-F1 strain did not replicate as well as expected in ferret lung cells. It may be that the virus has been replicated in cells other than lung and it is the inability to replicate in lung cells, not ferret cells, that is the cause of the low number of fluorescing cells observed.

Table 5. Specific parvoviral fluorescence in non-canine cells

Virus	Strain	Titer	Cells		
			Mink lung	Ferret lung	Vero
CPV	FLF1-2	2.5[a]	176 ± 37[b]	26 ± 8	5-10
CPV	KB	3.5	>1000	>1000	27 ± 7
CPV	A78-22	3.5	>1000	>1000	5-10
CPV	TN-233	2.8	56 ± 13	32 ± 9	<5
FPV	FPV-J1	3.5	<5	74 ± 13	<5
FPV	FPV-J2	3.0	NSF[c]	5-10	<5
FPV	FPV-C1	3.3	<5	11 ± 3	NSF
FPV	FPV-C2	3.8	NSF	<5	NSF
FPV	FPV-F1	3.5	NSF	<5	5-10
FPV	FPV-G1	3.5	<5	<5	NSF
FPV	ICK-33	2.8	NSF	<5	<5
MEV	MEV-1	3.8	28 ± 6	17 ± 8	5-10
MEV	MEV-2	2.5	30 ± 6	<5	NSF
MEV	MEV-3	2.5	<5	NSF	<5

[a]Infectivity titer of inoculum expressed in $\log_{10}$ $TCID_{50}$/0.1 ml. Titer was determined in CRFK cells.

[b]Average number of fluorescing cells/well. When the mean was between 11 and 1000, the mean ± standard deviation is given.

[c]No specific fluorescence.

GENERAL DISCUSSION

Although the fluorescent cross-reactivity with PPV conjugate and CPV has been confirmed, this study has shown that an equal reaction occurs with PPV conjugate and FPV and MEV. Thus, it can not be used to differentiate CPV, FPV and MEV. Likewise, the low level serum neutralization detected with high titered PPV antiserum is not unique to any of the 3 viruses.

The ability of CPV to agglutinate pig erythrocytes seemed to be affected less by an increase in pH than the agglutination capacities of FPV or MEV. It is this author's opinion, however, that the magnitude of the difference is not sufficient to allow HA reactions to be used to differentiate CPV from FPV or MEV. What is of interest, is the low HA results obtained with the Johnson strain of FPV (and MEV-3 if it is truly the Johnson strain of FPV) compared to the HA titers of the other strains of FPV. This may explain the negative HA results obtained by Johnson with his isolate and pig erythrocytes and the low HA results obtained by Carmichael et al., (1980) where the Johnson snow leopard strain was used as the type strain for FPV. The data presented in this study indicate that this strain is not representative of FPV.

Of the methods tested, in vitro species susceptibility appears to be the most reliable for differentiating CPV, FPV,

and MEV. However, since fluorescing cells can be detected by using inoculums with high infectivity titers, the mere presence of specific fluorescence in cells from a species is not sufficient for virus identification. Even at 1000 $TCID_{50}$, low numbers of fluorescing cells were detected with isolates of each virus in most cell types tested. Where no specific fluorescence was seen, an increase in the infectivity titer of the inoculum might have resulted in observed fluorescence. It is suggested that the relative abilities of a virus to replicate in certain cells be used to differentiate the 3 viruses. The results of simultaneous titrations in CRFK, mink lung, ferret lung, and A-72 cells should identify the viruses. Canine parvovirus should titer well in CRFK and A-72 cells; FPV should titer high only in CRFK cells; and MEV should titer high in CRFK cells and the titration results in mink lung cells should be greater than the results in ferret lung cells. It is this multiple titration system that is being used at the National Veterinary Services Laboratories, Ames, Iowa, for virus identification.

PART II. EVALUATION OF THE EFFICACY OF FELINE PANLEUKOPENIA VIRUS VACCINES FOR CANINE PARVOVIRAL ENTERITIS

INTRODUCTION

When CPV emerged in 1978 as a pathogen of dogs, it was a unique situation because a vaccine for the disease, in the form of FPV vaccine, already existed. Prior to being licensed for use in dogs, however, the efficacy of the heterotypic vaccine had to be established. This was difficult because, except in young pups, CPV produces a mild disease experimentally. A similar situation most probably occurs after natural exposure with most infections being subclinical or resulting in only a transient loose stool or diarrhea that is not recognized as significant by the owner (Osterhaus et al, 1980a; Pollock and McGregor, 1980).

Feline panleukopenia virus vaccine for use in cats is considered satisfactory by the USDA if it will induce protection against a challenge that can produce a leukopenia of $\leq$4000 cells/mm^3 or $\leq$25% of the prechallenge normal (Standard requirements, 1981). Leukopenia is not a prominent occurrence in CPV infection, so other measurable signs of infection and criteria of significance had to be established. This study deals with the development of these criteria and their subsequent use for evaluating the efficacy of FPV vaccines for CPV enteritis.

MATERIALS AND METHODS

Dogs

Fifty-five beagle dogs, 2 to 3 months of age, were obtained from sources known to be free of canine parvoviral infection.[1] Susceptibility to CPV was confirmed by the varying serum-constant virus SN test previously described, and all dogs had antibody titers <1:2.

Vaccines

Vaccines studied were commercially prepared FPV vaccines that contained either modified-live virus (MLV) or killed virus (KV). The HA titers of the vaccines were determined at a pH of 6.6 by the previously described procedure. The infectivity titers of MLV vaccines were determined in CRFK cells by fluorescent antibody staining 5 days postinfection. Each vaccine was administered intramuscularly to 4 or 5 dogs. Revaccination occurred at the shortest interval recommended by the manufacturer (2 or 3 weeks). Sera were collected at the time of each vaccination and antibody levels determined against the KB isolate of CPV by the SN test previously described. The vaccines were not diluted except where otherwise indicated.

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[1]Thirty-six dogs were from Cornell Dog Farm, Cornell University, Ithaca, New York; 15 dogs were from the Department of Laboratory Animal Science, Cornell University, Ithaca, New York; and 4 dogs were taken by Caesarian section from a dam obtained from Laboratory Research Enterprises, Kalamazoo, Michigan.

Challenge Material

Two separate CPV challenges were used in the study. The first[1] was a tissue culture preparation containing $10^{5.0}$ $TCID_{50}$/ml. The second was FLF1-2, previously described, which also contained $10^{5.0}$ $TCID_{50}$/ml. No extraneous viruses were detected in either challenge preparation after 2 passages in pDK cells, A-72 cells (a canine fibroblastic cell line used for the detection of CCV), and Vero cells. The abilities of the challenges to produce significant effects of CPV infection were similar.

One ml amounts of undiluted challenge were given orally to each dog 14 days after the last vaccination. Food was withheld from the dogs for 24 hours prior to challenge and the dogs were fed immediately after challenge. Each dog was housed in a separate cage to facilitate the evaluation and collection of feces.

Evaluation of CPV Infection

Rectal temperatures and total and differential leukocyte counts were obtained from 3 days prior to challenge to 14 days postchallenge. Fecal samples were collected on the day of challenge and each day thereafter for CPV hemagglutination testing; the physical appearance of the feces was recorded daily. Sera were collected on the

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[1]Obtained from Norden Laboratories, Lincoln, Nebraska.

day of challenge and antibody levels determined as previously described.

Leukocyte Counts

Total leukocyte counts were determined using a semi-automated electronic cell counter.[1] Duplicate counts were obtained for each sample. If the counts did not exceed the 3% error of the instrument, the 2 counts were averaged and corrected for coincidence. If the difference between the 2 counts was greater than 3%, the reading were disregarded and a new set of 2 counts was obtained.

Differential leukocyte counts were conducted on stained smears.[2] The absolute lymphocyte counts were calculated by multiplying the number of lymphocytes counted in 100 cells by the corrected total leukocyte count and dividing by 100.

HA testing of feces

Viral shedding was evaluated quantitatively by determining the amount of viral hemagglutinin present in the feces using a modification of the technique described by Carmichael et al, (1980). Two- to 3-gram samples of fecal

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[1]Coulter Counter Model ZB1, Coulter Electronics Inc., Hialeah, Florida.

[2]Wright's Stain, Fisher Scientific Company, Fair Lawn, New Jersey.

material were mixed with 5 ml of Dulbecco's phosphate buffered saline. The suspended samples were centrifuged at 1500 X g for 25 minutes, then 2 ml of the supernate from each sample was vigorously mixed with 0.2 ml chloroform. This chloroform treated sample was centrifuged at 1500 X g for 15 minutes. The supernate was removed and frozen at -70°C until tested for HA activity.

The HA test was conducted in 96-well U-bottom microtiter plates. Dulbecco's phosphate buffered saline, pH 7.0, with 0.5% bovine serum albumin was used as a diluent, and prewashed pig erythrocytes, 1.0% in the above diluent, were the agglutinated cells. Each fecal sample was titrated in duplicate with 1 titration series being incubated for 30 minutes at room temperature with CPV antiserum capable of neutralizing 2048 HA units and the other incubated with normal serum prior to making 2-fold dilutions. After the erythrocytes were added, plates were incubated at 4°C overnight. The highest dilution at which HA was detected was considered the endpoint titer. At least a 16-fold reduction in titer must have occurred after incubation with CPV antiserum before the HA was considered specific.

RESULTS AND DISCUSSION

Seronegative Control Dogs

Fifteen seronegative control dogs, in 3 groups of 5 dogs each, were given the challenge preparation. The highest daily rectal temperature obtained and the largest daily percent lymphocyte drop are presented in Table 6. In 11 of the 15 dogs, these 2 events occurred on the same day indicating they were not random fluctuations. Based on these results, a febrile response of $\geq 39.7^{\circ}C$ and a lymphopenia of $\geq 50\%$ of the prechallenge normal were considered criteria of significant CPV infection that could be reproduced by the challenge preparation at least 80% of the time.

The clinical signs of enteritis observed after administration of the challenge preparation varied considerably from dog to dog (Table 7). Some dogs had no signs or only a transient loose pancake-like stool while others developed a pronounced diarrhea accompanied by copious amounts of light brown mucus and frank blood. No enteric signs were noted prior to day 5 postchallenge except with dog 9261 which had a loose stool on days 1, 3, and 4 postchallenge. Vomiting was not a consistent observation, and was occasionally noted prior to challenge. Similarly, loose pancake-like stools were observed at times other than the postchallenge observation period. For this reason,

Table 6. Highest febrile response and lymphopenia in seronegative control dogs inoculated with CPV

Dog number	Temperatures °C	Percent lymphopenia
CI41	39.8 (5)[a]	82[b] (5)
CI45	39.8 (5,6)	79 (5)
CI46	39.7 (5)	78 (5)
CI49	40.6 (6)	79 (5)
CI50	41.0 (5)	59 (5)
9256	39.8 (5)	67 (5)
9261	39.3 (6)	71 (5)
9285	39.9 (6)	48 (5)
9251	39.9 (5)	72 (5)
9270	39.7 (6)	71 (6)
CJ94	40.0 (5)	63 (5)
CJ85	39.6 (3)	64 (7)
CK01	39.7 (7)	43 (7)
CJ90	39.9 (5)	58 (5)
CJ83	40.0 (6)	50 (6)

[a]Day postchallenge that highest temperature or percent lymphopenia was observed is given in parenthesis.

[b]Percent reduction in absolute lymphocyte count from prechallenge normal level.

Table 7. Clinical signs of enteritis[a] in seronegative control dogs inoculated with CPV

Dog number	Day postchallenge									
	5	6	7	8	9	10	11	12	13	14
CI41										
CI45		D	MBD	BD	L	L				
CI46		L	L	D						
CI49				L			L			
CI50				L	L		L			
9256		V	MBD	BL	L	L		L	L	
9261	L	L	L	L	L	L	D	L	L	
9285		L	DV	BD	D	LMB				
9251				DB	LMB	LMB	L			
9270				M	L	L	V			
CJ94	L		VLM	D	L			L		V
CJ85	L			L						
CK01			LMB	LMD	L	LMB			L	
CJ90		MB			MD	V				M
CJ83										V

[a]Clinical signs of enteritis were reported as follows: D = diarrhea; M = mucus in feces; B = blood in feces; L = loose pancake-like stool; V = vomitus.

neither vomiting nor the presence of a loose stool were considered as definitive signs of CPV infection. Those signs that were attributed to the challenge inoculum were diarrhea, defined as feces that flowed down the inclined pan on the bottom of the cage, or the presence of mucus or frank blood in the feces.

None of the fecal samples collected prior to day 4 or after day 8 postchallenge had HA titers above 1:16. The low HA titers that were seen were not reduced by incubation with CPV antiserum. Therefore, any HA activity seen prior to day 4 or after day 8 was considered nonspecific. Those titers for the 3 groups of controls that were considered the result of CPV infection are presented in Table 8. Hemagglutination titers of >1:64 were obtained in 14 of 15 dogs. Dog CJ94 developed an HA titer of 1:64, 8 days postchallenge, but the titer was only reduced to 1:16 by incubation with antiserum. Although the 1:64 titer was probably due to the presence of CPV, the agglutination did not meet the criterion of a 16-fold reduction in the presence of antiserum and was thus considered to be nonspecific.

Based on the results obtained with the challenge preparation of CPV in seronegative controls, 4 criteria of significant infection were established. They are: (1) rectal temperature $\geq$39.7°C, (2) lymphopenia of $\geq$50% of the prechallenge normal, (3) presence of enteric signs of

Table 8. Canine parvovirus hemagglutinin activity in feces of seronegative control dogs inoculated with CPV

Dog number	Day postchallenge				
	4	5	6	7	8
CI41	...[a]	2048[b]	≥4096	2048	...
CI45	2048	≥4096	≥4096	≥4096	≥4096
CI46	...	≥4096	≥4096	...	...
CI49	128	≥4096	≥4096	1024	...
CI50	...	...	≥4096	≥4096	...
9256	64	≥4096	≥4096	1024	...
9261	...	64	512	64	...
9285	...	1024	256	512	...
9251	128	512	≥4096	≥4096	...
9270	...	...	...	256	≥4096
CJ94	...	...	...	...	...
CJ85	...	...	≥4096	512	...
CK01	...	...	128	64	256
CJ90	...	512	≥4096	...	...
CK83	...	...	128	256	...

[a]No specific hemagglutination detected.

[b]Reciprocal of the highest dilution at which hemagglutination was detected.

illness which were limited to diarrhea or blood or mucus in feces, and (4) presence of CPV hemagglutinin in the feces at a level of $\geq$1:64. The occurrence of these 4 criteria in the 15 seronegative control dogs is summarized in Table 9. The challenge preparation was considered capable of producing at least 3 of the 4 criteria in at least 80% of the controls, and this then was established as the minimum acceptable challenge level for a valid vaccine immunogenicity test.

Modified-Live FPV Vaccines

The initial vaccine trial in dogs was conducted with a serial of modified-live FPV vaccine diluted to a level that would be minimally acceptable for cats. The diluted vaccine had a viral titer of $10^{3.1}$ $TCID_{50}$/dose. Fourteen days after a single vaccination only 2 of 5 vaccinated dogs developed measurable antibody levels (1:3 and 1:4). The immunity of the vaccinates was challenged at that time and all dogs developed at least 2 of the 4 criteria of CPV infection. These results indicate that a vaccine that met the minimum acceptable titer for immunizing cats did not contain sufficient antigenic mass initially, nor was it able to induce sufficient viral replication after inoculation, to immunize dogs.

Undiluted vaccines were also evaluated in dogs (Table 10). The vaccines were produced by 3 different manufacturers,

Table 9. Presence of challenge criteria in seronegative control dogs inoculated with CPV

Group	Dog number	Criteria Present[a]			
I	CI41	T	L		H
	CI45	T	L	C	H
	CI46	T	L	C	H
	CI49	T	L		H
	CI50	T	L		H
II	9256	T	L	C	H
	9261		L	C	H
	9285	T		C	H
	9251	T	L	C	H
	9270	T	L	C	H
III	CJ94	T	L	C	
	CJ85		L		H
	CK01	T		C	H
	CJ90	T	L	C	H
	CJ83	T	L		H

[a]Criteria present reported as follows: T = temperature $\geq 39.7^{\circ}C$; L = lymphopenia of $\geq 50\%$ of prechallenge normal; C = clinical signs of enteritis which were limited to diarrhea or blood or mucus in feces; H = hemagglutination titer $\geq 1:64$ in feces.

Table 10. Serological and CPV challenge results in dogs vaccinated with modified-live FPV vaccine

Vaccine	Titer	Dog number	Serology[a] One dose	Serology[a] Two doses	Results of challenge[b]
Firm A	$10^{5.2}$[c]	9281	<2	7	T L C H
Serial 2	128[d]	9252	≥32	≥243	Protected
		9253	3	2	L C H
		9288	8	≥243	Protected
		9283	2	2	T L C H
Firm B	$10^{5.3}$	CP12	<3	3	Protected
Serial 1	128	CP13	<3	21	Protected
		C096	5	47	Protected
		C097	7	36	Protected

[a]Reciprocal serum dilution that neutralized 100-300 $TCID_{50}$ of canine parvovirus.

[b]Results of challenge reported as follows: T = temperature ≥39.7°C; L = lymphopenia of ≥50% of prechallenge normal; C = clinical signs of enteritis which were limited to diarrhea or blood or mucus in feces, H = hemagglutination titer ≥1:64 in feces.

[c]Infectivity titer expressed in $TCID_{50}$/dose.

[d]Hemagglutination titer.

Table 10 (Continued)

Vaccine	Titer	Dog number	Serology[a] One dose	Serology[a] Two doses	Results of challenge[b]
Firm B	$10^{5.0}$	CM67	7	47	NC[e]
Serial 2	512	CM82	9	≥140	NC
		CM70	36	≥140	NC
		CM76	≥140	625	NC
Firm C	$10^{5.5}$	CM80	81	NA[f]	NC
	2048	CM71	47	NA	NC
		CM69	47	NA	NC
		CM75	≥107	NA	NC

[e]Not challenged.

[f]Not applicable.

and although the viral titers were comparable ($10^{5.0}$ to $10^{5.5}$ $TCID_{50}$/dose) there were greater differences in the HA titers of the vaccines (128 to 2048). The serological responses and subsequent protection from immunity challenge elicited by the vaccines appeared to correlate better with the HA titers than with the infectivity titers. For example, with the 2 serials from firm B, the better serological responses were seen with serial 2, the serial with the higher HA titer but the lower infectivity titer.

As data were evaluated from vaccination-challenge trials, it became apparent that as the antibody titer increased, the likelihood of protection from the effects of the challenge preparation also increased (Table 11). Since 1:11 was the highest antibody level shown to be non-protective, it was decided not to challenge the dogs vaccinated with serial 2 from firm B, or the serial from firm C. The lowest individual antibody titer induced by each vaccine was 1:47.

The possibility of a rectal temperature of $\geq 39.7^{o}C$ occurring by chance instead of as a result of the challenge exists and was seen in 3 of 20 vaccinates that were otherwise considered protected. The 20 dogs did not develope a lymphopenia of $\geq 50\%$ of the prechallenge normal, fecal hemagglutinin $\geq 1:64$, or clinical signs of enteritis following challenge. The 3 dogs with elevated temperatures were

Table 11. Comparison of prechallenge serological titers and the effects of CPV challenge

Prechallenge serology[a]	Results of challenge[b]			
<2	T			H
<2	T	L	C	H
<2	T	L	C	H
<2		L	C	H
<2		Protected		
<2			C	H
2	T		C	H
2	T	L	C	H
3		Protected		
3			C	H
3		Protected		
4			C	H
4	T		C	H
7	T		C	H
8		Protected		

[a]Reciprocal serum dilution that neutralized 100-300 $TCID_{50}$ of canine parvovirus.

[b]Results of challenge reported as follows: T = temperature $\geq 39.7^{\circ}C$; L = lymphopenia of $\geq 50\%$ of prechallenge normal; C = clinical signs of enteritis which were limited to diarrhea or blood or mucus in feces. H = hemagglutination titer $\geq 1:64$ in feces.

Table 11 (Continued)

Prechallenge serology	Results of challenge
10	Protected
11	L C
12	Protected
12	Protected
14	Protected
16	Protected
21	Protected
23	Protected
23	Protected
36	Protected
47	Protected
62	Protected
107	Protected
107	Protected
243	Protected
≥243	Protected
≥243	Protected

excitable animals that showed similar temperatures during the 3 days prior to challenge. Although none of the 20 vaccinates considered protected had lymphopenias of ≥50% of the prechallenge normal (mean maximum lymphopenia 31 ± 12%) 4 dogs had lymphopenias of >45%. To what extent viral replication in these clinically protected dogs may have influenced temperatures and lymphocyte counts is not known, but since elevated temperatures can occur in excited dogs, and fluctuations in lymphocyte counts of ≥50% seem possible, it was decided to consider a dog protected unless at least 2 criteria of significant parvovirus infection were present.

Inactivated FPV Vaccines

Preinactivation titers of the killed FPV vaccines evaluated in dogs were not available. The HA titers of the vaccines correlated positively with the serological responses and immunity to CPV infection produced by the vaccines. The results (Table 12) are ranked by ascending HA titer. Although the vaccine from firm X did not protect dogs with 2 doses, the manufacturer had previously demonstrated that 1 dose of vaccine would immunize cats against feline panleukopenia. It therefore appears that the minimum antigenic mass necessary to immunize dogs is greater than the amount necessary to immunize cats.

The dogs vaccinated with serial 2 from firm Z were not immunity challenged because the high neutralizing antibody

levels produced indicated protection (see above). These dogs were maintained in isolation to determine the duration of immunity produced by the vaccine. The antibody titers obtained at monthly intervals dropped sharply, and by 2 months after the second dose, the geometric mean antibody titer was only 1:169. After the initial drop, the antibody titers remained fairly constant (fluctuating within a 3-fold range). The geometric mean antibody titer 8 months after the second dose was 1:207. Control dogs from the same litter housed with the vaccinated dogs remained seronegative, and in vitro testing of the vaccine used to immunize the dogs failed to detect any viable virus.

Table 12. Serological and CPV challenge results in dogs vaccinated with inactivated FPV vaccine

Vaccine	HA titer	Dog number	Serology[a]		Results of challenge[b]
			One dose	Two doses	
Firm X	2	CJ87	<3	4	L H
		CK02	<3	2	L H
		CJ84	<3	<2	Protected
		CJ93	7	7	T L H
		CJ98	3	2	L H
Firm Y	128	CJ92	<3	10	Protected
Serial 1		CJ91	<3	8	Protected
		CJ95	4	23	Protected
		CJ96	<3	14	Protected
		CJ82	<3	3	Protected
Firm Z	512	CP15	4	12	Protected
Serial 1		CP16	<3	16	Protected
		C098	4	12	Protected
		C099	<3	62	Protected

[a]Reciprocal serum dilution that neutralized 100-300 $TCID_{50}$ of canine parvovirus.

[b]Results of challenge reported as follows: T = temperature $\geq$39.7°C; L = lymphopenia of $\geq$50% of prechallenge normal; C = clinical signs of enteritis which were limited to diarrhea or blood or mucus in feces, H = hemagglutination titer $\geq$1:64 in feces.

Table 12 (Continued)

Vaccine	HA titer	Dog number	Serology[a]		Results of challenge[b]
			One dose	Two doses	
Firm Y	1024	9282	5	23	Protected
Serial 2		9280	4	11	L C
		9260	32	243	Protected
		9286	14	107	Protected
		9250	>32	107	Protected
Firm Z	2048	14	280	≥925	NC
Serial 2		10	187	≥1398	NC
		7	187	≥1398	NC
		13	124	≥935	NC

GENERAL DISCUSSION

The establishment of the 4 criteria of significance for signs of experimental CPV infection made it possible to objectively evaluate the efficacy of FPV vaccines for CPV. It was demonstrated that when sufficient antigenic mass, as measured by the HA test, was present in either a MLV or KV vaccine, the vaccine induced protection against clinical CPV.

Whether a vaccine was capable of preventing infection by CPV was not established in this study. Other workers have indicated that HI titers of 1:256 (Smith et al., 1980) and 1:80 (Carmichael and Pollock, 1981) were necessary to prevent viral infection. Since the SN test has been shown to be more sensitive than the HI test (Pollock and Carmichael, 1982), it is doubtful that an SN titer of 1:12, which appeared in this study to be protective against the clinical manifestations of CPV, would actually prevent infection.

Presently, a serial of KV vaccine is considered satisfactory if 4 of 4 vaccinated dogs are protected from immunity challenge. Protection is defined as the presence of not more than 1 of the criteria of infection following exposure to a challenge that produces at least 3 of the 4 criteria in 80% of the susceptible control dogs. Early in the evaluation of vaccines for CPV, it became apparent that

there was a strong positive relationship between increasing antibody titers and protection from challenge. In cats, a neutralizing antibody titer of 1:4 is considered protective by the USDA (Standard requirements, 1981) and a serial of inactivated FPV vaccine that can generate neutralizing antibody titers of at least 1:8 in 3 of 4 vaccinates and not less than 1:4 in the remaining vaccinate is considered satisfactory for release without immunity challenge of the vaccinates. From the data generated by this study, it appears that a similar serological test can be instituted for KV vaccines for CPV. If a serial of vaccine can produce neutralizing antibody titers of at least 1:16 in 3 of 4 vaccinated dogs while the remaining vaccinate develops an antibody titer of at least 1:8, the serial could be considered satisfactory for release without immunity challenge of the vaccinates. A seronegative dog housed with the vaccinates would be required to remain free of antibody as a control against postvaccinal exposure to CPV.

An even better test for KV vaccines would be to release serials on their HA titer. The minimum post-inactivation HA titer necessary to immunize dogs could be determined in a large number of dogs to assure accuracy (20 vaccinates and 5 controls). Once the minimum HA titer is established, each subsequent serial of vaccine must have an HA titer equal to or in excess of the minimum level in order

to be considered satisfactory. Depending upon the reproducibility of the HA test, a reference preparation with an HA titer equal to the HA titer of the vaccine tested in dogs may have to be run simultaneously with the serial of vaccine under test.

It has been reported that FPV vaccine replicates in the dog, often producing high persistent antibody titers (Appel et al., 1980a). This might explain the extreme variability of the immune response seen with serial 2 from firm A. In the 2 dogs with high antibody titers, the virus apparently replicated. In the other 3 dogs, there appeared to be no viral replication and the antigenic mass present in the vaccine was not sufficient to produce immunity.

Although the duration of the antibody responses was not compared, the titers generated by the modified-live FPV vaccines used in this study were similar to those seen with the inactivated FPV vaccines. This is particulary true when vaccines with the same HA titers are compared. For this reason, it is felt that in a large proportion of dogs, modified-live FPV vaccines do not replicate, but act instead like KV vaccines.

It has been suggested by Carmichael and Pollock (1981) that the percentage of dogs in which FPV replication occurs can be increased by increasing the infectivity titer of the vaccine. In that study, 8 of 8 dogs given $10^{7.5}$ $TCID_{50}$ of

FPV, 5 of 8 dogs given $10^{5.5}$ $TCID_{50}$ of FPV, and 3 of 8 dogs given $10^{3.5}$ $TCID_{50}$ of FPV developed HI antibody titers of $\geq$1:80, the level the authors considered protective. However, since antibody titers of this level are possible after a single dose of inactivated FPV vaccine (Appel et al., 1979), the higher antibody titers seen with the higher titered vaccines may have been due to an increase in antigenic mass and not an increase in the number of dogs in which viral replication occurred. Carmichael and Pollock also stated that when viral replication does occur, the resulting antibody titers will persist longer. However, in the study cited, the persistence of the antibody response was not determined, and therefore, an increase in viral replication with an increase in infectivity titer was not proven.

Presently, a serial of modified-live FPV vaccine is considered satisfactory for marketing if its viral titer is at least 0.7 $\log_{10}$ above that required to induce protection from CPV challenge in at least 19 of 20 dogs with protection being defined as it was for KV vaccines. If viral replication can not be guaranteed, it seems more practical to consider a serial of vaccine satisfactory for marketing based on its total antigenic mass and not just its infectivity titer. With parvoviruses, the ability to agglutinate erythrocytes is a characteristic of mature infective virions, incomplete particles, and empty capsids,

all of which are antigenic (Siegl, 1976). The HA test would thus appear to be a reliable test for antigenic mass. Additional testing will determine its usefulness as an *in vitro* tests for release for marketing of serial lots of MLV as well as KV vaccines.

SUMMARY

In this study, methods of differentiating CPV, FPV, and MEV were examined and of the methods tested, *in vitro* species susceptibility was shown to be the most reliable. A procedure was proposed in which an unidentified virus would be titrated simultaneously in Crandell feline kidney, mink lung, ferret lung, and canine origin A-72 cells. Canine parvovirus should titer well in CRFK and A-72 cells; FPV should titer high only in CRFK cells; and MEV should titer high in CRFK cells and the titration results in mink lung cells should be greater than the results in ferret lung cells. It is this multiple titration system that is being used at the National Veterinary Services Laboratories, Ames, Iowa, for virus identification.

The close serological relationship between CPV and FPV suggested that existing FPV vaccines could be used to immunize dogs against the effects of CPV. A vaccination-challenge procedure was established to evaluate the efficacy of FPV vaccines. The results indicated that FPV vaccines could, indeed, be used to protect dogs against CPV enteritis, but the minimum effective dose for dogs was higher than the minumum effective dose for cats. It was also shown that the infectivity titer of a serial of MLV vaccine was less important than its total antigenic mass, as measured by the HA test, in predicting its efficacy. Minimum standard

requirements that utilized the HA test were proposed for FPV vaccines intended for use in dogs.

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