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CANINE PARVOVIRUS - LABORATORY DIAGNOSIS AND BIOLOGICAL CHARACTERISTICS

By

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ABSTRACT

CANINE PARVOVIRUS - LABORATORY DIAGNOSIS
AND BIOLOGICAL CHARACTERISTICS

By

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Presumptive hemagglutination (HA) tests and virus isolation procedures were used to examine 150 fecal and/or intestinal preparations for the presence of canine parvovirus (CPV). Samples with HA activity ≥1:32 were almost always positive for CPV isolation, and only a few preparations exhibited nonspecific agglutination of RBC. Virus isolation was more sensitive than HA, as evidenced by the recovery of CPV from 8 specimens with no demonstrable HA activity. Results of fluorescent antibody tests compared favorably with results of virus isolation and HA tests.

Canine parvovirus agglutinated erythrocytes of pig, rhesus monkey, cat, and horse, and the virus grew in cell cultures derived from cat, dog, cow, goat, monkey, and human. Replication of CPV was not enhanced by the presence of adenoviruses.

DEDICATED TO MY WIFE

JOY

AND TO MY CHILDREN

CURT AND LOURI

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Classification and Nomenclature

Parvoviridae is a family of viruses comprised of two recognized genera, Parvovirus and Densovirus, and an adeno-associated virus (AAV) group to which no genus name has been assigned (76). Virions are nonenveloped, isometric particles with a diameter of 20-30 nm and are unique in that the genome is single stranded (SS) DNA. A summary of physicochemical characteristics of Parvoviridae is provided in Table 1.

Parvoviruses and AAV are viruses of vertebrates and are placed in separate genera on the basis of the latter's inability to replicate without aid of helper viruses. The Densovirus genus includes those viruses infecting arthropods; their replication, like that of the Parvovirus genus, occurs autonomously. Further distinguishing characteristics of the genera are based on type of DNA which is encapsidated. Parvoviruses incorporate only one type of SS DNA (- strand), whereas the DNA encapsidated by individual virions of AAV and densoviruses are complementary (+ or - strand) and will, upon extraction, anneal spontaneously to form double-stranded products.

Numerous viruses isolated from a variety of sources are now considered to belong to the Parvoviridae family. These are listed in Table 2.

Table 1. Physicochemical characteristics of Parvoviridae

	Characteristic
Size (nm)	20-30
Symmetry	icosahedral
Capsomers	32?
Buoyant density	1.38-1.46 g/cm^3
Nucleic acid	
Type	SS DNA
Molecular weight	$1.5\text{-}2.0 \times 10^6$
g+c Content	41-53%
Infectivity	
Ether stable	yes
Heat stable	yes
Acid stable	yes

Table 2. Parvoviridae - member viruses

Parvoviruses	Source of Isolation	Reference
1. Genus: Parvovirus Type Species: Parvovirus r-1 (rat virus)		
Recognized Members		
Rat virus (RV)	rat liver sarcomas and transplantable leukemia	66
Antigenically related viruses		
H-3	human Hep-3 transplantable tumor	31
X-14	X-ray and MC-treated rats	88
L-S (Lum-Schreiner virus)	rat choroleukemic tumor	73
HER (hemorrhagic encephalopathy virus of rats)	CNS of cyclophosphamide-treated rats	36
KIRK	human Detroit-6 tumor cells	21
H-1	human HEP-1 transplantable tumor	114
Antigenically related viruses		
HT	human placentas and embryos	113
Minute virus of mice (MVM)	mouse adenovirus stock	29
Porcine parvovirus (PPV)	hog cholera virus stock	81
Antigenically related viruses		
KBSH	human KB tumor cells	44
Bovine parvovirus (BPV-Haden virus)	calf feces	1
Feline parvovirus (feline panleukopenia - FPL)	leopard spleen	57
Antigenically related viruses		
Mink enteritis virus (MEV)	mink liver and spleen	118
TVX	human tumor cell lines	44
LuIII	human LU106 cell line	44
RTV	rat AT cell line	44
Probable Members		
*Canine parvovirus (minute virus of canines - MVC)	dog feces	17
Goose hepatitis virus (GHV)	gosling liver and heart	67

Table 2 (continued)

Parvoviruses	Source of Isolation	Reference
Hepatitis A virus of man		77
Gastroenteritis virus of man		32
*Canine parvovirus (CPV)	dog feces	54
Aleutian mink disease	mink spleen	89
HB	human placentas and tumors	113
Lapine parvovirus	rabbit feces	75
2. Genus: Densovirus Type Species: Densonucleosis virus (galleria)		
Densonucleosis virus	*Galleria mellonella* larvae	84
Junonia virus	*Junonia colnis* larvae	64
3. Genus: Adeno-Associated Virus (AAV) Type Species: AAV type 1		
Recognized Members		
AAV type 1	simian virus 15 stock	7
AAV type 2	human adenovirus 12 stock	49
AAV type 3	human adenovirus 7 stock	49
AAV type 4	simian virus 15 stock	87
Bovine AAV (X7)	bovine adenovirus 1 stock	72
Avian AAV	quail bronchitis virus stock	35
Probable Members		
Canine AAV	canine hepatitis virus stock	33
Equine AAV	equine adenovirus stock	34

*The canine parvovirus (CPV) associated with the recent worldwide occurrence of enteritis and myocarditis in dogs is not related immunologically to minute virus of canines (MVC) but is related to feline panleukopenia virus (L. N. Binn, personal communication).

History of Isolations

Kilham and Oliver in 1959 (66) described the first partial characterization of a parvovirus. While attempting to demonstrate oncogenic properties of papovaviruses, they recovered from tumorous tissue a virus which produced cytopathic effects (CPE) in rat embryonic cultures and agglutinated guinea pig erythrocytes. The virus was referred to as rat virus (RV). In 1960, Toolan et al. (114) isolated a virus from a human transplantable tumor (Hep-1) and designated the virus H-1. When inoculated into young hamsters, the virus caused a variety of malformations, including dwarfism, flat face, domed head, protruding eyes, and fragile bones. In subsequent studies, Toolan (113) was able to isolate H-1 virus from hamster neonates inoculated with emulsions of various tissues of cancer patients and human embryos. He also recovered another parvovirus (HT), which was serologically related to H-1, and an additional virus (HB), which was unrelated serologically to H-1 and RV. Additional rodent parvoviruses were isolated (21,31,36,44,73,88) and, owing to their similar effects when inoculated into young hamsters, these viruses were often referred to as hamster osteolytic viruses.

Atchison et al. (7) isolated a 20- to 25-nm agent from stock simian adenovirus (SV-15) and proposed the name adenovirus-associated virus (AAV). Although other investigators (5,23) had previously noted by electron microscopy small particles associated with adenoviruses, Atchison and co-workers clearly demonstrated that these particles were distinct viruses unrelated immunologically and structurally to adenoviruses. They also demonstrated that the viruses contained DNA and were dependent on adenoviruses for replication, although replication was not limited to a particular adenovirus. Subsequently, it was shown that AAV of human and simian origin represented 4 serotypes (48). In

addition, AAV of avian, bovine, canine, and equine were described (33,34,35,72).

Although Johnson and Cruickshank (59) were first to present evidence that feline panleukopenia virus (FPV) was a parvovirus, the disease which it causes was known as early as 1900. It was thought to be caused by a virus after Verge and Christoferoni (116), in 1928, were able to produce the disease in healthy cats with bacteria-free filtrates prepared from eulsified organs of diseased cats. Later, Lawrence et al. (68) and Lucas and Riser (71) demonstrated that a variety of clinical syndromes, including agranulocytosis and feline distemper, were associated with the same virus. It was noted by Schofield (101) in 1949 and Wills (118) in 1952 that serious epizootics of enteritis which occurred in commercially raised mink in Canada exhibited clinical features similar to those observed with FPV. Subsequently, the virus was isolated and a close immunological relationship between FPV and mink enteritis virus was observed (58).

In 1961, Abinanti and Warfield (1) isolated a virus from the intestinal tract of normal calves. The virus grew in cell cultures of bovine embryonic kidney and agglutinated erythrocytes of guinea pigs and humans. Infected cell cultures were also noted to adsorb erythrocytes of these species, prompting Abinanti and Warfield to refer to the previously undescribed virus as hemadsorbing enteric (HADEN) virus of calves. Data obtained by Storz and Warren (117) and Bachmann (8) permitted the classification of HADEN virus as bovine parvovirus.

The electron microscopic observation of viral particles similar to Kilham rat virus in hog cholera infected pig kidney cell cultures was noted by Mahnel (74) in 1965. Cartwright and Huck (26) recovered a small DNA-containing virus from aborted and stillborn piglets, and

Mayr and co-workers (80) isolated similar viruses from "normal" cell cultures derived from pig kidneys. These latter investigators observed that these viruses shared many properties with viruses of the parvovirus group. Later, Cartwright et al. (27) suggested that the viruses described by Mayr et al., as well as those isolated by other investigators (18,51),were identical or at least immunologically closely related to the virus which she and Huck had earlier isolated.

Binn and co-workers (17) in 1967 reported the isolation of a small virus from feces of asymptomatic dogs. The virus was referred to as "minute virus of canines" (MVC), and in 1970 Binn et al. (18) provided evidence for classification of the agent as a parvovirus. The virus was difficult to cultivate and only agglutinated erythrocytes of rhesus and African green monkeys. Specific antibodies determined by hemagglutination inhibition tests were found in approximately 70% of dogs examined. In 1977, Eugster and Nairn (38) observed, by electron microscopy, parvoviruses in the feces of young puppies with diarrhea. No other clinical signs were present, and recovery was uneventful. Beginning in mid-1978, a severe gastroenteritis associated with parvovirus was described (2). Subsequent reports (3,4,19,28,37,40,41,55, 60,65,86,109) revealed the disease to be of pandemic proportions. Also, a fatal myocarditis associated with the virus was noted (24,47, 50,55,56,70,86,112). The canine parvoviruses (CPV) associated with enteritis and myocarditis were identical or at least closely related immunologically but were distinct from MCV (T. N. Binn, personal communication). Neither MCV nor CPV were related to canine adeno-associated virus, but CPV was closely related immunologically to feline panleukopenia virus (T. N. Binn, personal communication).

A virus associated with Aleutian disease of mink was recently isolated (89) and shown to have properties consistent with parvoviruses. The virus (ADV) could only be propagated on initial isolation at a reduced temperature of 31.8 C. After several subcultures at this temperature, optimum temperature for replication was 37 C. Shahrabadi and co-workers (103), using ADV purified from tissues of infected mink, provided evidence that the DNA was single stranded.

Matsunaga et al. (75) isolated and characterized a parvovirus from feces of rabbits. The virus grew in rabbit kidney cell cultures and agglutinated human group O erythrocytes at 4 C. No relationship to KRV was observed. The virus was not associated with disease, and a high proportion of commercially obtained rabbits exhibited antibody.

Virus Replication

DNA Structure

The DNA extracted from several autonomous parvoviruses was shown to be single stranded (SS) by formaldehyde reaction (30,99,115). As a result of hydrogen bonding between base pairs, double stranded (DS) DNA lacks free amino groups. On the other hand, free amino groups exist in SS DNA and react with formaldehyde resulting in an increase of absorption at 260 nm. Nearest neighbor analysis was used by Salzman et al. (100) and McGeoch and co-workers (82) to confirm the SS nature of H-1 and MVM viral DNA, and Mayor and Melnick (78) showed with acridine orange staining that the DNA of X14 was single stranded. Unequal ratios of A-T and G-C also indicated that DNA of autonomous parvoviruses was single stranded (98).

Originally, a controversy existed over the type of DNA present in the defective parvoviruses (AAV). Rose et al. (95) extracted DNA from

AAV and found evidence of DS DNA, but acridine orange staining performed by other investigators (12,78) indicated that the DNA was SS both inside cells and in intact virions. The controversy was resolved when Crawford et al. (30) proposed and Rose and co-workers (93) confirmed with density shift experiments that AAV contained two types of DNA, either + or - strands, which annealed after extraction to form DS products. The density shift experiment was conducted by growing virus in the presence of bromodeoxyuridine (Budr), which substitutes for thymidine in DNA, thus making the DNA more dense in cesium chloride (CsCl). When the DNA was extracted from such virions and mixed with DNA obtained from virions grown in the absence of Budr, hybrid density molecules were observed in CsCl gradients. Subsequently, Berns and Rose (16) were able to separate + and - strands by similar means. They grew virus in the presence of Budr and extracted DNA, which was then subjected to conditions to minimize annealing; heavy and light bands were noted in CsCl gradients. Analysis of the base composition of the DNA revealed the two strands to be of unequal thymidine content. The - strand contained approximately 27%, whereas the + strand contained about 21% (94). Berns and Adler (13) used a less cumbersome method to isolate + and - strands by separating intact virions (grown in the presence of Budr) with CsCl gradients and then extracting the DNA. More recently, agarose-acrylamide gels were used to separate + and - strands directly from denatured DNA (46).

DNA configuration in mature virions of both autonomous parvoviruses and AAV was determined to be linear. Salzman et al. (100) used exonuclease I and electron microscopic (EM) examination to verify the linear nature of KRV, and EM examination was used to determine linearity of DNA extracted from MVM and Lu III (30,104). Gerry et al. (42) demonstrated the linear nature of AAV DNA. Although the DNAs of

parvoviruses and AAV are linear, both termini of viral strands are able to form duplex hairpin structures. The terminal sequences of AAV are repetitious, and it was suggested by Gerry et al. (42) to represent a palindrome of the type 122'1...122'1; evidence to support such a model was provided by Fife et al. (39). Autonomous parvoviruses, unlike AAV, apparently lack terminal repetition but do contain sequences at both termini with properties of palindromes (22).

DNA Synthesis and Virus Reproduction

Berns and Hanswirth (14) suggested that these differences in terminal sequences of AAV and autonomous parvoviruses might account for replication and subsequent + or - strand encapsidation by AAV and only - strand by autonomous parvoviruses. It was suggested that DNA synthesis is primed by a 3'terminal hairpin structure and consequently AAV DNA would be expected to have identical termini in order for both strands to be synthesized equally. Since a distinction would need to be made for + and - strand synthesis with autonomous parvoviruses, their DNA would require nonidentical termini.

The precise mechanism by which autonomous parvoviruses replicate is unknown. They usually require rapidly dividing cells in order to replicate (105,110,111), and replication appears to be related to cellular DNA synthesis (9). Although Salzman (96) reported that KRV contained a virion associated DNA polymerase, this finding could not be confirmed by other investigators (9,91). Bates et al. (9) demonstrated a concomitant rise in viral infectivity of bovine parvovirus and cellular DNA polymerase α^* activity, suggesting that cellular DNA polymerase was involved in viral replication. They also noted a slight elevation of DNA polymerase β^*, but no increase in polymerase γ.*

*For nomenclature of eukaryotic DNA polymerases, see ref. 118.

Adeno-associated viruses are defective and require an adenovirus "helper" for productive infection (7). The nature of this helper function is unknown, but apparently it does not require adenovirus DNA synthesis, as DNA temperature sensitive mutants of adenovirus were also functional (54). Herpesviruses provide a partial helper function, permitting an abortive infection with AAV (6,20). The defect in herpesvirus helper function is believed to be at the level of DNA encapsidation (79).

The infectious cycle of autonomous parvoviruses and AAV appears to be essentially identical in the early stages. Adsorption is completed in approximately two hours for parvoviruses (11) as well as for AAV (15) and adsorption of AAV occurs without the aid of helper viruses (15). Also, AAV penetration to the cell nucleus and subsequent uncoating are unaffected by the absence of adenoviruses (15). AAV DNA will, in fact, at least in some instances, form a stable relationship within the cell and can be induced to form mature virions by subsequent infection with adenoviruses (45). This stable relationship was demonstrated to result from integration of the AAV genome into cellular DNA (45).

As previously mentioned, the cellular function responsible for autonomous parvovirus replication and the mechanisms by which adenoviruses aid AAV replication are unknown. Of interest was the observation that replication of some autonomous parvoviruses was stimulated by certain adenoviruses. Ledinko and Toolan (69) observed a pronounced effect with H-1 virus. They demonstrated that H-1 virus, which normally does not grow in human embryonic lung cells, does so in the presence of human adenovirus type 12.

Transcription and subsequent protein synthesis were reviewed by Carter (25) and Tattersall (108), respectively, and appear to be similar for both non-defective and defective parvoviruses. The RNA of both virus types undergoes several post-transcriptional modifications, including polyadenylation and methylation. The mRNA is small and can only specify one protein of approximately 100K (15). The three polypeptides that are evident in electrophoretic analysis of disrupted virions were shown to be related and presumably derived from a single translation product (61).

Pathogenesis of Parvoviruses

The *in vitro* observation that parvoviruses generally require rapidly dividing cells in order to replicate is consonant with their *in vivo* predilection for mitotically active tissues. As a consequence, these viruses have usually been recovered from the intestinal tract in adult animals. Most parvovirus infections in adult animals produce no overt clinical signs; exceptions are feline panleukopenia virus and canine parvovirus, both of which may produce a severe gastroenteritis in animals of all ages (2,63). Similarly, the "Norwalk" agent, which is probably a parvovirus, is associated with gastroenteritis in humans (32). Infection of pregnant animals may result in subsequent infection of the fetus, causing abortion and mummification, as evidenced strikingly by porcine parvovirus infection (83). The mongoloid deformities caused by hamster osteolytic viruses (114) inoculated into young hamsters result from virus replication in developing dental and skeletal tissues as well as in rapidly dividing cells of the cerebellar cortex. Likewise, spontaneous ataxia (cerebellar hypoplasia) associated with feline panleukopenia virus infection in young kittens is a result of viral

damage to the cerebellar cortex. The pathogenesis of the important parvoviruses is discussed more fully below.

Feline panleukopenia virus was initially recognized as a cause of feline infectious enteritis and was presumed to be of viral origin as early as 1928 (116). Later, the virus was associated with several other disease syndromes, including leukopenia, fetal death, and cerebellar ataxia. In acutely infected cats, the virus is present in saliva, feces, vomitus, and urine, and spread of the virus occurs by fomites and aerosols (43). Following infection, the virus is initially thought to replicate in pharyngeal lymphoid tissue (43), followed by a viremia and spread to secondary target organs. The organs invariably affected are those containing highly mitotically active cells such as lymph nodes, spleen, thymus, bone marrow, and Peyer's patches, as well as crypt cells of the intestinal mucosa (63). Viral damage to lymphoid tissues results in lymphopenia and neutropenia and may lead to mild immunodeficiency (102). Although absorptive cells of the resulting villi are not directly affected, extensive damage to crypt cells occurs. The inability of these damaged crypt cells to migrate and replace sloughed villous absorptive cells leads to malabsorptive disease (63). The enteritic form of the disease is less severe in germ-free cats and this is thought to be a result of depressed mitotic activity of intestinal crypt cells in the absence of bacterial flora (63). The pathogenesis of feline panleukopenia was recently reviewed by Kahn (63).

The clinical disease caused by canine parvovirus (CPV) is strikingly similar to the disease in cats caused by FPL (2). Enteritis with vomiting, diarrhea, and a marked leukopenia with resulting pathological changes similar to those observed with FPL suggest that the pathogenesis

of CPV is analogous to the pathogenesis of FPL. However, fetal infection and cerebellar hypoplasia have not been described for CPV. The myocarditis associated with CPV is difficult to explain, since myocardial cells have little or no mitotic activity. It has been suggested that changes in the myocardium may result from an autoimmune reaction and that infection with CPV may occur with the aid of a helper virus (90); however, the latter possibility seems unlikely, in that CPV was not influenced by canine adenoviruses *in vitro* (personal observation).

Porcine parvovirus causes no apparent clinical disease in young or adult animals but may result in reproductive failure in the sow or gilt (62). Transplacental infection occurs, and fetal mummification often results if the dam is infected before the 56th day of gestation (62,83), the time of fetal immune competence. Owing to the nature of the swine uterus, i.e., separate blood supply for each fetus, both infected and uninfected fetuses may be present in the same litter (62).

Although bovine parvovirus (BPV) has been isolated from the intestinal tract of calves on numerous occasions (1,10,52), its role in clinical disease has not been clearly defined. Storz and co-workers (106) administered a cell culture propagated strain of BPV orally and intravenously (IV) to young calves. Enteritis developed 24 to 48 hours after infection and was more severe in IV inoculated calves. Attempts to relate bovine parvoviruses to reproductive problems have generally been unsuccessful, although Moojen et al. (85) reported the electron microscopic observation of parvovirus-like agents in tissues of two aborted bovine fetuses.

Aleutian disease of mink is presently thought to be caused by a parvovirus (89). Infection is persistent, and the disease is characterized by plasmacytosis, highly elevated levels of immunoglobulins,

and the presence of circulating immune complexes. These circulating immune complexes result in glomerulonephritis and necrotizing arteritis. Severity of the disease can be alleviated by administering immunosuppressive drugs. Aleutian disease was reviewed by Ingram and Cho (53).

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ARTICLE

CANINE PARVOVIRUS

Laboratory Diagnosis and Biological Characteristics

to be submitted to

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SUMMARY

Presumptive hemagglutination (HA) tests and virus isolation procedures were used to examine 150 fecal and/or intestinal preparations for the presence of canine parvovirus (CPV). Samples with HA activity ≥1:32 were almost always positive for CPV isolation, and only a few preparations exhibited nonspecific agglutination of RBC. Virus isolation was more sensitive than HA, as evidenced by the recovery of CPV from 8 specimens with no demonstrable HA activity. Results of fluorescent antibody tests compared favorably with results of virus isolation and HA tests.

Canine parvovirus agglutinated erythrocytes of pig, rhesus monkey, cat, and horse, and the virus grew in cell cultures derived from cat, dog, cow, goat, monkey, and human. Replication of CPV was not enhanced by the presence of adenoviruses.

INTRODUCTION

Binn and co-workers (4) in 1967 reported the isolation of a small virus from feces of asymptomatic dogs. The virus was referred to as "minute virus of canines" (MVC), and in 1970 Binn et al. (5) provided evidence for classification of the agent as a parvovirus. The virus was difficult to cultivate and only agglutinated erythrocytes of rhesus and African green monkeys. Specific antibodies determined by hemagglutination inhibition tests were found in approximately 70% of dogs examined. In 1977, Eugster and Nairn (11) observed, by electron microscopy, parvoviruses in the feces of young puppies with diarrhea. No other clinical signs were present, and recovery was uneventful. Beginning in mid-1978, a severe gastroenteritis associated with parvovirus was described (1). Subsequent reports (2,3,6,10,11,13,14,17, 19,21,24,25) revealed the disease to be of pandemic proportions. Also, a fatal myocarditis associated with the virus was noted (9,15,16,17,18,22,24,26). The canine parvoviruses (CPV) associated with enteritis and myocarditis were identical or at least closely related immunologically but were distinct from MCV (T. N. Binn, personal communication). Neither MCV nor CPV were related to canine adeno-associated virus, but CPV was closely related immunologically to feline panleukopenia virus (T. N. Binn, personal communication).

At Michigan State University, we initially used a hemagglutination test (HA) directly on extracts of fecal material and intestinal contents to presumptively diagnose CPV infections. We later propagated the virus and prepared a fluorescent antibody (FA) conjugate which replaced our presumptive HA test. Electron microscopic (EM) examinations were performed on some cases. Occasional discrepancies between FA and EM examinations prompted the present report, in which we describe the

reexamination of 150 cases using isolation and HA procedures. Biological characteristics, including susceptibility of erythrocytes of different animal species to agglutination by CPV and the susceptibility of various cell cultures to infection with CPV, are also presented.

MATERIALS AND METHODS

Preparation of Specimens

An approximate 10% emulsion of feces or intestine was prepared with a mortar and pestle using balanced salt solution as diluent. As these preparations were used for both HA tests and viral isolation procedures, high concentrations of antibiotics (potassium penicillin-G, 1000 units/ml; polymyxin B sulfate, 500 units/ml; and streptomycin sulfate, 1000 µg/ml) were included in the diluent. Preparations were stored at -20 C and subsequently thawed and clarified by centrifugation at approximately 2000 x g for 5 min immediately before use.

Hemagglutination (HA) Tests

Porcine and rhesus monkey erythrocytes (RBC) were used routinely. Blood from pigs was collected in Alsever's solution and stored at 4 C; monkey RBCs were purchased commercially[a] as a 25% suspension in Alsever's solution. Two-fold dilutions (50 µl) of supernatant fluids from prepared specimens were made using standard microtiter procedures, and 50 µl of a cold 1% suspension of RBC was added to each well. Plates were shaken lightly to mix the contents and incubated at 4 C until controls settled, usually within 1 to 2 hours. The diluent used for making dilutions and preparing 1% RBC suspensions was 0.1% bovine serum

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*Microbiological Associates, Bethesda, MD.

albumin in 0.85% NaCl. Microtiter plates were 96 well flexible U plates,[b] and HA tests on each specimen were performed simultaneously with both pig and monkey RBC.

In subsequent comparative studies with selected isolates of CPV, RBCs from other animal species were used. The source of RBC and variations from the above procedure are reported in the results.

Virus Isolation

The cells used routinely for virus isolation were Crandell-Rees feline kidney cells (CRFK). Eagle's minimum essential medium with 0.5% lactalbumin hydrolysate, 1 mM sodium pyruvate, 2X concentration of non-essential amino acids, and 10% bovine fetal serum (BFS) was used for propagating the cells. Cells were seeded (1.5 ml) into 16 x 150 mm culture tubes containing coverglasses at a cell concentration of approximately 1.3-1.4 x 10^5 per ml or a split-ratio of 1 to 4. This concentration of cells resulted in about 80% confluency within 40 hours, at which time medium was replaced with fresh medium containing only 2% BFS. Freshly refed cells were inoculated with 0.2 ml of supernatant fluid from specimens prepared as described earlier. Two tubes were used for each specimen, and inoculated cells were incubated in a stationary position at 36 C and examined each day for cytopathic effects (CPE). Coverglasses were removed at day 2 and day 5 and stained with a fluorescent antibody conjugate prepared as described below. Positive cultures were frozen at day 7 or earlier if the CPE was severe.

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[*] Cooke Microtiter Plates, Microbiological Associates, Bethesda, MD.

Preparation of Hyperimmune Serum

Hyperimmune serum was prepared by inoculating a young adult New Zealand White rabbit with a cell culture isolate of CPV obtained from the intestine of a dog that died from gastroenteritis. The isolate (VC-13-79) had been subcultured 3 times in CRFK cells and had a HA titer of 1:4,096 per ml of cell culture fluid. One milliliter of a mixture of viral fluid and Freund's complete adjuvant[c] was administered at 2 subcutaneous sites and 1 intramuscular site. Two weeks later 0.5 ml of virus without adjuvant was administered intravenously. Serum collected at this time had a HA titer of 1:64 against 20 HA units of CPV. The HI titer was 1:2,048 5 days after the IV injection, and 50 ml of blood was collected via cardiac puncture. The rabbit was then humanely killed by injecting pentobarbital directly into the heart.

Preparation of FA Conjugate

Fluorescent antibody conjugate was prepared essentially as described elsewhere (7). Briefly, immunoglobulins were precipitated from cold serum by the addition of 1/2 volume of saturated ammonium sulfate. The globulin fraction was collected by centrifugation at approximately 2000 x g, resuspended in distilled water adjusted to pH 7.5 with 0.1 M NA_2HPO_4, and reprecipitated with ammonium sulfate. After 3 precipitations, the globulin was dialyzed overnight against several changes of 0.85% NaCl. Protein concentration was determined by the Kalb procedure (20), and 0.05 mg of FITC[d] per mg protein was added. Conjugation was allowed to proceed overnight at 4 C with constant stirring, after which free fluorescein was removed by passing

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[c] Difco Laboratories, Detroit, MI.

[d] Fluorescein isothiocyanate, Grand Island Biological Company, Grand Island, NY.

the conjugate through a column of coarse G-25 Sephadex. The conjugate was then absorbed with rabbit liver powder.

Performance of FA Test

Fluorescent antibody tests were performed on 8 μm thick frozen sections of intestine, fecal preparations made by rolling a swab with collected fecal material onto a microscope slide, and cell cultures growing on coverglasses. Preparations were fixed for 10 min at room temperature (∿23 C) with acetone, overlaid with conjugate, incubated for 30 min in a 35 C moist chamber, and washed with 0.85% NaCl for 10 min. Examinations were made using a Zeiss microscope equipped with a transmitted 100 watt halogen lamp source and FITC interference filter. Barrier filters were Schott OG 4 and OG 5 used in combination.

Growth Rate of CRFK Cells and Growth Rate of CPV

The growth rate of CRFK cells under conditions used for isolation of CPV was determined. Plastic flasks (25 cm^2) were seeded with 5 ml of a cell suspension containing 1.37×10^5 cells per ml and incubated at 36 C. Duplicate counts of cells collected from 2 flasks were made in a hemacytometer at 12 hour intervals for the first 48 hours and thereafter at 24-hour intervals. At 36 hours after seeding, when the cell monolayer was essentially 80% confluent, medium was replaced in a corresponding set of flasks with 0.5 ml of CPV (VC-13-79). The inoculum contained approximately 2,048 HA units per ml and was allowed to adsorb for 90 min at room temperature. Following adsorption, the monolayers were washed twice and refed with medium containing 2% BFS. Uninoculated control flasks used for growth rate study were treated in a similar manner. The amount of CPV HA activity was determined at 12 hour intervals for the first 24 hours and thereafter at 24-hour intervals.

Cell Culture Host Range of CPV

Susceptibility of cell cultures derived from a variety of animal species to CPV infection was determined. Growth conditions for the various cells were analogous to those described earlier for CRFK cells. Cell types used and their source are listed in Table 1. Plastic flasks (25 cm^2) and 16 x 150 mm culture tubes containing rapidly dividing cells were inoculated with 0.5 ml and 0.2 ml, respectively, of CPV (VC-13-79) containing approximately 2,048 HA units per ml. Inocula in plastic flasks were allowed to adsorb for 90 min, as described directly above; inocula were not removed from tubes. The ability of cell cultures to support the growth of CPV was determined by FA and HA examinations.

Effect of Adenoviruses on CPV Replication

The effect of canine adenoviruses types 1 and 2 on CPV replication was determined by the simultaneous inoculation of CPV (VC-13-79) and adenovirus onto rapidly dividing canine kidney cells (MDCK). The inoculum contained 2,048 HA units per ml of CPV and 10^5 $TCID_{50}$ per ml of the respective adenoviruses. Inoculation of plastic flasks and tubes was done as described under cell culture host range. Fluorescent antibody examinations were performed at 40 hours after infection, and cultures were frozen at 72 hours due to extensive adenovirus CPE. Hemagglutination activity and viral infectivity were determined. Infectivity analysis was performed in CRFK cells to avoid cellular destruction by adenovirus and was conducted using microtiter slide chambers[e] to achieve greater accuracy of the FA test used to ascertain the presence of infected cells. Controls consisted of cells infected

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[e]Bellco Glass, Inc., Vineland, NJ.

Table 1. Cell cultures, source, and conditions for growth analysis of CPV

Cell Culture	Source	"Helper" Virus
Feline kidney (CRFK)	W. A. Nelson-Rees[1]	none
Canine kidney (MDCK)	ATCC[2]	none
Canine kidney (MDCK)	ATCC	canine adenovirus 1
Canine kidney (MDCK)	ATCC	canine adenovirus 2
Monkey kidney (VERO)	ATCC	none
Monkey kidney (VERO)	ATCC	simian adenovirus (SV-37)
Human (HeLa)	ATCC	none
Human (HeLa)	ATCC	human adenovirus 8
Human (HeLa)	ATCC	human adenovirus 31
Mouse (L cells)	ATCC	none
Bovine kidney	MSU[3]	none
Bovine kidney	MSU	bovine adenovirus 3
Caprine kidney	MSU	none
Caprine kidney	MSU	caprine adenovirus (CH-8)[5]
Caprine kidney	MSU	caprine adenovirus (CH-38)[5]
Ovine kidney	MSU	none
Equine dermal	Kanitz[4]	none
Equine dermal	Kanitz	equine adenovirus (E-268)[5]

[1]Naval Biomedical Research Laboratory, Oakland, CA.

[2]American Type Culture Collection, Rockville, MD.

[3]Michigan State University, Clinical Microbiology Laboratory.

[4]College of Veterinary Medicine, Purdue University.

[5]MSU isolates: CH-8 and CH-38 are previously undescribed adenoviruses of goats. Other viruses were obtained from ATCC.

with CPV alone and adenovirus alone. The effect of additional adenoviruses on the replication of CPV in other cell types was also investigated. The particular virus and cell type are presented in Table 1.

RESULTS

Clinical Cases - Diagnosis of CPV Infection by HA and Virus Isolation

Of 150 specimens (intestine/feces) examined, 30 exhibited HA activity to both pig and rhesus monkey RBC with HA titers ranging from 1:8 to greater than 1:65,536. Thirteen additional samples at dilutions ≤1:4 reacted with pig RBC and 8 of these reacted similarly with monkey RBC. However, retesting of these samples yielded negative HA results in most instances. Of interest was the finding of one sample with HA activity of 1:4,096 for pig RBC but no activity for monkey RBC. Similar but less dramatic differences were noted in another sample in which pig RBC were agglutinated at 1:256 and monkey RBC were agglutinated at 1:16. Only 1 sample agglutinated rhesus monkey RBC but failed to agglutinate pig RBC. The HA activity was low (1:16) and incomplete. Results are summarized in Table 2.

Canine parvoviruses were isolated from 34 of the 150 specimens examined. Twenty-four of these positive samples corresponded to 24 of the 30 samples in which HA activity to both pig and monkey RBC was demonstrated. Five of the remaining 6 samples which were positive by HA but negative by isolation had HA titers of 1:16 or less. The other 10 specimens positive by isolation included the 1 sample which exhibited HA activity to only porcine RBC and 9 which were HA negative for both porcine and monkey RBC. Cytopathic effects (CPE) were directly related

Table 2. Canine parvovirus infection: results of hemagglutination and virus isolation

Case No.	HA		Isolation
	pig RBC	monkey RBC	
C-1549-78	1:64	1:128	+
C-1728-78	---	---	+
C-1992-78	1:64	1:32	+
VC-4-79	1:65,536	1:65,536	+
VC-13-79	1:256	1:256	+
VC-16-79	---	---	+
VC-17-79	---	---	+
VC-26-79	1:32	1:32	+
VC-27-79	1:65,536	1:65,536	+
VC-31-79	1:32,768	1:16,384	+
VC-42-79	1:4096	1:4096	+
VC-45-79	1:8192	1:8192	+
VC-52-79	1:1024	1:1024	+
*VC-56-79	1:16	1:16	-
VC-82-79	1:128	1:64	-
VC-90-79	1:1024	1:1024	+
VC-93-79	---	---	+
VC-101-79	---	---	+
VC-104-79	1:16,384	1:16,384	+
VC-109-79	1:512	1:512	+
VC-114-79	1:2048	1:2048	+
VC-116-79	1:1024	1:2048	+
VC-131-79	---	---	+
*VC-140-79	1:8	1:8	-
*VC-146-79	1:8	1:8	-
VC-150-79	1:256	1:16	
VC-160-79	---	---	+
VC-162-79	1:32,768	1:32,768	+
VC-169-79	---	---	+
*VC-177-79	1:8	1:8	-
VC-187-79	1:4096	1:8192	+
VC-191-79	---	---	+
VC-193-79	1:8192	1:8192	+
*VC-194-79	1:16	1:16	-
VC-200-79	1:4096	---	+
VC-202-79	1:1024	1:2048	+
VC-254-79	1:512	1:1024	+
VC-262-79	1:16,384	1:32,768	+
VC-272-79	1:4096	1:2048	+
*VC-277-79	---	1:16(inc.)	-
VC-126-80	1:64	1:64	+

*HA activity was not removed in the presence of specific CPV antiserum.

to degree of HA activity and were minimal or nil in low titer and negative HA samples. Results are summarized in Table 2.

Electron microscopic (EM) results were available for comparative purposes in 30 of the 150 cases, and FA results were available in 86. Electron microscopic results were in agreement with isolation and HA findings in 25 of the 30 cases, and FA examination of fecal smears or frozen sections of intestine correlated with HA and isolation results in 76 of the 86 specimens examined. Differences in FA, EM, HA, and isolation results are summarized in Table 3. Four samples were positive by EM which were negative by HA and isolation; the converse was true for the other sample. Six samples positive by FA were negative for HA activity and in 4 of these virus was not isolated. Of 4 samples negative by FA, 3 were positive for HA and all 4 were positive by isolation. Fecal preparations were occasionally difficult to interpret due to lack of intact cells, although a generalized fragmented fluorescence in association with fecal debris was often noted in samples with high HA activity (Figure 1).

Biological Characteristics of Canine Parvovirus

Erythrocyte Agglutination

The VC-13-79 isolate of CPV which had been subcultured 3 to 5 times in CRFK cells was used in preliminary HA studies. This particular isolate agglutinated RBC from pig, rhesus monkey, cat, and horse but not those of sheep, dog, goat, cow, rabbit, guinea pig, chicken, and human. Agglutination occurred at 4 C and at room temperature with RBC of pig and monkey, but only at 4 C with those derived from cat and horse. Erythrocytes from pig and monkey were agglutinated essentially to the same degree by CPV and approximately 2.0 to 4.0 times more

Table 3. Canine parvovirus detection: discrepancies noted with FA, EM, HA and isolation results

		HA Results		Isolation Results	
		+	-	+	-
EM Results	+(4)	0	4	0	4
	-(1)	1	0	1	0
FA Results	+(6)	0	6	2	4
	-(4)	3	1	4	0

Figure 1. Immunofluorescence of CPV antigen in association with fecal debris.

Figure 2. Immunofluorescence of CRFK cells infected with CPV.

efficiently than those from cat and horse. Erythrocytes of cat and horse eluted when test plates were removed from 4 C and placed at room temperature. No elution was noted with RBC of pig and monkey, except at the higher dilutions of antigen. The HA activity of CPV was not appreciably altered over a wide pH range (pH 5.5-7.5). Significant differences between RBC of different swine breeds were not noted, although RBC that failed to settle properly were occasionally encountered. Seven other CPV isolates subcultured 1 to 3 times in CRFK cells were compared with VC-13-79. Results are presented in Table 4.

Cell Culture Susceptibility

The ability of CPV (VC-13-79) to replicate in various cell cultures was examined. Successful replication, as determined by transfer ability of CPV through 3 subcultures, was noted in cell cultures derived from dog, cat, cow, goat, human, and monkey. Virus did not replicate in cell cultures derived from sheep, pig, horse, and mouse. The virus grew best in cells of cat (Figure 2) and dog, to a lesser extent in those derived from goat and cow, and poorly in human and monkey cells. Obvious CPE was not observed in cells other than those derived from cat.

Adenovirus "Helper" Function

Effect of canine adenoviruses, types 1 and 2, on CPV replication was nonexistent. No differences were noted in HA activity or infectivity. Similarly, CPV caused no positive or negative effect on canine adenovirus replication. Canine parvovirus replication in cell cultures derived from animal species other than dog (Table 1) was not benefited by the presence of other adenoviruses.

Table 4. Agglutination of pig, monkey, cat, and horse erythrocytes by isolates of canine parvovirus

Case No.	Erythrocytes From			
	Pig	Monkey	Cat*	Horse*
VC-13-79	1:2,048	1:4,096	1:64	1:16
VC-27-79	1:16,384	1:16,384	1:256	1:256
VC-31-79	1:2,048	1:2,048	1:128	1:32
VC-42-79	1:4,096	1:4,096	1:128	1:16
VC-45-79	1:4,096	1:4,096	1:128	1:16
VC-114-79	1:1024	1:512	1:32	1:8
VC-102-79	1:16,384	1:16,384	1:256	1:32
VC-128-80	1:512	1:512	1:32	1:16

*Erythrocytes from cat and horse did not agglutinate completely and appeared as a rough fringed button.

Growth Rate of CRFK Cells and CPV

The growth rate of CRFK cells is shown graphically in Figure 3. At a seeding rate analogous to that used in isolation studies, the lag period lasted for about 24 hours and maximum growth was obtained between 36 and 96 hours. Confluency was reached in approximately 60 hours and cells attained their saturation density at 96 hours.

Figure 3 also depicts the viral growth curve of CPV in a corresponding set of CRFK cells infected at 36 hours post-seeding. Approximately 50 to 70% of the virus was absorbed in 90 min at room temperature, as indicated by remaining HA activity in the inoculum. No intracellular HA activity was detected prior to 12 hours after infection, and HA activity in supernatant fluid did not appear until 24 hours had elapsed. Most CPV replication occurred within 72 hours, and maximum HA titers of intracellular and extracellular virus were obtained 120 hours after infection.

DISCUSSION

One hundred fifty fecal and/or intestinal preparations were examined for the presence of CPV using a presumptive HA test and virus isolation. In agreement with recently published results of Carmichael et al. (8), HA titers $\geq$1:32 correlated well with positive isolation results. Samples which exhibited HA activity of 1:16 or less and were negative by isolation were presumed to be nonspecific and not related to CPV, as specific antiserum failed to remove this activity in parallel hemagglutination inhibition (HI) tests. Similarly, the 2 samples (VC-82-79 and VC-150-79) with moderate HA activity and the 1 sample (VC-200-79) which agglutinated only porcine RBC were unaffected by the presence of specific antiserum. The cause of these nonspecific

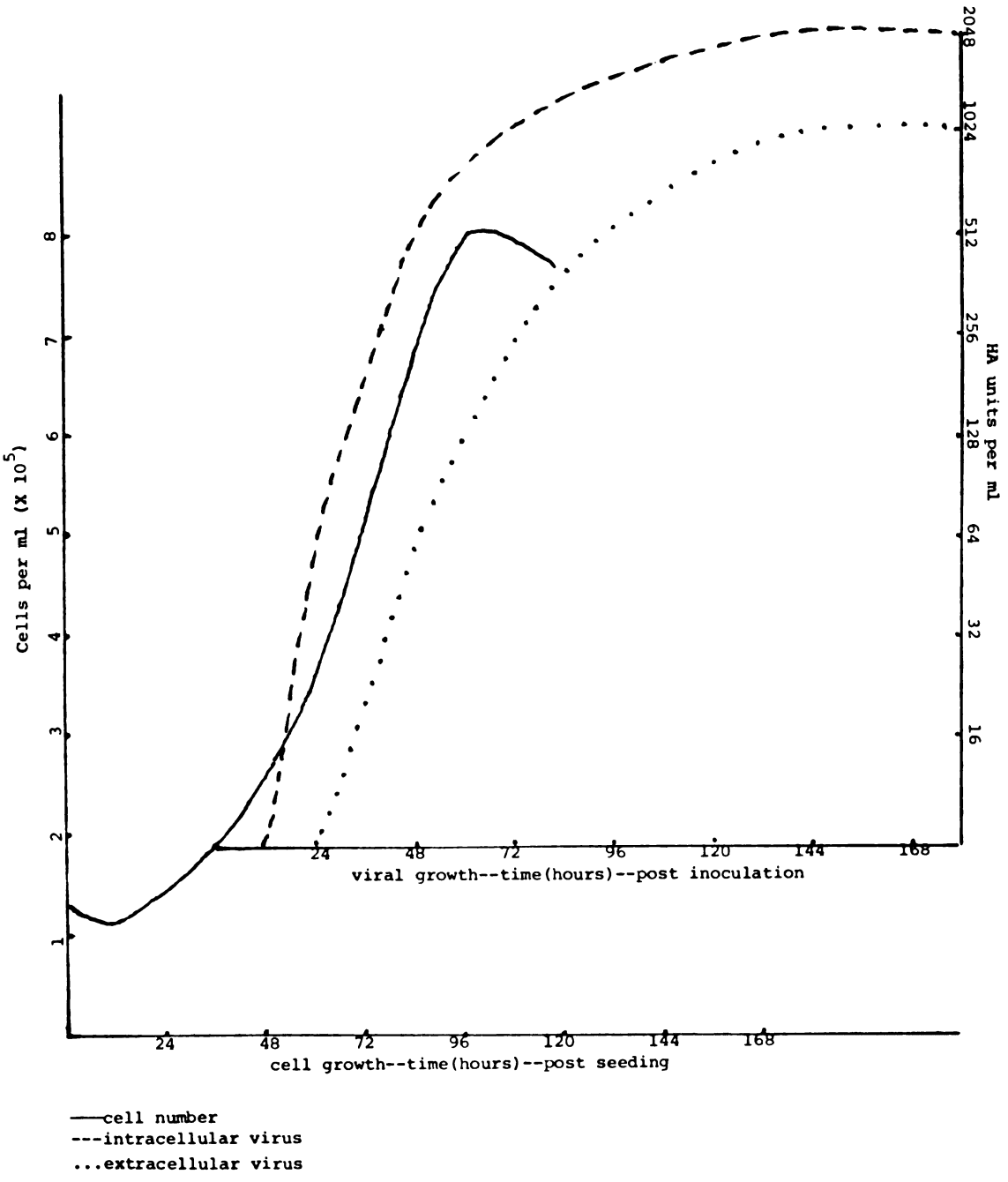

Figure 3. Growth curves of CRFK cells and canine parvovirus.

reactions was not determined, but it seems unlikely that these levels of HA activity, at least for VC-200-79, were due to nonspecific agglutinins. However, attempts to isolate viruses other than CPV using MDCK cells were unsuccessful (data not shown). The CPV isolated from VC-200-79 was apparently incidental to the HA activity. The reason for lack of CPV specific HA was attributed to the small amount of virus present in the specimen, as evidenced by the presence of only a few FA positive cells in inoculated cultures. The selective partial agglutination of 1 sample (VC-277-79) for rhesus monkey RBC may have been due to the presence of minute virus of canines (MVC), which agglutinates monkey RBC but not those of pig (5).

Viral isolation procedures were shown to be a more sensitive technique than direct HA for the diagnosis of CPV. Eight specimens with no demonstrable HA activity were positive by isolation. In each of these cases, only a few FA positive cells were noted in inoculated cultures, and HA activity of supernatant fluids was negligible. It is doubtful that these isolates represent CPV with different HA properties, although this possibility was not eliminated. In general, HA activity of fluid from infected cell cultures correlated well with the HA activity in the original inoculum, indicating that virus "output" was directly related to virus "input." Thus, lack of HA activity was thought to be a result of low amounts of virus in these preparations.

Positive EM results and negative HA/isolation results were likely due to the presence of CPV antibody in the intestinal tract; consequently, there was no free virus, or the presence of MVC, which is microscopically indistinguishable from CPV, may have interfered. Negative EM results and positive HA/isolation results might occur with samples containing small amounts of virus. However, the 1 sample in

this category had a HA titer of 1:8,192, which was considerably higher than some others that were positive by EM.

Negative FA results on the 4 specimens from which CPV was isolated were thought to be due to the type of specimen utilized. Fecal material was submitted in each of these cases, and difficulty was occasionally encountered in the interpretation due to the absence of intact cells. The presence of intact cells was not necessarily a prerequisite, as a generalized fragmented fluorescence was often observed in association with debris if virus concentration was heavy, i.e., high levels of HA activity. The 4 samples that were positive by FA but from which no virus was isolated may have been due to the presence of CPV antibody. The presence of antibody would not affect the FA test results, as viable cells would be impermeable, but emulsifying the preparation for isolation studies would disrupt the cells and effective neutralization of CPV could occur.

The ability of CPV to agglutinate RBC of pig, rhesus monkey, horse and cat at 4 C is in agreement with results reported by Carmichael and co-workers (8), but elution at 23 C did not occur with RBC of pig and monkey except at high dilutions of antigen. In fact, only minor differences were noted in HA activity with pig and monkey RBC when parallel tests were conducted at 4 C and 23 C. These findings are in agreement with Gagnon and Povey (14), who also reported HA activity of CPV at 23 C. There was no spontaneous agglutination of pig RBC at pH values below 6, as reported by Carmichael et al. (8), although occasional batches of RBC failed to settle properly. No major differences were noted in HA activity of 8 cell culture isolates of CPV.

Growth of CPV in cell cultures derived from cat, dog, mink, raccoon, and bovine has been reported (3), and we observed that

replication occurred in cells derived from goat, human, and monkey as well. Replication of CPV in monkey cells (vero) was in contrast to a previous negative report (3). Linser et al. (23) reported that the ability of minute virus of mice to infect cells was dependent upon specific receptor sites, and it is surprising that CPV does not exhibit the restricted host range observed with most autonomous parvoviruses.

Canine parvovirus is associated with myocarditis in young dogs (9,15,16,17,18,22,24), and it has been suggested that the ability of CPV to replicate in heart muscle may be due to a "helper" virus (6). However, we failed to demonstrate any enhancement of CPV replication in MDCK cells when CPV was co-cultivated with either canine adenovirus types 1 or 2. This finding does not necessarily rule out the possibility that other viruses could provide helper function, although it seems unlikely. We used an enteric isolate of CPV in our study, and the possibility remains that a myocardial isolate of CPV might behave differently.

The growth rate of CPV was maximum during the logarithmic growth of CRFK cells, as was expected. Under conditions which we used for general isolation purposes, maximum virus concentration was attained within 4 to 5 days after inoculation. Therefore, there was no reason for holding cultures for extended periods of time.

In conclusion, the results presented herein indicate that an accurate diagnosis of CPV infection is best achieved through virus isolation procedures. Although HA titers of 1:32 or greater are usually indicative of CPV infection, simultaneous HI tests should be conducted to rule out nonspecific reactions. Care should be taken in interpreting FA results of fecal smears, as the fragmented fluorescence

associated with fecal debris may be confusing. In such cases, a blocking test would be advisable. The primary advantage of EM examination is the detection of viruses other than CPV. No distinguishing differences were noted between different isolates of CPV.

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