Equine Parvovirus: Initial Isolation and Partial Characterization

F.C. Wong, J.G. Spearman, M.A. Smolenski and P.C. Loewen*

ABSTRACT

A viral agent was isolated from the fetal liver of an aborted equine fetus. The isolate hemagglutinated red blood cells from guinea pig, rhesus monkey and rooster. By hemagglutination inhibition tests, the isolate was shown to be antigenically distinct from parvoviruses of bovine and canine origin. Specific hemagglutination inhibiting antibody against the viral isolate was exhibited by 26 of 136 horse sera tested. The isolated virus showed properties compatible with those of an autonomous parvovirus including size, morphology, stability to ether treatment and heating to 56°C, the presence of a 5300 base DNA genome, characteristic protein composition and density (1.405 g/mL). The virus was classified as an equine parvovirus.

RÉSUMÉ

Les auteurs rapportent l'isolement d'un virus du foie d'un avorton équin. Ce virus agglutinait les hématies du cobaye, du singe rhésus et du poulet. Des épreuves d'inhibition de l'hémagglutination permirent de constater que le virus précité se distinguait des parvovirus d'origine bovine et canine, du point de vue antigénique; 26 des 136 échantillons de sérum équin éprouvés à cette fin contenaient des anticorps spécifiques, inhibiteurs de l'hémagglutination, contre la virus isolé du foie de l'avorton. Cet isolat possédait des propriétés compatibles avec celles d'un parvovirus autonome, à savoir: dimensions, morphologie, résistance, à l'éther et à 56°C, génome d'acide désoxyribonucléique de 5300 bases, composition protéique caractéristique et densité de 1,405 g/mL. Les auteurs le classifièrent comme un parvovirus équin.

INTRODUCTION

In the spring of 1982, a high rate of abortion occurred among nearly full term pregnant mares on a single farm in southwestern Manitoba when 16 out of 70 mares aborted. Upon diagnostic study of two of the fetuses, microbial infection by bacteria and chlamydiae was ruled out and, ultimately, viral agents were isolated from the livers of both fetuses. One viral isolate, labelled 21-1 was chosen for further characterization and this paper describes its initial characterization as a parvovirus.

MATERIALS AND METHODS

TISSUE CULTURES

Pig fallopian tube (PFT) cell line (1) was kindly supplied at the 60th passage level by Dr. A.M.P. Bouillant of the Animal Diseases Research Institute, Agriculture Canada. The cell line was used in this study below the 80th passage level. Continuous cell lines A-72, Vero, BHK-21, PK-15, EFTr and Hep-2 were obtained from the American Type Culture Collection. Primary GMK and HAM cells were obtained from Flow Laboratories of Mississauga, Ontario and Rhesus monkey kidney cells were obtained from Connaught Laboratories, Willowdale, Ontario. Primary embryonic kidney and lung cells of bovine and equine origin were prepared in this laboratory.

TISSUE CULTURE MEDIA

Routine tissue culture medium was Eagles' MEM supplemented with 100 units/mL penicillin, 50 μg/mL streptomycin and 50 μg/mL neomycin. The antibiotic concentration in this mixture is designated as antibiotics x 1. Where necessary, amphotericin-B was also added to a final concentration of 25 μg/mL. Growth medium was supplemented with 8% fetal calf serum which was reduced to 1% for maintenance medium. In experiments with 5-fluorodeoxyribouridine (FUDR), CMRL 1066 medium obtained from Connaught Laboratories was used. Organic buffer in a final concentration of 1 mM of N-2 hydroxyethylpiperazine-N'-2 ethanesulfonic acid (HEPES) was added to all tissue culture media.

VIRUS ISOLATION

Fetal liver was homogenized into a 20% suspension in brain heart infusion broth (Bacto) containing 2 x antibiotics plus amphotericin-B. The suspension was incubated at 4°C for one hour. The suspension was clarified by low speed, refrigerated centrifugation (approximately 500 x g) for 15 minutes. The supernatant was inoculated onto monolayer cultures of PFT cells in tubes, 0.5 mL inoculum per culture. After one hour adsorption at 37°C, the PFT cultures were washed four times each with 5.0 mL of Hanks balanced salt solution (Gibco) containing 2 x antibiotics. Each culture was refed with 2.0 mL growth medium. The PFT cultures were then incubated at 37°C for four hours at which time the cultures were each trypsinized and seeded into a fresh tube at a 1:1 splitting ratio. The PFT cultures were returned to 37°C incubation, and observed daily for a cytopathic effect (CPE). When a CPE appeared, tissue culture fluid was tested for hemagglut-

*Provincial Veterinary Laboratory, 545 University Crescent, Winnipeg, Manitoba R3T 2N2 (Wong and Spearman) and Department of Microbiology, University of Manitoba, Winnipeg, Manitoba R3T 2N2 (Smolenski and Loewen).

Reprint requests to P.C. Loewen.

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ination (HA) with guinea pig red blood cells (RBC).

INFECTIVITY ASSAY

Pig fallopian tube cultures, under 24 hours old, were each inoculated with 250 μL of appropriately diluted virus with five culture tubes being used for each dilution. One hour at room temperature was allowed for virus adsorption, after which each culture was washed three times with 5 mL of Hanks balanced salt solution containing 2 x antibiotics and then refed with maintenance medium. After incubation at 37°C for five days, the supernatant from each culture was tested for hemagglutination with guinea pig RBC. Positive HA was considered infectivity and the tissue culture infectious dose for 50% CPE ($TCID_{50}$) was calculated by the Reed-Muench method (2).

HEMAGGLUTINATION (HA) AND HEMAGGLUTINATION INHIBITION (HAI) TESTS

Both tests were carried out in v-bottomed microtiter plates. A suspension of 0.3% washed RBC in pH 7.0 phosphate-buffered saline (PBS) containing 0.6% bovine albumin was prepared. The HA titration, performed at room temperature, involved serial twofold dilutions in 50 μL volumes of PBS into which was added 25 μL of the RBC suspension. The HA endpoint was read one hour after the RBC solution was added. The hemagglutination titer value used is the reciprocal of the highest dilution at which hemagglutination was observed.

The serum used for hemagglutination inhibition assays was treated by the method of King and Hopkins (3) to remove nonspecific hemagglutination inhibitor. Specifically, 100 μL of undiluted serum was mixed with 400 μL of a kaolin suspension in buffer consisting of 5.96 g HEPES, 8.19 g NaCl and 0.15 g $CaCl_2$ per liter adjusted to pH 6.6 with NaOH and absorption proceeded at room temperature for 1 h during which the serum-kaolin was mixed by shaking at 15 minute intervals. The treated serum, recovered by low speed centrifugation, was further treated to remove any autohemmagglutinin by adsorption with 50 μL of 50% packed RBC. These absorption procedures diluted the serum fivefold. For the test, 25 μL of antiserum dilutions were each mixed with 25 μL of antigen containing four to eight HA units. A minimum period of 40 minutes at room temperature was allowed for the serum-virus mixture to react before 25 μL of indicator RBC were added. The hemagglutination inhibition titer value used is the reciprocal of the highest dilution at which activity was evident.

PHYSIOCHEMICAL PROPERTIES

Heat stability of the isolate was tested by heating the virus fluid in a 56°C water bath for 30 minutes. The isolate was tested for stability in pH 3 citrate-phosphate buffer for a period of 90 minutes at room temperature. Assays for infectivity and HA activity were carried out before and after these treatments. The isolate was tested for its resistance to the action of trypsin and sodium deoxycholate by procedures previously described (4). Resistance of the isolate to standard treatment with ether at room temperature for ten minutes was tested. The sensitivity of the isolate to 5′-fluorodeoxyuridine was tested by a standard method (5) and a known DNA virus, equine rhinopneumonitis, was included as a control.

ANTIGENICITY

Antisera to the isolate were prepared in young, pregnant rabbits each receiving an intravenous injection of 1.0 mL crude virus fluid from the seed pool between 13 to 15 days of their gestation period. Sera from these rabbits were removed 30 days postinoculation. Initially antigenic crossing was examined by HAI tests against parvoviruses of bovine, porcine and canine origin and their homologous antisera. The canine parvovirus was a local isolate made on a dog kidney cell line A-72. The antiserum to the canine parvovirus was kindly supplied by Dr. D.W. Key, Veterinary Laboratory Services, Ontario Ministry of Agriculture and Food. The bovine parvovirus was a local isolate grown on primary bovine embryonic lung cells. The antiserum to the bovine parvovirus was kindly supplied by Dr. A.W. McClurkin, National Animal Disease Center, Ames, Iowa. The porcine parvovirus was strain S3009 (England) kindly supplied by Dr. J.B. Derbyshire of the Ontario Veterinary College. Antisera to the porcine parvovirus were field serum samples having shown an homologous HAI titer of 32,000.

ELECTRON MICROSCOPY OF THE NEGATIVELY STAINED VIRION

Both crude virus fluid and CsCl purified preparations were examined with a Philips 201 electron microscope using a method described by Hammond *et al* (6).

PURIFICATION OF THE VIRUS

For characterization, the virus was prepared using PFT cells in 75 cm^2 square culture flasks following the absorption-trypsinization procedure. After five days growth the medium was removed and the infected cells were washed twice with PBS. Into each bottle of infected cells 2 mL of lysis buffer containing 15 mM Tris-HCl pH 7.8, 0.1 M NaCl, 1% Nonidet NP40, 0.1% sodium dodecyl sulfate and 2 mM phenylmethylsulfonyl fluoride was added. The infected cells were frozen and then thawed at 4°C three times followed by centrifugation at 10,000 g for ten minutes to remove any cell debris. The supernatant was then centrifuged at 164,000 g in a Beckman 60ti rotor for four hours and the pellet was resuspended in a thirtyfold smaller volume of a solution containing 50 mM Tris pH 7.6, 1 mM EDTA and 1 x antibiotics. This solution was mixed with a saturated CsCl solution to give a density of 1.37 g/mL and centrifuged for 48 hours at 105,000 g in a Beckman SW50.1 rotor. The bottom of the tubes were pierced and five-drop fractions were collected. The optical density at 280 nm was determined for each fraction using a Gilford spectrophotometer and the refractive index was determined for selected fractions using a Bausch and Lomb refractometer. The fractions exhibiting strong hemagglutination activity were pooled and dialyzed against 50 mM Tris pH 7.6, 1 mM EDTA. This solution was used for subsequent analysis of the protein and nucleic acid components.

PARTIAL CHARACTERIZATION OF THE NUCLEIC ACID AND PROTEIN COMPONENTS

Sodium dodecylsulfate (SDS) was added to the virus solution to a final concentration of 1%. For the nucleic acid isolation, the solution was incubated at 37° C for 30 minutes. Sodium acetate was added to a final concentration of 0.25 M and cold 95% ethanol was added to a final concentration of 70% with gentle mixing. After storing for 30 minutes at -60° C, the DNA was pelleted by centrifugation, dried under vacuum and resuspended in an appropriate buffer for nuclease digestions. Electrophoresis was carried out on 0.8% agarose gels (7).

For protein isolation, the SDS solution with 20 mM 2-mercaptoethanol added was incubated at 37° C for 30 minutes and then boiled for two minutes. The sample was electrophoresed directly on a 5% polyacrylamide gel. For trypsin cleavage, the virus solution was treated with 50 μg/mL trypsin for two hours at 37° C followed by the sodium dodecylsulfate extraction and electrophoresis as already described. Molecular weight references were electrophoresed on parallel gels.

RESULTS AND DISCUSSION

VIRUS ISOLATION AND REPLICATION

Diagnostic study of two aborted equine fetuses ruled out infection by bacteria, chlamydiae and equine rhinopneumonitis virus type 1 and type 2. In screening for another possible viral agent, a cytopathic effect was observed on PFT cell cultures in five days. There were no peculiar characteristics to the CPE with the infected cells becoming condensed and shedding, not unlike the effect of aging on uninfected PFT cells. The second blind passage of isolate 21-1 on PFT cells gave a $TCID_{50}$ of 2.0 x 10^6/mL. A seed pool was prepared on second passage for use as an inoculum for all experiments in this study.

The growth of the virus in viral isolate 21-1 on 3.5 hour old cells was compared with and without trypsinization after virus adsorption. There was good correlation between the infectious titer (Fig. 1a) and the hemagglutination titer (Fig. 1b) for the respective procedures. The main difference between the two procedures was the rate at which peak titer was reached.

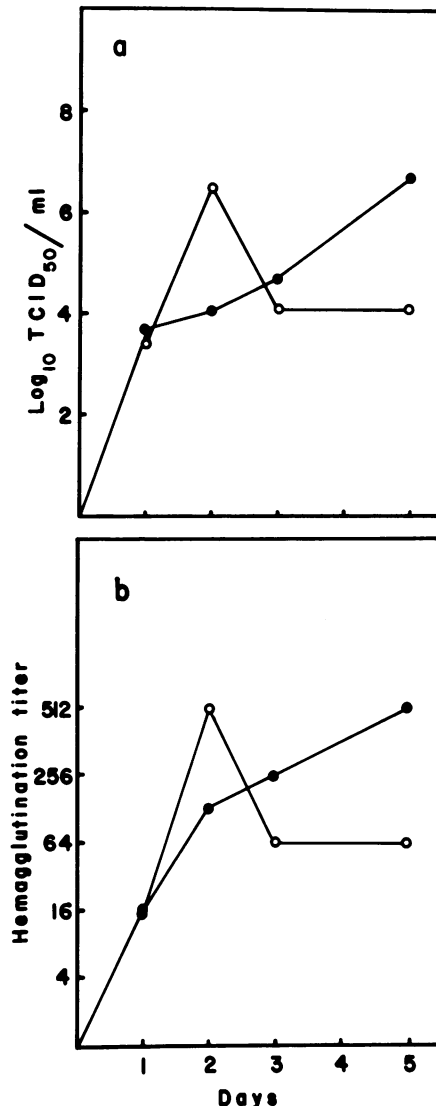

Fig. 1. Growth of virus on pig fallopian tube cell cultures. Isolate 21-1 was used to infect pig fallopian tube cells that were 3.5 hours old after which a portion of the cells were treated with trypsin. The tissue culture infectious dose for 50% cytopathic effect ($TCID_{50}$) was determined in *a* and the hemagglutination titer was determined in *b*. Cells were grown without (○) and with (●) trypsinization.

No viral growth was evident during two passages in cell lines Hep2, A72, Vero, HBK-21, PK-15, EBTr or the primary cell types bovine embryonic kidney and lung, porcine embryonic kidney, rabbit embryonic kidney, grevet and rhesus monkey kidney, or equine embryonic heart and kidney. Further studies should be carried out to confirm the absence of viral growth in these and other cell types.

HEMAGGLUTINATION

At room temperature and pH 7.0, the isolate was found to hemagglutinate red blood cells from guinea pig, rooster and rhesus monkey. Under the same conditions, no hemagglutination activity was demonstrable with red blood cells from sheep, rabbit, pig and human (type 0). The hemagglutination titer of the seed pool was highest with guinea pig red blood cells with a titer of 512, as compared to rhesus monkey red blood cells with a titer of 64 and rooster red blood cells with a titer of 16. The HA titers remained unchanged at 4° C.

HEMAGGLUTINATION INHIBITION

Of the four rabbits given intravenous injections of 1.0 mL of crude virus fluid, only two responded by showing a high homologous HAI titer: rabbit number 2 with a titer of 640, and rabbit number 4 with a titer of 160. Rabbit number 3 yielded an HAI titer of 40 while number 1 showed no detectable antibody at 1:10 serum dilution. By using rabbit sera number 2 and number 4 in HAI tests, the new equine isolate was shown to be unrelated antigenically to the three autonomous parvoviruses of bovine, porcine and canine origin (Table I).

A total of 136 individual horse sera, submitted for various diagnostic testings between June 1981 and July 1983, were tested against the isolate. Using a minimum HAI titer of 10 as a significant level, the sera from 26 horses were positive (Table II). Of these 26 positive sera, 9 gave titers below 80, and 17 showed titers between 80 and > 640. All 26 equine sera did not react at 1:10 dilution against the three parvoviruses of bovine, porcine and canine origin. It is not clear, at the moment, if a nonspecific hemagglutination inhibitor exerts an effect on hemagglutination by parvoviruses. The King and Hopkins method of serum treatment was adopted because this method has yielded reproducible results on routine testing of thousands of sera by HAI against infectious bronchitis virus. Indeed, without the kaolin treatment, all 136 horse sera tested gave HAI titers against the isolate. A sample of serum was taken from the mare on the day of abortion (the fetal tissue of which yielded 21-1), and another sample was taken from the same mare

TABLE I. Hemagglutination Inhibition Titers of Antisera to Various Parvoviruses

	Antisera Prepared Against				
	a Isolate 21-1		b Bovine	c Porcine	b Canine
Viral Antigen Tested	Rabbit #2	Rabbit #4	Parvovirus	Parvovirus	Parvovirus
21-1	640[a]	160	ND[b]	ND	ND
bovine parvovirus	ND	ND	320	ND	ND
porcine parvovirus	ND	ND	ND	32,000	ND
canine parvovirus	ND	ND	ND	ND	320

[a]Reciprocal of serum dilution
[b]Not detectable at 1:10 serum dilution

TABLE II. Sera from Manitoba Horses Showing a Hemagglutination Inhibition Effect against Viral Isolate 21-1

Year	Number of Horse Sera Tested	Number of Sera with a Titer of 10 or Higher
1981	71	6
1982	52	18
1983 (to July)	13	2

three months later. Without kaolin treatment, the HAI titers in the acute state was 160 and in the convalescent state was 320. After kaolin treatment, the acute state gave a titer of 20 and the convalescent state a titer 320. Three other isolates showing the same characteristics have been made from equine aborted fetal tissues, one from liver and two from lung.

PHYSIOCHEMICAL PROPERTIES OF THE ISOLATE

The isolate was stable to heating at 56°C for 30 minutes with no loss in both infectious and hemagglutination titer during the heating treatment. The isolate was also found to be stable during a 90 minute incubation at room temperature and pH 3 with no decrease in either the infectious or the hemagglutination titers. Treatment with trypsin, sodium deoxycholate and ether did not reduce the infectious titer. The deoxyribonucleoside analogue FUDR is an effective agent for reducing the infectious titers of DNA viruses, such as equine rhinopneumonitis virus from 5.0 x 10^5/mL to 3.1 x 10^2/mL and the isolate 21-1 was also sensitive to 50 µg/mL FUDR having its infectious titer reduced from 2.0 x 10^6/mL to 5.0 x 10^3/mL.

CHARACTERIZATION OF THE PURIFIED VIRUS

The optical density and hemagglutination profiles obtained following equilibrium centrifugation of the viral preparation in CsCl are shown in Figure 2. A prominent peak of hemagglutination activity with a density of 1.405 g/mL corresponding to a shoulder in the absorbance peak is evident and the presence of virus was confirmed by assaying for infectious particles. The peak of virus was examined in the electron-microscope following negative staining revealing a small spherical virion with a diameter of 22 nm (Fig. 3). This is consistent with the morphology and size of other parvoviruses (8).

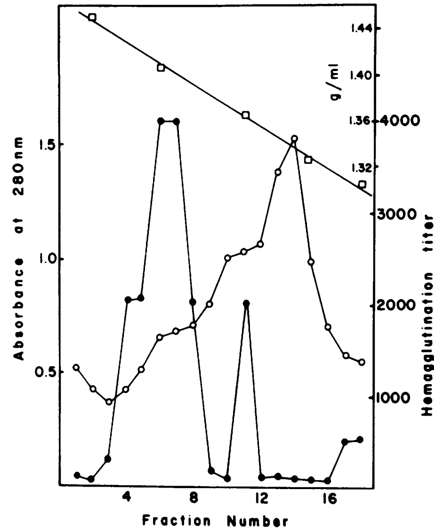

Fig. 2. Purification of the virus in isolate 21-1 by equilibrium centrifugation in CsCl. The absorbance at 280 nm (○), hemagglutination titer (●) and density (□) were determined. Fractions 4-8 with a median density of 1.405 g/mL were pooled for use in characterizing the viral component.

The nucleic acid extracted from the virus migrated as a single band on 0.8% agarose gels consistent with a size of 5300 bases and was sensitive to deoxyribonuclease but not ribonuclease (Fig. 4a). The protein extracted from the virus migrated as three bands during electrophoresis on SDS polyacrylamide gels. In Figure 4b the minor bands at 81,000 and 63,000 molecular weight are not as evident as they were on the actual gel but the main band is evident at 68,000 molecular weight. A characteristic of other parvoviruses is that the main protein component is converted to a protein of molecular weight 63,000 when the whole virus is treated with trypsin (9,10). As shown in Figure 4b the protein in the purified virus derived from

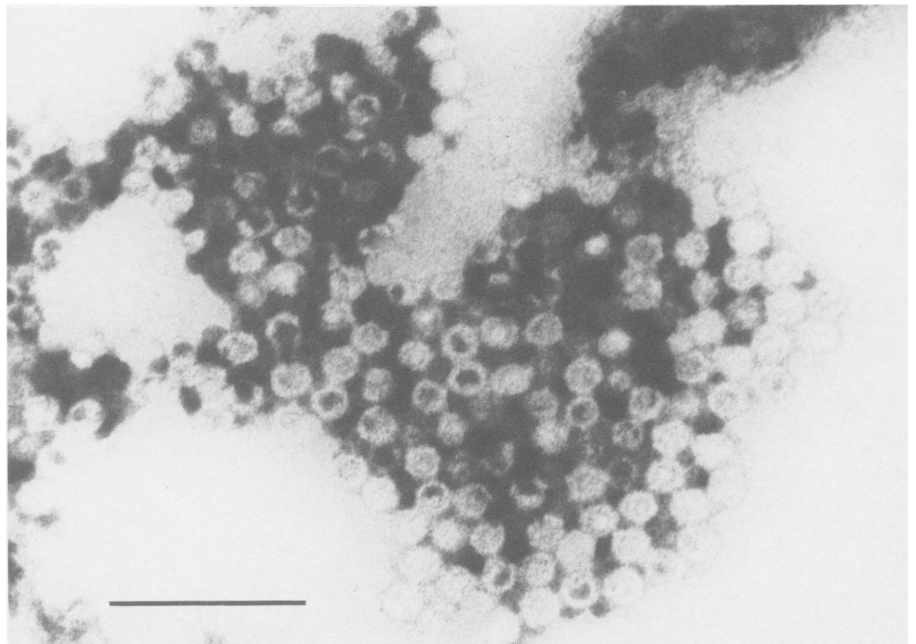

Fig. 3. Electronmicrograph of viral particles purified by equilibrium centrifugation magnified 250,000X. The bar represents 100 nm.

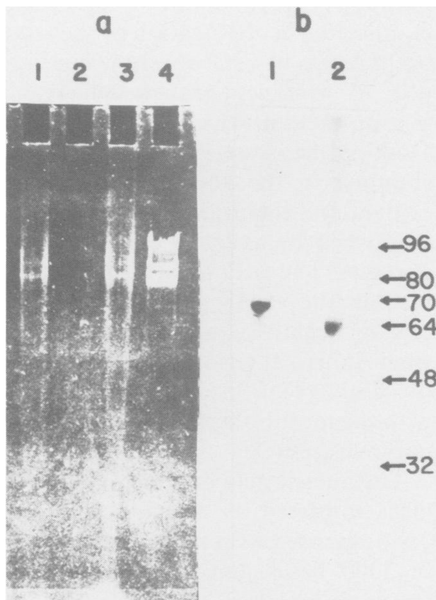

Fig. 4. Agarose gel electrophoresis of viral DNA and protein. (a) The viral DNA was electrophoresed on a 0.8% agarose gel untreated (lane 1), treated with 2 mg/mL pancreatic deoxyribonuclease for 30 min at 37° C (lane 2), and treated with 2 mg/mL Tl ribonuclease for 30 min at 37° C (lane 3). An EcoRI restriction endonuclease digest of bacteriophage λ DNA was run in lane 4 for size determination. (b) The viral protein was electrophoresed on 5% SDS polyacrylamide disc gels. Untreated virus was run in lane 1 after boiling SDS extraction and virus treated with 50 μg/mL trypsin for 2 h at 37° C was run in lane 2 after boiling SDS extraction. The numbers alongside indicate the location of molecular weight marker proteins with sizes in thousands. The gels were stained with Coomassie brilliant blue.

isolate 21-1 is modified in the same fashion.

On the basis of the morphology, size, density, nucleic acid content and characteristic protein content we have designated the active viral component in isolate 21-1 to be an equine parvovirus. This is the first report of a parvovirus from an equine source.

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