Preliminary report on the observation of a coronavirus in the intestine of the laboratory rabbit[1]

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Accepted July 4, 1980

LAPIERRE, J., G. MARSOLAIS, P. PILON, and J.-P. DESCÔTEAUX. 1980. Preliminary report on the observation of a coronavirus in the intestine of the laboratory rabbit. Can. J. Microbiol. **26**: 1204–1208.

Intestinal infections are a major cause of mortality in domestic rabbits. Although a few pathogenic organisms have been associated with this type of infection, it is often impossible to demonstrate the presence of any etiologic agents.

Recently viral particles were observed in the intestine of diseased rabbits submitted to our laboratory for diagnosis. A study was then initiated to evaluate the presence of viral agents in the intestine of rabbits showing clinical signs of enteritis. Viral particles with morphological characteristics of the Coronaviridae family were observed by electron microscopy in most specimens of fecal material obtained from sick rabbits. Such particles were not seen in the feces of healthy animals.

These particles, which have a density of 1.07–1.18 g/cm^3 on sucrose gradient, hemagglutinate rabbit red blood cells. Furthermore this virus has some antigenic relationship with the human coronavirus 229-E since immune serum to this virus blocks the hemagglutination of the rabbit erythrocytes.

Antibody titers to this virus were detected in rabbit sera obtained from colonies with a high incidence of intestinal infections.

When fecal material containing coronavirus particles were inoculated on various tissue culture systems, no cytopathic effects were observed.

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Les infections intestinales constituent la principale cause de mortalité chez le lapin domestique. Plusieurs agents pathogènes ont été associés à ce type d'infection cependant dans plusieurs épizooties, il est impossible de mettre en évidence la présence d'un agent quelconque.

Récemment des particules virales ont été observées dans l'intestin de lapins soumis à notre laboratoire pour des fins de diagnostic. Ces observations nous ont incités à entreprendre une étude dans le but d'évaluer l'incidence des virus dans l'intestin des lapins ayant des signes cliniques d'entérite. Les résultats des examens en microscopie électronique indiquent que des particules virales possédant des caractéristiques morphologiques des Coronaviridae sont observées dans la majorité des intestins de lapins malades. L'examen de matière fécales provenant d'animaux en santé n'a pas permis de démontrer la présence de ces particules.

La densité de ce virus sur gradient de sucrose a été établie à 1,07–1,18 g/cm^3. De plus cet agent viral possède la propriété d'agglutiner les erythrocytes de lapins. Ce pouvoir hémagglutinant est inhibé par un serum immun contre le virus corona humain 229-E.

Des titres d'anticorps hémagglutinants ont été démontrés dans des sérums de lapins provenant de colonies ayant une incidence élevée des infections intestinales.

Finalement aucun effet cytopathique n'a pu être observé lorsque l'agent viral est inoculé sur les différents systèmes de cultures cellulaires utilisés.

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[1]This study was supported by a grant from the Conseil des recherches et services agricoles du Québec (No. IAF-79-776).

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0008-4166/80/101204-05$01.00/0

Introduction

Intestinal infections are a major cause of mortality in the domestic rabbit (Greenham 1962). These infections are responsible for important losses for the breeder as well as for the researcher using these animals. Young animals at weaning age are most often affected and symptoms of watery diarrhea, distended abdomen, dehydration, and rapid death are generally observed (Whitney 1976). Outbreaks have also been reported in which animals died without showing any clinical signs (Richter and Hendren 1969). The pathogenic organisms most often associated with this type of infection include coccidia (Ostler 1961), *Escherichia coli* (Presscott 1978), and *Bacillus piliformis* (Ononiwu and Julian 1978). Unfortunately in many outbreaks, it is not possible to demonstrate the presence of an etiologic agent. Recently, corona-like particles were observed in the fecal material from a sick rabbit submitted to our laboratory for diagnostic purposes. With the exception of a rotavirus proposed recently as a possible cause of rabbit enteritis (Petric *et al.* 1978), viruses are seldom associated with this type of infection in these animals. Considering the importance of viruses in the pathogenesis of intestinal infections in different animal species (Appel *et al.* 1979; Kraft 1957; Storz *et al.* 1978; Chasey and Cartwright 1978; Caul 1979; Doyle and Hutchings 1946; Kienan *et al.* 1976) including man (Middleton *et al.* 1974), we were interested in investigating the presence of coronaviruses in the laboratory rabbit.

The present paper reports the results of our investigation.

Materials and methods

Specimens

A total of 21 rabbits ill with rabbit enteritis were obtained from four colonies with a high incidence of intestinal infections. The sick animals were generally 6–10 weeks of age; they were autopsied and fecal samples and sections of small intestines were collected. The specimens were shipped to the laboratory and kept at −70°C until used. Fecal material from 10 healthy rabbits was also collected from a colony free of intestinal infections. Those specimens were used as control.

Electron microscopy (EM)

For EM examination, fecal samples and mucosal scrapings were individually diluted 1:5 in phosphate-buffered saline (PBS), pH 7.2. The preparations were clarified by centrifugation at 2000 × *g* for 30 min. One aliquot of each sample was then placed on a carbon-coated grid, negatively stained with a 3% phosphotungstic acid solution (PTA) at pH 6.1, and examined with an electron microscope operating at an accelerating potential of 80 kV. Three characteristics of the coronavirus particles morphology were sought: the diameter of the particles, the presence of surface projections, and the length of those projections. The diameter of approximately 100 particles was measured. The length of the surface projections was measured from the boundary of the particles to the distal end of the projections.

Hemagglutination (HA) activity

The HA properties of the material collected were evaluated in the presence of rabbit, guinea pig, rat, mouse, chicken, sheep, and human type O red blood cells. The assay was performed in U-type microplates at room temperature with 1% erythrocytes concentration in 0.01 *M* PBS, pH 7.2. Tenfold dilutions of the samples prepared as described above for EM examination were used and an equal amount of erythrocyte suspension (0.025 mL) was added to each dilution. Titers were expressed as a reciprocal of the highest dilution of hemagglutinin that showed complete hemagglutination.

Purification of virus

Fecal material with a high HA activity was first centrifuged at 2000 × *g* for 30 min to remove bacteria and cell debris. The supernatant was layered on 5 mL of a 60% sucrose cushion and centrifuged at 90 000 × *g* for 2 h at 4°C. Fractions were collected from the bottom of the centrifuge tubes and assayed for HA activity. The fractions showing HA activity were examined by EM to confirm the presence of the virus and then layered over the top of a 20–60% (w/w) sucrose gradient and centrifuged at 90 000 × *g* for 16 h at 4°C in a refrigerated Beckman L 5-65 model using a SW27 rotor.

Serological tests

Twenty serum samples were obtained from three colonies in which the viral particles were observed. Ten rabbit sera were also obtained from a colony free of intestinal infections. The sera were first inactivated at 56°C for 30 min and tested by hemagglutination inhibition assay (HIA) for the presence of HI antibodies to the rabbit coronavirus. The HIA was performed as described by Palmer *et al.* (1975). Serial 10-fold dilutions were mixed with an equal volume of hemagglutinin containing four HA units, incubated for 60 min at 4°C, and then mixed with 0.025 mL of 1% rabbit erythrocytes. The preparation was incubated at 4°C for 60 min. The reciprocal of the highest dilution of sera that inhibited HA was determined as the HI titer. Immune sera to 229-E human coronavirus, porcine hemagglutinating encephalitis virus (HEV), and avian infectious bronchitis virus (IBV) were also used to evaluate the antigenic relationship of the rabbit coronavirus to other prototypes of coronaviruses.

Cell culture

Supernatants of fecal material previously prepared for EM examination were inoculated on various cell culture systems. Specimens showing an elevated HA titer were selected for this purpose; a total of four cases were used. Primary culture of rabbit kidney cells, whole rabbit embryo, and a continuous cell line of rabbit cornea (SIRC) were used. The cells were grown in equal parts of minimum essential medium (MEM) (Earle's base) and 199 medium (Hank's base) with 2% fetal calf serum and 1% antibiotic preparation containing 100 iu penicillin and 100 mg streptomycin. A 1-mL aliquot of the supernatant fractions selected was inoculated in 75 cm^2 tissue culture flasks containing 2% fetal calf serum and 1% antibiotic preparation. The preparations were incubated at 37°C for 7 days.

Four weekly passages were effected. The cell cultures inoculated were frozen and thawed three times and the supernates of each passage were ultracentrifuged at 90 000 × *g* for 90 min and were examined by EM for the presence of viral particles. The HA activity was also evaluated.

Results

Figure 1 shows electron micrographs of particles observed in specimens from sick rabbits. The virions were pleomorphic particles with an inner

diameter ranging between 60 and 220 nm. Large elongated forms were also seen. In most specimens examined, surface projections which measured approximately 20 nm in length were present. They were teardrop shaped and widely spaced. Finally, an inner tongue-shaped structure was present inside the virion. Similar viral particles were not seen in the feces from healthy rabbits.

The results of the determination of the HA properties of the rabbit coronavirus indicate that this viral agent has HA activity in the presence of rabbit erythrocytes but not in the presence of the other type of red blood cells used. Furthermore, 100% of the fecal samples from sick animals agglutinated rabbit erythrocytes while only 38% of the material from the mucosal scrapings collected from the small intestines of the same animals exhibited an HA activity. The titers of the fecal material varied between 4 and 1024 with a geometric mean of 57.9 whereas the HA activity of the material from the mucosal scrapings of the intestine ranged between 2 and 32 with a geometric mean of 7.33. Specimens from healthy rabbits exhibited HA titers less than 2.

When the fecal material from sick animals was centrifuged in a sucrose density gradient, HA activity was found in two different peaks. The distribution of those peaks is illustrated in Fig. 2. The density of the heavy peak was 1.18 g/cm^3 and that of the light peak was 1.07 g/cm^3.

Results of the serological evaluation of rabbit sera collected from different colonies are presented in Table 1. These results indicate that in colony A, all the sera evaluated had HI antibody titers to rabbit coronavirus. Those titers ranged between 10 and 160 with a geometric mean of 54.4. In colony B, six of the seven sera had HI antibody titers to the viral agent. The titers ranged between 80 and 160 with a geometric mean of 126.9. In colonies C and D all the sera tested were free of demonstrable HI antibody titers to the rabbit coronavirus.

The results of the HA assay in presence of

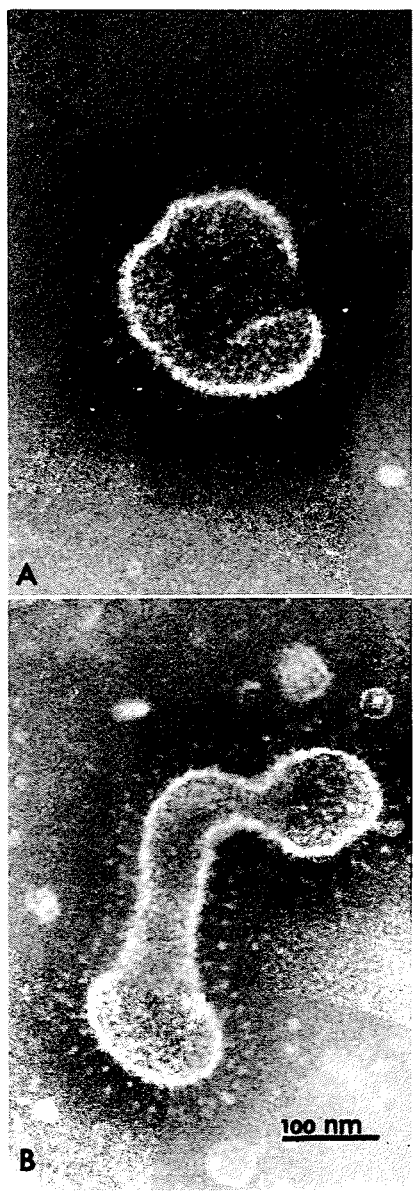

FIG. 1. Electron micrographs of negatively stained coronavirus particles in fecal samples from rabbits with clinical signs of enteritis. A, spherical particle; B, Elongated particle. Typical teardrop surface projections are seen in both A and B.

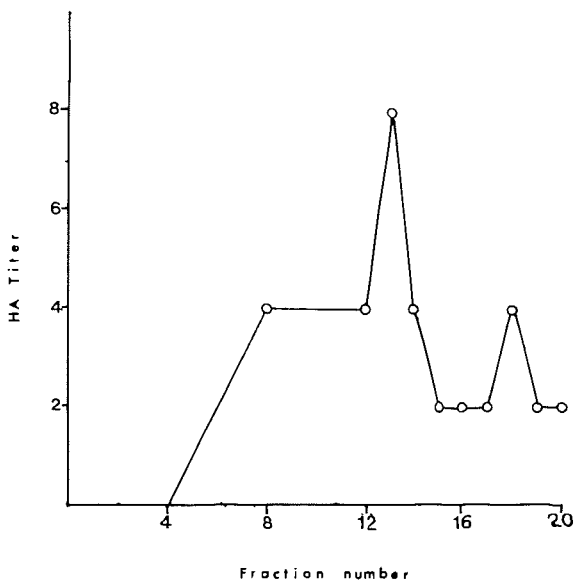

FIG. 2. Distribution of HA antigens of rabbit caronavirus isolate in a sucrose density gradient.

TABLE 1. Serological evaluation of rabbit sera from four different colonies for the presence of HI antibody titers to rabbit coronavirus

Colonies	HI positive/ total number	HI titers range*
A	9/9	10–160 (54.43)
B	6/7	80–160 (126.99)
C	0/4	—
D	0/10	—

*Geometric mean in parentheses.

TABLE 2. Relationship of rabbit coronavirus to prototypes of other hemagglutinating coronaviruses*

		Antisera		
Antigen	Samples	229-E	HEV	IBV
Fecal material	3	3/3	0/3	0/3

*The HIA was used to evaluate this relationship. Data are number of samples inhibited/total.

specific immune sera to other coronaviruses are presented in Table 2. These results indicate that immune sera to human coronavirus 229-E inhibited the HA activity of the rabbit coronavirus. Immune sera to the HEV and IBV did not prevent the hemagglutination.

No cytopathic effects were observed following inoculation of the fecal material on the different tissue culture systems used. Furthermore, the examination by EM of an aliquot of the supernatant of the culture media of each passage did not reveal the presence of any viral particles following ultracentrifugation. The supernatants of inoculated tissue cultures did not show any HA activity.

Discussion

The presence of viral agents in the intestine of laboratory rabbits is seldom reported. In the past, a parvovirus (Matsunaga *et al.* 1977) and a rotavirus (Petric *et al.* 1978) have been observed in the digestive tract of this animal species. To our knowledge, this is the first report on the presence of a coronavirus in the intestine of the rabbit.

The morphology and the diameter of the viral particles observed as well as the presence of surface projections are major points which classify this agent in the Coronaviridae family. Members of this virus family are pleomorphic organisms with a diameter ranging between 60 and 220 nm. Their density in sucrose gradient varies between 1.16 and 1.23 g/cm^3. The viral particles reported in this study are also pleomorphic and their diameter is approximately 60–220 nm. The density of the complete virion in sucrose gradient has been estimated at approximately 1.18 g/cm^3 which is within the ranges of the coronaviruses. The presence of virions with a density of 1.07 g/cm^3 could be explained by the absence of nucleic acid. On EM examination these particles correspond to PTA penetrated virions.

When compared with other coronaviruses, the morphology of the surface projections are similar to the projections of the human coronavirus 229-E. They both are bulbous and widely spaced on the virus envelope (Tyrrel *et al.* 1978). Finally the inner tongue-shaped membrane observed in the spherical forms of the virions is also reported with IBV (Tyrrell *et al.* 1978).

Some members of the Coronaviridae family agglutinate red blood cells (Tyrrell *et al.* 1978). The viral agent observed in this study also has an HA activity and rabbit erythrocytes are agglutinated at room temperature by a preparation of fecal material and by material obtained by mucosal scraping of the intestine of sick rabbits. This HA activity is limited to rabbit red blood cells. This property is an important characteristic because it allows rapid detection of the virus in the intestine of sick rabbits. The HA appears to be virus specific and not due to other hemagglutinating substances because the presence of HA titers in the material evaluated correspond to the observation of viral particles by EM. This HA property is maintained following sucrose gradient purification. Furthermore, rabbit sera from infected colonies prevent the HA activity as well as immune serum to the human coronavirus 229-E. These results confirm the HA property of the rabbit coronavirus. The results obtained with immune serum to human coronavirus 229-E also indicate some antigenic relations between the two viruses. Fecal material from healthy rabbit did not exhibit any HA activity and viral particles were not observed by EM.

The predominance of the HA activity was higher and the HA titers more elevated in the fecal material than in the mucosal scrapings. These data indicate that the hemagglutinin is present and available at higher concentration in the fecal material. Thus, the evaluation of the HA activity of the feces constitutes a reliable assay to detect the presence of the rabbit coronavirus.

The results of the serological evaluation indicate the presence of HI titers in colonies A and B. In these colonies, a high incidence of intestinal infections is reported and viral particles have been observed in fecal material from sick rabbits. This strongly suggests that the titers detected are due to the presence of the viral agent in those colonies.

In colony C, intestinal infections are also re-

ported; however a serological response has not been demonstrated. This could be attributed to the relatively small number of samples tested or it could indicate that the animals tested did not have any previous contact with the virus.

In colony D, the absence of serological response corresponds to the absence of intestinal infections. Furthermore, the absence of HI titer in colonies C and D suggest that the titers detected in colonies A and B were specific to the virus and not caused by the presence of a nonspecific inhibitor. Such inhibitors should have been detected in colonies C and D.

The serum samples tested were collected from adult animals because the majority of young rabbits affected died rapidly after the onset of the clinical manifestations. For this reason, it was impossible to show a seroconversion in the sera of these young animals.

Although fresh material with an elevated HA activity was used to infect different tissue cultures, no cytopathic effect was observed after four passages. These results were confirmed by the absence of HA activity in the supernatants of the culture media. The absence of *in vitro* growth made difficult any further characterization of this viral agent.

The role of the rabbit coronavirus in the pathogenesis of intestinal infection in this animal species is not yet known. However coronaviruses are responsible for enteritis in other species (Kraft 1957; Appel *et al.* 1979) and the results of this study also suggest an etiological role in diarrhea of rabbits.

Acknowledgements

The authors would like to thank Dr. L. Berthiaume and Mr. R. Alain from the Centre de recherche en virologie, Institut Armand-Frappier, for their assistance in the identification of the virus.

APPEL, M. J. G., B. J. COOPER, H. GREISEN, F. SCOTT, and L. E. CARMICHAEL. 1979. Canine viral enteritis. 1. Status report on corona- and parvo-like viral enteritides. Cornell Vet. **69**: 123–144.

CAUL, E. O. 1979. Coronavirus-like particles present in simian faeces. Vet. Rec. **104**: 168–169.

CHASEY, D., and S. F. CARTWRIGHT. 1978. Virus-like particles associated with porcine epidemic diarrhea. Res. Vet. Sci. **25**: 255–256.

DOYLE, L. P., and L. M. HUTCHINGS. 1946. A transmissible gastroenteritis in pigs. J. Am. Vet. Med. Ass. **108**: 257.

GREENHAM, L. W. 1962. Some preliminary observations on rabbit mucoid enteritis. Vet. Rec. **74**: 79–84.

KIENAN, K. P., H. R. JERVIS, R. H. MARCHWECKI, and L. N. BENN. 1976. Intestinal infection of neonatal dogs with canine coronavirus 1-71: studies by virologic, histologic, histochemical and immunofluorescent techniques. Am. J. Vet. Res. **37**: 247–256.

KRAFT, L. M. 1957. Studies on the etiology and transmission of epidemic diarrhea of infant mice. J. Exp. Med. **106**L: 743–755.

MATSUNAGA, Y., S. MATSUNO, and J. MUKOYAMA. 1977. Isolation and characterization of a parvovirus of rabbits. Infect. Immun. **18**: 495–500.

MIDDLETON, P. J., M. T. SZYMANSKI, G. D. ABBOTT, R. BORTOLUSSI, and J. R. HAMILTON. 1974. Orbivirus acute gastroenteritis of infancy. Lancet, **1**: 1241–1244.

ONONIWU, J. C., and R. J. JULIAN. 1978. An outbreak of Tyzzer's disease in an Ontario rabbitry. Can. Vet. J. **19**: 107–109.

OSTLER, D. C. 1961. The diseases of broiler rabbits. Vet. Rec. **73**: 1237–1253.

PALMER, D. F., W. R. DOWLE, M. T. COLEMAN, and G. C. SCHILD. 1975. Advance laboratory technics for influenza diagnosis. Immunology Series No. 6, U.S. Department of Health, Education and Welfare, Public Health Service, Washington. pp. 16–62.

PETRIC, M., P. J. MIDDLETON, C. GRANT, J. S. TAM, and C. M. HEWITT. 1978. Lapine rotavirus: preliminary study on epizoology and transmission. Can. J. Public Health, **69**: 69.

PRESSCOTT, J. F. 1978. *Escherichia coli* and diarrhea in the rabbit. Vet. Pathol. **15**: 237–248.

RICHTER, C. B., and R. L. HENDREN. 1969. The pathology and epidemiology of acute enteritis in captive cottontail rabbits (*Sylvilagus floridanus*). Path. vet. **6**: 159–175.

STORZ J., A. M. DOUGHRI, and I. HAJER. 1978. Coronaviral morphogenesis and ultrastructural changes in intestinal infections of calves. J. Am. Vet. Res. **173**: 633–635.

TYRREL, D. A. J., D. J. ALEXANDER, J. D. ALMEIDA, C. H. CUNNINGHAM, B. C. EASTERDAY, D. J. GARWES, J. C. HIERHOLZER, A. KAPIKIAN, M. R. MACNAUGHTON, and K. MCINTOSH. 1978. Coronaviridae: second report. Intervirology, **10**: 321–328.

WHITNEY, J. C. 1976. A review of non-specific enteritis in the rabbit. Lab. Anim. **10**: 209–221.