

## ISOLATION OF CORONAVIRUSES FROM NEONATAL CALF DIARRHOEA IN GREAT BRITAIN AND DENMARK

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### ABSTRACT

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Coronaviruses were isolated from neonatal calves with diarrhoea in Great Britain and Denmark. They were serially passed in gnotobiotic calves which developed acute diarrhoea. Pathological lesions were found in the small and large intestines. Coronaviruses were demonstrated by electron microscopic examination of the faeces and intestinal contents, immunofluorescent staining of sections of small and large intestine and by isolation in tracheal organ cultures. In early passages of the British coronavirus, particles of about 30 nm in diameter were observed in the faeces by electron microscopy. These particles were removed from the coronavirus preparations by cross-protection experiments in calves. The coronaviruses were morphologically and antigenically similar to the bovine coronavirus isolated in the United States and the British virus was adapted to replicate in calf kidney cell cultures.

### INTRODUCTION

Coronavirus-like particles have been associated with diarrhoea in calves in the United States (Mebus et al., 1972; Stair et al., 1972) and the virus was characterized as a member of the coronavirus group by Sharpee et al. (1976). The presence of the virus has been reported in Belgium on the basis of a serological survey (Zygraich et al., 1975) and in Canada on the basis of electron microscopy and immunofluorescence of tissue sections (Acres et al., 1975; Morin et al., 1976). However, apart from the studies in the United States, no further evidence for coronaviruses as a cause of diarrhoea in calves has been obtained.

During examination of faeces from field outbreaks of diarrhoea in the United Kingdom, coronavirus-like particles and small particles 27–32 nm in diameter were observed in the electron microscope (Woode et al., 1974). The presence of coronaviruses in outbreaks of diarrhoea was also suspected in Denmark by the use of immunofluorescent techniques. This paper reports the

isolation of coronaviruses from diarrhoeic calves in the United Kingdom and Denmark and describes the experimental infection in calves and culture of the virus *in vitro*.

## MATERIALS AND METHODS

### *Source of viruses*

Two British viruses, D544 and K418, and one Danish virus were studied. D544 was obtained from a 2-days old calf which died after suffering from diarrhoea. Coronavirus-like particles were seen in the faeces by electron microscopy and *Salmonella dublin* was also isolated. K418 was obtained from the faeces of a calf on a second farm. This calf had a mild diarrhoea and quickly recovered.

The Danish virus was obtained from the intestinal contents of a 2-days old Jersey calf in a herd with a high mortality rate from calf scours. Sections of the small intestine and colon revealed large numbers of fluorescent cells when stained with fluorescein-conjugated antiserum to the American coronavirus. No known pathogenic bacteria were isolated from the calf.

### *Animal inoculation*

Calf faeces containing either the British virus D544 or the Danish virus were diluted 1 : 3 with phosphate-buffered saline (PBS), pH 7.2, and passed through 0.45  $\mu$ m membrane filters. Gnotobiotic calves (Dennis et al., 1976) aged 1–42 days were given intranasal or intra-oral inoculations of 2–5 ml of each filtrate. Unfiltered faeces or intestinal contents were used for further passes in calves. In order to ensure freedom from rotaviruses, the filtered preparation of the Danish virus was incubated for 1 h at 37° C with an equal volume of undiluted antiserum prepared in a gnotobiotic calf against the British calf rotavirus (Woode et al., 1974) and with a neutralising titre of 1/80.

### *Virus detection by electron microscopy*

Approximately 5 ml of faeces or intestinal contents were mixed well with three volumes of PBS. After clarifying twice at 8000 *g* for 30 min, the supernatant fluid was centrifuged at 100000 *g* for 2 h. The resulting pellet was resuspended in a few drops of PBS, layered on top of a sucrose cushion consisting of 4 ml of 30% (*w/w*) and 4 ml of 40% (*w/w*) sucrose solution and centrifuged at 83000 *g* for 2 h. The pellet was resuspended in a few drops of PBS, a drop placed on a carbon-coated formvar electron microscope grid and examined after staining with 2% potassium phosphotungstate, pH 6.0.

Culture fluids were prepared by a similar procedure without the initial dilution with PBS.

Electron microscopy of faecal samples from calves inoculated with corona-

virus D544 showed the presence of small virus-like particles in addition to coronavirus particles. To separate the two different viruses, calf 5 (Table I) was inoculated at 2 days of age with a filtrate prepared from the faeces of calf 4 by treatment with ether and nonidet P-40. Forty days later calf 5 was inoculated with a faecal filtrate from calf 2 in which both coronaviruses and small viruses were present. Ether and nonidet treatment was conducted by mixing 1.0 ml of diarrhoeic faeces from calf 4, taken 2 days after inoculation, with 50 ml of PBS. After centrifugation at 8000g for 30 min the supernatant fluid was passed through clarifying and 1.2  $\mu$ m membrane filters, mixed with an equal volume of Analar diethyl ether and mechanically shaken for 10 min. The aqueous layer was removed and ether extraction repeated twice. The remaining ether was allowed to evaporate from the final aqueous layer before nonidet P-40 was added to a final concentration of 1% (*v/v*). After passage through a 0.45  $\mu$ m filter, 4.0 ml were inoculated intranasally into calf 5.

### *Antisera*

Serum samples were taken from experimentally infected calves before inoculation and 3 weeks after infection.

Rabbit antiserum to the American coronavirus isolated from calf diarrhoea (virus neutralising titre of 1/512) was kindly supplied by Dr. N. Zygraich, R.I.T., Rixensart, Belgium. Fluorescein-conjugated rabbit antiserum to the same virus was supplied by Professor C.A. Mebus, Lincoln, Nebr., U.S.A.

### *Pathology*

Tissues for histopathological examination were removed under pentobarbitone sodium anaesthesia. Short lengths of upper, middle and lower small intestine were tied off and filled with 12% neutral buffered formalin; fixation was completed by immersion in fixative. Blocks of fixed tissues were dehydrated and embedded in paraffin wax; sections were cut at 5  $\mu$ m and stained with haematoxylin and eosin. For villus-crypt ratio determinations, five to eight villi per section, which were sectioned through their entire length, were selected.

### *Immunofluorescence of small intestine and spiral colon*

Calves infected with either the British or Danish virus were sacrificed 24–48 h after diarrhoea began. Sections were cut from frozen segments of tissue, fixed in acetone and stained either by the direct method with fluorescein-conjugated rabbit antiserum to the American coronavirus or by the indirect method with convalescent antisera to the British (calves 1 and 7, Table I) or the Danish viruses (calf 12) and fluorescein-conjugated rabbit anti-bovine gamma globulin antiserum (Nordic Laboratories).

TABLE I

Results of inoculation of coronaviruses into gnotobiotic calves

Calf	Age (days)	Calf from which inoculum prepared	Development of diarrhoea	Coronavirus in faeces or gut contents		Fluorescence in small intestine and spiral colon
				Electron microscopy	Organ culture	
1	13	British virus D544	-	+	+	NT
2	1	1	+	+	+	NT
3	28	1	+	+	+	+
4	4	2	+	+	+	NT
5	42	2	-	+	+	NT
6	3	5	+	+	+	+
7	1	6	+	+	+	NT
8	3	7	+	+	NT	NT
9	4	Danish virus	+	NT	NT	+
10	2	Danish virus	+	+	NT	+
11	1	9	+	+	+	+
12	1	9	+	NT	NT	NT
13	36	9	-	+	NT	NT
14	38	9	-	+	NT	NT

NT = not tested.

*Cell culture*

Primary calf-kidney (CK) cell cultures were prepared in tubes with flying coverslips. Growth medium was Earle's salt solution with galactose 0.1% (*w/v*) in place of glucose, lactalbumin hydrolysate (Nutritional Biochemicals) 0.5% (*w/v*) and foetal calf serum 10% (*v/v*). For maintenance of infected cultures 3% foetal calf serum was used initially, but in later experiments foetal calf serum was replaced with tryptose phosphate broth 5% (*w/v*), bovine albumin 0.09% (*w/v*) and Hepes buffer. For titration of viruses, dilutions were made in PBS and 0.2 ml of each dilution inoculated into 1.8 ml of medium in each coverslip tube.

*Virus isolation in cell culture*

Bacteria-free filtrates of faeces or intestinal contents were inoculated into coverslip cultures at dilutions of  $3 \times 10^{-2}$  and further ten-fold dilutions up to  $3 \times 10^{-7}$ . The cultures were fixed in acetone 24 h to 7 days after inoculation and examined for evidence of infection by immunofluorescence using the methods and sera described above. In a later attempt to culture these coronaviruses, the small intestine of a calf killed on the 2nd day of diarrhoea follow-

ing administration of the British virus was ground with sterile sand in Earle's balanced salt solution. Dilutions of this material from  $3 \times 10^{-2}$  up to  $3 \times 10^{-4}$  in PBS were inoculated into CK coverslip cultures with 3% foetal calf serum in the medium.

#### *Virus isolation in tracheal organ cultures*

Organ cultures of foetal trachea were prepared and maintained as described by Stott et al. (1977). Faecal filtrates were prepared as described for the preparation of calf inocula.

Three to five tracheal rings, contained in one 6 oz bottle, were immersed for 1.5 h at 37° C in a mixture of 0.5 ml of faecal filtrate and 0.5 ml maintenance medium. Unadsorbed virus was removed by washing the rings with 5 ml of medium. The maintenance medium was changed every 3 or 4 days for 21 days, stored at -70° C, then assayed for haemagglutinating activity.

At 10 and 21 days one ring from each bottle was frozen in liquid nitrogen, sectioned and stained for immunofluorescence as described above.

#### *Haemagglutination tests*

One per cent (*v/v*) washed rat erythrocytes were added to samples diluted in micro-titre plates with PBS containing 0.1% bovine serum albumin. End points were read after the erythrocytes had settled for 1-1.5 h at 4° C. The haemagglutination inhibition test was conducted by incubating 4 HAU of virus with dilutions of antiserum for 1 h at 37° C.

## RESULTS

#### *Clinical signs of disease*

Of the 14 gnotobiotic calves inoculated with the British coronavirus D544, the Danish coronavirus, or with serial passages of these viruses, ten developed diarrhoea which varied in colour from yellow to dark green (Table I). The diarrhoea was sometimes accompanied by anorexia. The incubation period varied between 2 and 5 days and diarrhoea persisted for 4 days. All the calves allowed to recover, did so without treatment. Three calves (5, 13 and 14) of the four which did not develop diarrhoea were over 35 days of age when challenged but were shown to have multiplied to coronavirus by the presence of particles in their faeces.

#### *Electron microscopic examination*

Coronavirus particles were identified in faecal or intestinal samples of all inoculated calves examined (Table I). Particles ranged in diameter from 110 to 180 nm and were characterized by the presence of projections 17 to 24 nm

in length (Fig.1). Occasional particles appeared to have lost some of their projections leaving areas with either no projections or a narrow fringe of shorter projections. On some particles this narrow fringe was clearly visible (Fig.1B). Coronavirus particles were not seen in pre-inoculation faecal samples although particles with fringes of about 10 nm were often seen in pre- and post-challenge faecal samples. Hence diagnosis of coronavirus particles by electron microscopy was made only when particles with distinct, approximately 20 nm long projections were identified.

In addition to coronavirus particles, other virus-like particles, approximately 30 nm in diameter, were seen in faecal samples from calves 1 to 4 which were inoculated with the British virus or serial passes of it. These particles were seen only in preparations from calf 1 after extensive examination in the electron microscope but were readily seen in clumps of 2–100 or more particles in daily faecal samples taken from calves 2, 3 and 4. They were not seen in pre-inoculation faecal samples. Although their morphology was sometimes indistinct, there appeared to be two morphological types present, one type appeared calicivirus-like (Fig.2A) and the other astrovirus-like (Fig.2B) (Madeley et al., 1977).

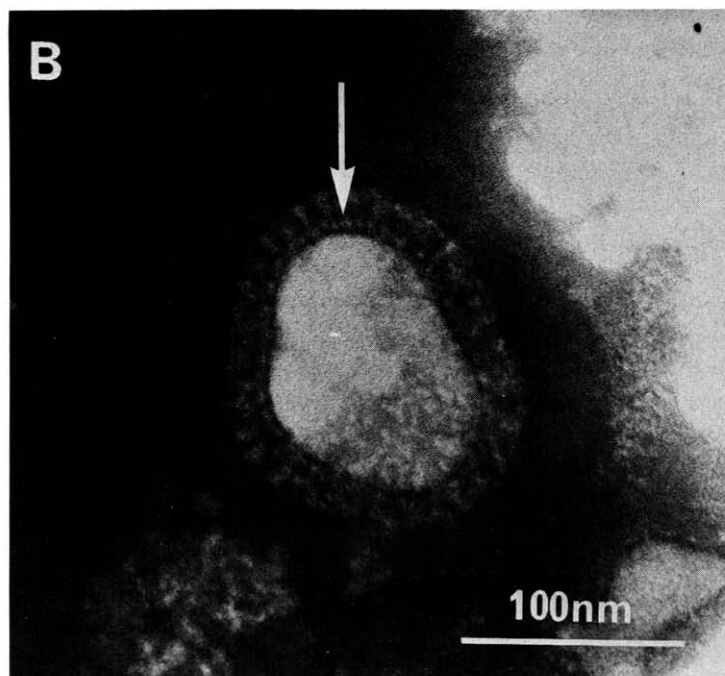
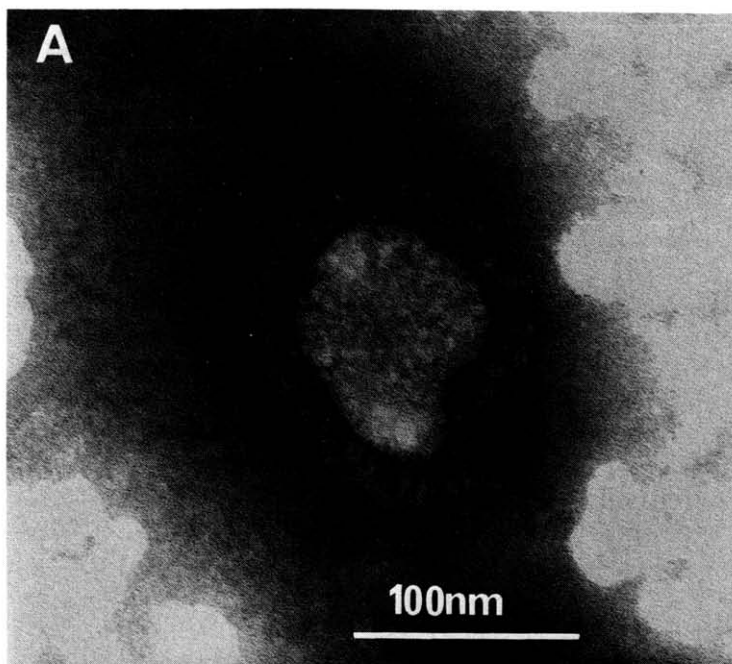
The attempt to separate these 30 nm particles from the British coronavirus by calf inoculation was successful. A faecal filtrate, in which coronaviruses and 30 nm particles were present, was treated with ether and nonidet to destroy the coronaviruses. After inoculation of calf 5 with this filtrate, 30 nm particles were excreted in the faeces on days 3–5 after inoculation but coronavirus particles were not seen by electron microscopy. A serum sample taken 25 days after infection contained no detectable fluorescent or haemagglutination inhibiting antibodies to the coronavirus. Forty days after the first inoculation, calf 5 was inoculated with the untreated filtrate of the British virus. Coronavirus particles but not 30 nm particles were observed 3–5 days after infection. Further passes of this coronavirus preparation caused diarrhoea in calves 6 to 8 and only coronaviruses were detected in the faeces.

Electron microscopy of faecal samples from calves 9 to 14 inoculated with the Danish isolate or serial passes of it, did not reveal viruses other than coronaviruses.

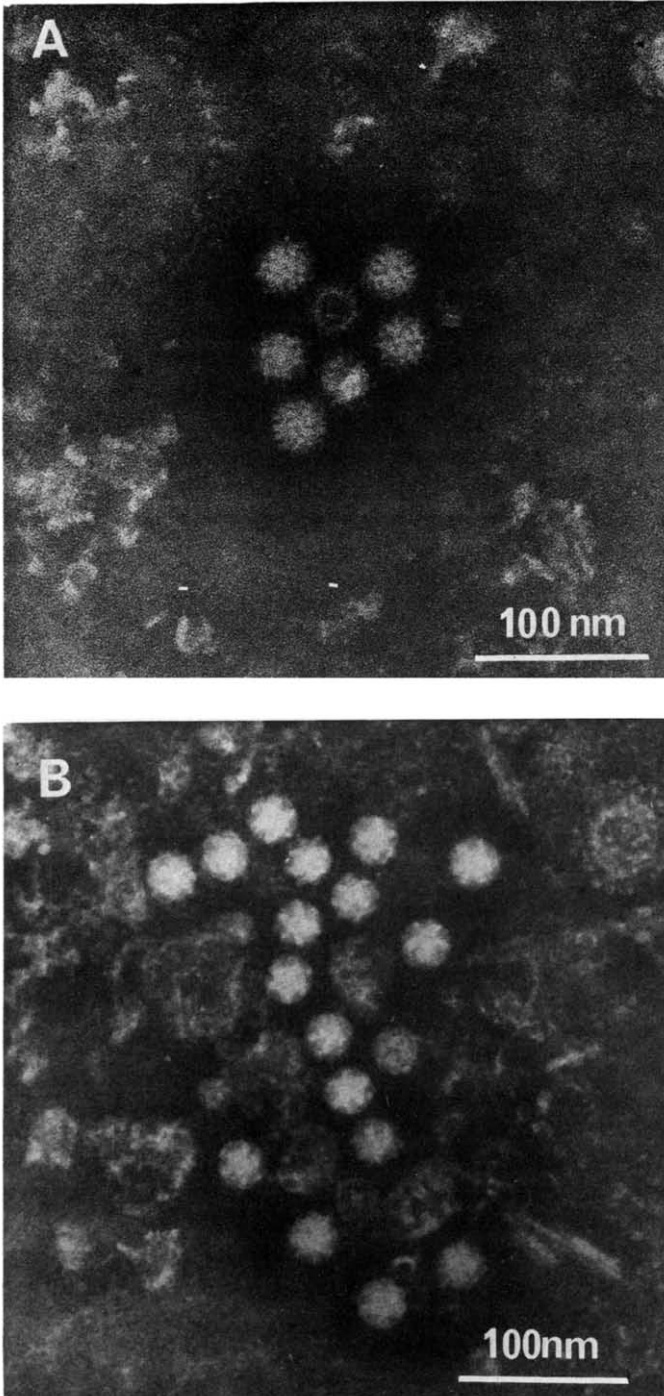
### *Histopathology*

The villi of the upper and middle small intestine of control calves were long and thin and lined by columnar epithelial cells whose nuclei were found near the luminal surface of the cell. They had well defined brush borders. The villi of the lower small intestine were also long but the nuclei of the columnar epithelial cells were at the base of the cell and large vacuoles were present in the cytoplasm. There were few cells in the lamina propria of the upper, middle and lower small intestine.

In all five infected calves studied histologically the lesions were most prominent in the middle and lower small intestine. This was reflected in the lower



**Fig.1. Negatively stained coronavirus particles in a faecal sample from a diarrhoeic calf. Some particles show a narrow fringe of shorter projections (arrowed) and 20 nm projections (Fig.1B).**



**Fig. 2.** The two morphological types of approximately 30 nm virus-like particles seen in faecal samples of calves 1 to 4. (A) Calicivirus-like particles. (B) Astrovirus-like particles.



villus to crypt ratios in these areas compared to control calves (Table II). The range of measurements for the villus to crypt ratios of the upper small intestine tended to overlap those of the control calves, although some villi were thickened and lined by cuboidal epithelial cells. In the middle and lower small intestine the villi observed were distinctly shortened and thickened. They appeared ragged at the luminal surface and were either lined by cuboidal to squamous epithelial cells with poorly defined brush borders or were devoid of epithelium with the lamina propria exposed at the villous tips. There was an increase in the number of cells in the lamina propria.

The colonic epithelium of infected calves had fewer goblet cells and the epithelial cells tended to be cuboidal to squamous in some areas but there were no well defined lesions.

TABLE II

Villus to crypt ratios in the small intestine of calves inoculated with the British and Danish coronaviruses

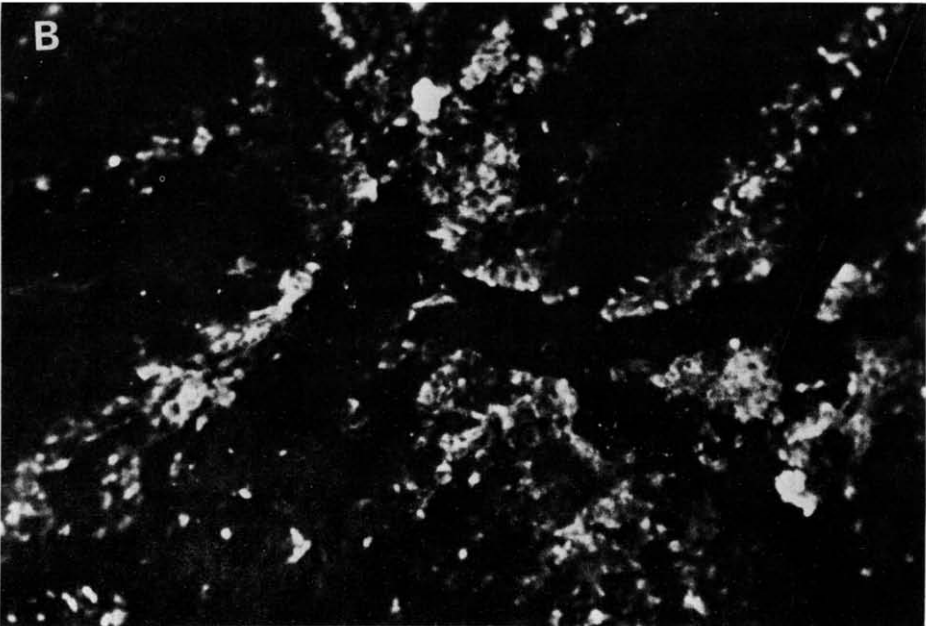
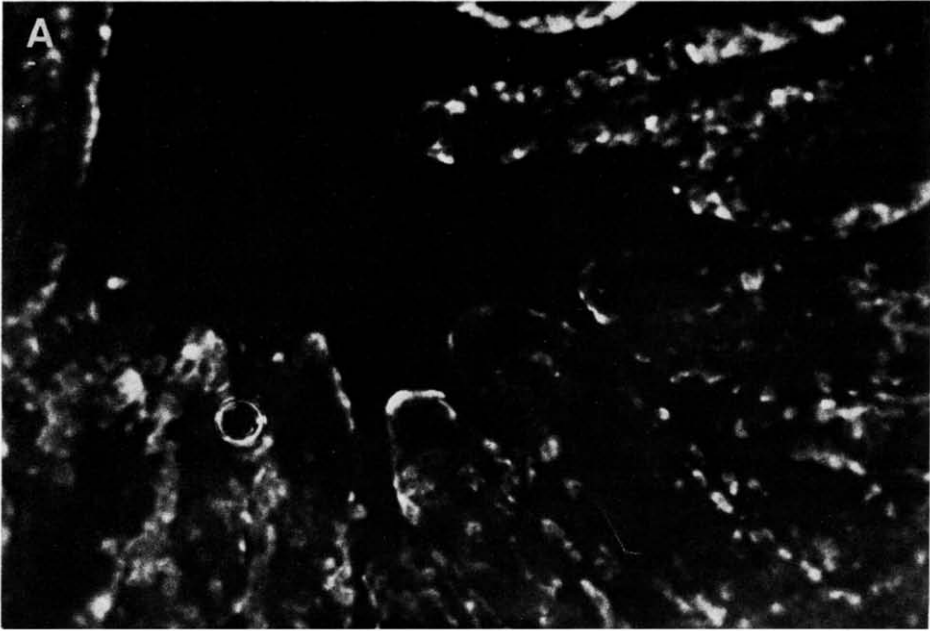
Calf No.	Age inoculated (days)	Age necropsied (days)	Villus : crypt ratio in small intestine		
			Upper	Middle	Lower
6	3	7	3.4* (3.0-3.8)**	2.0 (1.2-3.0)	1.6 (1.2-1.8)
8	3	7	NT	2.25 (2.0-2.25)	1.3 (1.2-1.5)
9	4	10	4.4 (3.3-4.7)	1.8 (1.3-2.5)	1.2 (1.0-1.4)
10	2	5	2.67 (1.7-2.9)	2.0 (1.3-2.3)	1.9 (1.7-2.1)
11	1	5	3.0 (2.2-3.5)	2.5 (2.1-2.9)	1.6 (1.3-2.0)
Control calf 1	—	7	4.1 (3.4-4.5)	5.6 (4.5-7.6)	4.3 (3.5-5.5)
Control calf 2	—	6	4.2 (3.0-4.8)	5.7 (4.2-6.5)	5.1 (5.0-5.2)

\*Average of five to eight measurements.

\*\*Range of measurements observed.

### *Immunofluorescence of small intestine and colon*

In calves infected with the British and Danish viruses the villous epithelial cells of the middle and lower small intestine fluoresced with antisera to the British, Danish and American coronaviruses (Fig.3A). Fluorescence was not observed in crypt cells. In some sections fluorescence was confined to the tips of the villi but in other sections the majority of the villous cells fluoresced. Fluorescent cells were either absent or very few in the upper small intestine but in the spiral colon, most of the cells of the epithelium and crypts fluoresced (Fig.3B).



**Fig.3.** Immunofluorescence of sections of (A) small intestine from calf 11, and (B) spiral colon from calf 9, both sacrificed 24 h after the commencement of diarrhoea.

### *Cell culture of the virus*

Attempts to cultivate the Danish coronavirus in cell cultures proved unsuccessful. Several attempts were made to propagate the British virus in cell culture with intestinal and faecal filtrates from the infected gnotobiotic calves and with tracheal organ culture fluids. Although these preparations were infectious to calves and tracheal organ cultures respectively, they failed to infect CK cultures at dilutions of  $3 \times 10^{-2}$  to  $3 \times 10^{-7}$  when examined by immunofluorescence 1–7 days after inoculation. Nor was fluorescence seen after three further passes in cell culture. The only successful adaptation was achieved following inoculation of a ground intestinal preparation from calf 2 into CK cells at a dilution of  $3 \times 10^{-4}$ . Dilutions of  $3 \times 10^{-2}$  to  $3 \times 10^{-3}$  were not infectious. A few large plaques of immunofluorescent cells were observed, 7 days after infection, by staining with antisera to the British, Danish or American coronaviruses. The virus was then passed at 3- to 5-day intervals in primary CK cells but the titre of infectious virus was low in early passes.

Electron microscopy of preparations from the eighth pass in tissue culture revealed the presence of coronavirus particles which were indistinguishable from those seen in faecal preparations. No other virus-like particles were observed.

A mixture of culture fluids from the eighth and ninth passes of the British coronavirus was inoculated intranasally into a gnotobiotic calf. Eight days after infection the calf developed diarrhoea, coronavirus particles were observed in the faeces and coronavirus antibody was detected in the serum 3 weeks post inoculation. This virus was serially passed in two gnotobiotic calves which developed diarrhoea and excreted coronaviruses in the faeces.

### *Virus isolation in tracheal organ cultures*

Haemagglutinating activity and fluorescent antigens were detected in tracheal organ cultures inoculated with faecal filtrates of two British viruses, D544 and K418, filtrates from experimental calves 1 to 7 and a filtrate from calf 11 inoculated with the Danish virus (Table I). Damage to ciliary activity was not observed. Haemagglutinating titres ranged from 2 to 32 in the organ culture fluids. The time of maximum production varied between 1 and 12 days after infection and, sometimes, haemagglutinating activity was still detectable at the end of the experiment, 21 days after infection. Coronavirus D544 was serially passaged four times. Haemagglutinating activity produced in cultures infected with D544 and K418 was inhibited by a 1/64 dilution of rabbit antiserum to the American bovine coronavirus and by 1/320 dilutions of convalescent antisera from calves inoculated with the British and Danish viruses. The pre-inoculation sera did not show inhibitory activity. Whenever haemagglutinating activity was detected fluorescent antigens were found in the epithelial cells of the trachea by immunofluorescent tests using antisera to the British and American coronaviruses.

Electron microscopy of fluids from cultures inoculated with the three coronaviruses revealed the presence of coronavirus particles which were indistinguishable from those seen in faecal preparations. No other virus-like particles were observed.

## DISCUSSION

Two bovine coronaviruses were shown to cause diarrhoea in gnotobiotic calves. The pathological lesions observed were similar to those reported by Mebus et al. (1973) for the American coronavirus isolated from calf diarrhoea. The two viruses were shown to be related antigenically to the American virus by immunofluorescent staining techniques and haemagglutination inhibition tests. In the few older calves inoculated with both the British and the Danish coronaviruses there appeared to be a decrease in susceptibility with age since older calves excreted virus but did not develop diarrhoea.

Some of the calves inoculated with early passes of the British virus appeared to be infected with more than one virus. Although coronaviruses and approximately 30 nm viruses were co-transmitted in calves 1 to 4 it appeared that the British coronavirus was a pathogen in its own right as no evidence of infection of calves 6, 7 and 8 with the 30 nm viruses or any other virus was obtained. From preliminary work, however, it seems that filtrates containing the 30 nm particles are capable of inducing diarrhoea in calves (to be published) and thus, at present, it appears that viral diarrhoea in calves can be caused by several quite different types of viruses.

The determination of the cause of an individual outbreak of enteritis depends on the availability of sensitive diagnostic tests. Although both British coronaviruses were identified in field material by electron microscopy, this method is unsuitable for routine diagnosis because of the length of time required to prepare and examine each sample and as the numbers of virus particles in watery faecal samples can be low. The fringed particles which are found in normal faeces make it more difficult to visualize coronavirus particles than, for example, particles of rotavirus. As the coronaviruses could not be isolated readily in cell culture from diarrhoeic material, the other two methods, immunofluorescent staining of gut sections and isolation in tracheal organ cultures, may be more suitable for diagnosis. Immunofluorescent staining of sections from the spiral colon of infected animals has the advantage that fluorescent antigen persists for several days (Mebus et al., 1973) but this method can only be used on calves which have been killed and is therefore limited to a herd diagnosis. Isolation in tracheal organ cultures was successful for the three viruses studied in this paper and the American virus (Stott et al., 1977) and may prove a useful diagnostic test to determine the incidence of bovine coronavirus infection in outbreaks of enteritis.

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