Viruses and virus-like particles detected during examination of feces from calves and piglets with diarrhea

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Abstract

A total of 763 fecal or intestinal samples from diarrheic calves and piglets were examined for viral content by immunofluorescence, electron microscopy or cell culture. Routine fluorescent antibody and cultural tests detected rotavirus (n = 126), coronavirus (n = 80) and bovine viral diarrhea virus (n = 13). Electron microscopy detected rotaviruses (n = 24) and coronaviruses (n = 17) not identified by standard fluorescent antibody tests. Other viruses detected by electron microscopy included Breda virus-like particles (n = 49), astroviruses (n = 1), caliciviruses (n = 1), rhabdoviruses (n = 1), parvoviruses (n = 2), enteroviruses (n = 3), togavirus-like particles (n = 2), and "chained" particles (n = 5). Mixtures of several of the viruses were detected in a number of fecal samples.

The survey emphasized the value of electron microscopy as a broad-spectrum diagnostic tool.

Résumé

L'identification de virus et de particules virus-like durant l'examen de fèces de veaux et de porcelets avec de la diarrhée

Un nombre total de 763 échantillons de fèces ou de prélèvements intestinaux survenant de veaux et de porcelets diarrhéiques furent examinés pour la présence de virus par immunofluorescence, microscopie électronique ou culture cellulaire. Les tests d'anticorps fluorescents et de culture ont détecté le rotavirus (n = 126), le coronavirus (n = 80) et le virus de la diarrhée bovine (n = 13). La microscopie électronique a détecté les rotavirus (n = 24) et les coronavirus (n = 17) qui n'avaient pas été identifiés par la technique d'anticorps fluorescents. Entre autres, la microscopie électronique a mis en évidence d'autres agents tels que les particules virus-like Breda (n = 49), les astrovirus (n = 1), les calcivirus (n = 1), les rhabdovirus (n = 1), les parvovirus (n = 2), les enterovirus (n = 3), les particules toga-like (n = 2) et des particules en chaîne (n = 5). Un mélange de plusieurs virus fut identifié dans de nombreux cas.

L'étude a démontré l'utilité diagnostique de la microscopie électronique.

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Introduction

Rotaviruses and coronaviruses are well established as causes of diarrhea in young animals (1–3). Although bovine viral diarrhea (BVD) virus is usually associated with disease in older animals, it has occasionally been incriminated as a cause of diarrhea in neonatal calves (4,5). Breda virus (6,7) and an unnamed syncytium-forming virus (8) have also been reported as causes of diarrhea in calves. Other viruses such as astroviruses (9,10), caliciviruses (9,10), parvoviruses (11), and enteroviruses (12) have been recovered from cases of diarrhea in calves, piglets, and lambs, but are generally thought to cause only mild disease under normal conditions (9,11,12).

Diagnosis of rotavirus and coronavirus infection is usually made by immunological methods such as the fluorescent antibody (FA) test or enzyme-linked immunosorbent assay (ELISA). The limited availability of electron microscopes has tended to restrict the use of this equipment for diagnosis, though they are capable of detecting a wide range of viruses.

We have been disturbed by the number of outbreaks of diarrhea in calves and piglets in Saskatchewan where no causative diagnosis has been made. Hence, increased use was made of electron microscopy to supplement routine virological, bacteriological, and parasitological procedures, especially where the outbreaks were severe or recurring. Our aim was to check existing virological techniques and to investigate the possibility that other undetected viral agents were involved in causing diarrhea.

Materials and methods

Processing of samples

Samples of feces or intestines from young calves and piglets with diarrhea were received as routine diagnostic submissions. The ages of the animals ranged from two days to six weeks, though most were one to two weeks old. Fecal smears and frozen sections of intestines were tested by FA procedures for rotavirus and coronavirus. Where the history indicated the possibility of BVD virus infection, isolation was attempted in cell culture. When initial results were negative or suspicious, and the history indicated a severe or continuing problem, samples were examined further by electron microscopy. In some cases, samples that were positive by FA were checked further if the disease was unusually severe.

Fluorescent antibody procedures

Fecal smears or frozen sections of intestine were dried and fixed with acetone. Bovine coronavirus and calf and piglet rotavirus were diagnosed by indirect FA tests, using bovine coronavirus and rotavirus monoclonal antibodies (kindly supplied by Dr. L. Babiuk, Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan) and goat antimouse fluorescein conjugate (Organon Teknica-Cappel, Malvern, Pennsylvania). Transmissible gastroenteritis (TGE) coronavirus was detected by a direct FA procedure using a

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TABLE 1
Results of various test procedures on fecal or intestinal samples from calves or piglets

Procedure	Virus or virus-like particles	Calves[a] Tested	Calves[a] Positive	Piglets[b] Tested	Piglets[b] Positive
Fluorescent antibody test	Rotavirus	578	124	99	2
	Coronavirus	541	79	117	1
	BVD	134	13	NA	NA
Cell culture (only)	BVD	132	1	NA	NA
	Enterovirus	132	1	NA	NA
Electron microscopy	Rotavirus (FA neg)	221	21	72	3
	Coronavirus (FA neg)	221	11	72	6
	Astrovirus	221	0	72	1
	Calicivirus	221	1	72	0
	Rhabdovirus	221	1	72	0
	Togavirus-like	221	2	72	0
	Parvovirus	221	2	72	0
	Enterovirus	221	1	72	2
	Breda virus-like	221	42	72	7
	"Chained" particles	221	5	72	0

[a]634 specimens tested from 459 calves
[b]129 specimens tested from 94 piglets

conjugated porcine antiserum (Institut Armand-Frappier, Laval, Quebec). Bovine viral diarrhea virus was detected using an indirect fluorescent antibody test with a sheep antiserum (Moredun Research Inst., Edinburgh, UK) to BVD virus and a rabbit antisheep conjugate (Organon Teknika-Cappel, Malvern, Pennsylvania).

Isolation procedures

Where BVD virus was suspected in calves with primary enteric disease, feces or intestines were homogenized, clarified by low speed centrifugation, and passaged twice at weekly intervals in near confluent embryonic bovine tracheal epithelial cells. Cultures were maintained in Earle's minimal essential medium (Gibco, Grand Island, New York) with 1% nonessential amino acids, 0.5% lactalbumin hydrolysate, 5% BVD antibody free fetal calf serum and antibiotics. Second passage cultures were then subjected to an indirect fluorescent antibody procedure for BVD.

Similar cultural procedures were used to further identify enteroviruses and parvoviruses demonstrated by electron microscopy. Enteroviruses were presumptively identified on the basis of their cytopathic effects (CPE) and parvoviruses on the basis of their CPE and hemagglutinating activity with guinea pig erythrocytes.

Electron microscopy procedures

Five percent suspensions of fecal samples or intestinal contents were prepared in saline, sonicated for 30 s in an ultrasonic cleaner (Bransonic B-12, Branson, Shelton, Connecticut) and clarified by centrifugation at 12,000 *g* for 8 min (Eppendorf 5414, Hamburg, West Germany). The supernatant was then ultracentrifuged at 122,000 *g* for 12 min through a 20% sucrose cushion (Airfuge, Beckman Instruments, Palo Alto, California). The pellet was resuspended in a small volume of saline, briefly sonicated, and applied to carbon-coated Formvar grids. The grid was washed three times with 1% potassium phosphotungstate (pH 6.8) to remove residual sucrose, and then left to stain in the same solution for 30 s. After drying, the samples were examined for virus content by electron microscopy (EM 410, Phillips, Eindhoven, Holland). Although most of the viruses were identified on the basis of size and morphology, it was sometimes necessary to include hemagglutinating activity and cultural properties.

TABLE 2
Combinations of viruses detected in fecal and intestinal samples from calves by fluorescent antibody test

Rotavirus + Coronavirus	10
Rotavirus + BVD	2
Coronavirus + BVD	3
Rotavirus + Coronavirus + BVD	1

TABLE 3
Combinations of viruses and virus-like particles detected in fecal and intestinal samples from calves by electron microscopy

Rotavirus + Coronavirus	12
Rotavirus + Breda virus-like particles	23
Coronavirus + Breda virus-like particles	1
Coronavirus + Parvovirus	1
Coronavirus + "Chained" particles	2
Breda virus-like particle + "Chained" particles + Rotavirus	1

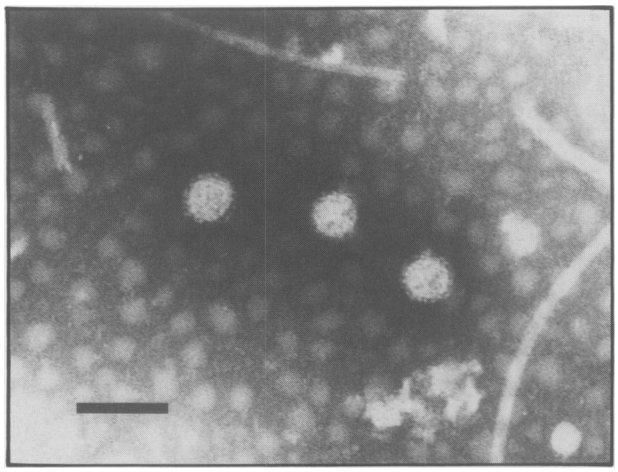

Figure 1. Togavirus-like particles from feces of a three-week-old calf. Bar = 100 nm.

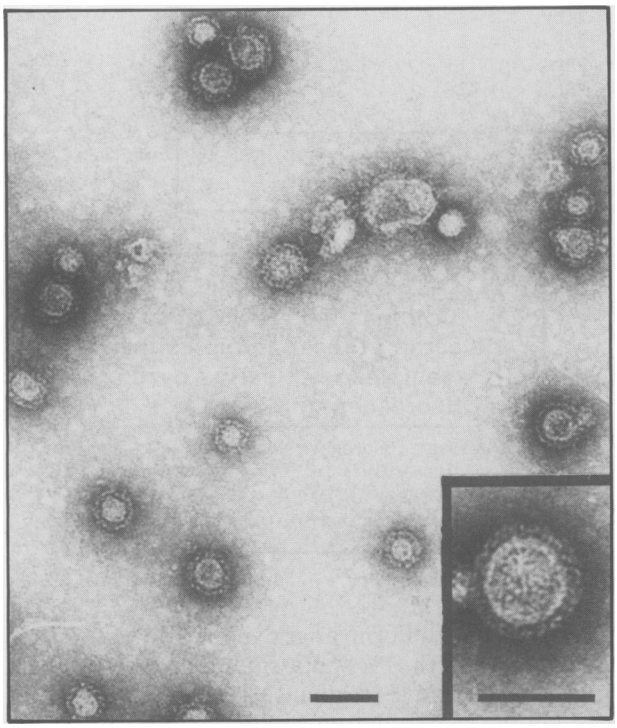

Figure 2. Electron micrograph of negatively stained Breda virus-like particles in calf feces. Inset shows single particle at higher magnification. Bars = 100 nm.

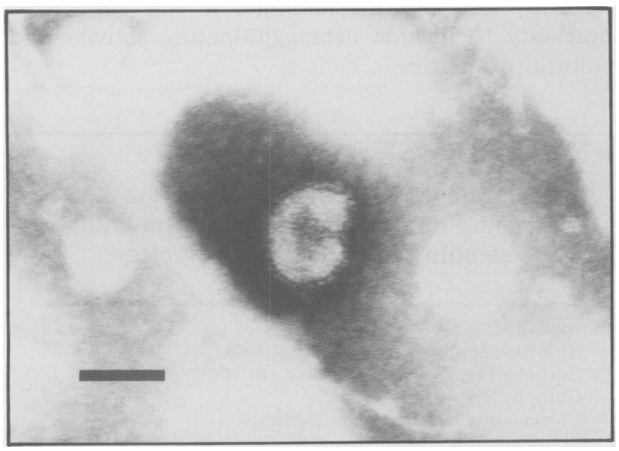

Figure 3. Breda virus-like particle with kidney-shaped morphology. Bar = 100 nm.

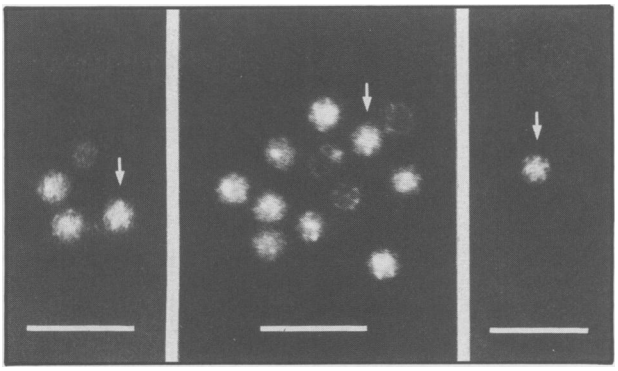

Figure 4. Astroviruses from piglet feces. Arrows indicate particles showing star-like appearance. Bars = 100 nm.

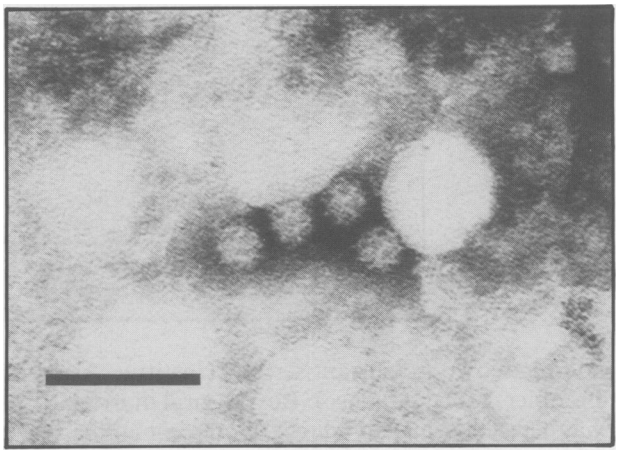

Figure 5. Calicivirus particles in calf feces, showing typical surface structure. Bar = 100 nm.

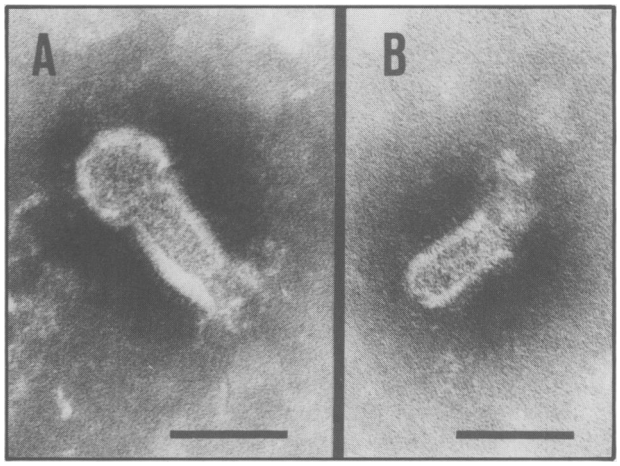

Figure 6. Fringed rhabdovirus particles from calf feces showing (A) typical helical structure and (B) characteristic bullet shape. Bar = 100 nm.

Results

The results are summarized in Table 1. Mixtures of viruses were also detected, as shown in Tables 2 and 3.

Numerous rotavirus and coronavirus positive samples were detected using immunofluorescent procedures. In a considerable number of samples from both species, rotaviruses were detected only by electron microscopy. Some of these samples contained large numbers of rotaviruses and still failed to react by FA on retesting. A number of samples also contained coronaviruses that were only detected by

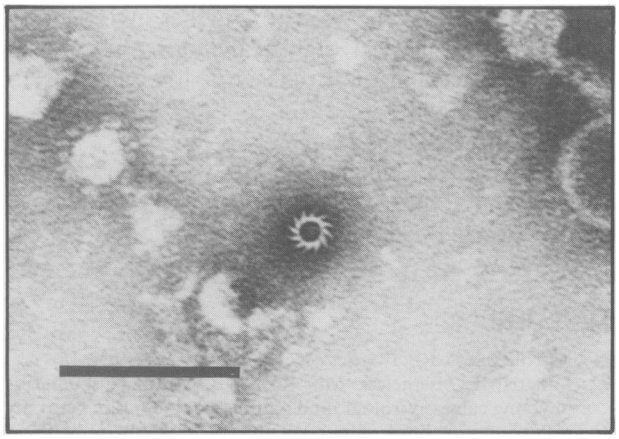

Figure 7. "Pinwheel" particle in calf feces, showing well defined surface projections. Thought to be bacteriophage sheath in cross-section. Bar = 100 nm.

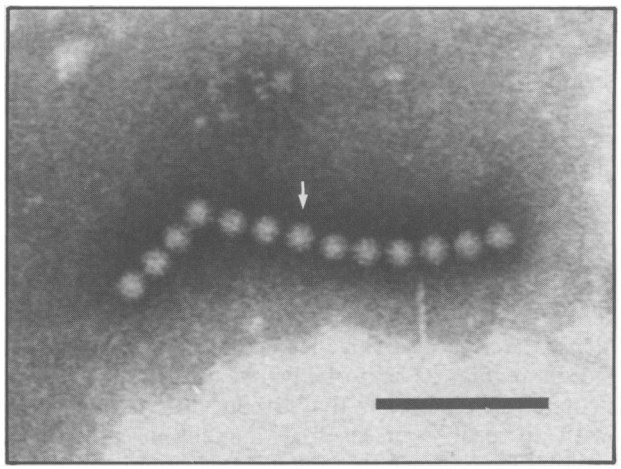

Figure 8. Chained array of small virus-like particles. Some surface detail is visible which occasionally gives a star-like appearance (arrow). Bar = 100 nm.

electron microscopy, though virus content was generally low.

The BVD virus isolated by culture of feces was from a two-day-old calf with diarrhea, the isolate being a cytopathic strain. Pathology data were not available. In two fecal samples, 50 nm particles were seen with a togavirus-like morphology (Figure 1), but no virus was isolated in cell culture.

The Breda virus-like particles seen in the feces generally possessed a spherical shape with a diameter between 40 nm and 160 nm (Figure 2). They often had an electron translucent center. Some particles were flattened on one side or indented, and in very occasional specimens, kidney-shaped particles were seen (Figure 3). The particles were surrounded by a fringe with a length between 6 nm and 14 nm.

Astroviruses were found in large numbers in a single fecal specimen (Figure 4) received from a property with a substantial outbreak of watery diarrhea in two- to five-day-old piglets. Caliciviruses were found in a fecal sample from a single calf (Figure 5), but were only seen in small numbers. Numerous rhabdovirus-like particles were present (Figure 6) in the feces of a seven-day-old calf with depression, moderate diarrhea and some dysentery of two days duration. No other details are known.

A number of hollow pinwheel-shaped particles with a 12 pointed margin and a diameter of 25 to 35 nm were seen in six bovine fecal samples (Figure 7).

Numbers of "chained" particles were seen in five feces. They had a diameter of about 20 nm, and often showed a five- or six-sided appearance, with some evidence of surface structure (Figure 8). Their most outstanding feature was alignment into a chain-like formation. Occasionally, clusters of free particles of a similar nature were seen in the same specimens.

Discussion

The detection of rotaviruses and coronaviruses in numerous specimens was to be expected, as these viruses are well-documented causes of diarrhea in young animals (1–3).

Fluorescent antibody procedures failed to detect the presence of rotavirus in a number of fecal and intestinal specimens that were positive by electron microscopy. In some cases this could have been due to the small numbers of virus particles present. Some of the samples, however, contained large numbers of the virus and still failed to react by FA on retesting. Antigenically distinct rotaviruses have been previously reported in cattle and pigs in Japan and Eastern Canada (13,14), but the differences are normally only detected using procedures such as neutralization or hemagglutination-inhibition tests. As rotavirus group antigen is normally detected by the FA test, it is possible that the different reactivity reflects the specificity of the monoclonal antibody used in this test, suggesting some variation in internal capsid proteins.

The FA test also failed to detect coronaviruses in a considerable number of fecal samples and intestinal sections from calves and piglets that were positive by electron microscopy. This discrepancy could have been caused by low content of virus. It is possible however, that some of the coronaviruses seen in the piglet fecal samples could belong to serogroups other than TGE virus. Coronaviruses that are antigenically distinct from TGE virus have been recorded in pig feces in Europe, Japan, and Eastern Canada (15–17). These atypical coronaviruses have been shown to cause diarrhea experimentally (15,18), but their clinical importance remains to be determined. In the present survey, the numbers of coronaviruses in pigs that were detected only by electron microscopy substantially outnumbered those that reacted by FA.

Although BVD virus was demonstrated in intestinal tissues by FA procedures on a number of occasions, its presence was only confirmed by isolation in one two-day-old calf. Bovine viral diarrhea virus is more typically found in older calves and yearlings, but has been reported to cause diarrhea in young calves (4,5,19). The infection in the two-day-old calf may represent a late intrauterine or neonatal infection, or possibly a tolerant carrier animal, though tolerance to BVD virus is usually caused by noncytopathic strains (20). As BVD virus was not cultured from the specimens containing togavirus-like particles, their identities remain unknown.

Breda viruses are a recently recognized group of viruses that have been reported in calves with diarrhea

in the USA and France (6,21). Along with similar viruses isolated in a horse (22) and man (23), they have been provisionally classified into a group of viruses known as Toroviruses (24). They have also been associated with outbreaks of human enteric disease (23). The virus has a variable morphology, ranging from spherical to kidney-shaped (6,21,25). Two different serotypes of the virus have been described (24) to date. The viruses show some similarity to certain types of cell debris and also to coronaviruses and myxoviruses. They differ in that their fringe is shorter and more dense than that described for coronaviruses (6,21) and not as regular as that of myxoviruses (21). A report from France suggests that Breda virus is associated with up to 60% of cases of diarrhea in calves (20). Studies have shown that the virus is capable of inducing profuse watery diarrhea in experimental calves (7,24). The particles seen in the present survey appear very similar in size and morphology to those described in the literature (6,21,22) and were frequently present in large numbers. Precise identification will require further work.

Enteroviruses are common inhabitants of the intestinal tract. They are generally thought to have fairly low pathogenicity (12,26), though studies have shown that they are capable of causing mild enteric disease (12,26,27). Experimental and field studies have also suggested that the diseases caused by astroviruses and caliciviruses are generally fairly mild (9,28,29,30), though only a limited number of investigations have been carried out.

The numerous rhabdovirus-like particles that were detected in the seven-day-old calf possessed a morphology and size similar to published descriptions of rhabdoviruses (31). They do not appear to have been reported previously in cattle feces. Their significance in this case is unknown.

Based on isolation reports and serological studies, bovine parvovirus appears to be widespread in the cattle population in many countries (11). Parvoviruses are generally thought to cause little disease in cattle because most of the population possesses sufficient antibody to give protection. They have been shown to cause moderate to severe diarrhea in calves under conditions that promote enhanced mitotic activity in the gut (32).

The "pinwheel" particles were uniform in size and shape, though the 12 points were not always well defined. They are thought to be cross-sections of contracted sheaths of bacteriophage (33), and should not be confused with animal viruses.

The "chained" particles showed some resemblance to viruses, due to their five- or six-sided appearance, uniform size, and suggestion of surface substructure. Their alignment into a chain-like arrangement is highly unusual, however. They do not appear similar to any described cell ultrastructural component. Considering their small size, it is possible they are structural subunits derived from larger viruses. Their significance is unknown.

The present investigations confirm the value of electron microscopy as a broad-spectrum tool for detection of viruses, and emphasize its usefulness where other techniques fail to provide an answer. The studies also demonstrated the need for further investigation into the pathological significance of the viruses detected, especially the Breda virus-like particles.

Acknowledgments

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