

Development of Protein A–Gold Immunoelectron Microscopy for Detection of Bovine Coronavirus in Calves: Comparison with ELISA and Direct Immunofluorescence of Nasal Epithelial Cells*

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ABSTRACT

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A protein A–colloidal gold immunoelectron microscopy (PAG–IEM) technique was developed for the detection of bovine coronavirus (BCV) in the feces and nasal secretions of infected calves. Feces or nasal swab fluids were incubated sequentially with hyperimmune bovine anti-bovine coronavirus serum and protein A–gold, negatively stained, applied to formvar-coated copper grids and viewed using an electron microscope. The PAG–IEM method specifically identified BCV particles and possible subviral particles in feces and nasal-swab fluids from infected calves. The PAG–IEM method did not label other enveloped enteric viruses or morphologically similar fringed particles commonly found in feces. Detection of BCV using PAG–IEM was compared with ELISA and direct immunofluorescence (IF) of nasal epithelial cells by monitoring fecal and respiratory tract shedding of BCV from two experimentally infected and two naturally infected calves from birth to 3 weeks of age. PAG–IEM and ELISA detected shedding of BCV in fecal (4/4 animals) and nasal (3/4 animals) samples for an average of 5.25 days each. The observed agreement of BCV detection by PAG–IEM and ELISA was 85%. PAG–IEM may be a more sensitive immunoassay for the detection of BCV in diagnostic specimens from infected neonatal calves than ELISA. BCV infection of nasal epithelial cells was detected by immunofluorescence in 4/4 calves, persisted for the duration of the study in 2/4 calves and was sporadic in the other two animals. The observed agreement of BCV detection by PAG–IEM and IF was 57%.

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INTRODUCTION

Bovine coronavirus (BCV) is a pneumoenteric virus which causes a mild upper respiratory tract infection and a severe lower intestinal enteritis in 3–30-day-old calves, resulting in diarrhea, dehydration and often death (Stair et al., 1972; Sharpee et al., 1976; Langpap et al., 1979; Phillips and Case, 1980; Thomas et al., 1982; McNulty et al., 1984; Reynolds et al., 1985; Saif et al., 1986; Bosgiraud and Nicolas, 1986; Heckert et al., 1986). The virion is pleomorphic (65–210 nm) and has 20–25-nm long club-shaped peplomers anchored in a lipid envelope (Sharpee et al., 1976; Roseto et al., 1982).

Diagnosis of BCV enteric infections in live animals is often done by electron microscopy (EM) or immunoelectron microscopy (IEM) of material from diarrheic animals (Stair et al., 1972; England et al., 1976; McNulty et al., 1981). Diagnosis of BCV upper respiratory tract infections is not routinely done but is possible by EM, IEM, virus isolation methods and direct immunofluorescence examination of nasal epithelial cells (Thomas et al., 1982; McNulty et al., 1984; Reynolds et al., 1985; Bosgiraud and Nicolas, 1986; Heckert et al., 1986; Saif et al., 1986).

Diagnosis of BCV infection by EM or IEM is dependent upon morphological identification of virus particles or immune complexes. Diarrheic material often contains particles which resemble BCV in morphology, making the positive identification of the virus difficult. In one study, a number of laboratories were sent known human coronavirus specimens for examination by negative-staining EM. Only 51% made a correct diagnosis; 33% found no virus and 16% reported a wrong virus in the specimen (Almeida, 1983). The majority of these so-called coronaviruses are membrane fragments with a fringed surface. Unfortunately, endoplasmic reticulum, bacteria and mycoplasma, when broken down, yield fragments that assume spherical form and frequently display a fringe of surface projections. Similarly, the disrupted cristae of broken mitochondria have distinctive surface projections and can also appear in the viral size range (Almeida, 1983).

Protein A–colloidal gold immunoelectron microscopy (PAG–IEM) has been used to specifically detect rotavirus and enterovirus antigens in fecal samples (Hopley and Doane, 1985; Doane et al., 1987). This paper reports the development of a PAG–IEM method for the rapid and specific identification of BCV in feces and nasal secretions of infected calves. To evaluate its use as a diagnostic test, this method was used to follow the shedding of BCV in fecal and nasal samples from two naturally infected (NI) and two experimentally infected (EI) calves. The PAG–IEM method was compared with ELISA and direct immunofluorescence staining of nasal epithelial cells for the diagnosis of BCV infection in these calves.

MATERIALS AND METHODS

Experimental design

Two groups of Holstein calves were used in this study. The first group consisted of two colostrum-deprived bull calves (Nos 213 and 214), procured, housed at birth in isolation facilities and challenged as described by Saif et al., (1983). At 2 days of age each animal was inoculated with a 20% suspension of virulent DB2 strain of BCV in phosphate buffered saline (PBS), 2 ml per nostril and 46 ml orally. The inoculum consisted of large intestinal contents from a gnotobiotic calf previously infected with the DB2 strain of BCV (Saif et al., 1986). The inoculum contained approximately 50 virions per grid square by IEM and was known to cause diarrhea in 100% of challenged animals. Feces and nasal swabs were collected and rectal temperatures recorded daily for the first week of life and then three times per week until 3 weeks of age (EI group).

In the second group, two naturally infected heifer calves (Nos. 4682 and 4609) housed on a commercial dairy farm under conventional management conditions, were sampled (as above) three times per week from birth until 3 weeks of age (NI group). The severity of diarrhea was scored on a scale of 0–3, with 0 representing firm feces; 1, pasty feces; 2, semi-liquid; 3, liquid. Upper respiratory tract signs were scored on a similar scale with 0 representing a normal amount and consistency of lacrimal, nasal and salivary secretions; 1, excessive clear secretions; 2, cloudy thick secretions.

PAG-IEM procedures

Fecal and nasal specimens

Diarrheic material from calves containing Bredavirus (a Torovirus) (Saif et al., 1981; Woode et al., 1982; Horzinek et al., 1987), fringed particles or no viruses and pig feces containing transmissible gastroenteritis virus (TGE, a coronavirus of swine) by IEM were examined by PAG-IEM. All fecal samples and nasal swab supernatants from the NI and EI groups of calves were also examined by PAG-IEM.

PAG-IEM

Fecal samples were stored at -20°C after collection and processed for PAG-IEM using procedures similar to those described previously (Saif et al., 1977). Briefly, samples were diluted 1:5, sonicated, clarified at 4°C and the supernatants passed through $0.45\ \mu\text{m}$ filters (Uniflo, Schleicher and Schuell, Keene, NH). Nasal swab supernatants were filtered ($0.45\ \mu\text{m}$), concentrated 10-fold by ultracentrifugation ($99\ 000\times g$, 2 h, 4°C) and resuspended in $200\ \mu\text{l}$ PBS. Fecal or nasal samples ($200\ \mu\text{l}$) were incubated overnight at 4°C with appropriately diluted hyperimmune bovine anti-bovine coronavirus serum ($800\ \mu\text{l}$)

as described by Saif et al. (1986). The immune complexes were pelleted twice ($69\,000\times g$, 35 min, 4°C), resuspended in $50\ \mu\text{l}$ sterile distilled H_2O (EM water) and incubated for 45 min at 37°C with $25\ \mu\text{l}$ of 10 nm protein A-gold (PAG) (AuroProbe, EM protein-A G10, Janssen Life Sciences, Piscataway, NJ) diluted 1:20 in gold buffer (0.1% BSA in Tris-buffered saline, pH 8.2). In select specimens, the PAG binding was blocked by the addition of $25\ \mu\text{l}$ ($5\ \text{mg ml}^{-1}$) of unlabeled protein A in gold buffer, as a control. Further incubations were carried out in microtiter plate wells as described by Hopley and Doane (1985). The virus-antibody-PAG suspension ($50\ \mu\text{l}$) was transferred to a formvar carbon-coated copper EM grid (300 mesh) on top of 1% agar in a microtiter well and allowed to diffuse into the agar for 30 min at room temperature. Grids were washed by sequential immersion into two drops of gold buffer and one drop of EM water followed by negative staining with 1.5% phosphotungstic acid containing 0.2% sucrose (pH 7.2) for 30 s. Grids were blotted dry and examined at 80 kV in an electron microscope (Model 201, Philips Norelco, The Netherlands). The viral particles in a specimen were estimated on the basis of the following scale measured in number per grid square: < 1 ; $\geq 1 \leq 10$; $\geq 11 \leq 50$; $\geq 51 \leq 100$; ≥ 101 .

ELISA

All nasal swab supernatants and fecal samples collected were tested for BCV antigen by ELISA using procedures similar to those previously described for detection of bovine rotavirus (Saif et al., 1983). Briefly, 96-well polystyrene plates (Immulon I, Dynatech Laboratories, Alexandria, VA) were coated with $200\ \mu\text{l}$ hyperimmune (Rows A-F) or pre-immune (Rows G-H) ammonium sulfate-precipitated gnotobiotic calf anti-bovine coronavirus serum ($25\ \mu\text{g ml}^{-1}$) in carbonate coating buffer (pH 9.6), at 37°C for 1 h and 4°C overnight. Plates were rinsed twice in PBS-Tween (Dynawasher II, Dynatech Laboratories, Alexandria, VA) and $100\ \mu\text{l}$ of feces [diluted 1:25 and processed as described previously (Saif et al., 1983)] or undiluted nasal swab supernatants were added, each to four wells of positive capture serum and duplicate wells of negative control serum. Each set of duplicate samples (on positive capture antibody) received a further $100\ \mu\text{l}$ of a 1:100 dilution of hyperimmune or pre-immune ammonium sulfate precipitated bovine anti-bovine coronavirus serum, as a blocking test, followed by incubation overnight at 4°C . Plates were rinsed and $100\ \mu\text{l}$ hyperimmune guinea pig anti-bovine coronavirus serum (1:3000) in 2% BSA-PBS-Tween was added and incubated for 1 h at 37°C . After rinsing, $100\ \mu\text{l}$ of rabbit anti-guinea pig alkaline phosphatase conjugate (1:400) (ICN ImmunoBiologicals, Lisle, IL) in 2% BSA-PBS was added and the plates were incubated for 1 h at 37°C . Plates were rinsed twice, first with PBS-Tween, then with distilled H_2O , and $200\ \mu\text{l}$ of the substrate, *p*-nitrophenyl phosphate disodium, (Sigma Chemical Company, St. Louis, MO) in 10% diethanolamine

was added. Color development was read at 405 nm (Titerteck Multiskan, Flow Laboratories, McLean, VA) when positive control wells reached an absorbance value of approximately 1.0. Fecal and nasal swab supernatants, previously determined as positive or negative for BCV by IEM, were included on all plates as controls. A cutoff absorbance value for each sample was determined by adding three standard deviations (of all the negative control samples and all samples on negative control serum) to the background absorbance value of the sample on negative control serum. Mean values were determined for each set of duplicate samples tested. A sample was considered positive if it was above the cut-off value for that sample and if its absorbance value was reduced (in the blocking test) by > 50% of the difference between the unblocked absorbance value and the cut-off absorbance value.

Direct immunofluorescence (IF) assay

Nasal epithelial cells were collected with 16.5-cm cotton-tipped swabs (1 per nostril) and fixed onto glass slides for immunofluorescence staining as described by Saif et al. (1986). Nasal swab supernatants and fixed cells were stored at -20°C until further testing.

Nasal cells were stained using fluorescein-conjugated bovine anti-bovine coronavirus serum, bovine anti-bovine rotavirus serum (control) or PBS as described previously (Saif et al., 1986). Wells were considered positive when they contained at least two specific fluorescing cells per well. The percentage of cells

TABLE 1

Clinical signs, BCV shedding by PAG-IEM and ELISA, and nasal-cell IF for experimentally infected Calf 213

	Days of age														
	0	1	2	3	4	5	6	7	8	9	11	13	16	18	20
Fever ^a	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-
Fecal BCV															
Fecal score ^b	0	0	0	3	0	0	3	3	2	2	2	0	0	0	0
PAG-IEM ^c	0	0	0	<1	<1	1-10	51-100	11-50	51-100	0	0	0	0	0	0
ELISA	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
Nasal BCV															
Nasal score ^d	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
PAG-IEM ^c	0	0	0	0	1-10	51-100	11-50	11-50	11-50	1-10	0	0	0	0	0
ELISA	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-
Nasal cell IF ^e	0	0	0	0.1	6	15	5	5	5	3	5	1	0.1	0.1	0.1

^a> 39.5°C.

^b0-3 as described in text.

^cVirus particles per grid square.

^d0-2 as described in text.

^e% immunofluorescent nasal cells.

positive for coronavirus antigen was estimated for each well by examining five different fields of view (200 ×) and averaging the results.

RESULTS

Clinical signs of infected calves

All calves in both the EI and NI groups developed profuse watery diarrhea and became febrile (rectal temperature >39.5°C) following BCV infection during the 3-week study period (Tables 1–4). The average durations of diarrhea for calves in the EI and NI groups were 4.5 and 5.5 days, respectively. Only one (NI Calf 4609) of the four calves showed moderate signs of a clinical upper respiratory tract infection for 3 days; two calves (NI Calf 4682 and EI Calf 214) showed only slight signs for 2–3 days and the fourth calf (EI Calf 213) did not develop clinical signs of respiratory tract infection (Tables 1–4).

PAG-IEM evaluation of specimens

The PAG-IEM method specifically labeled BCV viral particles in IEM positive fecal samples or supernatant fluids from IF positive nasal cells (Fig. 1). PAG particles primarily bound to antibody coating the virus peplomers and gave low background levels of free PAG. Fecal or nasal samples positive by

TABLE 2

Clinical signs, BCV shedding by PAG-IEM and ELISA, and nasal-cell IF for experimentally infected Calf 214

	Days of age														
	0	1	2	3	4	5	6	7	8	9	11	14	16	19	21
Fever ^a	-	-	-	-	+	+	+	+	+	+	+	+	+	-	-
Fecal BCV															
Fecal score ^b	0	0	2	0	0	3	3	2	2	1	0	0	0	0	0
PAG-IEM ^c	0	0	0	0	0	<1	51-100	11-50	11-50	0	0	<1	0	0	0
ELISA	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-
Nasal BCV															
Nasal score ^d	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0
PAG-IEM ^c	0	0	0	<1	1-10	11-50	1-10	11-50	11-50	0	<1	<1	<1	0	0
ELISA	-	-	-	+	+	+	+	+	+	+	-	-	-	-	-
Nasal cell IF ^e	0	0	0	7	25	25	10	8	10	15	1	0.1	0.1	0.1	0

^a>39.5°C.

^b0-3 as described in text.

^cVirus particles per grid square.

^d0-2 as described in text.

^e% immunofluorescent nasal cells.

TABLE 3

Clinical signs, BCV shedding by PAG-IEM and ELISA, and nasal-cell IF for naturally infected Calf 4609

	Days of age									
	0	3	5	7	10	12	14	17	19	21
Fever ^a	-	-	-	-	+	+	+	-	-	-
Fecal BCV										
Fecal score ^b	0	0	3	0	2	3	1	0	2	3
PAG-IEM ^c	0	0	0	0	0	<1	<1	<1	0	0
ELISA	-	-	-	-	-	-	-	-	-	-
Nasal BCV										
Nasal score ^d	1	0	0	0	0	0	2	2	0	1
PAG-IEM ^c	0	0	0	0	0	0	0	0	0	0
ELISA	-	-	-	-	-	-	-	-	-	-
Nasal cell IF ^e	0	1	2	1	3	0.1	0.1	0.1	0.1	0.1

^a> 39.5°C.

^b0-3 as described in text.

^cVirus particles per grid square.

^d0-2 as described in text.

^e% immunofluorescent nasal cells.

TABLE 4

Clinical signs, BCV shedding by PAG-IEM and ELISA, and nasal-cell IF for naturally infected Calf 4682

	Days of age									
	0	3	5	7	10	12	14	17	19	21
Fever ^a	-	-	-	-	-	+	-	-	-	-
Fecal BCV										
Fecal score ^b	0	0	1	3	2	3	0	2	0	0
PAG-IEM ^c	0	0	>10	<1	0	0	<1	0	0	0
ELISA	-	+	+	+	-	-	-	-	-	-
Nasal BCV										
Nasal score ^d	0	0	0	1	1	0	0	0	0	0
PAG-IEM ^c	0	0	0	0	<1	0	<1	0	0	0
ELISA	-	-	+	-	-	-	+	-	-	-
Nasal cell IF ^e	0	0	0	2	2	0.1	0	0	0.1	0

^a> 39.5°C.

^b0-3 as described in text.

^cVirus particles per grid square.

^d0-2 as described in text.

^e% immunofluorescent nasal cells.

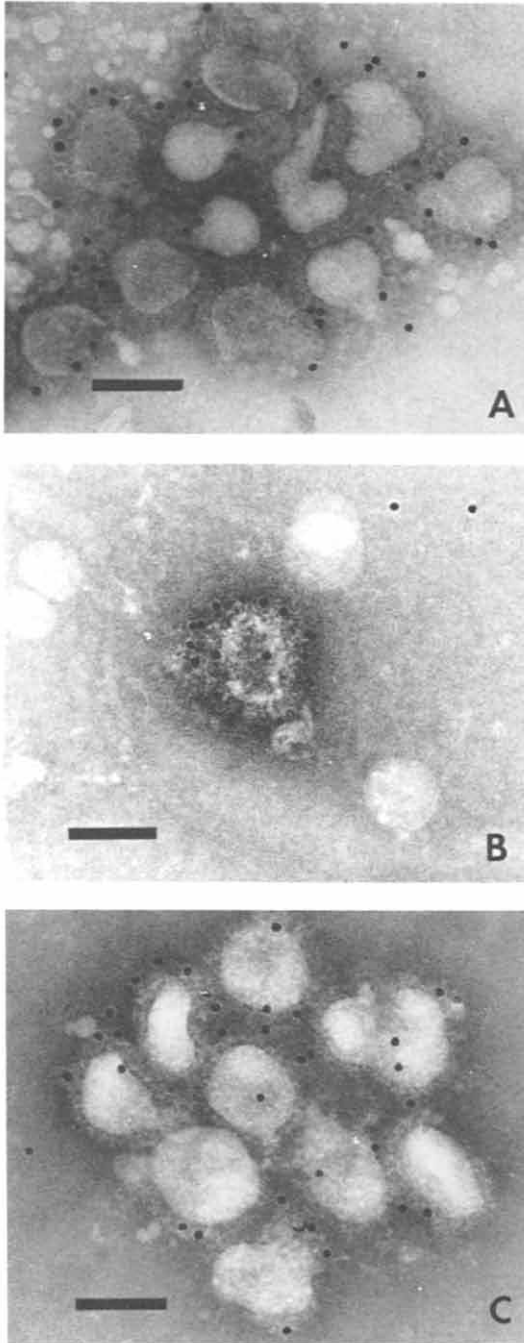


Fig. 1. PAG-IEM of labeled BCV from feces and nasal swab fluids. (A) PAG-IEM of a labeled BCV aggregate from feces; (B) PAG-IEM of a labeled individual BCV from feces; (C) PAG-IEM of labeled BCV from nasal swab fluids. (Bar represents 100 nm.)

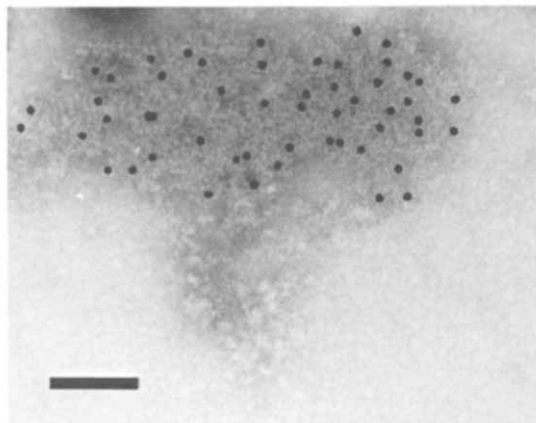


Fig. 2. PAG-IEM of labeled BCV antigen of undefined morphology (bar represents 100 nm).

PAG-IEM also contained a variable amount of labeled free viral antigen of undefined morphology (Fig. 2). The specificity of the labeling of this possible subviral material was demonstrated by comparative assays using positive and negative controls. Labeled free antigen of undefined morphology was never seen in PAG-IEM negative samples, but frequently seen in PAG-IEM positive samples. Labeling of the viral and subviral-antibody complexes by PAG was blocked by the addition of protein A, indicating that non-specific PAG aggregates were not occurring.

The PAG-IEM method did not label the two enveloped viruses, Bredavirus or transmissible gastroenteritis virus (Fig. 3), which are antigenically unrelated to BCV. Fringed particles and cellular debris in fecal samples IEM negative for BVC or supernatant fluids from cells IF negative for BCV were also unlabeled (Fig. 3). The background level of free PAG was low in all specimens examined.

Detection of BCV fecal and nasal shedding by PAG-IEM

Shedding of BCV in feces and nasal swab fluids from the EI group of calves was detected by PAG-IEM. The average duration of BCV shedding in the feces and nasal swab fluids detected by PAG-IEM was 5 and 6 days, respectively, with levels of viral particles ranging from < 1 to > 101 per grid square (Tables 1 and 2).

In the NI group of calves, fecal shedding of BCV was detected by PAG-IEM, but little or no nasal shedding was evident. The average duration of BCV shedding in the feces detected by PAG-IEM was 4.5 days with numbers of viral particles ranging from < 1 to > 101 per grid square. The average duration of

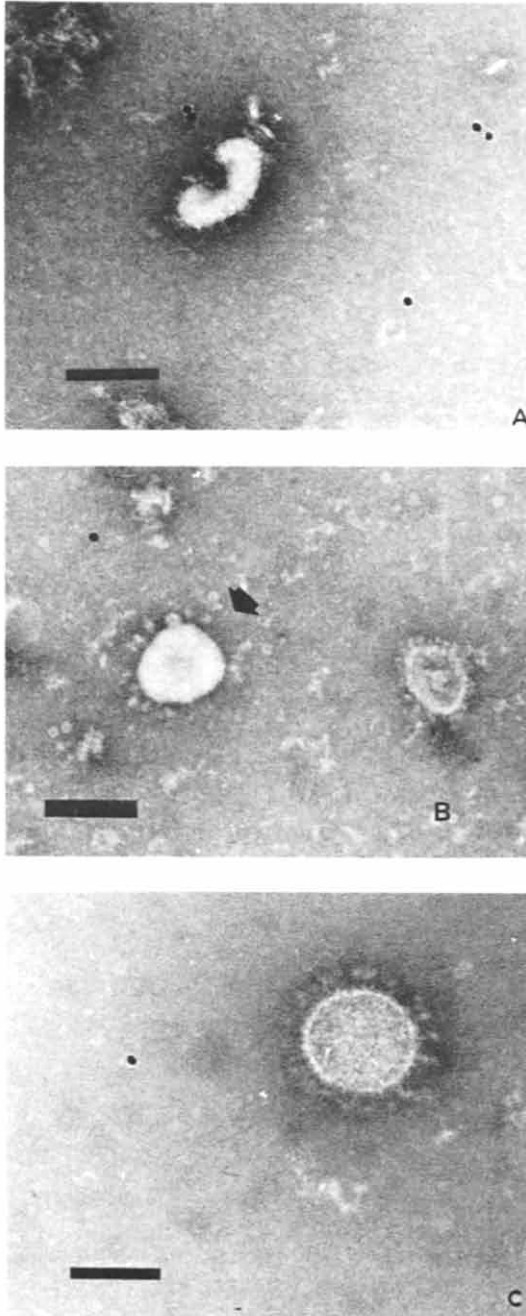


Fig. 3. Unlabeled viruses and fringed particles detected by PAG-IEM. (A) PAG-IEM of unlabeled Bredavirus; (B) PAG-IEM of unlabeled transmissible gastroenteritis virus (arrowhead); (C) PAG-IEM of unlabeled fringed particles. (Bar represents 100 nm.)

BCV shedding in the nasal swab fluids by PAG-IEM was 0.5 days with numbers of viral particles ranging from 0 to < 1 per grid square (Tables 3 and 4).

Detection of BCV fecal and nasal shedding by ELISA

Shedding of BCV in feces and nasal swab fluids was detected by ELISA for an average duration of 4 and 6.5 days, respectively, from the EI group of calves and 2.5 and 0.5 days, respectively, from the NI group (Tables 1-4).

Detection of BCV shedding in feces or nasal swab fluids by PAG-IEM and ELISA were in good agreement (observed agreement of 85%) for both EI and NI groups. By PAG-IEM and ELISA 23.5% of the fecal and nasal swab samples were positive, 61.2% were negative, 11.2% were PAG-IEM positive /ELISA negative and 4.1% PAG-IEM negative/ELISA positive (Table 5). This yields a Kappa value of 0.7. Kappa values > 0.75 represent excellent agreement and values < 0.45 represent poor agreement (Sackett et al., 1985).

TABLE 5

Comparison of PAG-IEM and ELISA detection of bovine coronavirus

	PAG-IEM	
	Positive	Negative
ELISA		
Positive	23 (23.5%)	4 (4.1%)
Negative	11 (11.2%)	60 (61.2%)

TABLE 6

Comparison of PAG-IEM and IF detection of bovine coronavirus infection

	PAG-IEM	
	Positive	Negative
IF		
Positive	16 (32.7%)	20 (40.8%)
Negative	1 (2.0%)	12 (24.5%)

Immunofluorescence of nasal epithelial cells

Both EI calves had BCV-infected nasal epithelial cells, as detected by IF, within 24 h of BCV inoculation. The percentage of infected nasal epithelial cells increased rapidly, peaked at 2–3 days post-inoculation in both animals (15–25%), then decreased over the next 6–8 days to a low level (0.1%), which persisted for the duration of the study (3 weeks) in Calf 213 and almost to the end in Calf 214 (Tables 1 and 2).

In contrast, the age at which BCV-infected nasal epithelial cells were first detected in the NI calves was variable (3 or 7 days of age). The percentage of BCV-infected cells (2–3%) and the period of peak detection (3–5 days) was less than the EI group. The nasal epithelial cell infection again persisted at a very low level (0.1%) for the duration of the study (although sporadic in Calf 4682) (Tables 3 and 4).

Detection of BCV upper respiratory tract infection by PAG-IEM and IF was in poor agreement (observed agreement of 57%) for both EI and NI groups. By PAG-IEM and IF 32.7% of the nasal swab samples were positive, 24.5% were negative, 2% PAG-IEM positive/IF negative and 40.8% PAG-IEM negative/IF positive (Table 6). This yields a Kappa value of 0.14, representing very poor agreement.

DISCUSSION

In the present study, protein A-gold immunoelectron microscopy was successfully used to detect bovine coronavirus in the feces and nasal secretions of infected calves. This technique did not specifically label an antigenically unrelated porcine coronavirus (TGE), a morphologically similar, but antigenically unrelated enveloped bovine enteric virus (Bredavirus) or morphologically similar fringed particles commonly found in feces. PAG-IEM clearly labeled only intact BCV or free BCV antigens of undefined morphology in calves infected with BCV but not coronavirus-like particles found in negative control or pre-challenge fecal samples. This ability of PAG-IEM to detect small subviral antigens has also been shown by others (Stannard et al., 1982; Louro and Lesemann, 1984; Hopley and Doane, 1985). This is a distinct advantage over IEM since coronaviruses are fragile and peplomers easily break off making the identification of the virus difficult. The PAG-IEM method for detecting BCV in feces or nasal secretions compared well to ELISA with a Kappa value of 0.7. This study did not compare the sensitivities of the two assays, although others have shown that PAG-IEM is 1000 times more sensitive than direct electron microscopy and consistently more sensitive than standard IEM methods (Doane et al., 1987). The increased sensitivity of the PAG-IEM method is probably related to the ability of PAG particles to bridge small antigen-antibody complexes (Hopley and Doane, 1985). In the light of the re-

ports that IEM is at least as efficient as ELISA, PAG-IEM appears to be one of the most sensitive of the immunoassays currently available (Nicolaiëff et al., 1980; Obert et al., 1981; Kjeldsberg and Mortensson-Egnund, 1982; Rubenstein and Miller, 1982; Svensson et al., 1983; Hughes et al., 1984; Hopley and Doane, 1985).

PAG-IEM did not compare well with IF for the detection of upper respiratory tract infections in the calves studied. This would be expected since IF detects BCV-infected cells and not free BCV virions or antigens, as does PAG-IEM. As seen in Table 2 the majority of discrepant results were IF positive/PAG-IEM negative, indicating that BCV upper respiratory tract infections do not always result in free virion release in numbers detectable by PAG-IEM.

The ability of BCV to replicate in both the enteric and respiratory tracts of neonatal calves was confirmed as reported previously (Reynolds, 1983; Reynolds et al., 1985; Saif et al., 1986). All animals developed a fever and profuse watery diarrhea, but only mild or no symptoms of upper respiratory tract infection. However, the virus did replicate in the upper respiratory tract as indicated by IF positive nasal cells and virus detection from nasal swab supernatants by PAG-IEM and ELISA. It was not clear from this study whether the respiratory tract infection preceded the enteric infection or vice versa. There appeared to be a persistence of viral infection in the upper respiratory tract, as IF positive nasal epithelial cells were detected (at a very low percentage) in three of the animals for at least 2 weeks after viral shedding was first detected. This was a considerably longer period of shedding than reported by Reynolds et al. (1985), from challenged gnotobiotic calves, but similar to that reported by Saif et al. (1986).

Owing to the high electron density of the (PAG) gold marker it can be detected at relatively low magnifications permitting rapid scanning of grids and improving the feasibility of doing viral diagnosis by EM methods. Although PAG-IEM may not be suited to large-scale screening of specimens, it is however, a highly sensitive and specific assay for the detection of viral particles or antigens in selected or questionable specimens.

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