

JVM 00694

Evaluation of an immunogold electron microscopy technique for detecting bovine coronavirus

A.A. El-Ghorr, D.R. Snodgrass and F.M.M. Scott

Moredun Research Institute, Edinburgh, Scotland, U.K.

(Accepted 15 December 1987)

Summary

A solid phase colloidal gold immunoelectron microscopy (IGEM) technique for detecting bovine coronavirus (BCV) was developed and shown to be specific. This test was compared with three other diagnostic tests using fifteen faecal samples. Bovine coronavirus was detected in 2 samples by direct electron microscopy (DEM), in 3 samples by immunosorbent electron microscopy, in 5 samples by haemadsorption-elution-haemagglutination and in 6 samples by IGEM. Ninety four faecal samples were tested by DEM and IGEM. Of 26 samples found to contain BCV by IGEM only 14 were positive by DEM. The IGEM technique is simple, efficient and less susceptible than others to non-specific reactions.

Bovine coronavirus; Immunogold; Electron microscopy, Diagnosis

Introduction

Neonatal calf diarrhoea is a syndrome of complex aetiology in which several infectious agents interact with environmental influences to determine the health of the young calf. Bovine coronavirus (BCV) is an endemic virus known to be involved in calf diarrhoea (Bridger et al., 1978; Mebus et al., 1973; Patel et al., 1982; Reynolds et al., 1986; Saif et al., 1986; Snodgrass et al., 1986; Stair et al., 1972), and has been isolated also from the respiratory tract (McNulty et al., 1984; Saif et al., 1986). In two recent studies BCV was detected in 4% of 302 diarrhoeic calves in Scotland and the north of England (Snodgrass et al., 1986) and 14% of 490 diarrhoeic calves in the south of England and Wales (Reynolds et al., 1986).

Correspondence to: D.R. Snodgrass, Moredun Research Institute, 408 Gilmerton Road, Edinburgh, EH17 7JH, Scotland, U.K.

Several methods have been used to detect BCV in faeces. With direct electron microscopy (DEM) (Reynolds et al., 1984) it is often difficult to differentiate pleomorphic coronavirus particles from membranous material or other fringed bodies. The characteristic morphology of the core virion surrounded by a fringe of peplomers is not always apparent (Dea et al., 1981; Crouch and Acres, 1984).

An alternative method, the haemadsorption-elution-haemagglutination assay (HEHA) (Van Balken et al., 1978/79) relies on the virus' selective adsorption to and elution from rat red blood cells at different temperatures. The HEHA occasionally suffers from non-specific reactions due to the complex nature of faeces (Viscidi et al., 1984).

Additionally, several enzyme linked immunosorbent assays (ELISA) (Reynolds et al., 1984; Crouch et al., 1984) have been reported for detecting BCV but require highly specific antisera.

The detection of coronavirus antigen in cells of the infected gut using a fluorescent antibody technique has been described by Woode et al. (1978), but is dependent on tissue being removed very shortly after death and most antigen is present early in the course of disease. Coronavirus isolation in cell or organ cultures has been attempted but is also of limited value as many coronavirus strains do not grow readily *in vitro* (Bridger et al., 1978; Mebus et al., 1973).

In this paper an immunogold electron microscopy (IGEM) technique is described which was developed in an attempt to provide a rapid sensitive test that would be less susceptible to non-specific reactions. Immunogold labelling has already been shown to improve the detection of faecal viruses by increasing specificity and sensitivity (Kjeldsberg, 1985; 1986).

Materials and Methods

Virus preparation

Faeces from gnotobiotic calves experimentally and separately infected with BCV or rotavirus were used as positive and negative control samples respectively. Faecal samples were diluted 1 in 4 in phosphate buffer (PB), pH 7.2, mixed and ground with carborundum powder, centrifuged at $10\,000 \times g$ for 3 min and the clarified supernatant fluid was examined for the presence of BCV.

Bovine coronavirus was also grown *in vitro* on fetal tracheal organ culture as described by Stott et al. (1976); and in human rectal tumour (HRT 18) cells as described by Laporte et al. (1979). Culture fluid was clarified by centrifugation at $10\,000 \times g$ for 1 min and the supernate examined.

Antisera

Antiserum was produced in a gnotobiotic calf which had been infected orally with BCV at 4 days of age and given an intramuscular injection of BCV in Freund's complete adjuvant 4 wk later. Serum was collected 2 wk later and IgG extracted by affinity chromatography (Hudson and Hay, 1980). In addition, IgG from an anti-BCV serum produced in a gnotobiotic piglet was extracted by ion exchange chro-

matography (Hudson and Hay, 1980). The bovine and porcine sera had neutralising antibody titres of 1/1280 and 1/3840 respectively and were used at a dilution of 1/100 in all immunosorbent electron microscopy (ISEM) and ISEM tests.

Direct electron microscopy

Formvar carbon-coated 400 mesh copper EM grids were used throughout. A 3.5 μ l drop of poly L-lysine was added to each grid and excess fluid was blotted with filter paper. A 3.5 μ l drop of fluid to be examined was placed on each grid as appropriate and the excess removed with filter paper. The grids were negatively stained using phosphotungstic acid and ammonium molybdate (2:1).

A set pattern of examining EM grids was adhered to in order to standardise the counting of virus particles and facilitate comparison between different preparations. Virus particles were counted around the 4 edges of a grid square and across one diagonal. A total of ten grid squares from different areas of 2 duplicate grids was examined. The grids were coded and examined at a magnification of 20000 on a Siemens Elmiskop 1A electron microscope.

Immunosorbent electron microscopy

Copper EM grids were floated on a 10 μ l drop of bovine anti-BCV IgG on a block of dental wax and incubated. The grids were then washed twice in PB and re-floated on a 10 μ l drop of antigen. After a second incubation the grids were negatively stained as previously described for DEM.

All incubations were performed in a moist chamber at 37°C for 1 h.

Immunogold labelling and the ISEM test

A copper EM grid was coated with the porcine antibody by floating the grid on a drop of the pig anti-BCV IgG and was incubated for 1 h. The grid was washed twice with PB and reacted with a drop of antigen. After incubation for 1 h the grid was washed as described earlier and then floated on a drop of bovine anti-BCV IgG, reincubated for a further 30 min and washed as described previously. The grid was then placed on a drop of pig anti-bovine IgG which was conjugated to 5 nm colloidal gold. The conjugation was performed following the technique described by De Mey (1984). This conjugate was stored at 4°C and was diluted 1/5 in PB immediately before use. Any aggregates which may have formed on storage were removed by centrifugation of the diluted conjugate at 10000 \times g for 2 min. After incubation with the gold conjugate for 1 h excess fluid on the grid was removed by blotting the side with filter paper and the grids negatively stained as described.

All incubations were performed in a moist chamber at 37°C, and a standard drop of 10 μ l was used throughout.

During examination only virus particles with more than 20 gold particles attached to them were counted as positive to reduce the possibility of confusing light background staining with specific labelling.

Sucrose gradient fractionation of BCV

A volume of 60 ml of BCV grown in HRT18 cells was clarified by centrifugation at $3000 \times g$ for 20 min to remove gross debris. Virus in the supernate was pelleted through a 10% (w/w) sucrose cushion by ultracentrifugation at $100\,000 \times g$ for 45 min at 4°C in a Beckman L5 Ultracentrifuge using an SW28 rotor. The pellet was resuspended in 0.3 ml TNE buffer (0.01M tris-hydrochloride (pH 7.5) – 0.1 M NaCl–1.0 mM EDTA), layered onto a 20–55% (w/w) sucrose gradient and centrifuged at $52\,000 \times g$ in an SW40 Ti rotor overnight at 4°C . The gradients were fractionated on an ISCO Density Gradient Fractionator and 0.5 ml fractions collected. Each fraction was examined by ISEM and the haemagglutination (HA) titre measured by the method described by Sato et al. (1977), using rat red blood cells.

Comparison of diagnostic tests

Fifteen bovine faecal samples were examined for BCV by DEM, ISEM, HEHA and ISEM. The HEHA test was performed as described by Van Balken et al. (1978/79). A further 94 faecal samples were compared by DEM and ISEM.

Results

Direct electron microscopy

It was often difficult to visualise intact virions and differentiate these from other fringed bodies by DEM. The low numbers of virus particles in addition to the presence of faecal debris compounded these difficulties and made unequivocal diagnosis of the presence of coronavirus difficult. Fig. 1A illustrates a coronavirus particle in a bovine faecal sample and highlights the problem of differentiating coronavirus-like particles.

Immunosorbent EM

The virus was concentrated approximately one thousand-fold using ISEM. The faecal background debris was markedly reduced but fringed bodies and virus-like particles were still detected in around 30% of samples, sometimes making interpretation difficult. The concentration effect of ISEM is illustrated in Fig. 1B.

Immunogold EM

Using ISEM BCV particles were both concentrated and specifically labelled. As illustrated by Fig. 1C, most of the gold particles were closely associated with the virus particles, with minimal gold background staining.

Fig. 1D illustrates a negative sample in which relatively low gold background staining was observed and debris or other particles which may have been mistaken for coronavirus were not labelled. Compared to DEM the background debris was also substantially reduced.

Control experiments utilised cultured and faecal BCV, and a faecal rotavirus preparation (Table 1). These control experiments showed that all the virus particles in the test (Table 1, column 1), were specifically labelled. In the coating an-

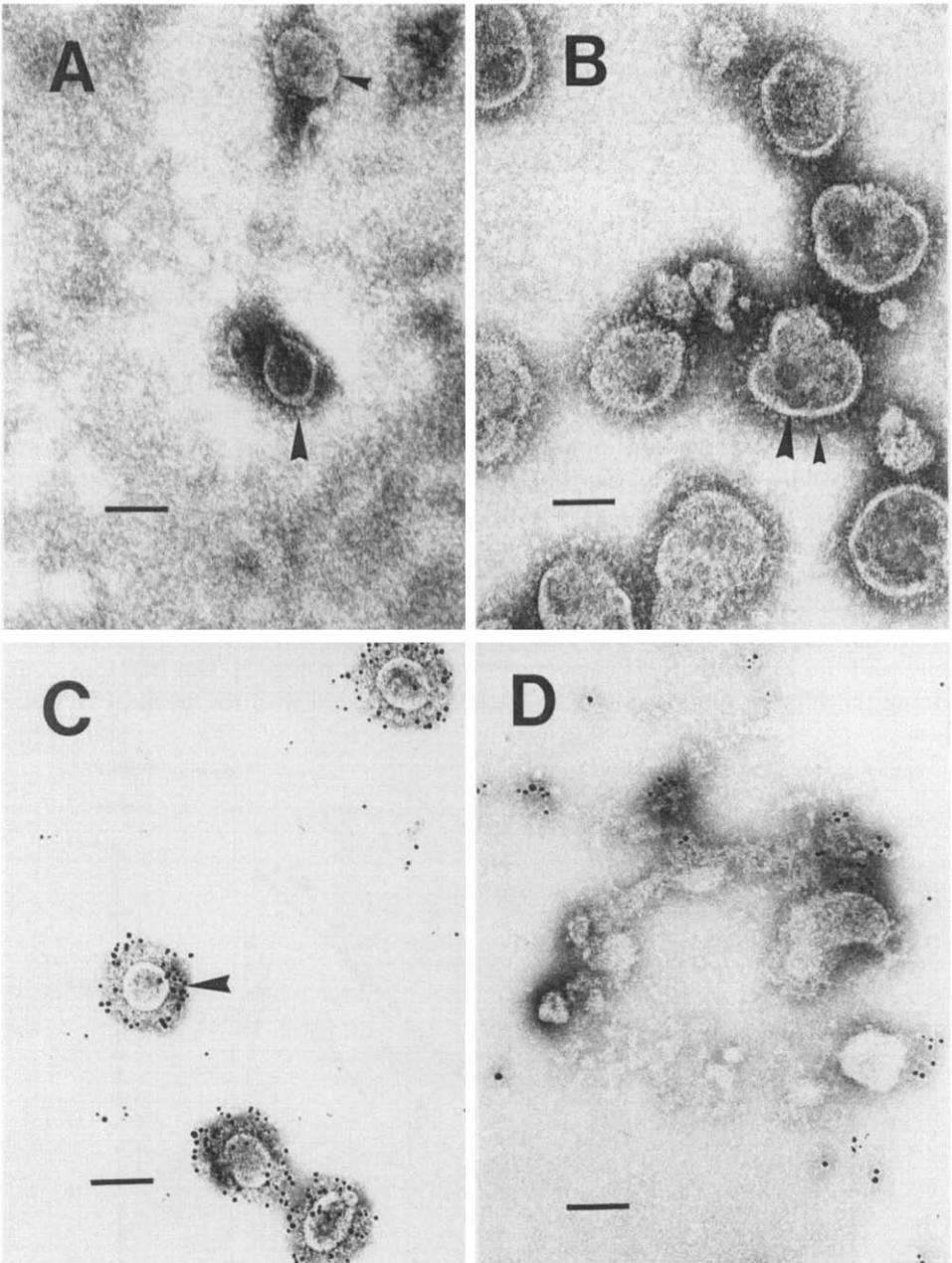


Fig. 1. (A) A particle considered to be BCV (large arrow) and a coronavirus-like particle (small arrow) observed by DEM. The potential for misdiagnosis is clear. (B) BCV particles detected by ISEM. The pleomorphic coronavirus particles (large arrow) are concentrated onto the antibody coated grid. In addition, many intact peplomers (small arrow) are obvious. (C) An IGEM preparation of a bovine faecal sample containing BCV. The 5 nm colloidal gold particles are mainly bound to the coronavirus particles (arrowed). (D) A bovine faecal sample, negative for BCV, as observed by IGEM. Fringed coronavirus-like particles are not labelled. Bars represent 100 nm.

TABLE 1

Number of coronavirus and coronavirus-like particles in 10 grid squares observed in IGEN and control preparations.

Sample	Technique			
	IGEM	IGEM performed without		
		1st Ab	2nd Ab	1st and 2nd Ab
Cultured BCV	3,556/0	0/1	0/2,943	nt
Faecal BCV	6,225/0	6/3	0/1,344	0/0
Faecal rotavirus	0/0	0/4	0/1	0/4

Ab : antibody. nt : not tested. -- : no. of labelled particles / no. of unlabelled particles

tibody control, the results were similar to DEM examination, with no concentration of particles (column 2). Where the second antibody was omitted (column 3), results similar to ISEM were obtained, with concentration of the sample but no specific labelling. Finally by omitting both antisera (column 4), no concentration and no specific labelling were observed. These results demonstrate the specificity of the IGEN test.

Sucrose gradient

The number of labelled virus particles in sequential fractions of a sucrose gradient and the HA activity of each fraction are given in Fig. 2. The fractions containing the highest numbers of virus particles coincided with the peak of HA ac-

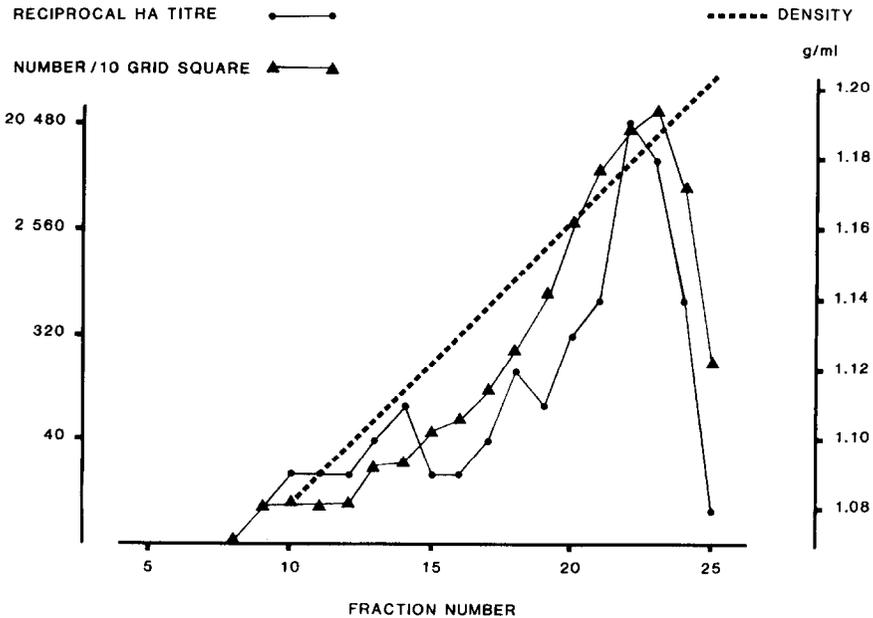


Fig. 2. HA and IGEN results on a 20-55% (w/w) sucrose gradient BCV preparation.

tivity. The density of this region was 1.18 g ml^{-1} which corresponds with that of BCV in sucrose (Siddell et al., 1983).

Comparison of diagnostic tests

Of 15 samples examined, coronavirus was detected in 2 samples by DEM, in 3 samples by ISEM, in 5 samples by HEHA and in 6 samples by IGEM (Table 2).

In the comparison of 94 faecal samples, 68 samples were found to be negative for BCV by both DEM and IGEM. Of the remaining 26 only 14 were positive when examined by DEM, whereas all were found to be positive in the IGEM test (Table 3).

Discussion

It has been noted that BCV peplomers are fragile and easily sheared from the outer envelope leaving a much less distinctive particle (Stair et al., 1972). Our results confirmed that when faecal samples containing BCV were examined by DEM coronavirus particles were usually observed to be widely spaced and the background heavily contaminated with debris. Equivocal results were compounded by the presence in faeces of other fringed particles which could be mistaken for coronavirus (Dea et al., 1981).

In this study a higher sensitivity and specificity were observed in ISEM prepa-

TABLE 2

Comparison of four tests for diagnosis of BCV in faeces.

Faecal sample	Detection of BCV by			
	DEM	ISEM	HEHA	IGEM
1	+	+	+	+
2	+	+	+	+
3	-	+	+	+
4	-	-	+	+
5	-	-	+	+
6	-	-	-	+
7-15	-	-	-	-
Total +	2	3	5	6

TABLE 3

Comparison between DEM and IGEM in the diagnosis of BCV in 94 faecal samples.

Results	No. of samples
DEM + IGEM +	14
DEM + IGEM -	0
DEM - IGEM +	12
DEM - IGEM -	68

rations. However around 30% of faecal samples still contained virus-like particles which could not be unequivocally determined to be BCV.

An attempt was made to further increase the sensitivity and specificity of BCV diagnosis by combining the concentrating effect of ISEM with the specific labelling of antibody conjugated to colloidal gold in an ISEM test. This was successfully achieved, with the ISEM allowing coronavirus-like particles to be diagnosed accurately as BCV or debris. The results of the coronavirus purification on a sucrose gradient gave further confirmation of specificity. The peplomers of coronavirus particles were rarely seen in ISEM preparations as the antibody coating seemed to obscure these projections. The main disadvantages of ISEM are the expense of reagents and equipment and the limited throughput of samples. ISEM should detect different BCV strains as all isolates examined so far have been serologically similar (Dea et al., 1982).

The principle of this immunogold detection technique can be applied to other antigens which are not morphologically distinct and need to be labelled or localised. The technique therefore has widespread applications (Horisberger, 1981; Ducatelle et al., 1984) and has been used to label rotaviruses and adenoviruses (Kjeldsberg, 1985) in human faeces by an indirect labelling test where the viruses were directly adsorbed onto EM grids. Caliciviruses (Kjeldsberg, 1986) in human faeces have been labelled in suspension and the complexes adsorbed onto EM grids.

This novel technique should prove useful in the epidemiological study of disease associated with BCV infection, and as a standard against which to evaluate the development of other diagnostic techniques, such as the new dot-blot hybridization assay to detect viral RNA using a cDNA probe (Shockley et al., 1987).

Acknowledgements

The authors gratefully acknowledge Mr. A. McL. Dawson for IgG extraction; Mr. E.W. Gray and Miss L. McGee for expert advice and assistance with the electron microscope; Mr. A. Inglis for the photography; Mr. J.D. Menzies for producing the gold conjugate; Dr. Janice Bridger at the Institute for Animal Health, Compton Laboratory, for providing the porcine serum; and Dr. E. Davies at the PHLS, Bristol, for supplying HRT18 cells.

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