Monoclonal antibodies to bovine coronavirus and their use in enzymoimmunoanalysis and immunochromatography

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ABSTRACT: Two monoclonal antibodies (MAb) to the outer structural protein E2 (spike peplomeric protein) and two MAb to the inner capsid protein N of bovine coronavirus (BCV) were prepared and identified by Western blotting to be used for increasing the specificity and sensitivity of BCV detection. The MAb were checked by the haemagglutination inhibition test and immunoperoxidase tests and no cross reactivity with rotavirus was demonstrated by the immunoperoxidase test and ELISA. A mixture of all the four MAb at predetermined optimum concentrations was first used in sandwich ELISA and then, in combination with an anti-coronavirus polyclonal antibody, for the development of a simple and rapid immunochromatographic test (ICT). The results of which can be read visually within 10 min. The inclusion of MAb into ELISA and ICT allows the detection of both intact and incomplete BCV virions. ELISA and ICT were used in the examination of a set of 74 faecal samples collected from calves suffering from diarrhoea. ELISA, used as the golden standard verified by electron microscopy, detected BCV in 15 samples (20.3%) and ICT in 16 samples. Three of the ICT-positive samples were negative by ELISA. On the other hand, two of the 58 ICT-negative samples were positive by ELISA. Sensitivity and specificity of ICT were 94.9% and 86.7%, respectively. ***For free full paper in pdf format see*** *http://www.vri.cz/vetmed.asp*

Keywords: Immunochromatographic test (ICT); ELISA; bovine rotavirus; bovine coronavirus (BCV)

List of abbreviations: **BCV** – bovine coronavirus, **MAb** – monoclonal antibody, **ELISA** – enzymoimmunoanalysis, **ICT** – immunochromatographic test, **HRP** – horse radish peroxidase, **HT** – haemaglutination test, **HIT** – haemaglutination inhibition test, **TMB** – tetramethyl benzidine, **DAB** – 3,3'-diaminobenzidine

INTRODUCTION

Intestinal infections, affecting mostly calves in the first weeks after birth, rank highly among the major causes of increased morbidity in cattle herds. The identification of the causative agent in individual outbreaks is often difficult, because the effects of viral, bacterial, or parasitic agents can combine with those of other factors, such as nutrition, stress, or immunological status. One of the most frequently occurring viral agents causing neonatal calf diarrhoea (NCDV) is bovine coronavirus (BCV) associated also with winter dysentery (WD) in adult animals (Millane *et al.*, 1995; Saif *et al.*, 1990). High concentration of enteropathogenic viruses in faeces along with their resistance to environmental effects are responsible for permanent contamination of housing premises and infection of animals that, in the case of coronavirus, can occur orally or nasally. The severity of the infections depends on characteristics of the agent,

its virulence, inoculum size, and age and immunity of the patient. Infections of the intestinal tract begin in the proximal part of the small intestine and extend therefrom affecting progressively jejunum, ileum, and sometimes also colon. The virus replicates in enterocytes causing their destruction and desquamation of the intestinal mucosa resulting in diarrhoea and dehydration which can be lethal (Babiuk, 1985; Clark, 1993). Shortening of villi (villous atrophy), observed in the affected intestine, is interpreted as a defence reaction that shall reduce the surface area and thus the water loss. Villous atrophy is a phenomenon that is specific for viral diarrhoea only.

Coronavirus is widely distributed in cattle populations in many countries and specific antibodies can be found in a majority of adult animals (Rodák *et al.*, 1982). Its prevalence in calves developing diarrhoea and clinically normal calves ranges from 8 to 69% and from 0 to 24%, respectively (Clark, 1993; Reynolds *et al.*, 1986). The reason for that is probably chronic virus shedding

by animals in which the development of diarrhoea, but not virus replication, was blocked by colostral or active immunity. In addition to BCV, calves affected by diarrhoea often shed rotavirus.

Viral diarrhoea can affect whole herds. Transmission of infectious diseases is enhanced by high concentration of animals on large farms. Rapid and reliable diagnostic methods are the primary prerequisite for timely and effective implementation of therapeutic and preventive measures. Direct virus demonstration in faeces is the most often used method for the diagnosis of intestinal BCV infections. The potential of methods based on the antigen – antibody reaction has been increased by implementation of monoclonal antibodies (MAb), which react specifically with individual antigenic determinants. So far four structural proteins have been identified in BCV: envelope peplomeric glycoprotein E2, which is present in both cleaved and noncleaved forms (180 to 190 kDa and 90 to 120 kDa, respectively); integral transmembrane glycoprotein E1 (23 to 26 kDa); nucleocapsid protein N (50 to 54 kDa) and its trimer (160 kDa); haemagglutinin E3 present as a dimer (124 to 140 kDa) a with disulphidic bond which can be reduced to two identical subunits (62 to 65 kDa) (Deregt and Babiuk, 1987; Deregt *et al*., 1987; Hussain *et al*., 1991; Tsunemitsu and Saif, 1995).

The objective of this study was to prepare MAb to bovine coronavirus, to identify them, to describe their characteristics and to test selected combinations thereof in direct ELISA and the newly developed single-step immunochromatographic test (ICT) in which the antigen-antibody reaction proceeds between the mobile phase and the stationary phase on a nitrocellulose membrane.

MATERIALS AND METHODS

Faecal sample processing

Faecal samples ($n = 74$) were collected in several herds affected by neonatal diarrhoea. The samples were suspended in fivefold volume of 0.15M PBS, pH 7.2, homogenised, and centrifuged at 7 000 rpm in the centrifuge B4i (Jouan, France) for 20 min. The supernatant was separated and kept at –20°C.

Coronavirus

Bovine coronavirus strain C-197, maintained in the Collection of Animal Pathogenic Microorganisms at the Veterinary Research Institute, Brno, under reg. No. CAPM V-326 was propagated in the MDBK cell line maintained by passaging in the growth medium Eagle MEM supplemented with 5% of bovine foetal serum.

The inoculum volume for the infection of the monolayer was 5 ml containing 10^4 $TCID_{50}$ per 0.1 ml. After adsorption at 37°C for 1 h, the inoculum was removed and the cell culture was further grown in serum-free Eagle MEM for three to four days until CPE became apparent. The culture was frozen-thawed and cell debris was separated by centrifugation at 7 000 rpm using the centrifuge J2-21 and the rotor JS 7.5 (Beckman Instruments, Palo Alto, USA). The viral suspension was condensed by ultracentrifugation (Czerny and Eichhorn, 1989) at 25 000 rpm and 10°C for 1.5 h using the rotor Sw 28 (Beckman Instruments, Palo Alto, USA). The sediment was resuspended in 0.15M PBS, pH 7.2, transferred onto a layer of 30% saccharose and centrifuged at 35 000 rpm and 10°C for 2 h using the rotor Sw 55 Ti (Beckman Instruments, L8-80M). The obtained virus suspension was used for the immunisation of BALB/c mice and rabbits.

Preparation of polyclonal antibodies to coronavirus

Purified coronavirus was mixed with complete (1st treatment) or incomplete (2nd treatment) Freund adjuvant at 2 : 1. California rabbits aged 3 months were immunised subcutaneously three times at 21-day intervals with 0.5 ml of the virus suspension containing 50 µg of protein and exsanguinated one week after the last treatment. IgG fraction containing the antibody activity was separated from blood serum by precipitation with ammonium sulphate to 33% saturation. The content of antibodies was checked by ELISA.

Preparation of monoclonal antibodies

Eight-week-old BALB/c mice were immunised with three doses of BCV (HA titre 1 : 25 000, 30 µg of protein) at 21-day intervals. The first two doses were administered intraperitoneally and the third dose, containing 10 µg of protein, intravenously. The mice were exsanguinated three days thereafter and hybridomas were prepared as described by Galfré and Milstein (1981) using the myeloma cell line Sp 2/0 Ag14 (Institute of Molecular Genetics, Prague). Selected hybridomas, tested by ELISA and identified by Western blotting, were administered intraperitoneally to pristane-treated mice (Galfré and Milstein, 1981). Ascitic fluid was collected, precipitated with ammonium sulphate up to 50% concentration, and dialysed against PBS, pH 7.2.

Western blotting

Purified BCV was solubilised in sample buffer under both reducing and non-reducing conditions and proteins separated in 10% polyacrylamide gel were trans-

ferred onto nitrocellulose membrane (Towbin *et al.*, 1979). After drying, individual nitrocellulose strips were incubated with ascitic fluids containing MAb at the dilution 1 : 1 000. The reaction was detected with swine or mouse IgG antibodies prepared by affinite chromatography and conjugated with horse radish peroxidase (HRP) by the periodate method (Farr and Nakane, 1981). Hydrogen peroxide and 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, USA) were used as the substrate.

Immunoperoxidase test

The specificity of MAb was checked by the immunoperoxidase test using BCV-infected MDBK monolayers grown on slides. Slides with non-infected monolayers were used as negative controls. The cell cultures were fixed with acetone. HRP-labelled goat antibodies to mouse IgG were used as the conjugate (Farr and Nakane, 1981).

Haemagglutination inhibition test

The method developed by Vautherot and Laporte (1983) was used. Twofold dilution series of MAb starting from 1 : 100 were prepared with PBS supplemented with BSA at 2 mg/ml in U-type microtitre plates (GAMA, České Budějovice, Czech Republic). Each MAb dilution was completed with four HA units of BCV and the plates were incubated at 37°C for 60 min. Then, 0.8% suspension of mouse erythrocytes was added into each well and the plates were incubated for another 60 min. MAb titre was defined as the highest dilution inhibiting the agglutination of erythrocytes.

ELISA

A modified ELISA procedure (Czerny and Eichhorn, 1989) was used for the selection of hybridoma fluids and titration of MAb to coronavirus. Wells of microtitre plates (GAMA, České Budějovice, Czech Republic) were coated with purified BCV at a dilution established in preliminary titrations. Hybridoma fluids were tested at the dilution 1 : 2 only. Twofold dilution series of MAb starting from 1 : 100 were prepared in wells of microtittre plates. HRP-labelled swine antibodies to mouse IgG were used as the conjugate. Enzymatic reaction induced by the addition of hydrogen peroxide and tetramethyl benzidine (Sigma Chemical Co., St. Louis, USA) was stopped with 1M sulphuric acid. MAb were tested also with noninfected cell cultures to eliminate possible nonspecific reactions.

The presence of coronavirus in faecal samples was demonstrated by ELISA in microtitre plates (GAMA, České Budějovice, Czech Republic) with well bottoms coated with an MAb mixture at the concentration 15 µg per ml of each. The processed faecal samples were tested in twofold dilution series starting from 1 : 2 and MAb to coronavirus labelled with HRP were used as the conjugate. The enzymatic reaction was visualised by addition of TMB with hydrogen peroxide and stopped with 1M sulphuric acid. Positive and negative control samples were included into each test series. The intensity of yellow colour was measured spectrophotometrically at 450 nm (SLT Spectra, Schoeller, Austria). Samples, or dilutions, showing absorbances > 0.100 were classified as positive.

Rotavirus in faeces was demonstrated by standardised ELISA described earlier (Reschová *et al.*, 2000).

Electron microscopy (EM) of faecal samples

Negative staining with 2% ammonium molybdate at pH 7.0 (Brenner and Horne, 1959) and the electron microscope Tesla BS 500 were used.

Immunochromatography

MAb – colloid gold bond

Colloid gold was prepared by a modified method (Reschová *et al.*, 2000) from 0.02% solution of gold trichloride (Sigma Chemical Co., St. Louis, USA) in sodium citrate solution. Only colloid gold solutions showing spectrophotometric absorption maximums between 525 and 535 nm were used for conjugation with antibodies (Dar *et al.*, 1994; Gupta *et al.*, 1992).

The pH of the colloid gold solution was adjusted to 6.5 with 1% potassium carbonate and 1 ml of it was mixed with 10 µg of a pooled MAb sample. The mixture was incubated at 37°C for 10 min and then mixed with 6 µl of 20% human albumin. This step was followed by centrifugation at 6 000 rpm for 10 min in the centrifuge B4i (Jouan, France). Supernatant was removed leaving about 50 µl in the tube. The contents of the tube were stirred thoroughly and the finished conjugate was distributed at 5 µl into wells of a divided microtitre plate (GAMA, České Budějovice, Czech Republic) and freeze-dried. The coated microtitre plates were kept in a dry place at 4°C until used, but not longer than 4 months.

Immunochromatographic test

Nitrocellulose membrane with pore size 8 µm (Sartorius, USA), was fixed onto a plastic plate 0.5 × 5.0 cm.

Then 2 μl of polyclonal rabbit antibody to coronavirus at the concentration 100 μg/ml was applied in the form of a patch or a strip onto the lower end of the membrane (test zone) and the same volume of polyclonal antibody to mouse IgG at the same concentration at a distance of 5 mm from the former (control zone). After drying, the surface of the membrane was blocked with an inert protein – 2% lactalbumin hydrolysate (Difco Laboratories, Detroit, USA).

PBS supplemented with 0.1% of Triton X-100 and 10% of saccharose and the tested suspensions were distributed at 40 μl and 50 μl, respectively, into wells of the divided microtitre plates containing freeze-dried conjugate and a test strip with the activated membrane was put into each well.

The results were read after 5 to 10 min. A single colour patch in the upper (control) zone indicated a negative result, and patches in both the test and the control zones indicated a positive result.

RESULTS

Monoclonal antibodies to bovine coronavirus

Four hybridomas producing IgG monoclonal antibodies, in which affinity to structural proteins of BCV was demonstrated by Western blotting, were obtained. MAb D7/F4 and D9/H1 reacted with the protein E2 and MAb G2/D10 and G2/F7 with the protein N (Figure 1). Their specificity was further verified by the immunoperoxidase test. Haemagglutination inhibition titres ranged from 16 000 to 128 000 and ELISA yielded a uniform titre of 204 800. No cross reactions with rotavirus were found in any of the MAb (Table 1).

Table 1. Characteristics of monoclonal antibodies

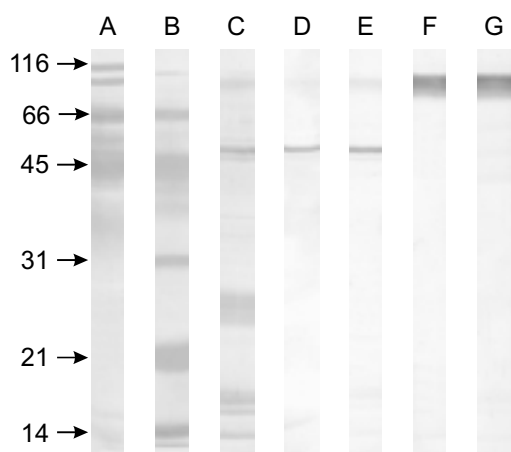| MAb type	Reactivity with BCV antigen	Concentration (mg/ml)	Titre ELISA	Titre HIT	Cross-reactions with rotavirus
G2/D10	N	15	204 800	16 000	neg.
D7/F4	E2	12	204 800	128 000	neg.
G2/F7	N	16	204 800	32 000	neg.
D9/H1	E2	18	204 800	64 000	neg.

Figure 1. Identification of MAb to bovine coronavirus by Western blotting

A – high molecular weight standard
B – low molecular weight standard
C – anti-BCV mouse serum
D – MAb G2/D10 – N
E – MAb G2/F7 – N
F – MAb D7/F4 – E2
G – MAb D9/H1 – E2

ELISA

A direct sandwich MAb ELISA was developed and used for testing a set of 74 calf faecal samples collected in 7 herds. BCV was demonstrated in 15 of them (20.3%). Simultaneous tests for rotavirus were positive in 23 of the 74 samples. Positive for both BCV and rotavirus were 10 samples (Table 2).

Forty-one samples were tested for BCV also by electron microscopy. Agreement of results was found in 36 (87.7%) samples of which 11 were positive and 25 negative by both ELISA and EM. Three samples were ELI-

Table 2. Numbers of positive and negative results in tests for the presence of bovine coronavirus (MAb ELISA) and rotavirus (ELISA) in 74 faecal samples

	Rotavirus		Coronavirus	
	+	–	+	–
	23	0	10	13
	0	51	5	46
Total	74		15	59

Table 3. Numbers of positive and negative results in tests for the presence of bovine coronavirus in 41 faecal samples by MAb ELISA and electron microscopy

	MAb-ELISA		EM	
+	−		+	−
14	0		11	3
0	27		2	25
Total	41		13	28

SA-positive and EM-negative and two samples were EM-positive and ELISA-negative (Table 3).

Immunochromatography

A single-step ICT for the demonstration of BCV using the MAb described above with visual reading of results (Figure 2) and a modified method of MAb conjugation with colloid gold particles were developed. The specificity of the conjugate was confirmed by EM demonstration of MAb-coronavirus complexes (Figure 3). The same set of 74 faecal samples was tested by ICT and the results were compared with those of MAb ELISA (Table 4). Three of the 16 ICT-positive samples were negative by MAb ELISA and 2 of the 58 ICT-negative samples were

Tab. 4. Numbers of positive and negative results in tests for the presence of bovine coronavirus in 74 faecal samples tested by immnochromatography and MAb ELISA

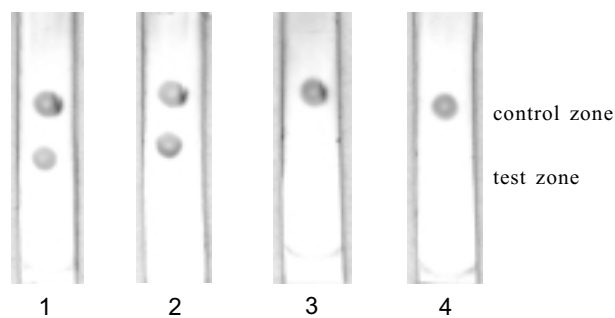|	ICT		MAb-ELISA	
+	−		+	−
16	0		13	3
0	58		2	56
Total	74		15	59

Figure 2. Immunochromatographic detection of bovine coronavirus

1, 2 – positive result
3, 4 – negative result

positive by MAb ELISA. Taking MAb as the golden standard, the sensitivity and specificity if ICT were 94.9% (56 of 59) and 86.7% (13 of 15), respectively.

DISCUSSION

BCV infections can be diagnosed by a variety of methods, all are based on direct detection of the agent in faecal samples collected from calves suffering from diarrhoea (Clark, 1993). The currently used methods include BCV isolation in cell cultures, BCV demonstration by electron microscopy, haemagglutination test, and several variants of enzymoimmunoanalysis. Each of the methods has its merit, but also several drawbacks. Isolation in cell cultures is time-consuming. Electron microscopy requires expensive equipment and identifies unambiguously only complete BCV virions. Partial or complete loss of spikes on the viral envelope, which occurs during virus maintenance or sample processing, can result in false interpretations of findings (Smith *et*
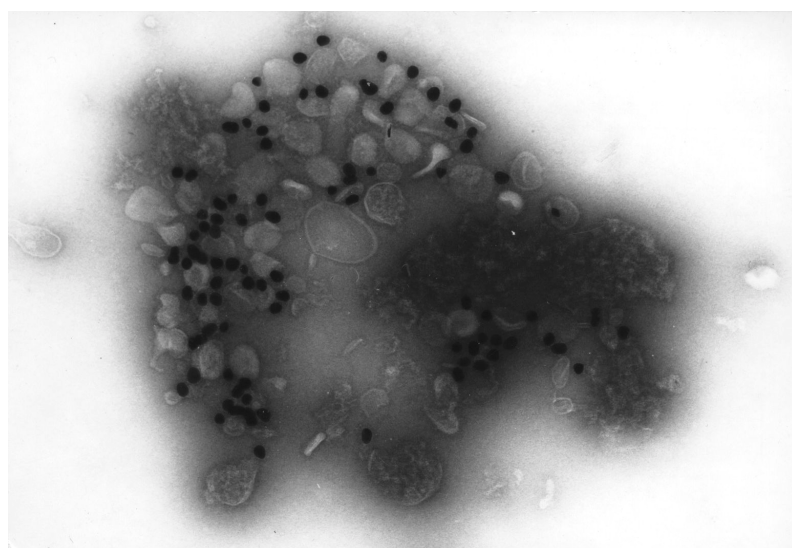

Fig. 3. Specific complexes of gold-labelled MAb with bovine coronavirus (photo made in the laboratory of electron microscopy of the Veterinary Research Institute, Brno) magn. 72 000×

al., 1996). In spite of its drawbacks, electron microscopy is still used as the basic test method. The value of results of the haemagglutination test is affected by possible presence of nonspecific agglutinins in faecal samples which are responsible for false positive reactions (Clark, 1993). The probability of their presence is increased by a high content of ballast components in processed faecal samples. Such complication was observed also in our laboratory and therefore the use of HT within this study was limited to the identification of purified BCV propagated in cell cultures which was free of such impurities. The currently preferred method for the demonstration of BCV infections is ELISA, the sensitivity and specificity of which depends largely on the quality of reagents. Polyclonal antibodies prepared by immunisation of laboratory animals with purified BCV are used commonly (Reynolds *et al*., 1986). Because of the low virus stability, the purification procedures are rather sophisticated and time-consuming and the specificity of the test is not proportional to the expended effort. In our and other authors' experience, polyclonal antibodies to BVC react with cell components and are responsible for a high level of nonspecific background, cross-reactions with rotaviruses, and low sensitivity in comparsion to ELISA for other intestinal pathogenic agents (Crouch *et al*., 1984; Czerny and Eichhorn, 1989).

A solution to these problems is suggested in this study. This consist in the use of monoclonal antibodies to structural BCV proteins, in a sandwich ELISA, and in the development of rapid MAb ICT. We succeeded in the preparation of two MAb to the peplomeric envelope antigen E2 and two MAb to the inner capsid protein N. The molecular masses of these proteins, as determined by immunoblotting, are consistent with the data published by Deregt and Babiuk (1987), Deregt *et al*. (1987), and Hussain *et al*. (1991).

Direct sandwich ELISA using a mixture of MAb for the demonstration of BCV in faecal samples was developed. Compared with the use of a single MAb, the combination of four MAb at 15 μg/ml each assured a higher sensitivity of the test. The reproducibility of the system was verified by multiple testing of standard samples positive for BCV by electron microscopy. The developed MAb based ELISA was used for the demonstration of BCV in field samples collected from calves suffering from diarrhoea. The specificity of the two MAb for the E2 antigen and another two MAb for the N protein of BCV allowed us to detect both complete and incomplete virions. The use of the MAb mixture in ICT and ELISA assures a higher avidity than that of a single MAb (Crouch *et al*., 1984; Smith *et al*., 1996). The use of a mixture of several MAb in the developed antibody – BCV system is also important considering the known antigenic variability of some BCV strains (Vautherot and Laporte, 1983; Saif, 1990). The specificity and sufficient avidity of the mixture of MAb prepared within this

study was confirmed by formation of immune complexes of BCV with gold-labelled MAb as documented by electron micrographs.

A comparison of MAb ELISA with electron microscopy demonstrated agreement of results in 87.7% of the 74 tested samples. Positive by MAb ELISA and negative by electron microscopy were 7.3% of the samples. Negative by ELISA were 4.9% of samples in which only solitary virus particles were detected by electron microscopy. This finding is consistent with the data published by Schoenthaler and Kapil (1999). Therefore, MAb ELISA was used as a standard for the assessment of results obtained by ICT, as previously dane for rotaviruses (Reschová *et al*., 2000).

ITC has been included into our study because it is currently preferred as a simple method for antigen or antibody demonstration in both human and veterinary medicine (Bhaskar *et al*., 1996; Dar *et al*., 1994; Gupta *et al*., 1992; Klingenberg and Esfandiari, 1996).

Like in other methods, the results of ICT depend largely on the principle of its design and on reagent quality. In the case of ICT, this applies particularly to the selection of membrane with the optimum pore size allowing passage of immune complexes without affecting the irreversibility of the link of both antibodies (Bonzom, 1996). Also important is the connection between the membrane and the absorption material and the type of fixation of both onto the plastic plate. In our experiments, these conditions were met to such an extent that the use of previously prepared, stored, and activated strips and plates allowed us to obtain the result within ten minutes.

BCV was demonstrated in 20.2% of the samples. This proportion corresponds to the mean prevalence of BCV in Czech cattle herds in the past ten years. The small discrepancy between the results of ICT and ELISA, manifested in three ICT-positive and ELISA-negative samples was probably caused by the presence of coproantibodies forming immune complexes with the antigen that are more readily detected by ICT because of a different principle of this method. Two samples negative by ICT showed ELISA titres near the detection limit. Similar results were obtained also in the demonstration of bovine rotavirus by ICT (Reschová *et al*., 2000). The lower sensitivity, compared to ELISA does not reduce the diagnostic value of ICT, because calves developing clinical coronavirus-induced gastroenteritis show almost invariably high antibody titres in ELISA. The somewhat lower sensitivity and specificity of ICT (94.9 and 86.7%, respectively) have been previously described (Sato *et al*., 1996) and this is offset by its benefits, including speediness and simplicity, allowing its use in laboratories lacking more expensive equipment, or even in the field. It can be concluded that ICT is an effective tool for a pilot survey of the prevalence of coronavirus-induced gastroenteritis in cattle herds.

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