

## Detection of bovine enteric coronavirus in clinical specimens by hybridization with cDNA probes

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Molecular hybridization, previously optimized for purified bovine coronavirus (BCV), was adapted for detection of virus in clinical specimens. For this purpose, the accuracy of the existing tests had to be improved and suitable means for removal of extraneous molecules had to be established. Six radioactive probes were used to obtain adequate detection signals. These probes, containing the complete N and E1 gene sequences and other sequences, hybridized to about  $\frac{1}{4}$  of the total length of the viral RNA.

Genomic RNA could be detected after direct spotting of samples, but prior Freon-extraction or centrifugation of specimens on a cushion of sucrose improved considerably the positive identification of virus containing samples. RNA detection in positive clinical specimens was significantly better by hybridization than immunological detection of BCV by ELISA, although differences were slight after two passages of the virus on HRT-18 cell monolayers. Consequently, the reliability of positive and negative test results in hybridization tests on Freon extracted specimens was better than in ELISA. However, results after extraction with other organic solvents were inferior. The accuracy of ELISA was surpassed by hybridization assays.

Background signals, due to vector homology were found to be negligible in all the samples analyzed.

**KEYWORDS:** BCV (bovine coronavirus), DNA–RNA hybridization, sample-processing, test parameters.

### INTRODUCTION

Bovine enteric coronavirus (BCV) particles have frequently been observed in samples of diarrhoeic neonatal calves in different parts of the world.<sup>1–3</sup> BCV is, together with other infectious agents such as rotavirus and enterotoxigenic *Escherichia coli* as well as environmental conditions, responsible for the cause of

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endemic neonatal diarrhoea.<sup>4-7</sup> Economical losses due to this disease are considerable,<sup>8</sup> implying a need for reliable detection methods of the causative agents.

Detection of the pleomorphic BCV particles in faecal samples was initially done by direct electron microscopy (DEM),<sup>5</sup> although the poor preservation of the peplomers<sup>9</sup> and the presence of particles with morphological resemblance to BCV make DEM less suitable for unambiguous identification of BCV-positive or -negative specimens. The recently described colloidal-gold immunoelectron microscopy for BCV detection in faecal samples increases the accuracy of detection.<sup>10</sup> Alternative methods, based on the serological detection<sup>11,12</sup> or on the specific haemagglutinating activity of the virus,<sup>3,13</sup> have been described. Further diagnosis has also been done by immunofluorescence staining of either post-mortem colon sections,<sup>9,14</sup> or cell cultures inoculated with BCV-positive specimens.<sup>15</sup> In these methods, highly specific antisera are required,<sup>11,12</sup> whereas haemadsorption virus capture and release assays lack sufficient sensitivity,<sup>3</sup> and cell cultures are time-consuming.

Recently, a report was published on the detection by cDNA probes of viral RNA, isolated from tissue culture propagated virus.<sup>16</sup> We described the detection of tissue-culture propagated BCV by means of radioactive and biotinylated cDNA probes and demonstrated that direct virus fixation and denaturation on nitrocellulose is preferable to prior RNA isolation. Furthermore, conditions for optimal detection by hybridization and for detection signal amplification were established.<sup>17</sup>

Here, we describe investigations on the potential use of cDNA probes for BCV detection in faecal samples, applied either directly or after treatment to the nitrocellulose membrane. The results were compared to those obtained by ELISA and a first and second passage on tissue culture.

## MATERIALS AND METHODS

### Reference virus

The NCDC strain (Mebus) of BCV was obtained from the American Type Culture Collection (cat. # VR 874) and propagated in human rectal tumor (HRT-18) cells, as described by Laporte *et al.*<sup>18</sup> The virus was purified from the supernatants of infected cell cultures by differential and isopycnic ultracentrifugation on sucrose gradients.<sup>19</sup> Purified virus was obtained at a buoyant density of 1.18–1.20 g ml<sup>-1</sup>, as determined from the coincidental peak of haemagglutinating activity and optical density at 280 nm, diluted in TBS (0.05 M Tris-HCl, pH 8.0, and 0.15 M NaCl), and concentrated by ultracentrifugation for 2 h at 100,000 g in a Beckman SW 27.1 rotor. Sedimented virus was resuspended in TBS and frozen at -70°C until further use.

### Clinical specimens

Faecal samples from 1 to 2 weeks old diarrhoeaic calves were obtained from dairy herds in Southern Quebec. The specimens were homogenized in ten volumes of TBS buffer and clarified by centrifugation at 5000 g for 20 min at 4°C. The supernatants were filtered through Millipore membranes (0.45 µm) and stored at -70°C.

## Virus isolation in cell cultures

Monolayers of HRT-18 cells, grown in 6-well Linbro tissue culture plates, were rinsed twice with PBS (0.01 M phosphate-buffered saline, pH 7.2) and inoculated with 0.5 ml of clarified clinical samples, diluted in RPMI 1640 medium containing twice the normal concentration of antibiotics.<sup>18</sup> The inoculum was discarded after adsorption for 1 h at room temperature and infected cell cultures were maintained in medium without serum. The monolayers were checked daily for the appearance of cytopathic effects. About 4–5 days after initial inoculations, cells and supernatants were frozen and thawed twice before clarification by centrifugation. These clarified samples were then used as inoculum for further subpassages to HRT-18 cell monolayers, grown in 25 cm<sup>2</sup> tissue culture flasks (Corning).

## Processing of faecal specimens for hybridization

Faecal samples, 20 µl diluted with 180 µl TBS, were mixed with equal volumes of either phenol/chloroform (1:1), Freon (1,1,2-trichloro-1,2,2-trifluoroethane), carbon tetrachloride or chloroform and vortexed for 30 s, followed by phase-separation by centrifugation for 2 min at 14,000 g. The aqueous phases were collected and adjusted to a final concentration of 1 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.2) using a stock solution of 20 × SSC. Alternatively, samples (100 µl with the same final dilutions) from the different faecal specimens were applied to a 100 µl cushion of 30% sucrose in 0.05 M Tris-HCl, pH 7.6, and centrifuged for 30 min at 30 psi in a Beckman Airfuge. The pellets were resuspended in 100 µl of 1 × SSC.

## Probes

Construction of the genomic cDNA library of BCV-RNA has been described previously.<sup>17</sup> Briefly, isolated RNA from purified virions was used for the synthesis of first-strand cDNA according to the methods described by Gubler & Hoffman<sup>20</sup> and Binnns *et al.*<sup>21</sup> Second-strand synthesis was done by the use of RNase H and the Klenow fragment of *E. coli* DNA polymerase I<sup>20</sup> followed by dC-tailing of the double-stranded cDNA molecules<sup>22</sup> and cloning into a *Pst* I linearized 3' oligo-dG tailed pUC-9 vector. Recombinant plasmids were labelled by nick translation,<sup>23</sup> using [ $\alpha$ -<sup>32</sup>P]dCTP, and added to the prehybridization solution after heat denaturation.

## Probe selection

One recombinant plasmid contained as probe an insert (1.63 kbp) representing the polyadenylated 3' end of the genome and the complete translational reading frame of the nucleocapsid protein (N), as verified by sequence analysis and comparison to the sequence published by Lapps *et al.*<sup>24</sup>

Another probe, containing the translational reading frame of the matrix protein (E1) and a part of the N-gene, was obtained by cloning a dC-tailed 810 bp fragment,

amplified by the polymerase chain reaction (PCR; 25), into a *Pst* I linearized dG-tailed pUC-9 vector. PCR amplification was done after cDNA synthesis by use of a primer complementary to the RNA from position 1526 to 1550 (5' GAA CAT TTC TAG ATT GGT CGG ACT G) and an RNA-sense primer annealing to the cDNA complement of the RNA from the beginning of the translational startcodon of E1 at position 2336 to position 2313 (i.e. 5' ATG AGT AGT GTA ACT ACA CCA GCA). The numbering of the nucleotides is from the 3' end of the genome. The two probes described above are the only ones that contain a small overlap of 108 bp of the cloned viral cDNA. Four other probes, obtained from cloned random-primed cDNA, were selected by their capacity to hybridize strongly in colony-filter hybridization assays<sup>17</sup> and their absence of overlapping sequences as established by Southern blot analysis (data not shown). Inserts of 664 and 1120 bp of two of these recombinant plasmids were subcloned into the RF of bacteriophage M13mp18 and mp19, and completely sequenced in both directions using the dideoxy chain termination technique,<sup>26</sup> with [ $\alpha$ -<sup>35</sup>S]dATP as a radioactive precursor. Computer analysis (IBI Pustell sequence analysis programs) of these sequences did not reveal any significant homology with other published coronavirus sequences as recently reviewed by Spaan et al.<sup>27</sup> The other two probes, used in the probe-pool for BCV detection, contained insert sizes of 0.7 and 1.4 kbp, respectively, as established by agarose-gel electrophoresis analysis, but were not further subjected to sequence analysis.

The probe-pool consisting of six radiolabelled recombinant plasmids, largely containing non-overlapping sequences, would thus cover about  $\frac{1}{4}$  of the BCV-genome.

### Sample application and molecular hybridization

One hundred microliters of the processed or of the diluted original specimens were directly spotted by means of a 72-well Minifold apparatus (Schleicher and Schuell Inc.) onto nitrocellulose membranes (0.20  $\mu$ m), equilibrated in 20  $\times$  SSC. Denaturation and RNA fixation were achieved by baking the blots for 2 h at 80°C under vacuum. The blots were then rehydrated in a 6  $\times$  SSC solution and prehybridized for 4 h or overnight at 42°C in a solution containing a final concentration of 55% formamide, 5  $\times$  Denhardt's components (0.5 g Ficoll, 0.5 g polyvinylpyrrolidone and 0.5 g BSA per 500 ml H<sub>2</sub>O), SSPE salts (0.9 M NaCl, 0.05 M sodium phosphate and 0.005 M EDTA, pH 7.4), 0.1% SDS, and 100  $\mu$ g of denatured sheared calf thymus DNA per ml. The probes were directly added to the prehybridization solutions at concentrations indicated in the different experiments, followed by an incubation period of 48 h at 42°C. Washing of the blots was done according to standard procedures,<sup>28</sup> followed by exposure, using intensifying screens, of the air-dried blots to Kodak-X-Omat X-ray films at -70°C overnight.

### Detection signal amplification

Up to six probes (recombinant plasmids), were used simultaneously to hybridize to different locations on the genome, both for the detection of purified virus and virus present in faecal specimens. About 600 ng probes with a specific activity of

$10^7$ – $10^8$  cpm  $\mu\text{g}^{-1}$  was added to 2 ml prehybridization solution, which covered 24 cm<sup>2</sup> of nitrocellulose membrane on which 24 samples were spotted.

### Antisera

Anti-BCV hyperimmune sera were obtained after immunization of rabbits<sup>29</sup> and guinea pigs with the sucrose-gradient purified Mebus strain of BCV. Inoculation doses consisted of 400  $\mu\text{g}$  and 100  $\mu\text{g}$  of purified viral antigen, as determined by the method of Bradford,<sup>30</sup> for rabbit and guinea pigs respectively. Guinea pig and rabbit hyperimmune sera inhibited the haemagglutinating activity of BCV. Their haemagglutination-inhibition titres were 1:640 and 1:5120, respectively. The rabbit hyperimmune sera had a titre of about 1:10,240 by seroneutralization.

### Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA for detection of BCV antigens in faecal specimens from diarrhoeic calves was performed using modifications of previously described methods.<sup>31,32</sup> All washes were done three times with TBS containing 0.05% Tween 20 (TBS-T). A total reaction volume of 100  $\mu\text{l}$  was used in all microtitration wells.

To evaluate the specificity of the antisera to BCV, flat-bottomed microtitre plates (Flow Laboratories) were coated with 0.5  $\mu\text{g}$  of protein well<sup>-1</sup> of density gradient-purified viruses in 0.05 M sodium carbonate buffer, pH 9.6, by overnight incubation at 4°C. Both guinea pig and rabbit anti-BCV hyperimmune sera, diluted 1:100, were shown to be free of reactivity against bovine rotavirus, bovine herpes virus type 1 (strain Colorado), and the coronaviruses responsible for transmissible gastroenteritis (strain Purdue) and avian infectious bronchitis (strain M-41).<sup>31</sup> The optimum dilutions of the anti-BCV antisera were determined by checkerboard titrations.<sup>33</sup>

For clinical assays, plates were first coated with the guinea pig hyperimmune sera, diluted 1:500 in sodium carbonate buffer, for 3 h at 37°C followed by overnight incubation at 4°C. Free sites were blocked by incubation for 1 h at 37°C with TBS supplemented with 1% bovine serum albumin (BSA, fraction V, Sigma). The plates were then dried and stored refrigerated until required. The solid-phase antibody was subsequently incubated for 3 h at 37°C with ten-fold dilutions ( $10^{-1}$ – $10^{-3}$ ) of clarified faecal samples prepared in TBS-T, washed, and incubated for 2 h at 37°C with 1:1,000 dilution of the rabbit anti-BCV hyperimmune serum. After a further washing step, a 1:1000 dilution of peroxidase labelled goat anti-rabbit IgG (Boehringer Mannheim) was added to each well. The plates were then incubated for 90 min at 37°C, washed, and placed for 30–40 min at room temperature after addition of the enzyme-substrate solution (1  $\mu\text{g}$  ml<sup>-1</sup> of 5-aminosalicylic acid (Sigma)) and 0.005% hydrogen peroxide, pH 6.0. The absorbance was measured at 474 nm using a multichannel photometer (Titerteck Multiskan, Flow). A positive/negative ratio was determined as follows: Positive/negative = (A{sample} – A{blank})/A{negative} – {blank}, where A{sample} and A{blank} are A<sub>474</sub> values of clinical specimens tested and a Tris-buffered saline solution control, respectively, and A{negative} is the mean A<sub>474</sub> value of the supernatant fluid of uninfected HRT-18 cellcultures. A clinical sample was considered positive when the positive/negative

ratio was  $\geq 3$ . This high cut-off value was chosen since variation in clinical specimens was observed to be higher than those from tissue culture.

## Definitions

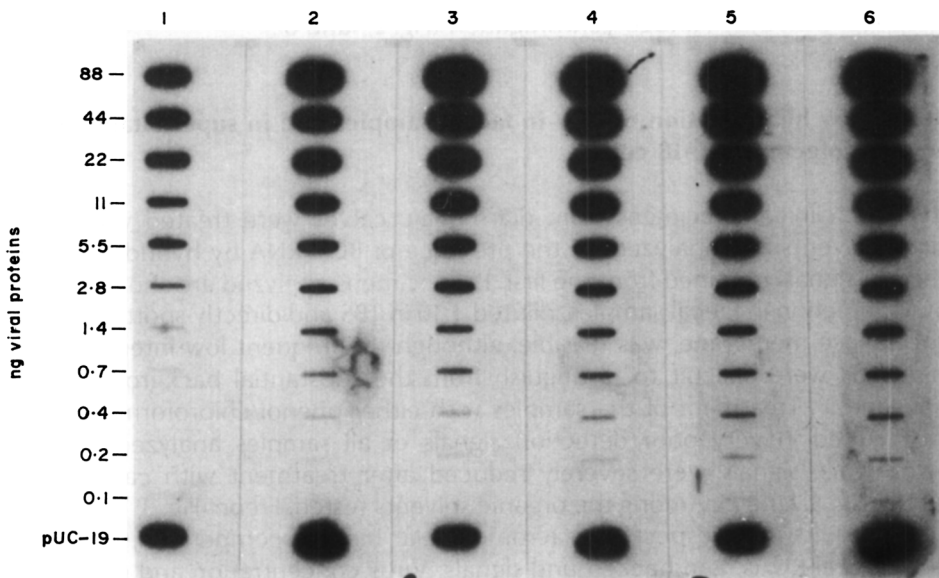
To avoid confusion in the terminology of test parameters (in %), definitions are as in Tijssen.<sup>33</sup> The ability of a test to give a positive test result for specimens from an infected animal ('sensitivity') was expressed as the detectability index of positives ( $DI(+)$  = true positives/true positives + false negatives). Similarly, the ability of a test to give a negative test result for specimens from non-infected animals ('specificity') is expressed as the detectability index of negatives ( $DI(-)$  = true negatives/true negatives + false positives). The ability of a test to recognize correctly specimens from infected and non-infected animals ('accuracy') was defined as  $DI(+, -)$  by the equation  $DI(+, -) = DI(+)$   $\times$  prevalence +  $DI(-)$   $\times$  (1-prevalence). Generally, prevalence is defined as the number of true positives (TP) plus false negatives (FN) among all test results ((TP + FN)/n). Here, specimens were considered to have a positive status (PS = TP + FN) when they were yielding either (i) a positive result with both virus detection by hybridization on original or processed samples and ELISA performed on the original specimens, or (ii) a positive hybridization result after second passage of the sample on HRT-18 cell monolayers. For a given detection method, the reliability of a positive test result (RT(+)) was defined as the ratio of true positives over the total number of positive test results. The reliability of a negative test result (RT(-)) was defined as the ratio of true negatives over the total numbers of negative test results. Sensitivity is here defined as the slope of the dose-response curve (dR/dC) and the detectability as the minimum amount of virus that can be detected.

## RESULTS

### Amplification of the detection signal

Combinations of one to six probes were used in a final concentration of 600 ng blot<sup>-1</sup> of 24 cm<sup>2</sup> to amplify the hybridization detection signal (Fig. 1). Serially-diluted purified virus was spotted onto nitrocellulose and virus denaturation as well as RNA fixation was achieved by baking of the blots. Figure 1 demonstrates that the amounts of detected viral RNA were not directly proportional to the number of different probes added, as insert sizes varied from 0.6–1.6 kbp. However, an increase of about 10–14-times in detectability was obtained when using six probes (Fig. 1, lane 6) instead of one (Fig. 1, lane 1), whereas the sensitivity increased four to six times. A minimum of about  $6 \times 10^4$  viral genomes could be detected after the detection signal amplification.

Labelled pUC-9 sequences without inserts did not contribute to signals in BCV-RNA detection,<sup>17</sup> emphasizing the specificity of the detection obtained. pUC-19 DNA (10 ng) was spotted as a control on the hybridization itself (Fig. 1).



**Fig. 1.** Amplification of the hybridization signal by multiple probes, containing non-overlapping viral cDNA sequences. Column-numbers indicate the number of different probes, in a final concentration of 600 ng per 2 ml, used for the individual blots. Hybridization was for 48 h at 42°C and autoradiography was done by exposure of the blots to X-ray film for 20 h at -70°C, using intensifying screens. The indicated amounts of viral proteins refer to the quantity of proteins measured according Bradford.<sup>30</sup> An amount of 100 pg viral proteins correspond to about  $1.14 \times 10^5$  virus particles or about 1.1 pg genomic RNA. 10 ng pUC-19 DNA was spotted as a control on the hybridization itself.

### Analysis of the influence of virus-treatment with different organic solvents on the detection of BCV-RNA by hybridization

Simulation models in which equal amounts of purified virus were added to several BCV-negative faecal samples, diluted 1:10 in TBS, showed strong competition in binding between virus particles and other macromolecules to the nitrocellulose membrane, as different detection signals were obtained for the same amounts of virus (data not shown). Therefore, it was attempted to eliminate or reduce interfering molecules by the extraction with organic solvents. In a test model, equal quantities of several purified-virus dilutions and of the individually processed samples were spotted directly onto the nitrocellulose membrane for analysis by hybridization, using the probe-pool of six probes. The detection signals from these samples, and from dilutions of purified virus, concentrated on a cushion of sucrose, are shown in Fig. 2. Although some virus loss was obtained during all processing procedures, when detection signals are compared with those obtained after spotting purified virus dilutions (Fig. 2, lane 1), almost no detection signals were seen after phenol/chloroform extractions (Fig. 2, lane 3), most probably due to uncoating of the RNA and the presence of RNases. Both chloroform and tetrachloride treatment of the samples resulted in virus loss, particularly in case of low virus quantities (Fig. 2, lane 2 and 5). The best recovery, especially of low virus quantities,

was achieved when extractions were done with Freon (Fig. 2, lane 4) or when virus was concentrated by Airfuge centrifugation (Fig. 2, lane 6).

### **Detection by hybridization of BCV in faecal samples and in supernatants of specimen infected-HRT-18 cells**

Thirty-four clinical specimens from diarrhoeaic calves were treated with several organic solvents and analyzed for the presence of BCV-RNA by hybridization. The detection signals obtained from the first 12 specimens analyzed are shown in Fig. 3.

BCV detection in faecal samples, diluted 1:10 in TBS and directly spotted onto the nitrocellulose membrane, was feasible, although the frequent low-intensity detection signals were difficult to distinguish from the substantial background signals (Fig. 3, lane 3). Treatment of the samples with either phenol/chloroform or chloroform resulted in very poor detection signals of all samples analyzed (data not shown), while signals were severely reduced after treatment with carbon tetrachloride (Fig. 3, lane 2). Among the organic solvents tested, Freon (Fig. 3, lane 1) gave the best results with respect to macromolecular binding competition, the preservation of viral RNAs, and background signals. Virus concentration and gross debris elimination was found to be optimal after centrifugation of the specimens on a cushion of 30% sucrose by an Airfuge (Fig. 3, lane 4).

Background signals, due to vector homologies with nucleic acid present in the specimens, were negligible (Fig. 3, lanes 'a').

### **Passage of samples on HRT-18 cells**

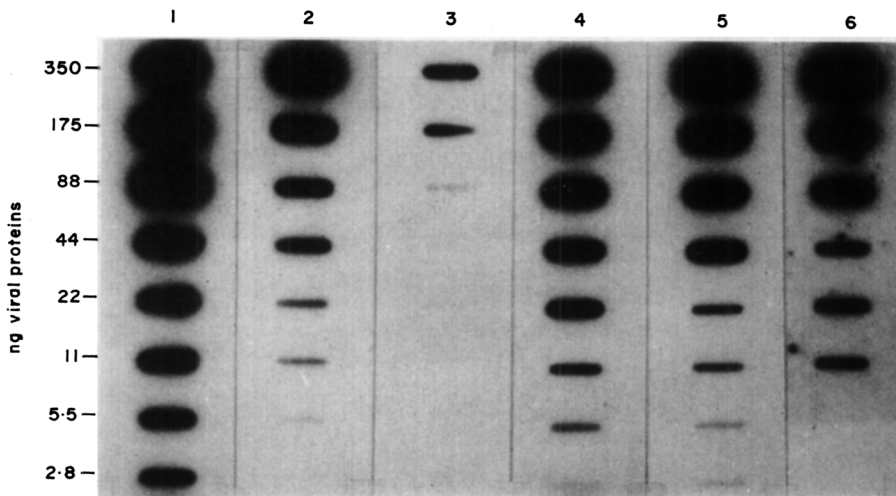
Potentially-present BCV in faecal specimens was subpassaged twice on HRT-18 cell monolayers and analysed by hybridization (Fig. 3, lane 5) and ELISA (Table 1) in order to test the presence of viable virus in the original specimens and to amplify virus quantities. As a result strong detection signals were obtained (Fig. 3, lane 5), which were also specific, because labelled vector sequences did not hybridize to nucleic acid isolated from HRT-18 cells.<sup>17</sup>

### **Detection by ELISA**

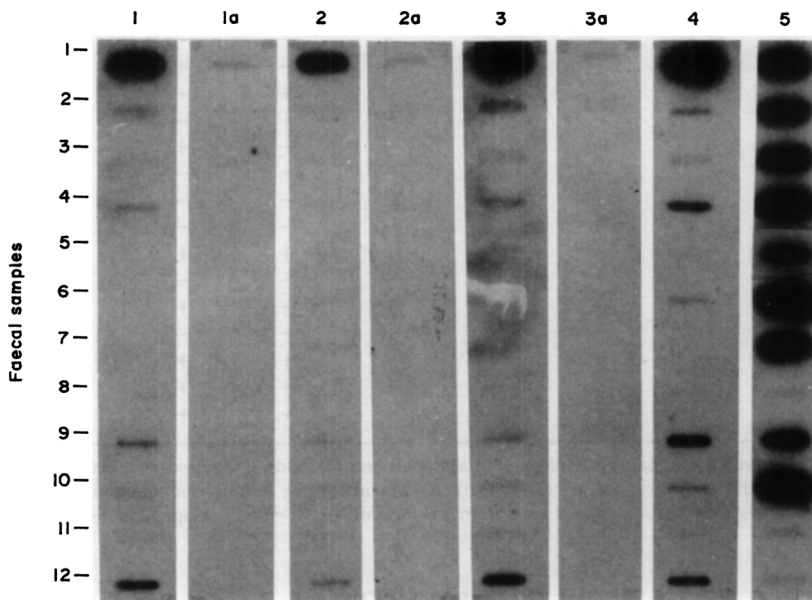
Clinical samples, which yielded an ELISA signal of at least three times that of the background, were recorded as positive (see Materials and Methods for specifications; Table 1). A comparison of the test parameters, as specified in Definitions is shown in Table 2.

Clinical samples were also subjected to ELISA after concentration on a cushion of sucrose by Airfuge-centrifugation or after Freon treatment. Although an improvement in the recognition of positive samples ( $DI(+)=56.5$ ) was obtained after centrifugation, the number of false positives increased dramatically ( $DI(-)$  decreased from 81.8 to 45.5). Freon treatment of samples prior to ELISA decreased the  $DI(+)$  from 43.5% to 34.8%, whereas the other test parameters remained about the same (data not shown).





**Fig. 2.** Influence of organic solvent extraction of purified virus on the detection by hybridization. Individual samples of serially-diluted purified virus were applied directly to nitrocellulose (1) or treated before blotting with chloroform (2), phenol/chloroform (3), Freon (4), carbon tetrachloride (5), respectively, or concentrated on a 30% sucrose cushion by means of an Airfuge (6), (only six samples are spotted in lane 6). Autoradiographs were exposed for 20 h at  $-70^{\circ}\text{C}$ .



**Fig. 3.** BCV detection by hybridization of faecal samples. Samples diluted 1:10 in TBS, were extracted either with Freon (1) or with carbon tetrachloride (2), or applied directly to the nitrocellulose (3). Airfuge-concentrated samples (4), or 100  $\mu\text{l}$  from 25 ml supernatants of HRT-18 infected cell monolayers (5), after a second passage of the specimens were also analysed. The 'a' slots refer to duplicates of correspondingly numbered blots, but incubated with 600 ng of labelled pUC-9 per 2 ml hybridization solution as a reference for background hybridization signals. Autoradiographs were exposed for 20 h at  $-70^{\circ}\text{C}$ .

**Table 1.** Comparison of cDNA hybridization with other methods for BCV detection in faecal specimens

No. faecal specimen	ELISA	Hybridization					ELISA	
		Faecal specimen processing			TC		TC	Status
		Direct	Freon	Airfuge	1st	2nd	2nd	
1	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+
3	-	+	-	+	-	+	+	+
4	+	+	+	+	-	+	+	+
5	-	-	-	-	-	+	+	+
6	-	-	-	+	-	+	+	+
7	-	-	-	-	-	+	+	+
8	-	-	-	-	-	-	-	-
9	-	+	+	+	+	+	+	+
10	-	-	-	+	-	+	+	+
11	-	-	-	-	-	-	-	-
12	+	+	+	+	-	-	-	+
13	-	-	-	-	-	+	-	+
14	-	-	-	-	-	-	-	-
15	+	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-
17	+	-	-	-	-	+	+	+
18	+	-	-	-	-	+	+	+
19	+	-	-	-	-	-	-	-
20	-	-	-	+	+	+	+	+
21	-	-	+	+	-	+	+	+
22	-	-	+	+	+	+	+	+
23	+	-	+	-	+	+	+	+
24	-	-	+	-	-	+	+	+
25	-	-	-	-	-	-	-	-
26	-	-	-	+	-	-	-	-
27	-	+	-	-	-	-	-	-
28	-	+	+	+	+	+	+	+
29	-	-	-	-	-	-	-	-
30	-	+	+	+	-	+	+	+
31	-	+	+	+	-	-	-	-
32	+	+	+	+	+	+	+	+
33	+	+	+	+	+	+	+	+
34	+	+	+	-	+	+	+	+

+ = positive; - = negative; Status is as defined in Materials and Methods, (Definitions); Direct = faecal sample directly applied to nitrocellulose; Freon = Freon extracted faecal sample; Airfuge = airfuge concentrated virus from faecal samples; TC = tissue cultured virus from faecal samples; 1st = first passage; 2nd = second passage.

## DISCUSSION

Detection amplification, using multiple recombinant plasmids was significant (Fig. 1). Frequently, the use of only one probe did not appear to be sufficient in BCV diagnosis (data not shown). A significant 30 to 50 times detection amplification was obtained using biotinylated probes.<sup>17,34</sup> However, it was established in preliminary experiments that biotin showed high affinity for substances in faecal specimens,

**Table 2.** Comparison of the results for BCV detection in clinical specimens by cell culture, ELISA, and cDNA hybridization

	ELISA	Hybridization				ELISA	
		Faecal specimen processing			TC		TC
		Direct	Freon	Airfuge	1st	2nd	2nd
DI(+)	43.5	47.8	60.9	65.2	43.5	95.7	91.3
DI(-)	81.8	81.8	90.9	81.8	100.0	100.0	100.0
RT(+)	83.3	84.6	93.3	88.2	100.0	100.0	100.0
RT(-)	40.9	42.9	52.6	47.4	45.8	91.7	84.6
DI(+, -)	55.9	58.8	70.6	70.6	61.8	97.1	94.1

DI(+) = detectability index of positives; DI(-) = detectability index of negatives; RT(+) = reliability of positive test results; RT(-) = reliability of negative test results; DI(+, -) = Accuracy of the test. (See Materials and Methods for definitions and Table 1 for further specifications)

resulting in strong background signals (even in the case of incubation of blots with biotin analogs alone; data not shown).

Detection of viral RNA, isolated from purified virus by means of SDS-Proteinase K digestion, was feasible, although some loss, especially of low virus quantities was obtained.<sup>17</sup> Similar treatment of virus-containing supernatants of infected cultured HRT-18 cells, significantly increased the sensitivity and slightly reduced the detectability,<sup>17</sup> indicating the positive influence of macromolecule elimination on virus detection. Therefore, the virus was bound to the nitrocellulose membrane either directly or after extraction with several organic solvents. Freon appeared to be the most favourable for debris elimination and nucleocapsid preservation, whereas sucrose cushion concentration was at least equally successful but more time-consuming, as only six samples per half-hour could be processed.

Extraction of the specimens or purified virus by the other organic solvents tested, resulted in substantial loss of the virus, likely due to progressed uncoating of the nucleocapsids followed by degradation of the templates, whereas RNA-loss might also have occurred due to accumulation in the interphase. Hybridization techniques, used for direct BCV detection in clinical specimens, clearly surpassed ELISA, regarding their ability to yield a positive result for virus-containing samples (DI(+)), the reliability of the test results (RT(+)) and RT(-)) as well as the accuracy DI(+, -); (Table 2). In general, accuracy may be very misleading (e.g. a blind statement that a test is negative for a rare disease will have a high accuracy), but it is meaningful here as a comparison of diagnostic techniques on the same set of samples with a fixed prevalence. In addition, tests may have similar accuracies but perform quite differently, i.e. some techniques may tend to give false positives, whereas inaccuracy in others may arise predominantly through false negatives. It is noteworthy that among the direct detection methods used, ELISA tended to give relatively more false negatives and equal numbers of false positives as the hybridization assays.

The effects of gross debris reduction by either Freon extraction or centrifugation on a cushion of sucrose by means of an Airfuge, increased the DI(+) about 13–17% and the accuracy (DI(+, -)) of the test about 12% when compared to direct sample application (Table 2), whereas the reliability of the negative test results (RT(-)) was also significantly better. Adaptation and a first passage of virus from

specimens on cell monolayers did not significantly improve detection by hybridization, compared to direct virus detection, whereas a second passage improved detection by both hybridization and ELISA (Table 2). A second passage of virus from faecal specimens on cell monolayers provided for hybridization the highest accuracy (DI(+, -)) and reliability of the negative samples (RT(-)), but is too expensive and the time required of about two weeks is unacceptable for quick diagnosis. Moreover, the simpler ELISA gave similar detectabilities, reliabilities and accuracy after a second passage on HRT-18 cells. The relatively low values obtained by ELISA, performed on the original specimens may be due to the presence of proteases, virus-specific secretory immunoglobulins, or other interfering factors.<sup>35</sup> On the other hand, the increase of positive ELISA readings after Airfuge-centrifugation of samples, which were negative for virus by hybridization or tissue culture, indicated the presence and concentration of macromolecules able to yield false-positive reactions. Freon treatment decreased the overall number of positives, presumably due to disruption of the viral envelope.

Previous studies<sup>36,37</sup> have shown that vector homology in diagnostic nucleic acid hybridization may cause problems, however, no significant background signal contribution was observed in our experiments (Fig. 3, lane 1a, 2a, 3a). Nevertheless, control hybridizations were always included as a precaution to circumvent possible misinterpretations.

Direct spotting of diluted and clarified faecal samples, by means of a slot-blot apparatus was often difficult as debris blocked the wells of the apparatus thus preventing sufficient virus binding. Furthermore, interpretation of the detection signals obtained after direct spotting was difficult due to blurred detection spots (Fig. 3, lane 3), which were most likely a result of non-specific probe-trapping by spotted clinical specimens, and may be responsible for the false-positive result of sample 27 and 31. One of the 34 samples analysed (# 12) was identified as true positive, but virus could not be detected after culturing on cell monolayers, indicating the presence of either inactivated virus or a different virus strain, that was not capable of adapting to HRT-18 cells. One of the specimens (# 13) was positive by hybridization after passage in cell culture twice, but negative by ELISA, suggesting the isolation of a different BCV serotype.

In this paper we demonstrated that molecular hybridization can be a valuable tool for direct diagnosis of BCV, particularly after suitable processing of the clinical specimens. Faecal sample processing was fairly simple, rapid and cheap when compared to the more accurate tissue culture method. However, the use of radioactivity is one of the major disadvantages of the technique.

Studies are currently underway for BCV diagnosis by means of virus-specific sequence amplification, using the polymerase chain reaction (PCR).<sup>25,28</sup> Diagnosis by means of PCR alone or in combination with hybridization techniques, might increase or even surpass test parameters to levels obtained by tissue culture.

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