

## Biotinylated and radioactive cDNA probes in the detection by hybridization of bovine enteric coronavirus

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cDNA, synthesized on bovine coronavirus (BCV) genomic RNA templates, could be used to detect very small quantities (i.e. 1 pg) of viral RNA by hybridization with either radioisotopic-labelled or biotinylated recombinant plasmids. Virus was optimally attached to nitrocellulose membranes when spotted in  $1 \times \text{SSC}$ , whereas  $20 \times \text{SSC}$  was superior for viral RNA. Denaturation and RNA fixation of both RNA, still encapsidated in virus particles and isolated genomic RNA, was achieved by baking of the blots in vacuum. Virus detection in the supernatant of infected HRT-18 cells was feasible, but improved significantly after proteinase K treatment. No homology was observed between virus cDNA with either plasmid DNA or nucleic acid isolated from non-infected HRT-18 cells. Hybridization with radioisotopic-labelled probes in higher formamide concentrations (up to 60%) increased the detection signals, possibly by reducing reassociation of the probe. Significant detection amplification (30–50 times) was achieved in the case of biotinylated probes by stimulation of hyperpolymer formation on already hybridized target sequences, by additional hybridization with biotinylated pUC-19. A detection amplification was also obtained when hybridization was done with two probes (pBC-52 and pBC-247), containing non-overlapping viral sequences. Although the detectability was surpassed by biotinylated probes, sensitivity was superior in radioisotopic virus detection.

**KEYWORDS:** BCV (bovine coronavirus), cDNA probes, hybridization conditions, detection amplification.

### INTRODUCTION

Bovine enteric coronavirus (BCV) particles are pleomorphic, 60–150 nm in diameter, and bear a double fringe of peplomers.<sup>1</sup> The genome of BCV is a single-stranded polyadenylated RNA molecule of about 20 kb possessing a positive polarity.<sup>2</sup> The viral structural proteins have been well characterized,<sup>3</sup> and recently two genes, encoding the nucleocapsid and matrix proteins, have been sequenced.<sup>4</sup>

Coronaviruses are associated with a wide variety of diseases in both humans and animals.<sup>5</sup> BCV, rotavirus and enterotoxigenic *Escherichia coli* appear to be the major causative agents in neonatal diarrhoea, a disease causing severe economic losses to the cattle industry.<sup>6</sup> A sensitive and specific diagnosis of BCV is imperative for a

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proper recognition of the disease-causing agent in order to undertake justified decisions in treatment. BCV in faecal samples of diseased cattle has been detected by electron microscopy.<sup>7</sup> Other techniques based on the haemagglutinating activity of the virus, such as haemadsorption–elution–haemagglutination assays (HEHA)<sup>8</sup> and reversed passive haemagglutination (RPHA),<sup>9</sup> have been introduced. BCV antibodies have also been applied successfully in the detection of BCV in faecal samples, using ELISA<sup>8</sup> or immunofluorescence staining of cell cultures inoculated with specimen.<sup>10</sup>

The use of cDNA diagnostic probes should be a powerful tool for the detection of BCV genomic RNA as the latter is single-stranded, thus providing a convenient substrate for hybridization purposes. Biotinylated probes are preferred over radioactive probes due to their safety, long-term stability and similar sensitivity.<sup>11</sup> Numerous examples can be found where radioactive DNA probes have been successfully applied in viral diagnosis;<sup>12</sup> however, the use of biotinylated probes<sup>13–15</sup> is often still in an experimental phase.

In this paper, we describe the synthesis of cDNA probes, the selection according to their capacity to hybridize with genomic RNA and the optimization of hybridization conditions and amplification of the hybridization signal, both for biotinylated and radioactive probes. The purpose of the optimization of the virus-detection limit is that further dilution of samples, in order to decrease background hybridization, may increase specificity without sacrificing sensitivity. Several conditions were studied which would be of importance for virus detection in clinical samples.

## MATERIALS AND METHODS

### Virus and cells

The NCDC strain (Mebus) of BCV was obtained from the American Type Culture Collection (ATCC, no. VR874). BCV was grown on HRT-18 cells, originally derived from an adenocarcinoma of the human rectum, using methods described by Laporte *et al.*<sup>16</sup>

### Virus purification

BCV was purified essentially by the method described by Dea *et al.*<sup>17</sup> Briefly, tissue culture supernatants of infected HRT-18 cells were clarified by centrifugation at 5000 *g* for 20 min at 4°C. The sedimented cells were resuspended in a small volume of RPMI medium followed by two cycles of freezing and thawing. The suspension was clarified by centrifugation, and the supernatant was combined with the one previously obtained. BCV was prepurified and concentrated by centrifugation of the supernatants on a 10 ml cushion of 35% sucrose in 0.05 M Tris-HCl, pH 7.6, at 30,000 × rpm (T50) for 3 h at 4°C.

Virus-containing pellets were resuspended and layered on a 20–55% continuous sucrose gradient, in 0.05 M Tris-HCl, pH 7.6, followed by centrifugation at 24,000 rpm (SW 27.1) for 16 h at 4°C. Finally, virus-containing fractions were

collected, diluted 5–8 times in  $1 \times \text{TE}$  (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and subjected to a 1.5 h centrifugation at 35,000 rpm (SW 50) to concentrate the virus. Purified virus was stored at  $-70^\circ\text{C}$ .

### Extraction of genomic RNA

An equal volume of a solution containing  $1 \times \text{TNE}$  (10 mM Tris-HCl, 100 mM NaCl and 1 mM EDTA, pH 8.0), 1% SDS and 0.4 mg proteinase K  $\text{ml}^{-1}$ , was added to the virus suspension. The mixture was incubated at  $50^\circ\text{C}$  for 5 min followed by an incubation at  $25^\circ\text{C}$  for 30 min. The solution was extracted twice with phenol-chloroform and once with chloroform, followed by RNA precipitation at  $-20^\circ\text{C}$  with ethanol, under which the RNA was stored until further use.

### cDNA synthesis, tailing and cloning

BCV genomic RNA was denatured by adding methyl-mercuric-hydroxide to a final concentration of 10 mM. The mixture was incubated for 10 min at room temperature, after which 2-mercaptoethanol (100 mM final concentration) and RNasin (4 U  $\mu\text{l}^{-1}$ ; Promega) were added.<sup>18</sup> The mixture was kept for 15 min at room temperature before adding all the components required for first-strand synthesis, which was essentially according to the method described by Gubler & Hoffman,<sup>19</sup> except for an extra addition of reverse transcriptase (1500 U  $\text{ml}^{-1}$ ; Pharmacia) after the first incubation period, followed by another 30 min incubation at  $43^\circ\text{C}$ .

Two sets of clones were obtained using this protocol; one set in which only oligo(dT) primers were used, thus representing most probably sequences at the 3' end of the genome, whereas for the other set, calf thymus oligonucleotide random primers were used.<sup>20</sup> Second-strand synthesis was also done as described by Gubler & Hoffman<sup>19</sup> in a 100  $\mu\text{l}$  reaction mixture, except that no  $\beta\text{-NAD}^+$  was added since T4-DNA ligase (Pharmacia) was used instead of *E. coli* DNA ligase. RNase H was purchased from Pharmacia. Fully blunt-ended cDNA molecules were generated by incubation with T4-DNA polymerase (Pharmacia; 18). The cDNA quantity was estimated from TCA-precipitate radioactivity data.

Homopolymer tailing of the double-stranded cDNA was essentially done by the method of Roychoudhury & Wu.<sup>21</sup> The dried DNA was resuspended in a small volume of double-distilled water, followed by subsequent addition of stock solutions to a final concentration of 0.13 M K-cacodylate, 0.03 M Tris-HCl, pH 7.6, 0.1 M DTT, 1 mM  $\text{CoCl}_2$  and 50  $\mu\text{M}$  dCTP into a final volume of 100  $\mu\text{l}$ . The mixture was incubated at  $37^\circ\text{C}$  for 5 min before the addition of 0.8 U of terminal deoxynucleotidyl transferase (BRL) per microlitre reaction mixture. A quarter of the reaction volume was mixed with phenol at 2, 3, 4 and 5 min after enzyme addition. The products were twice extracted with phenol-chloroform and precipitated at  $-20^\circ\text{C}$  with ethanol. C-tailed double-stranded cDNA was annealed to a *Pst* I linearized 3' oligo(dG)-tailed pUC-9 vector (Pharmacia) followed by transformation of *E. coli* strain JM101 according to the method described by Hanahan.<sup>22</sup>

## Preparation of cDNA probes

Chimeric plasmids were isolated from cultured bacteria using the alkaline lysis method<sup>18</sup> and purified by centrifugation on CsCl gradients or by chromatography using Sephacryl S-1000 SF (Pharmacia; 23). Insert DNA was excised from the plasmids by digestion with *Pst* I (Pharmacia) and purified by preparative gel electrophoresis on a 0.8% agarose gel and electro-elution.

Probes were labelled with  $\alpha^{32}\text{P}$ -dCTP (3704 Ci mmol<sup>-1</sup>; ICN) by nick translation according to Rigby *et al.*<sup>24</sup> to a specific activity of 10<sup>7</sup> cpm  $\mu\text{g}^{-1}$ .

Biotinylated probes were obtained, using the method described by Leary *et al.*,<sup>11</sup> in a reaction mixture containing: 1  $\mu\text{g}$  DNA, 0.02 mM of dATP, dGTP and TTP, 50 mM Tris-HCl, pH 7.8, 5 mM MgCl<sub>2</sub>, 10 mM 2-mercaptoethanol, 5  $\mu\text{g}$  nuclease-free BSA (Pharmacia), 20  $\mu\text{M}$  biotin-11-dUTP (BRL), 2 U DNA polymerase I and 200 pg DNase I (Sigma). Incubation was done at 15°C for 90 min followed by spun column chromatography using Sephadex G-50 (Pharmacia). The probe was directly used in hybridization assays.

## Hybridization

Colony filter hybridization<sup>25</sup> was performed on about 1000 clones with a radioactive probe, produced by cDNA synthesis in the presence of radioactive precursors, with random primers and purified virus RNA. Strong signal clones, obtained from both oligo(dT) as well as randomly primed cDNA, were further analysed with respect to their specificity and ability to detect virus. Purified virus was used to select and optimize conditions for hybridization to assure specificity. Virus was diluted in 1 $\times$ , 10 $\times$  and 20 $\times$  SSC (20 $\times$  SSC = 175.3 g NaCl and 88.2 g sodium citrate per 1 l H<sub>2</sub>O) and directly spotted onto nitrocellulose (0.20  $\mu\text{m}$ , BA 83; Schleicher & Schuell, Inc.) which was equilibrated in 20 $\times$  SSC. One hundred microlitre samples were applied to the nitrocellulose using the 72-well Minifold II apparatus (Schleicher & Schuell, Inc.). Denaturation and RNA fixation was achieved by baking the filters in vacuum for 2 h at 80°C. Filters were then rehydrated in 6 $\times$  SSC for 5 min and sealed in plastic bags to which the prehybridization solution was added. The prehybridization solution consisted of: X% formamide (X = amount of formamide indicated in different tests), 5 $\times$  Denhardt's solution (0.5 g Ficoll, 0.5 g polyvinyl-pyrrolidone and 0.5 g BSA per 500 ml H<sub>2</sub>O), 5 $\times$  SSPE (744 mM NaCl, 50 mM sodium phosphate and 6.3 mM EDTA, pH 7.4), 0.1% SDS and 100  $\mu\text{g}$  of denatured sheared calf thymus DNA per ml. Prehybridization was done for at least 4 h, or overnight, at 42°C, whereafter the probe was added without a change of solution. Incubation at 42°C was continued for another 24–48 h. Washing of the filters was done four times for 10 min at room temperature with 2 $\times$  SSC + 0.1% SDS and twice with 1 $\times$  SSC + 0.1% SDS for 30 min at 70°C, when a radioactive probe was used. The air-dried blots were exposed to Kodak-X-OMAT RP X-ray films at -70°C for 20–30 h using intensifying screens. When biotinylated probes were used, processing of the blots was done as described by Leary *et al.*<sup>11</sup>

## Amplification of virus detection signals

Hybridizations were performed as mentioned earlier, but with a combination of different probes (100 ng blot<sup>-1</sup> of 24 samples) in an attempt to increase the signal from a single genome copy. In other experiments, 200 ng biotinylated pUC-19 was added per blot of 24 samples, 20 h after incubation with the virus-specific probe followed by a continued incubation for another 12 h to increase network formation on the hybridized probe and thus amplify the signal. Blot processing was done as described earlier.

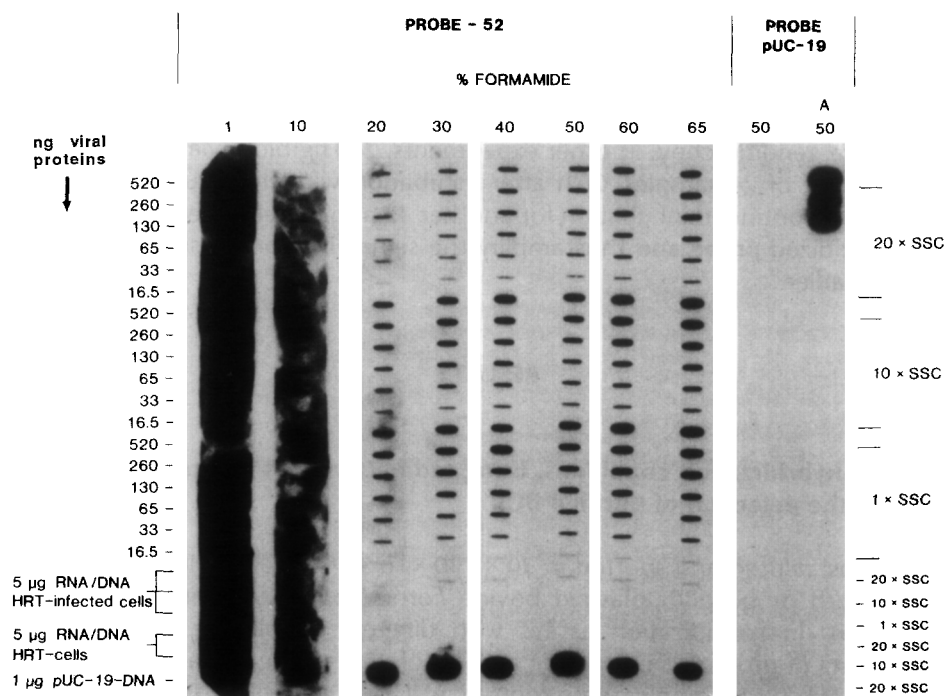
## RESULTS

### Analysis of hybridization conditions, using radioactive and biotinylated pBC-52, in the detection of purified BCV

Purified virus, diluted and spotted in 20 ×, 10 × or 1 × SSC on nitrocellulose, was detected with probe pBC (plasmid bovine coronavirus)-52, (containing a 600 bp cDNA insert) in hybridization assays with different formamide concentrations. About 100 ng of probe with a specific activity of 10<sup>7</sup> cpm µg<sup>-1</sup> was added per blot (48 cm<sup>2</sup>), on which 24 samples were spotted. pUC-19 DNA with a specific activity of 10<sup>7</sup> cpm µg<sup>-1</sup> was added to an identical blot as a control for potential background hybridization. Furthermore, nucleic acid, isolated from non-infected HRT-18 cells, was tested to detect potentially present homologies with the probe, whereas pUC-19 DNA was spotted, as a control for the hybridization itself. Fig. 1 demonstrates that concentrations of 1 or 10% formamide could not be used to determine the signal intensity obtained with the virus since the background was too high, as expected for low stringencies. The probe could detect the virus under all the other conditions indicated in Fig. 1, although higher concentrations of 60–65% formamide appeared to be optimal.

Different salt concentrations, used in diluting and spotting the virus, strongly influenced the amount of viral RNA detected. Virus diluted and spotted in 1 × SSC, as compared to 20 × SSC, resulted in an at least two to three times stronger detection signal with a simultaneous increase in detectability of smaller amounts of virus (Fig. 1). A similar result was observed when biotinylated probe pBC-52 was used, with the difference that the optimal formamide concentration was reduced to about 40% (Fig. 2). These data thus demonstrate that high salt concentrations reduced virus binding to nitrocellulose, when compared to low salt concentrations.

In addition to pBC-52, two other cDNA-clones (pBC-225 and pBC-247, containing insert sizes of 1.1 and 1.6 kbp, respectively), obtained from randomly primed cDNA and the inserts of which did not contain overlapping sequences with the insert of pBC-52, were selected for their capacity to hybridize strongly in colony filter hybridization assays (data not shown). These probes (pBC-225 and pBC-247) were tested in the same hybridization conditions, indicated in Fig. 1, as for pBC-52. The optimal formamide concentration for probes pBC-225 and pBC-247 in radioisotopic virus detection was lower than for pBC-52, i.e. 50% (data not shown), but spotting conditions for optimum detection remained the same. pUC-19 sequences did not



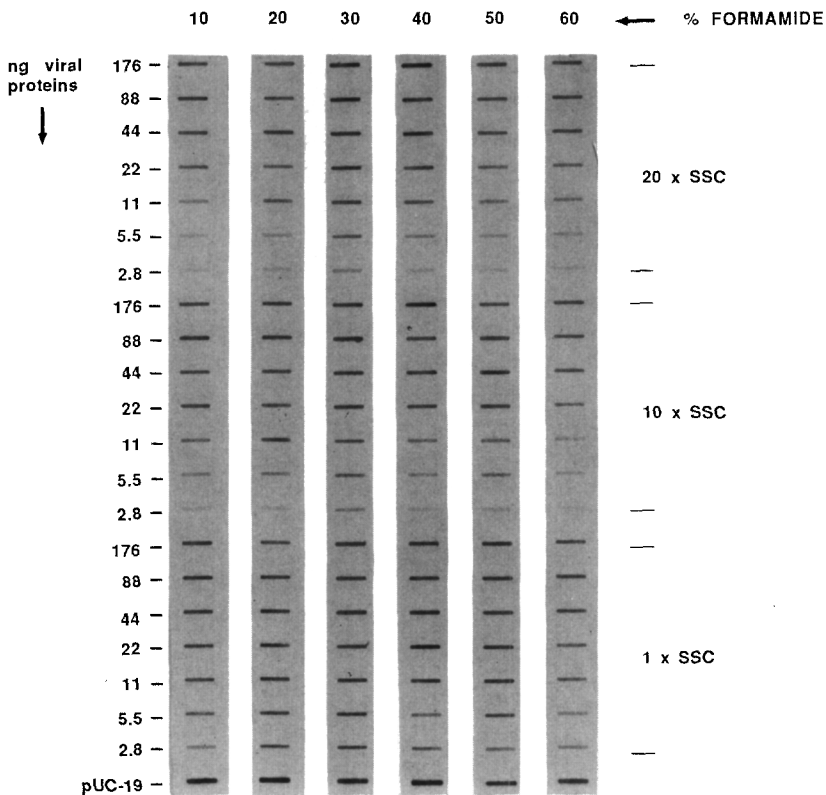
**Fig. 1.** Analysis of the effect of formamide and SSC concentrations on the detection by hybridization of BCV by  $^{32}\text{P}$ -labelled pBC-52. Hybridization was done at  $42^\circ\text{C}$  for 30 h with  $100\text{ ng probe ml}^{-1}$  using  $2\text{ ml}$  per blot ( $48\text{ cm}^2$ ), containing 24 samples. Viral samples spotted in different salt conditions were also incubated in 50% formamide with a nick-translated pUC-19 probe (lane pUC-19, 50) and showed the absence of background hybridization. In lane A50, pUC-19 DNA ( $1\text{ }\mu\text{g}$  each) was spotted in  $20\times$ ,  $10\times$  and  $1\times$  SSC, respectively, from upper to lower detection signal and hybridized in 50% formamide. No other samples are present in this lane. The amount of viral proteins indicated refers to the quantity of protein measured in the original virus sample multiplied by the dilution factor. One microgram pUC-19 DNA was spotted as a control for the hybridization of pBC-52 (= probe 52) itself, for all formamide concentrations tested. Autoradiography was done by exposure of the blots to X-ray film for 20 h at  $-70^\circ\text{C}$  using intensifying screens.

show any homology with BCV-RNA, since no hybridization signal was obtained in different spotting conditions (Fig. 1). Furthermore, probe pBC-52 did not hybridize to nucleic acid isolated from HRT-18 cells, whereas a slight detection signal could be seen with nucleic acid, obtained from infected HRT-18 cells (Fig. 1).

The replicative form (RF) of M13-mp19, containing the insert of pBC-52 in its *Pst* I site, was used in the same combinations of hybridization conditions, as mentioned in Fig. 1, but resulted in a very poor virus detection, although the control hybridization with the RF of M13-mp19 itself was very strong (data not shown).

### Determination of virus detection limit with biotinylated and radioactive probe pBC-52

BCV was diluted and spotted in  $1\times$  SSC, followed by subsequent baking, prehybridization overnight at  $42^\circ\text{C}$  using 60% formamide and hybridization for 30 h



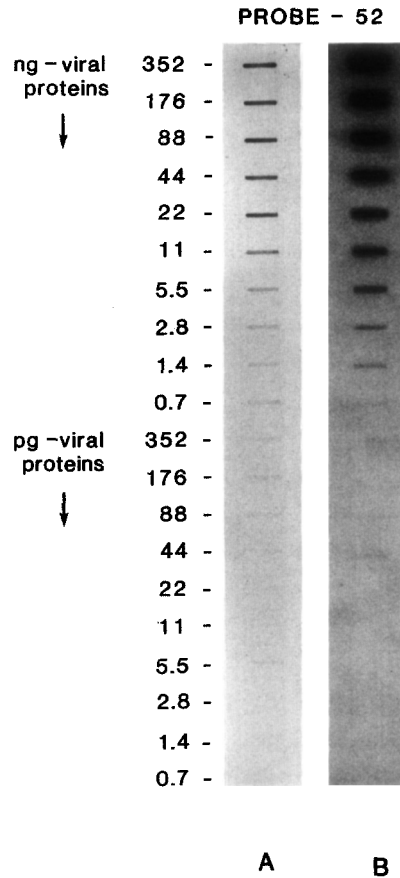
**Fig. 2.** Analysis of the effect of formamide and SSC concentration on the detection by hybridization of BCV with biotinylated pBC-52. Hybridization was done as mentioned for Fig. 1, followed by washing and enzymatic revelation for 1 h before drying of the membranes.

at 42°C after addition of 100 ng of  $\alpha^{32}\text{P}$ -dCTP-labelled pBC-52, with a specific activity of  $10^7$  cpm  $\mu\text{g}^{-1}$ , to the prehybridization solution. The other blot, to which 100 ng of biotinylated probe pBC-52 was added, was further incubated in 40% formamide. The difference in detectability of radioisotopic and biotinylated probes is shown in Figs, <sup>26</sup> 3(a) and (b). The detectability was superior for the biotin system (a minimum amount of virus containing about 150 pg, instead of about 1000 pg, viral protein could be detected; protein quantification according to Bradford), <sup>26</sup> whereas the sensitivity (dR/dC), <sup>27</sup> indicating the rate of change in the response signal by changing the concentration of the radioisotopic assay was superior.

About 1.1 pg of RNA (corresponding to  $10^5$  genomes and about 88 pg viral proteins) could, therefore, be detected without amplification procedures.

### **The influence of proteinase K treatment on purified virus and virus-containing supernatants of infected HRT cells on the detection of BCV using radioactive probe pBC-52**

Proteinase K/SDS treatment on purified virus was done as described in Materials and



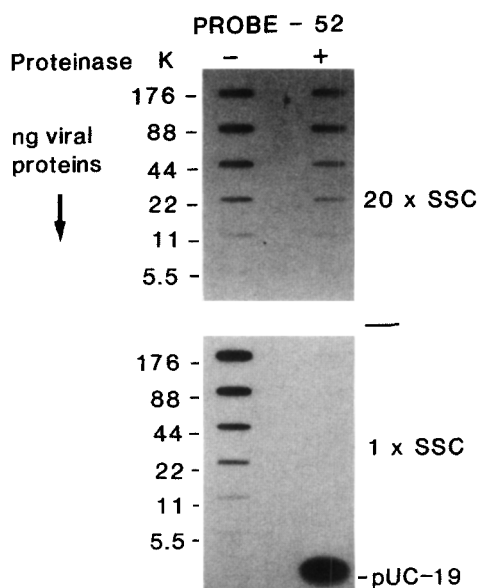
**Fig. 3.** BCV detection limit determination, with biotinylated pBC-52 (A) and radioisotopic-labelled pBC-52 (B). In each case, 100 ng probe  $\text{ml}^{-1}$  was added per blot ( $48 \text{ cm}^2$ ) containing 24 samples. The autoradiograph was exposed for 24 h at  $-70^\circ\text{C}$  using intensifying screens.

Methods for the isolation of genomic RNA. These tests were performed in anticipation to detect virus in faecal samples, for which these treatments may be required to eliminate binding competition onto nitrocellulose between contaminating proteins or nucleic acid and viral RNA. Similar amounts of RNA were detected with and without proteinase K treatment when spotting was done in  $20 \times \text{SSC}$ , whereas RNA could not be detected after proteinase K treatment when  $1 \times \text{SSC}$ , under which the virus detection was optimal, was used (Fig. 4).

Virus could also be detected in the supernatant of infected HRT cells (Fig. 5, lane B), whereas the hybridization signal increased strongly after proteinase K/SDS treatment (Fig. 5, lane C). The poor detection of virus in the supernatant is probably due to competition for binding sites between virus and cellular components onto the nitrocellulose. These results were confirmed with a simulation model in which purified virus was added and diluted in supernatant fluid of non-infected HRT-18 cells (data not shown).

The signal intensity (Fig. 5; spot C-1) obtained after proteinase K treatment of the supernatant, equalled the intensity of spot A-5, in which virus was diluted to 22 ng





**Fig. 4.** Influence of proteinase K treatment on BCV detection by pBC-52. Purified virus and RNA, isolated from purified virus by means of proteinase K, was diluted and spotted in 20 × SSC (upper panel), or in 1 × SSC (lower panel). The amount of viral nucleic acid is expressed indirectly by measuring the amount of viral proteins as in Fig. 1. Hybridization was done for 20 h at 42°C, and the autoradiographs were exposed for 10 h at -70°C using intensifying screens.

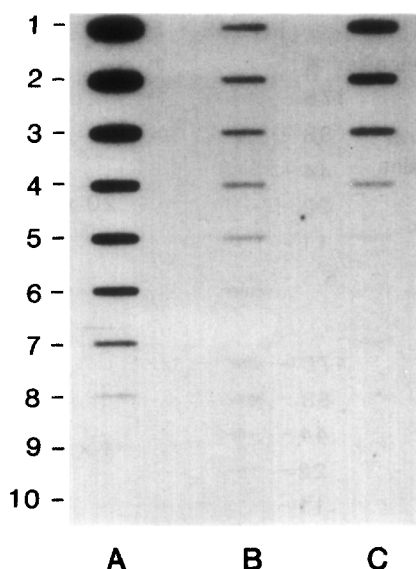
with respect to viral proteins. This amount corresponds to about  $2.5 \times 10^8$  virus particles per millilitre culture fluid.

### Amplification of the detection signal

A biotinylated agarose-purified insert from pBC-52, pBC-52, or a combination of pBC-52 (0.6 kb insert), pBC-225 (1.1 kb insert) and pBC-247 (1.6 kb insert) were hybridized with viral RNA immobilized on nitrocellulose. In addition, 20 h after incubation with the first probe(s), 200 ng of biotinylated pUC-19 was added to two of the blots (Fig. 6, 2nd hybridization), followed by another incubation at 42°C for 12 h before further processing. Fig. 6 demonstrates that this addition of pUC-19 significantly increased sensitivity, and that the detection limit decreased about 30–50 times, corresponding to about 3000 and 1800 genome copies, respectively. However, substituting the biotinylated probes in this system by radioisotopic probes resulted only in a slight amplification of the detection rate (data not shown).

The formation of nucleic acid hyperpolymers, which could explain at least the slight amplification, was suggested by the relative mobilities during electrophoresis of *nick-translated* pUC-19, before and after denaturation, and after denaturation and renaturation (Fig. 8).

No significant signal enhancement was obtained when a combination of three different biotinylated probes was used, two (pBC-225 and pBC-247) of which contain overlapping viral sequences, compared to the signal obtained with pBC-52 alone



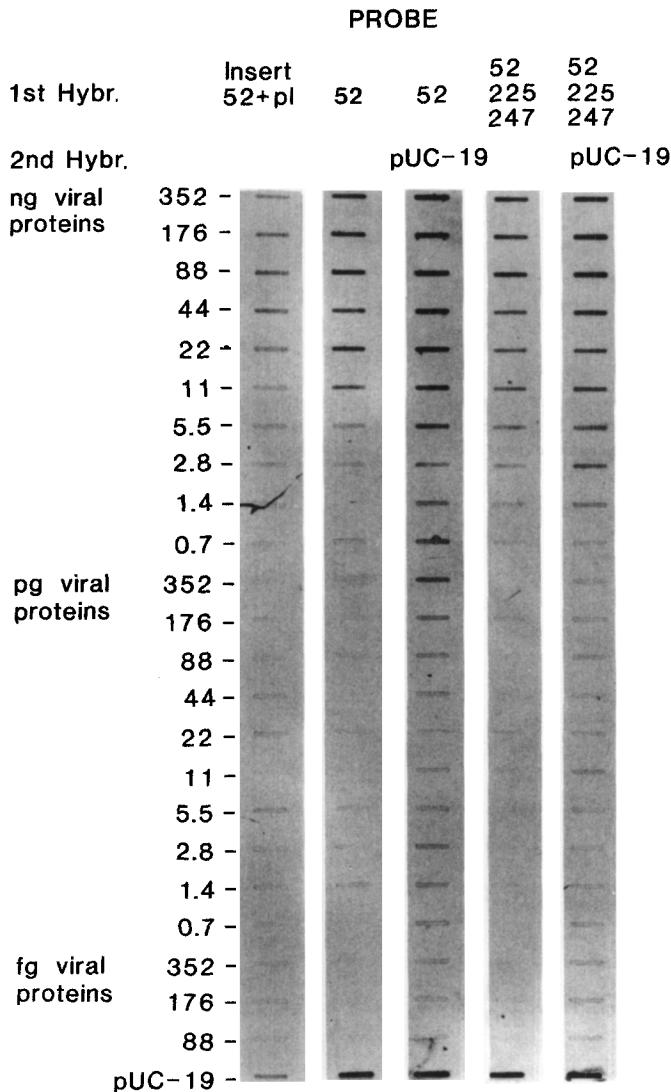
**Fig. 5.** Influence of proteinase K treatment of tissue culture supernatant of infected cells on BCV detection by pBC-52. Lane A:  $1:2^n$  ( $n = 1-10$ ) dilutions of non-treated purified virus (1 corresponds to 352 ng viral proteins). Lane B:  $1:2^n$  ( $n = 1-10$ ) dilutions of 100  $\mu$ l non-treated supernatant of HRT-18 infected cells. The samples were diluted and spotted in  $1 \times$  SSC. Lane C: as lane B, but after subsequent incubation of the supernatant with proteinase K, phenol/chloroform extraction and precipitation with ethanol. The precipitate was diluted and spotted in  $20 \times$  SSC. Hybridization was done for 30 h, and the blots were exposed to X-ray film for 30 h at  $-70^\circ\text{C}$  using intensifying screens.

(Fig. 6). In contrast, a definite signal enhancement was obtained when a combination of two probes (pBC-52 and pBC-247), lacking overlapping viral sequences, was used (Fig. 7). Amplification by an additional biotinylated pUC-19 hybridization was obtained, as mentioned above, despite the presence of overlapping viral sequences in the primary probes (Fig. 6).

## DISCUSSION

The data presented in this paper demonstrate that cDNA probes could be applied to detect very small quantities of BCV-RNA, i.e. approximately 1 pg ( $\approx 10^5$  genomes) without any further detection amplification.

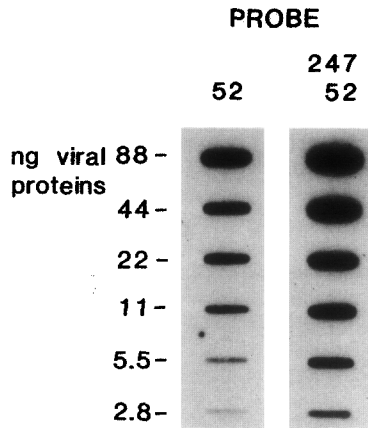
Direct spotting of the virus is preferred since it is simple and eliminates time-consuming preparation of RNase-free glassware and buffers, laboursome RNA extraction procedures, especially when many samples have to be processed, and exposure of the viral genome to potentially present RNases. Virus binding onto nitrocellulose was found to be feasible, but the detection signal was two to three times higher if spotting was done in low salt concentrations instead of the usual  $20 \times$  SSC (Figs 1 & 2). In contrast, RNA extracted from the virus was optimally immobilized on the nitrocellulose filters in high-salt conditions (Fig. 4). This differential effect of salt concentration might be due to their influence on the net charge of both the nitrocellulose membrane and the macromolecules. The negative



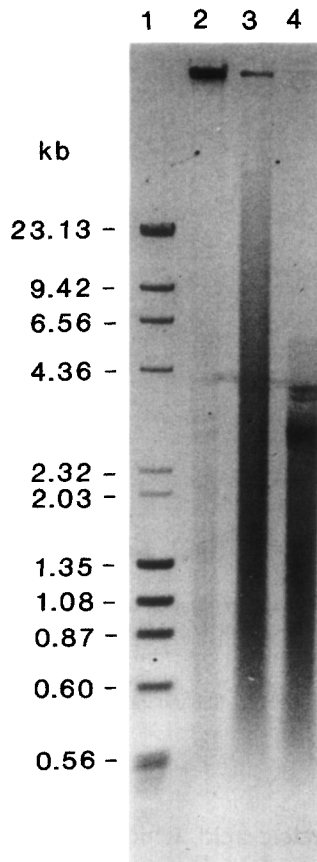
**Fig. 6.** Detection amplification by an additional hybridization to target sequences. Biotinylated probes or a combination of probes were first hybridized to BCV genomic RNA for 20 h at 42°C (1st hybridization), whereafter 200 ng (2 ml)<sup>-1</sup> biotinylated pUC-19 was added to two blots (48 cm<sup>2</sup> blot<sup>-1</sup>) each containing 24 samples. Incubation of the blots was continued for another 12 h (2nd hybridization) before further processing of the membranes. Probes 225 and 247 possess overlapping sequences (not with probe 52); pl = polylinker.

charge of nitrocellulose and nucleic acid, which would result in repulsion, can be shielded by high salt concentrations, whereas for the virus, hydrophobic attachment was optimal in low-salt conditions.

Optimal formamide concentrations for hybridization were found to differ slightly for the various radioisotopic probes. Formamide concentrations of 60–65% for pBC-52 were found to be optimal, most probably due to the preferential formation of



**Fig. 7.** Amplification of the detection signal using two radioisotopic-labelled probes that contain non-overlapping viral sequences. First lane: 50 ng pBC-52 ml<sup>-1</sup>; second lane: (25 ng pBC-52 + 25 ng pBC-247) ml<sup>-1</sup>. Hybridization was done for 30 h at 42°C and exposure to the X-ray film for 24 h at -70°C using intensifying screens.



**Fig. 8.** Visualization of probe-network formation by electrophoresis on a 0.8% agarose gel. Lane 1: lambda DNA-Hind III/ØX-174 DNA-Hae III. Lane 2: 1 µg nick-translated pBC-52 DNA after denaturation for 5 min at 100°C and renaturation by cooling to room temperature. Lane 3: 1 µg nick-translated pBC-52 DNA after denaturation for 5 min at 100°C and immediate cooling on ice. Lane 4: 1 µg nick-translated pBC-52 DNA.

RNA-DNA hybrids over that of DNA-DNA duplexes in these conditions.<sup>28,29</sup> Therefore, DNA-DNA reassociation in solution decreases, whereas the relative concentration of probe available for DNA-RNA hybridization increases. Moreover, background hybridization with immobilized DNA is decreased. This effect can be observed in Fig. 1 for the hybridization signals obtained with the probe and immobilized pUC-19, respectively, in the different concentrations of formamide tested. Not only external hybridization conditions may be of importance, but also intrinsic values such as the complexities and probe length. Reassociation in solution of nick-translated M13 probes was expected to be lower than that of pUC probes due to the relative higher complexities. However, a poor detection of the target sequences was obtained with these probes. This might be due to the relatively lower concentration of the M13 insert sequences, possibly resulting in a deviation from pseudo first-order kinetics and reduced thermal diffusion of single-stranded insert to the target sequences.

Virus could also be detected directly in the supernatant of infected HRT-18 cells, although proteinase K treatment strongly increased the sensitivity due to elimination of macromolecules competing for binding sites on the membrane. The detectability did not change significantly, probably since the amount of competing molecules was also decreased by further dilution (Fig. 5). This signal was specific for BCV, as vector sequences did not hybridize with nucleic acid isolated from non-infected HRT cells (Fig. 1). Proteins and lipids from purified virus did not seem to interfere with RNA fixation when purified virus was spotted, in contrast to the macromolecules of virus-containing supernatant of HRT cells (Figs 4 & 5).

Detection amplification with radioisotopic probes was obtained when multiple probes were used, hybridizing to separate locations on the genome (Fig. 7), but no significant signal amplification was seen when (biotinylated) probes, containing overlapping viral sequences, were used in virus detection (Fig. 6). The presence of overlapping sequences in the insert DNA most probably promoted a quick reassociation in solution. The use of different probes may also help to establish the existence of possible recombinations, such as reported for another coronavirus (murine hepatitis virus).<sup>30,31</sup>

Hybridization with biotinylated pBC-52, followed by a second hybridization with biotinylated pUC-19 resulted in a signal amplification of 30–50 times. This increase in signal was likely due to a network formation. Most probably a definite range of DNase concentrations, determining the number of nicks during nick translation,<sup>32</sup> is important for optimal amplification by network formation,<sup>33</sup> although this was not further investigated. About 1.1 pg RNA can be detected without amplification when using biotinylated probes [Fig. 3(a)], whereas a quantity of 20–35 fg RNA could be detected after signal amplification, which is about 900 times less than described recently for the detection of isolated BCV-RNA.<sup>34</sup> In the latter study, a cDNA probe of about 2160 bases, containing the genes coding for the N and M proteins,<sup>4</sup> was used.

In our experiments with biotinylated probes, signals obtained were not always strictly inversely proportional to dilution [Figs 3(a) & 6], although in controls (similar blots incubated with biotin analogues or biotinylated plasmids) no background staining was observed. Thus, detection limits even lower than mentioned above may be feasible, although difficult to determine.

The use of chimeric plasmids as molecular probes in virus detection using clinical

specimens may lead to misinterpretations due to background hybridization.<sup>35,36</sup> When this occurs, a strong amplification of the virus detection signal, allowing high dilutions, may reduce this problem. Amplification of the detection signals by the methods described here should be applicable to any kind of virus detection by hybridization.

Studies with both biotinylated and radioisotopic probes to establish conditions required for optimal virus and minimal background detection of virus in faecal samples are currently underway.

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