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Radioactive and enzymatic cloned cDNA probes for bovine enteric coronavirus detection by molecular hybridization

J. Collomb[1], **C. Finance**[1], **S. Alabouch**[1] and **J. Laporte**[2]

[1] Laboratoire de Microbiologie Moléculaire, Faculté des Sciences Pharmaceutiques et Biologiques, Université de Nancy I, Nancy
[2] Station de Virologie et d'Immunologie Moléculaires, INRA, Jouy-en-Josas, France

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Summary. Genomic RNA of F15 strain bovine enteric coronavirus (BECV) was cloned in *E. coli*. Three clones (174, 160, PG 78), selected in the cDNA library, including a large portion of the nucleocapsid (N), matrix (M) and peplomeric (S) protein genes, were used as probes for a slot blot hybridization assay. Two probe labelling techniques were compared, radiolabelling with ^{32}P and enzymatic labelling through covalent linkage to peroxidase and chemiluminescence detection. The radioactive probe 174 detected as little as 1 to 3 pg of viral RNA, while the less sensitive enzymatic probe could not reveal more than 100 pg of RNA. No significant detection amplification was achieved when a mixture of the three probes was used. Probe 174 allowed specific identification for BECV. No hybridization was noticed either with rotaviruses or even with other antigenically unrelated members of the family *Coronaviridae* such as transmissible gastroenteritis virus. The test proved valid for detection of BECV in the supernatant of infected HRT-18 cells: genomic RNA could be detected after direct spotting of samples, but prior nucleic acid extraction after proteinase K treatment improved virus detection. BECV diagnosis in faecal samples using enzymatic probe was compared with conventional diagnostic methods.

Introduction

The bovine enteric coronavirus (BECV), a member of the family *Coronaviridae*, is a pleiomorphic enveloped spherical particle (120 nm in diameter) surrounded by a characteristic fringe of "club-shaped" peplomers. The viral genome is a single-stranded polyadenylated RNA of approximately 20 kb in length, coding for a 3'-coterminal "nested set" of mRNAs and 4 major structural proteins: a phosphorylated nucleocapsid protein N (50 k), a transmembrane matrix glycoprotein M (20 k), a peplomeric glycoprotein S (present on the virion as 105 k

and 95 k subunits) and a haemagglutinin HE (125 k). Recently genes encoding for these proteins have been cloned and sequenced [1, 6, 10, 24].

BECV is implicated as one of the aetiological agents of neonatal calf diarrhoea [3, 15]. It is found alone or in association with rotaviruses, enterotoxigenic *Escherichia coli* and *Cryptosporidia* [9]. During the acute stage of infection, virus particles are excreted in large amounts. They grow in the brush border of intestinal epithelial cells causing villous atrophy, diarrhoea, dehydratation and often death. Recent reports also suggest they possess a tropism for respiratory epithelial cells [19, 21].

A major problem regarding coronavirus infections is the lack of suitable diagnostic techniques for a specific and rapid identification of the virus in biological samples. Among the methods used for routine diagnosis, electron microscopy (EM) of faecal samples [4] is time-consuming. It has also often proved difficult to differenciate pleiomorphic coronavirus particles from the fringed bodies normally found in faeces. Immunofluorescent staining of intestinal sections can only be used for post mortem diagnosis [14]. Alternative methods using the haemagglutinating activity of BECV, such as haemadsorption-elution-haemagglutination assay (HEHA) or haemagglutination inhibition assay (HAI), lack specificity and sensitivity [22, 23, 27]. Enzyme-linked-immunosorbent assays (ELISA) have been developed for the detection of BECV in faeces. The first of them employed polyclonal sera with low specificity [20]. Better results were obtained with a mixture of monoclonal antibodies coated on microplates for antigen capture [5, 7], but very little information on this has been made available yet. Recently, radioactive cDNA probes have been reported to be sensitive and specific for the detection of the Mebus strain of BECV by molecular hybridization [25, 29, 30].

This paper describes investigations for the development of a highly specific and sensitive slot blot hybridization assay to detect the F15 strain of BECV by means of radioactive and enzymatic cDNA probes. The enzymatic probe, labelled through covalent linkage to peroxidase using the "enhanced chemiluminescence (ECL) gene detection system", was chosen because of its long-term stability and also of its sensitivity reputed to be practically as great as that of a radioactive probe. The ultimate aim was to use the enzymatic probe for routine epidemiological investigation. An attempt has been made to determine the optimal conditions for virus detection in biological samples.

Materials and methods

Viruses and cells

The F15 strain of BECV, propagated in the human rectal tumor (HRT-18) cell line [17], and the Purdue-115 strain of TGEV, propagated in swine testicule (ST) cell line [2], were obtained from Dr. J. Laporte (INRA, Jouy en Josas, France). The simian-SA11 strain of rotavirus was grown in MA-104 cells.

Extraction of BECV genomic RNA

The methods used for infection of HRT-18 cells, titration, and BECV purification by differential and isopycnic ultracentrifugation on sucrose gradients, were identical to those previously described [16, 17, 28]. The virion RNA was extracted using the proteinase K-sodium dodecyl sulfate (SDS)-phenol method. An equal volume of a solution containing 2 × TNE buffer (20 mM Tris-HCl, 200 mM NaCl and 2 mM EDTA, pH 8.0) and 400 µg/ml proteinase K was added to the purified virus suspension. The mixture was incubated for 30 min at 37 °C and an equal volume of the same buffer containing 2% SDS was added. The incubation was continued for 5 min at 50 °C then for 30 min at 25 °C. The suspension was extracted with phenol/chloroform, followed by RNA precipitation at −20 °C with cold absolute ethanol. The dried pellet was dissolved in diethylpyrocarbonate-treated water and handled in autoclaved siliconised containers. The amount of viral RNA was quantitated spectrophotometrically, assuming that an OD of 1 at 260 nm corresponds to 40 µg/ml of single-stranded RNA.

cDNA synthesis and cloning

The construction of the cDNA library of BECV genomic RNA, the characterization and sequence analysis of the clones obtained, have been described previously [1, 24]. Briefly, the virus genome was used as a template for first-strand cDNA synthesis using as a primer either oligo dT or the nucleotide sequence corresponding to the *Bam* HI cleavage site (GGATCC). RNase T2-treated cDNA-RNA heteroduplexes were cloned into the *Pst* I site of the pBR322 plasmid by homopolymeric tailing and used to transform competent RRI *Escherichia coli*. The tetracycline-resistant, ampicillin-susceptible colonies were then selected. Recombinant plasmids were isolated from cultured bacteria by alkaline lysis [13]. DNA insert was excised from the purified plasmid using restriction endonuclease *Pst* I, then purified by electrophoresis on a 1% agarose gel and electroelution. Viral DNA inserts 174 (2 kb), 160 (2.1 kb), PG 78 (2.3 kb), were selected to prepare probes for this study (Fig. 1). Inserts PG 78 and 160 yielded two fragments of respectively 1.4 kb–0.9 kb and 1.8 kb–0.3 kb because of an internal *Pst* I cutting site. This is why most experiments were made using insert 174.

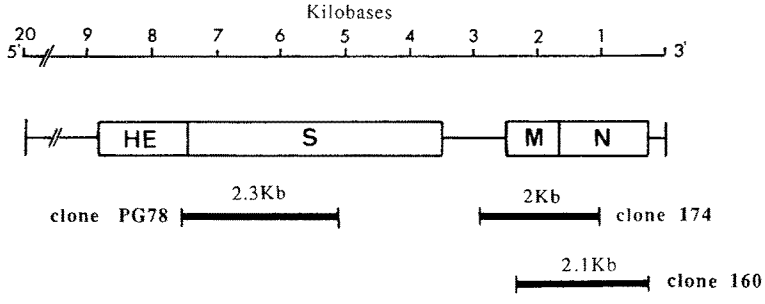

Fig. 1. A schematic diagram of the BECV genome and location of cDNA probes. The 20 kilobases coronavirus genome with a polyadenylated 3′ end at the right is represented by the top line. Regions representing the genes coding for nucleoprotein (*N*), matrix glycoprotein (*M*), peplomeric glycoprotein (*S*) and haemagglutinin (*HE*) are shown by boxes. Regions represented by clones PG 78, 174, 160 are shown by heavy lines. The numbers above each line represent the length of each probe in nucleotide base pairs

Preparation of BECV cDNA probes

Purified inserts were labelled by random priming according to Feinberg and Vogelstein [8] using [α-^{32}P]dCTP and a commercial "Multiprime DNA labelling systems" (Amersham). The specific activities ranged from 2 to 5 × 10^8 dpm/µg.

Enzymatic probes were obtained by covalent linkage of horseradish peroxidase to single-stranded DNA by means of glutaraldehyde, using the labelling procedure developed by Renz and Kurz [18] and the "ECL gene detection system" (Amersham) according to the manufacturer's instructions.

Preparation of samples for slot blot hybridization

BEVC genomic RNA

Varying amounts of purified genomic RNA made in siliconised containers, were denatured at 95 °C for 5 min in 20 µl of TE buffer (10 mM pH 7.4 Tris-HCl, 1 mM EDTA), mixed with 100 µl of 20 × SSC (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0) and applied directly to nitrocellulose filters.

Cell culture propagated virus

Cell cultures were inoculated with the appropriate virus at a multiplicity of approximately 1 PFU per cell. Cell culture fluids were harvested at 72 h post infection and clarified by centrifugation at 3,000 **g** for 10 min. Supernatant dilutions were made in 25 µl of TE buffer, then three different methods were applied: (*i*) each sample was denatured at 95 °C for 5 min, mixed with 100 µl of 1 × SSC and applied directly to nitrocellulose filters. (*ii*) Viruses were lysed by the addition of an equal volume of 1% Nonidet P-40 (NP-40) in TE buffer as described by Shockley et al. [25]. Samples were incubated for 10 min at 0 °C and mixed with an equal volume of a freshly prepared solution of two parts of 37% (wt/wt) formaldehyde and three parts of 20 × SSC, denatured at 60 °C for 15 min and applied to nitrocellulose filters. (*iii*) Nucleic acids were extracted using the proteinase K-SDS-phenol method as described above for extraction of genomic RNA. After extraction and precipitation with ethanol, nucleic acids were dissolved in 20 µl of TE buffer, denatured at 95 °C for 5 min, mixed with 100 µl of 20 × SSC and applied to nitrocellulose filters.

Faecal samples

From diarrhoeal neonatal calves were kindly provided by the Regional Veterinary Laboratory (Nancy, France). Eleven of samples were examinated for coronavirus haemagglutination inhibition [22] and eight by using both ELISA (commercial TETRA-KIT Diagnostic Laboratory LTP) and the standard electron microscopy.

To prepare samples for slot blot hybridization, 750 µl of PBS were added to 500 µl of faecal material, then vortexed and clarified by Microfuge centrifugation for 1 min. Supernatants were treated as described above for cell culture-grown virus using the proteinase K-SDS-phenol method.

Slot blot hybridization assay

Samples prepared as described above were spotted by means of a 24-well slot blot vacuum manifold (slot blot PR 600-Hoefer scientific instruments) onto nitrocellulose filters (BA 85; Schleicher and Schuell) equilibrated in 20 × SSC and then baked at 80 °C for 2 h.

When a radioactive probe was used, the nitrocellulose filters were prehybridized for 2 hours at 42 °C in plastic bags containing a solution (4 ml/100 cm^2) of 50% formamide-5 × Denhardt's solution (1 × is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum

albumine)-0.1% SDS-5 × SSC and 200 μg/ml of sonicated calf thymus DNA. Hybridization was then performed under the same conditions with fresh hybridization buffer containing denatured cDNA probe (0.5 to 1 × 10^6 cpm/cm^2) for 16 h at 42 °C. The nitrocellulose filters were washed twice in 2 × SSC, 0.1% SDS for 15 min at room temperature then twice in 0.1 × SSC, 0.1% SDS for 15 min at 52 °C. The air dried blots were autoradiographed on X-ray films (X Omat AR-5 Kodak) at −80 °C for 24–48 h using intensifying screens.

With an enzymatic probe, the hybridization assay was performed using the "ECL gene detection system" (Amersham) according to the manufacturer's instructions. Prehybridization was done for 10 min at 42 °C in sealed plastic bags containing a commercial hybridization buffer (0.25 ml/cm^2) which included 6 M urea and 0.5 M NaCl. The enzymatic probe was then added at a concentration of 20 ng/ml without a change of buffer and the hybridization was continued overnight at 42 °C. After washing the filters twice in 6 M urea, 0.5 × SSC, 4 g/l SDS for 20 min at 42 °C then twice in 2 × SSC for 5 min at room temperature, the detection signal was elicited by performing an enhanced chemiluminescent (ECL) reaction by means of peroxidase-catalyzed oxydation of luminol where the probe was bound. The filters were immersed for 1 min in a detection reagent (0.125 ml/cm^2) and then exposed to hyperfilm-ECL (Amersham) for between 1 and 5 min.

Results

Sensitivity of the cDNA probes in slot blot hybridization

To evaluate the sensitivity of the slot blot hybridization assay, varying amounts of genomic RNA extracted from purified BECV and ranging from 100 ng to 0.1 pg were first denatured, then applied to nitrocellulose filters in high salt concentration and finally hybridized. Two probe labelling techniques were compared: radiolabelling with ^{32}P and enzymatic labelling through covalent linkage to peroxidase and detection by enhanced chemiluminescence ("ECL gene detection system"). Insert 174 was used alone as probe or in association with inserts 160 and PG 78 in order to amplify the detection signal by hybridization on different locations of the viral genome. Inserts 174 and 160, selected in the cDNA library, correspond to a large portion of the matrix (M) and nucleocapsid (N) protein genes and include overlapping sequences covering about half their length. Insert PG 78 is the 5′ terminus of the peplomeric glycoprotein (S) gene and does not include any overlapping sequence with inserts 174 and 160 (Fig. 1).

The results, shown in Fig. 2, indicate that a positive hybridization signal can be obtained with either radioactive (Fig. 2 I) or enzymatic (Fig. 2 II) probes although intensity of detection is superior with radioactive probe.

The radioactive probe 174 (Fig. 2 I, column A) detects viral RNA at a concentration of 3 pg after 24 h autoradiography and can even reach 1.5 pg when exposure time is longer (48 to 72 h or more). This is the equivalent of 1 to 2 × 10^5 viral genomes, assuming that the BECV genomic size is 6.9 × 10^6 [12]. Preliminary assays conducted by applying genomic RNA samples to nitrocellulose filters by manual dot blot had previously indicated that the radioactive probe detects viral RNA at a concentration of 11 ng (data not shown). It appears therefore that if a slot blotting apparatus is employed, whereby samples can be concentrated on a small slot membrane area, the sensitivity increases significantly and the detection limit is lowered about 5 × 10^3 times.

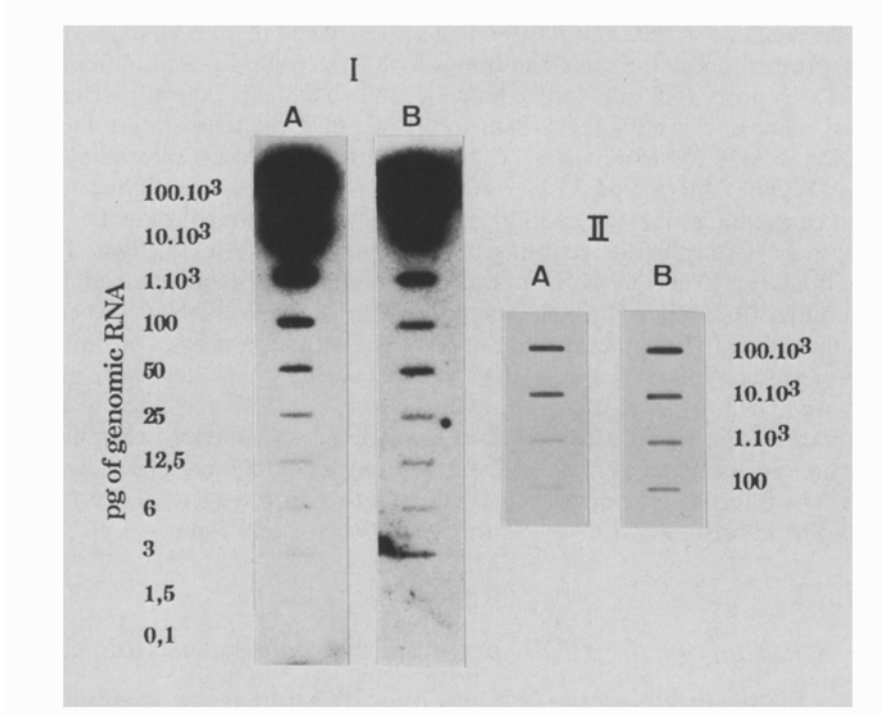

Fig. 2. Sensitivity of cDNA probes for BECV genomic RNA. Different dilutions of purified genomic RNA were applied to nitrocellulose in 100 µl of 20 × SSC. *A* cDNA probe 174 alone. *B* Mixture in equal quantities of three cDNA probes 174, 160 and PG 78. **I** Hybridization was performed at 42 °C for 16 h with ^{32}P labelled probe (5×10^7 cpm/nitrocellulose filter containing 24 wells) and autoradiography was done at −80 °C for 24 h with intensifying screen. **II** Hybridization was performed at 42 °C for 16 h with enzymatic probe (200 ng/10 ml hybridization buffer/nitrocellulose filter containing 24 wells). Time exposure on hyperfilm-ECL was 2 min

With the enzymatic probe 174 (Fig. 2 II, column A), less sensitive than the radioactive one, detection is limited at 100 pg of genomic RNA, i.e., 10^9 genomes. The result is obtained on hyperfilm-ECL after a very short exposure time (1 to 2 min). If exposure time is increased to 5 to 10 min (or more), the sensitivity of the hybridization assay is not improved and some background noise appears which may confuse results (data not shown).

The amount of detectable viral RNA is not greater when a combination of three probes (174-160-PG 78) is added, whether radioactive or enzymatic labelling is used (Fig. 2, columns B). The intensity of detection is slightly enhanced when an enzymatic probe-pool is used, particularly for blots having the lowest genomic RNA concentration. But signals are not significantly enhanced with a radioactive probe-pool.

Detection of BECV RNA in supernatant of infected HRT-18 cells and analysis of cDNA probe 174 specificity

BECV-infected HRT-18 cells were used to develop a detection procedure for the presence of BECV in faecal samples. For this purpose the influence of

sample treatment on the detection of BECV-RNA by slot blot hybridization was studied: various dilutions of clarified supernatant of BECV-infected HRT-18 cells with a titre of 2.1×10^8 PFU/ml were treated in three different ways. Some were denatured then directly spotted to the nitrocellulose filters. Others were spotted after virus lysis through NP-40 treatment. Some again were spotted after nucleic acid extraction using the proteinase K-SDS-phenol method as described in Materials and methods. Hybridization assays were then performed with either radioactive or enzymatic probe 174.

At the same time, the specificity of the cDNA probe was analysed. In order to detect potential homologies with the probe, slot blot hybridization was performed in the same conditions as above, with noninfected HRT-18 cells and two other types of virus propagated in cell culture and associated with gastroenteritis disease: rotavirus and another member of the family *Coronaviridae*, porcine transmissible gastroenteritis coronavirus (TGEV).

The results are shown in Figs. 3 and 4. BECV can be detected directly without preliminary treatment of the samples in the supernatant of infected HRT-18 cells. In this case the samples were spotted to nitrocellulose filters in low salt concentration according to the observations published by Verbeek and Tijssen [30]. These authors have demonstrated that the hydrophobic attachment of the viral envelope was optimal in low salt conditions whereas nucleic acids are usually spotted in 20 × SSC. In such conditions, with a radioactive probe 174, a hybridization signal is obtained up to a 10^{-2} dilution, equivalent to 2.1×10^4 PFU (Fig. 3 I, column A).

With NP-40 treated samples, only a poor BECV detection is obtained (Fig. 3 II, column A) which is different from what was found by Shockley et al. [25]. Such discrepancy may by due to filtration problems in spotting particularly viscous samples to nitrocellulose.

On the contrary, the proteinase K-SDS-phenol method increases detection sensitivity tenfold relatively to direct sample spotting. A hybridization signal is obtained up to a 10^{-3} dilution, equivalent to 2.1×10^3 PFU (Fig. 3 III, column A). Such a signal indicates that there are at least 3 pg of viral RNA on the blot equivalent to 2.6×10^5 viral particles. The titre of the viral suspension, calculated from these data, is therefore 2.6×10^{10} PFU/ml. But the titre obtained by plaque assay titration is 2.1×10^8 PFU/ml, which is the same as the titre usually obtained for BECV growth on HRT-18 cells. This interesting result shows that, in a three-day-old viral suspension, there would be 100 non-infectious particles to one titration-detected particle. This observation agrees with Shockley et al. [25] and Laporte (data not shown).

Identical results are given by slot blot hybridization with the enzymatic probe 174, with a slight difference, though: the sensitivity is 10 times lower than that of the radioactive probe, whatever the mode of sample treatment (Fig. 4, columns A).

The radioactive probe 174 binds specifically to BECV-RNA. No hybridization is observed with either non-infected HRT-18 cells or rotaviruses or even

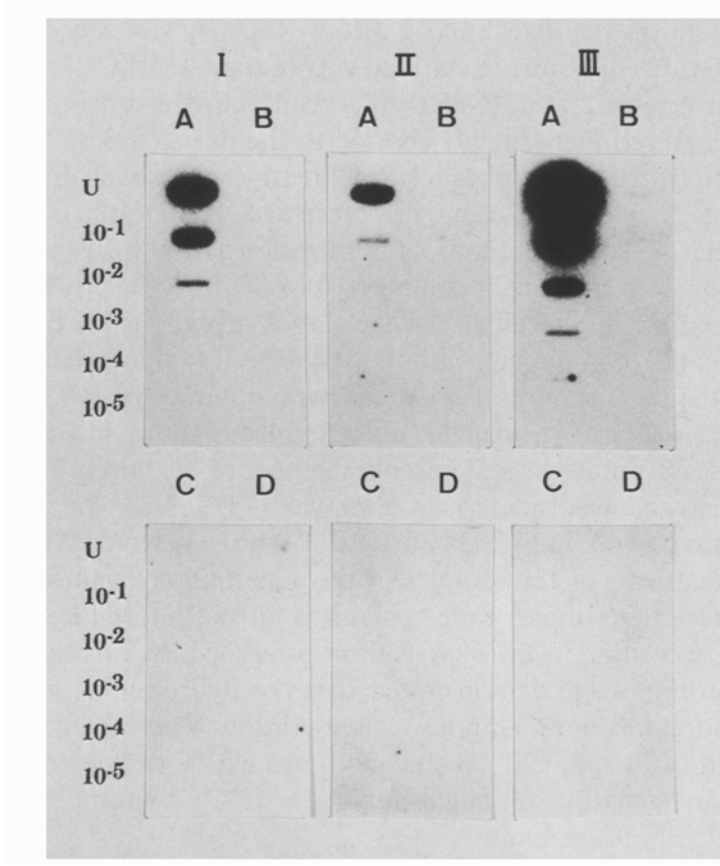

Fig. 3. Influence of various treatments on detection of viral RNA in cell culture fluids by use of radioactive probe 174. **I** Denatured samples directly spotted in 1 × SSC to nitrocellulose. **II** NP-40 treated samples. **III** Extraction of nucleic acids using proteinase K-SDS-phenol method and spotting in 20 × SSC to nitrocellulose. *A* Dilutions of BECV-infected HRT-18 cells (undiluted sample was 2.1×10^6 PFU). *B* Dilutions of non-infected HRT-18 cells (undiluted as for *A*). *C* Dilutions of rotavirus-infected MA 104 cells (undiluted sample was 2.5×10^6 PFU). *D* Dilutions of TGEV-infected ST cells (undiluted sample was 5×10^5 PFU). Hybridization was performed at 42 °C for 16 h with ^{32}P labelled probe 174. Autoradiography was done at −80 °C for 24 h with intensifying screen

TGEV, whatever the treatment or the dilution (Fig. 3, columns B, C, D). If these three types of samples are hybridized with the enzymatic probe 174 when they are directly spotted or after NP-40 treatment to nitrocellulose filters, then false positives appear (Fig. 4 I and II). This is probably due to the great affinity of peroxidase for macromolecules contained in samples. Such an artifact, which may confuse results, is indeed avoided by nucleic acid extraction using the proteinase K-SDS-phenol method (Fig. 4 III).

Detection of BECV RNA in faecal samples and comparison of slot blot hybridization with other diagnostic methods

To evaluate the usefulness of gene-based detection of coronavirus infections with other diagnostic methods, faecal samples from diarrhoeal neonatal calves

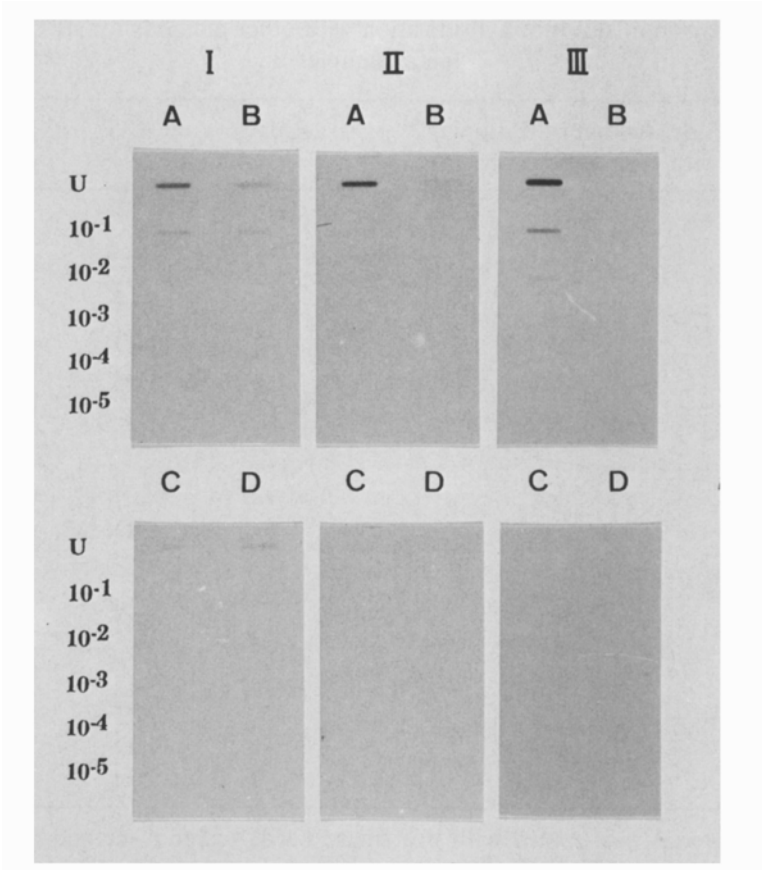

Fig. 4. Influence of various treatments on detection of viral RNA in cell culture fluids by use of enzymatic probe 174. **I** Denatured samples directly spotted in 1 × SSC to nitrocellulose. **II** NP-40 treated samples. **III** Extraction of nucleic acids using proteinase K-SDS-phenol method and spotting in 20 × SSC to nitrocellulose. *A* Dilution of BECV-infected HRT-18 cells (undiluted sample was 2.1×10^6 PFU). *B* Dilutions of non-infected HRT-18 cells (undiluted sample as for *A*). *C* Dilutions of rotavirus-infected MA 104 cells (undiluted sample was 2.5×10^6 PFU). *D* Dilutions of TGEV-infected ST cells (undiluted sample was 5×10^5 PFU). Hybridization was performed at 42 °C for 16 h with enzymatic probe 174. Time exposure on Hyperfilm-ECL was 2 min

were analyzed by slot blot hybridization using enzymatic probe 174 as described in Materials and methods.

The results are shown in Table 1 and compared with those obtained by conventional methods: haemagglutination inhibition (HAI), ELISA and electron microscopy (EM). On examination, the following points seem worth mentioning. (*i*) The sensitivity of the hybridization test using enzymatic probe and chemiluminescence detection is sufficient to allow BECV diagnosis in diarrhoeal neonatal calves. (*ii*) The proteinase K-SDS-phenol method is well adapted to the treatment of faecal samples, the interpretation of results is not hindered by any background signal. (*iii*) Comparison between the slot blot hybridization test and other diagnostic methods reveals that the best correlation is found

Table 1. Comparison of slot blot hybridization with other methods for BECV detection in faecal samples

Sample no.	Slot blot hybridization[a] with enzymatic probe 174	EM[b]	HAI[b]	ELISA[b]
1	+	ND	+	ND
2	+ +	ND	+	ND
3	+	ND	+	ND
4	−	+	ND	−
5	−	+	ND	−
6	−	+	ND	+
7	+ + +	ND	+	ND
8	+	ND	+	ND
9	+ + +	ND	+	ND
10	+ +	ND	+	ND
11	+ +	+	ND	−
12	+	ND	+	ND
13	+	ND	+	ND
14	+ + +	+	ND	−
15	−	ND	+	ND
16	−	−	ND	−
17	+ +	+	ND	+
18	+ +	+	ND	+
19	+	+	ND	−

[a] Faecal material was treated with proteinase K-SDS-phenol method as described in Materials and methods. Hybridization was performed at 42 °C for 16 h with enzymatic probe 174. Time exposure on Hyperfilm-ECL was 2 min. Estimated viral RNA concentrations: − no hybridization signal; < 100 pg homologous RNA per slot; + 100 pg to 10 ng; + + 10 ng to 100 ng; + + + > 100 ng

[b] Faecal material was examinated by electron microscopy (EM), haemagglutination inhibition (HAI) and ELISA: + coronaviruses detected; − no coronaviruses detected; *ND* not done

with the HAI, the two tests disagreed in only one case. The lower correlation observed with EM may be due to difficulty to differentiate true coronaviruses from the coronavirus-like particles frequently observed in faeces. Alternatively, as molecular hybridization was performed several days after EM, it is conceivable that the viral RNA should have been degraded by nuclease in faecal specimens and therefore left undetected by the probe. Finally, molecular hybridization proves better than ELISA in yielding more positive results.

Discussion

The research presented in this paper shows that it is possible to detect BECV by means of molecular hybridization using cDNA probes prepared from viral genomic RNA. The points which were considered were the sensitivity and

specificity of the hybridization test, and the conditions of its potential application for BECV detection in biological samples.

The cDNA probe 174 labelled with ^{32}P allows for the detection of quantities of homologous RNA as small as 1 to 3 pg, equivalent to 2×10^5 genomes. The ECL gene detection system, including DNA labelling with peroxidase combined with enhanced chemiluminescence, provides a level of sensitivity which is 50 times lower than radioactive labelling. However, the enzymatic procedure has the advantage of being easy and quick to use. The labelling takes 10 min and the results are obtained on hyperfilm-ECL in 2 min, whereas autoradiography takes between 24 and 48 h.

No significant signal amplification is obtained when a combination of three radioactive or enzymatic probes (174, 160, PG 78) is used. A possible explanation is the presence of overlapping sequences in inserts 174 and 160, which may foster the reassociation of DNA fragments in solution at the expense of hybridization to the nitrocellulose filter. Such reassociation probably occurs more easily with the radioactive probe, as labelling does not change the DNA configuration, than it does with the DNA probe covalently linked to peroxidase. This may account for the very small signal amplification obtained with the enzymatic probe.

The specificity of slot blot hybridization for the detection of BECV in biological samples has been clearly shown. No detectable binding was observed with the heterologous nucleic acid of rotavirus and non-infected HRT-18 cells. It is expected that the probe 174 which specifically identifies the F15 strain of BECV will also identify the Mebus strain, as in both BECV strains the nucleotide sequences of genes encoding for M and N proteins display almost 100% homology with only minor changes [6, 24]. The probe 174 does not allow the identification of all the members of the family *Coronaviridae*, as no detectable binding is observed with TGEV. This can easily be explained by the small percentage of homology (under 50%) existing between these two viruses [11, 25]. For the same reasons, similar results could probably be obtained for RNA detection of avian coronavirus IBV with which homology is also small [26]. This observation is to be linked to the fact that these coronaviruses belong to three separate antigenic subgroups and it opens the way for a possible use of the hybridization test to identify coronavirus subgroups.

BECV can be detected in supernatant of infected HRT-18 cells directly spotted onto the nitrocellulose filter. The procedure is simple and rapid to perform. With it, it is no longer necessary to prepare RNase-free glassware and buffers, whereas this is compulsory for the particularly delicate extraction of viral RNA. It is true, however, that the extraction of nucleic acids by using the proteinase K-SDS-phenol method greatly increases sensitivity. Some of these nucleic acids get lost in the process, but viral RNA binding is improved when proteins and macromolecules competing with viral RNA on the binding sites of nitrocellulose are eliminated. Moreover this process does not produce any false positives when the enzymatic probe is employed. Although it is more time-

consuming, the proteinase K-SDS-phenol method has proved preferable to direct spotting for BECV detection in faecal samples. The latter are loaded with various debris which may stop the wells of the slot blotting apparatus and prevent viral RNA binding.

In summary, the slot blot hybridization assay analysed in this study is sensitive and specific with both radioactive and enzymatic probes. The test is relatively rapid and can be conducted in less than 48 h, particularly if enzymatic probe is employed. Many samples can be examined in a short time. This method therefore will be useful as an epidemiological tool. Preliminary experiments on faecal specimens from diarrhoeal neonatal calves have proved that the molecular hybridization test with enzymatic probe is valid for general BECV diagnostic use. The sensitivity of the test appears to be at least as high as with the ELISA, HAI and EM diagnostic methods.

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Authors' address: Dr. J. Collomb, Laboratoire de Microbiologie Moléculaire, Faculté des Sciences Pharmaceutiques et Biologiques, Université de Nancy I, BP 403, F-54001 Nancy Cedex, France.

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