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Genomic relationship between turkey and bovine enteric coronaviruses identified by hybridization with BCV or TCV specific cDNA probes

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Summary. Genomic relationships between turkey and bovine coronavirus (TCV and BCV), which are currently placed in distinct antigenic groups, were demonstrated by hybridization using specific cDNA probes. BCV-specific recombinant plasmid probes p 52, p 27, and p 247, holding inserts derived from (probably nonstructural) genes, and plasmids pN 17 and pN 9 holding the N and M gene, respectively, permitted the detection of isolates of both BCV and TCV with similar sensitivities. Similarly, probing supernatants of cell cultures infected with several isolates of TCV, using probes pN 17 and pM 78, respectively holding the N gene of BCV and TCV, resulted in equally intense detection signals. Only a slight detection of MHV-3, which is antigenically related to BCV, was observed, whereas the probes did not allow the detection of IBV, TGEV, and HCV-229E, which are placed in antigenic groups separate from those of BCV and TCV. Detection of TCV was improved by hybridization with BCV-specific single-stranded (ss) probes holding sequences of the N and M genes and synthesized by the polymerase chain reaction. Diagnosis of TCV in 134 clinical samples by hybridization was better with PCR-produced ss BCV-specific probes than with ds PCR-produced probes or a combination of six recombinant plasmid probes holding non-overlapping BCV-specific cDNA sequences. Detection signals were absent when probing clinical samples with ^{32}P-labelled pUC-DNA.

Introduction

Coronaviral enteritis (Bluecomb disease) causes significant economic losses to the turkey industry in the United States and Canada [6, 24, 28]. Turkey coronavirus (TCV) infects and destroys the major absorbtive cells of the small and large intestines, leading to severe diarrhea in young poults but mild symptoms in adults [1, 22, 24].

Initially, the disease was diagnosed by oral infection of susceptible poults with filtered intestinal contents. Subsequently, detection of the virus was attempted by direct electron microscopy of clarified fecal samples [17] or detection of viral antigens by immunofluorescence in the cytoplasm of mucosal cells in frozen intestinal sections [19, 21, 23]. These techniques, however, are time-consuming when large numbers of samples have to be screened and lack sufficient sensitivity. Recently, an indirect ELISA has been employed for detection of TCV in clinical samples [7]. Rabbit hyperimmune serum produced against purified egg-adapted and tissue culture-adapted Minnesota strain of TCV cross-reacted strongly with bovine coronavirus (BCV) and murine hepatitis virus type 3 (MHV-3) although BCV and TCV are classified in different antigenic groups [20]. Subsequently, the existence of a strong antigenic relationship between BCV and TCV was confirmed by monoclonal antibodies produced against either virus [9].

In the current study, we investigated the genomic relationship between BCV and TCV by hybridization. Several recombinant plasmids, containing BCV-specific cDNA sequences [33, 34], were radioactively labelled by nick translation and used alone or in combination for the detection of TCV. Polymerase chain reaction (PCR)-produced probes which were highly efficient in detection of BCV [35], were also used to detect purified TCV or virus in supernatants of infected cell cultures. The use of one recombinant plasmid, either BCV or TCV specific, was usually unsufficient for the detection of TCV in clinical samples from diarrheic poults. Therefore, a combination of BCV-specific probes or inherently more sensitive PCR-produced probes were used to detect virus in clinical samples to confirm the positive detection signals obtained with tissue culture-propagated virus. These results emphasized a genomic relatedness between the two viruses and ruled out the possibility of the activation in vitro of a latent BCV homologous coronavirus.

Material and methods

Reference viruses and isolated

The prototype egg-adapted Minnesota strain of TCV [26], kindly supplied by Dr. B. S. Pomeroy (College of Veterinary Medicine, St. Paul, MN, U.S.A.), was initially propagated by inoculation into the amniotic cavity of 22- to 24-days old embryonating turkey eggs and serially propagated in HRT-18 cells, a continuous cell line derived from a human rectum adenocarcinoma [8, 14]. The origin of the tissue culture adapted TCV Quebec isolate 1713 has been described previously [9]. The NCDC (Mebus) strain of BCV was obtained from the American Type Culture Collection (ATCC, VR 874). The Mebus strain and Quebec BCV isolates, obtained from diarrheic calves, were also propagated in HRT-18 cells [14]. Turkey and bovine coronaviruses were purified from the supernatant fluids of infected cell cultures by differential and isopycnic ultracentrifugation on sucrose gradients as described previously [6, 7]. The Purdue strain of transmissible gastroenteritis virus (TGEV, ATCC, VR 763) and serotype 3 of murine hepatitis virus (MHV-3, ATCC, VR 262) were propagated, respectively, in swine testicle and mouse fibroblast L 2 cells [7]. The Beaudette, Hollande, and Massachusetts strains of infectious bronchitis virus (IBV) were propagated by inoculation into embryonating chicken eggs [7]. Purified human coronavirus

HCV-229 E was a gift from Dr. P. Talbot (Virology Research Center, Institut Armand-Frappier).

Clinical specimens and virus cultivation

Intestinal contents from 1- to 8-week-old turkey poults with mild to severe diarrhea were obtained from commerical flocks from different locations in Quebec, Canada. Specimens from 5 to 10 poults from the same flock were pooled and used to make ten percent homogenates in 0.05 M Tris-HCl, pH 8.0. The homogenates were clarified by centrifugation in an Eppendorf centrifuge for 2 min at room temperature. Supernatants were stored at − 70 °C until further use. Virus from clinical specimens of diarrheic turkey poults was propagated by one to three passages on HRT-18 cells as described previously [8].

Preparation and cloning of cDNA

The preparation of BCV-specific clones (covering about 1/4 of the genome and dispersed over its total length) has been described elsewhere [33, 34]. To prepare TCV clones, the tissue culture-adapted Minnesota strain of TCV [8] was purified on sucrose gradients (20 to 55%) and pelleted by ultracentrifugation for 2 h at 25,000 rpm in a Beckman SW 27.1 rotor. Genomic RNA was extracted from concentrated virus and copied into cDNA for cloning as described for BCV [33]. Briefly, purified RNA, denatured by methyl mercuric hydroxyide treatment was used as template for first-strand cDNA synthesis using Molony murine leukemia virus reverse transcriptase (Pharmacia) in the presence of either oligo-d(T) or random primers (BRL) [3, 11, 16]. DNA polymerase I and RNase H were used for second-strand synthesis [11]. Homopolymer tailing of the cDNA molecules [27], using dCTP precursors, was followed by insertion into *Pst* I linearized (dG)-tailed pUC-9 (Pharmacia) and transformation of JM 101 cells [13].

Selection of recombinant plasmids for probes in detection of TCV

About 300 TCV-clones from the cDNA library were screened for the presence of the genomic 3′-end and the N gene by colony filter hybridization [16] using BCV-specific radiolabelled recombinant plasmids, containing sequences of the N gene [34, 35]. One TCV-specific recombinant plasmid (pM 78) with an insert of 1.7 kbp containing the entire sequence of the TCV N gene (Fig. 1) (manuscript submitted), was selected as a probe to detect TCV. BCV-specific recombinant plasmids p 52, p 27, and p 247, which served previously for the optimization of hybridization conditions for BCV-RNA detection [33], and probes pN 17 and pN 9, holding respectively the BCV N and M gene (Fig. 1), were used in the detection of several coronaviruses in order to establish the presence of potential genomic homologies. The cDNA sequences of p 52 and p 27 of 664 bp and 1120 bp, respectively, were determined (unpubl. data) and compared with sequence data available for all structural genes of BCV [4, 15, 18], using the IBI Pustell sequence analysis programs. The translated sequence of the inserts revealed that they are parts of open reading frames. Although both probes were found to be specific for BCV [33], no sequence homologies were found between these inserts and the structural genes of BCV, suggesting that these inserts represent fragments of non-structural genes. Recombinant plasmid p 247, obtained by random priming of cDNA and holding an insert of 1.6 kbp was selected for its capacity to strongly detect BCV-RNA in colony filter hybridization assays. The insert of p 247 could be detected by probe p 27 in Southern blot analysis (not shown) and might represent at least a part of a non-structural gene.

Clinical diagnosis of TCV was assayed with a pool of six ^{32}P-labelled BCV specific recombinant plasmids (pBCV-pool), holding non-overlapping cDNA sequences, in order

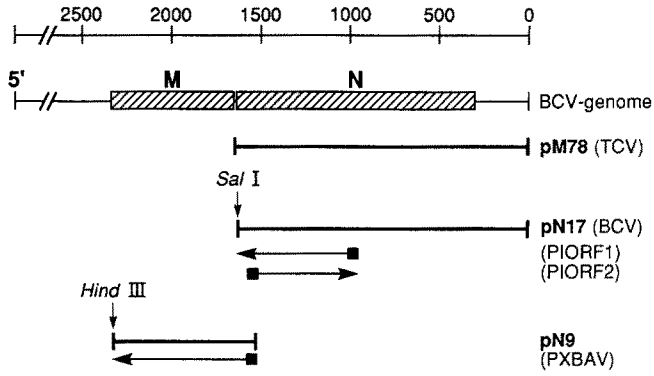

Fig. 1. Location of cloned cDNA with respect to the genomic RNA. Heavy bars represent cDNA inserts of recombinant plasmids pM 78 (TCV), pN 17 (BCV), and pN 9 (BCV), respectively. Solid squares represent primers used in PCR-probe synthesis, whereas arrows define the direction of synthesis and the regions of amplification. *N* and *M* Nucleocapsid and matrix protein genes of BCV. *Sal* I and *Hind* III Vector polylinker restriction endonuclease sites used to linearize plasmids pN 17 and pN 9, respectively

to amplify the detection signal. This pBCV-pool was superior in clinical diagnosis of BCV when compared with EM and ELISA [34].

Probe-labelling

Recombinant plasmids were isolated from bacteria by alkaline lysis, purified on CsCl gradients [16], and labelled by nick translation in the presence of [α^{32}P]dCTP (3000 Ci/mmol; 25) to specific activities of 5 to 10 × 10^7 cpm/μg.

Probe synthesis by means of PCR

Recombinant plasmid pN 17 was linearized in the vector polylinker with *Sal* I (Fig. 1) and served as a template for single-stranded (ss) probe synthesis [35]. Primer PIORF 1,
5′ TTA CAC CAG AGG TAG GGG TTC,
complementary to the sequence located 950 to 970 nucleotides from the 3′-end of the viral genome, was used in repeated primer extension using the PCR thermal cycler to synthesize probe molecules of approximately 680 bases, corresponding to the internal open reading frame inside the N gene. Double-stranded probes to the same sequence were synthesized by PCR using pN 17 as template and PIORF 1 and PIORF 2,
5′ ATG GCA TCC TTA AGT GGG CCG,
1553 to 1573 nucleotides from the 3′-end, as primers. Plasmid pN 9, containing the BCV M gene (Fig. 1), was restricted in the polylinker with *Hind* III for repeated primer extension in PCR, using the BCV-specific primer pXBAV,
5′ GAA CAT TTC TAG ATT GGT CGG ACT G,
1526 to 1550 nucleotides from the 3′-end, resulting in probe molecules of approximately 800 bases.

PCR mixtures for ss probe synthesis consisted of 110 ng template DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 200 μM of dATP, TTP, and dGTP, 50 pmol primer, 13 μl [α^{32}P]dCTP (3000 Ci/mmol, 3.3 μM) and 1.25 U *Taq* DNA polymerase (Cetus) in a final reaction volume of 20 μl. About 10 ng template was used in case of ds probe synthesis, whereas the final dCTP concentration was adjusted with "cold" dCTP to 20 μM. The reaction mixtures were overlaid with 30 μl paraffin oil and subjected

to 15 amplification cycles. Each cycle included a denaturation for 1 min at 94 °C, an annealing for 1 min at 52 °C, and a primer extension period for 3 min at 72 °C. The last cycle included a 10 min incubation at 72 °C, whereafter the products were separated from non-incorporated radionucleotides by spin-column chromatography, using Sephadex G-50. Ss and ds probes were labelled to specific activities of about 10^9 and 10^8 cpm/μg, respectively.

Sample preparation and hybridization

Clarified clinical samples from diarrheic turkey poults were extracted once with Freon (1,1,2-trichloro trifluoroethane) before application to nitrocellulose membranes (pore size 0.20 μm), previously equilibrated in a 6× SSC solution (20× SSC = 175.3 g NaCl and 88.2 g sodium citrate dihydrate per liter). Samples were spotted using a Minifold II slot-blot apparatus (Schleicher and Schuell, Inc.) followed by baking of the blots at 80 °C for 1 to 2 h under vacuum. Membranes were then rehydrated in a 6× SSC solution and prehybridized at 42 °C for 4 h or overnight in a standard hybridization solution consisting of: 60% formamide (or as indicated in the individual experiment), 5× Denhardt's components, 5× SSPE salts, 0.1% SDS, and 100 μg/ml of denatured, sheared calf thymus DNA [16]. ^{32}P-labelled recombinant plasmids and ds PCR-produced probes were heat-denatured and added to the prehybridization solution to a final concentration of 100 ng probe/ml. PCR-produced ss probes were added without previous heat-denaturation at concentrations indicated in individual experiments. Hybridization was for about 36 h at 42 °C, followed by blot-washing according to standard procedures [16].

Enzyme-linked immunosorbent assay (ELISA)

The double antibody-sandwich ELISA was used for the detection of TCV in clinical specimens from diarrheic turkey poults as described elsewhere [7].

Results

Detection of several coronavirus strains with ^{32}P-labelled BCV specific recombinant plasmids

Suspensions of purified virus of several coronavirus strains were adjusted to approximately 3×10^{11} virus particles per ml as determined by EM by admixture with known amounts of latex spheres. 100 μl of each suspension was spotted on nitrocellulose membranes and hybridized separately in triplicate under relatively stringent conditions (i.e., 65% of formamide) with ^{32}P-labelled BCV specific recombinant plasmids p 52, p 27, and p 247. The three probes allowed individually the detection of the tissue culture-adapted Mebus strain of BCV as well as two BCV Quebec isolates. In addition, strong hybridization detection signals were obtained with the tissue culture-adapted Minnesota strain and Quebec 1713 TCV isolates (Fig. 2). Similar results were obtained when probing identical blots with pN 17 and pN 9, respectively, holding sequences of the BCV N and M genes (data not shown). These BCV-specific probes allowed also individually the detection of twenty other Quebec isolates of TCV (data not shown). Only slight detection signals were seen with the antigenically related MHV-3, whereas no detection signals were obtained with several strains of IBV and the human coronavirus HCV-229 E (Fig. 2), both of which are classified

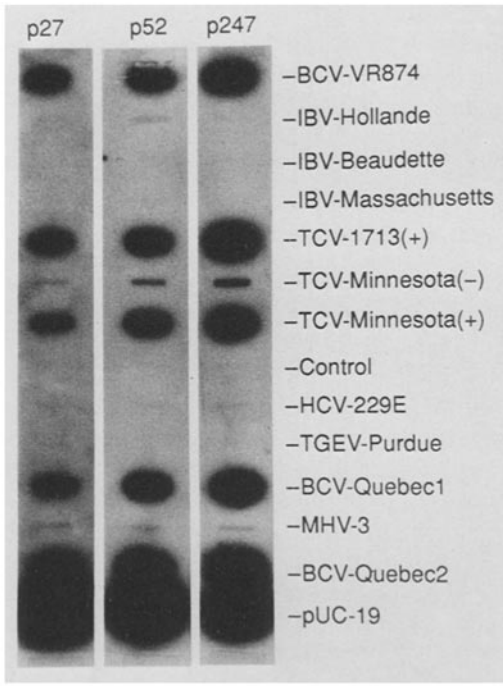

Fig. 2. Detection by hybridization of several coronavirus strains with BCV-specific probes. Hybridization in 65% of formamide with radiolabeled recombinant plasmids (p 27, p 52, and p 247) was done to examine possible genomic homologies amongst the different virus strains. Hybridization was for two days and autoradiography for overnight at −70 °C, using intensifying screens. *BCV* Bovine coronavirus; *IBV* infectious bronchitis virus; *TCV* turkey coronavirus; *HCV* human coronavirus; *MHV* murine hepatitis virus, *TGEV* transmissible gastroenteritis virus. Control refers to spotted supernatants of non-infected HRT-18 cells. pUC-19 DNA (100 ng) was spotted as a positive control for hybridization itself. + Virus propagation in the presence of trypsin; − no trypsin was added to the culture medium

in antigenic groups distinct from the BCV and TCV groups. Detection signals were absent when testing spotted supernatant fluids of non-infected HRT-18 cells (Fig. 2) and when probing identical blots with ^{32}P-labelled pUC-DNA (not shown), confirming the specificity of the hybridization signals obtained.

TCV detection with BCV-specific ss probes, synthesized by PCR

Aliquots of serially diluted suspensions of purified tissue culture-adapted Minnesota strain of TCV were spotted on nitrocellulose membranes. Figure 3, lane 1, shows the detection signals obtained with the PIORF 1-primed probe, synthesized on linearized plasmid pN 17, whereas lane 2 represents the detection signals obtained with the PXBAV-primed probe, synthesized on pN 9. TCV detection, using a combination of both probes, resulted in an about 3-fold increase in sensitivity, as determined by the slope of the dose-response curve [32], whereas detectability was increased about 5-fold (Fig. 3, lane 3). A minimum corresponding to about 2×10^4 viral genomes (as estimated via protein content), could be detected by combining both probes. As expected, detection of spotted pUC-19-DNA was significantly reduced with the PCR-produced probes (Fig. 3) when compared to nick-translated recombinant plasmid probes (Fig. 2), therefore increasing the specificty of detection.

Comparison of PCR-produced BCV-specific probes with BCV and TCV nick-translated probes in the detection of several isolates of TCV

The efficacity of TCV- and BCV-specific probes in detecting TCV was established using seventeen clinical isolates, propagated by three passages in HRT-

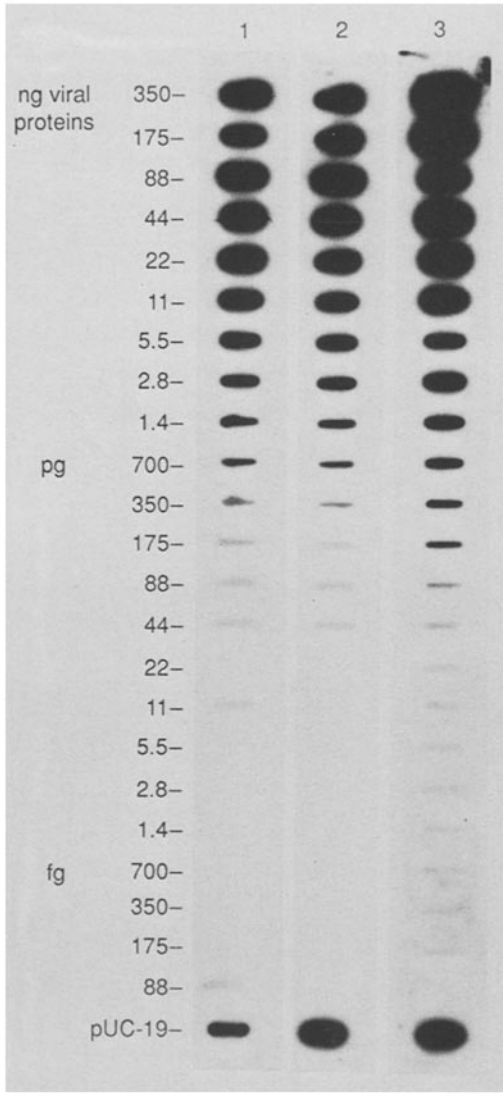

Fig. 3. TCV detection-limit determination with PCR-synthesized ss probes. *1* and *2* Detection signals obtained by hybridization with PCR-probes synthesized on pN 17 and pN 9, respectively. About 50 µl of the PCR probe reaction mixture, that was diluted with water to 100 µl final, was added per individual blot. *3* Hybridization signals obtained with a combination of both ss probes. Here, about 25 µl of each diluted reaction mixture was added to the prehybridization solution of the individual blot. The virus quantity was calibrated by the estimation of protein content rather than RNA due to the unstability of the latter. The amount of viral protein refers to the quantity of proteins measured according to Bradford [5]. An amount of 100 pg viral proteins is estimated to correspond to about 1.14×10^5 virus particles or about 1.1 pg genomic RNA. 100 ng pUC-19 DNA was spotted as a control on the hybridization itself. Exposure was overnight at − 70 °C

18 cells. Hybridization of virus-containing supernatants with probes pN 17 and pM 78, respectively, holding the N gene sequences of BCV and TCV resulted in similar detection signals (Fig. 4, lanes 1 and 3), confirming the genomic relatedness between BCV and TCV in this gene as suggested by serological evidence. Three cultured isolates (i.e., 32, 35, and 42), identified as weak-positive by ELISA [7] were only slightly positive or negative by hybridization with either of the two nick-translated recombinant plasmid probes. Hybridization with a combination of the PXBAV- and PIORF 1-primed PCR-produced ss probes showed a strongly increased detection sensitivity and resulted also in the identification of all 17 samples as positive (Fig. 4, lane 2).

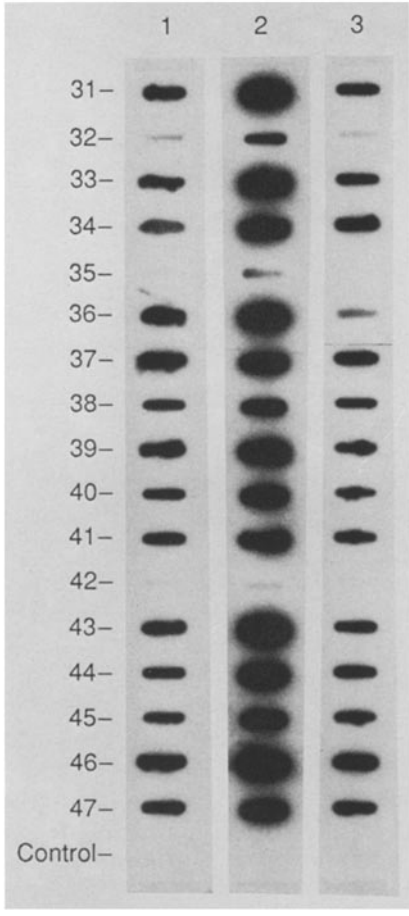

Fig. 4. Detection of TCV after passage in tissue culture. *1* and *3* Detection by hybridization on spotted third-passage supernatants from specimen-infected cell monolayers using probes pN 17 and pM 78, respectively, holding the N gene sequences of BCV and TCV, respectively. *2* Signals obtained with the combination of two PCR-synthesized ss probes. 25 µl of each probe reaction mix was added to the individual blot. Control is a spotted culture supernatant from non-infected HRT-18 cells. Exposure was overnight at − 70 °C

Clinical diagnosis of TCV with different BCV-specific probes

Ten clinical samples, which were TCV-positive by ELISA only after virus cultivation in HRT-18 cell monolayers, were also positive in hybridization assays using one BCV-specific probe (p 52) on the supernatant fluid from the first passage (not shown). TCV in the original clinical specimens could hardly be detected with probe p 52 alone (not shown). Clinical diagnosis was attempted with either the pBCV-pool of recombinant plasmids to amplify the detection signal or with probes, synthesized by PCR as a result of labelling to specific activities about 20 times higher than nick-translated probes.

A total of 134 clinical samples from birds with diarrhea were extracted with Freon to eliminate binding competition between the virus and macromolecules present in the samples for sites on the nitrocellulose. Treated samples were then spotted on nitrocellulose membranes for hybridization with BCV-specific probes (Fig. 5). Among these, 36 samples were identified as positive with ss PCR-produced probes, whereas 28 of these 36 were positive by hybridization with ds PCR-synthesized probes. Only 20 positive hybridization signals were obtained when the samples were probed with the pBCV-pool of recombinant plasmids.

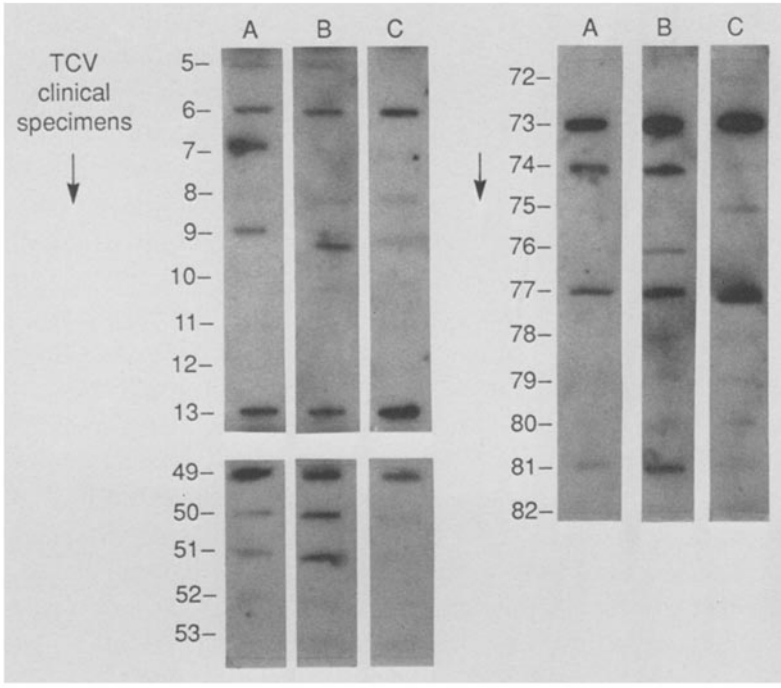

Fig. 5. Detection of TCV in clinical specimens. Samples, diluted 1 in 10 in TBS, were extracted with Freon and in quadriplicate spotted to nitrocellulose membranes. Blots were hybridized with PIORF 1 and 2 primed PCR-produced ds probes (*A*) and a combination of PXBAV- and PIORF 1-primed PCR-produced ss probes (*B*). *C* Hybridization detection signals with the pBCV-pool of recombinant plasmid probes. Similar blots with spotted clinical samples were also incubated with radiolabeled pUC 19-DNA as a control on possible background hybridization by vector sequences but were negative in all the samples tested (not shown). Exposure was for 2 days at − 70 °C

Three of these samples were not identified as positive with either of the two PCR-produced probes and may have been false-positives. However, similar blots, incubated with radioactive-labelled pUC-19-DNA to examine possible background hybridization with vector DNA did not reveal any significant hybridization detection signals, supporting the specificity of the signals obtained.

An optimized ELISA [7] on all clinical specimens performed rather poorly (only 9 samples were identified as positive; data not shown), may be as a result of repeated freezing and thawing of the samples.

Discussion

In the present study, genomic relatedness between BCV and TCV was demonstrated as BCV-specific probes to both structural and nonstructural genes could be used to detect isolates of TCV under relative stringent hybridization conditions. These results are in agreement with and extend previous findings on the serological relatedness between structural proteins of these two enteric coronaviruses as established by ELISA and Western immunoblotting [9].

Our BCV-specific probes could not detect the BCV-antigenically unrelated coronaviruses IBV and TGEV, whereas only slight detection signals were seen with MHV-3 (Fig. 2), which is placed in the same antigenic group as BCV [20]. Shockley et al. [31] also reported a weak detection with another strain of MHV (MHV-A59), using a ^{32}P-labelled insert probe corresponding to the BCV N and M genes [15, 31]. Their BCV-specific probe could in addition detect the serologically-related porcine hemagglutinating encephalitis virus and human coronavirus strain OC43.

Clinical diagnosis of TCV with a BCV specific ^{32}P-labelled probe (p 52) was initially hampered by both the low concentration of virus in samples and by macromolecules, which clog the wells of the slot-blot apparatus and compete with the virus for binding sites on the nitrocellulose. In a previous report on the clinical diagnosis of BCV [33], we investigated several options for the elimination of these macromolecules in bovine diarrheic samples and for the amplification of detection signals. Similarly, TCV in clinical specimens, that were treated with Freon before spotting, could be detected when using the pBCV-pool of recombinant plasmid probes (Fig. 5). Although not observed in this study, plasmid probes may in some cases cause difficulties in clinical diagnosis due to vector homology to plasmids in the sample [2, 10]. Therefore, PCR [12, 29, 30] was applied to synthesize probe molecules from inserts in linearized recombinant plasmids to reduce labelling of vector sequences. PCR-produced ss probes were more efficient than nick-translated recombinant plasmid probes in detection of purified TCV and extracellular virions present in TCV-infected cell culture supernatant fluids (cf. Figs. 3 and 4). In combination, the PCR probes allowed the detection of a minimum amount of about 10^4 viral genomes (Fig. 3). Hybridization signals obtained with spotted pUC-DNA (Fig. 3) were likely caused by molecules synthesized by polymerase-readthrough into vector sequences of incompletely digested templates. Detection of spotted pUC-DNA was, however, significantly reduced with PCR-probes when compared to recombinant plasmid probes (Figs. 2 and 3), improving the specificity of the detection assay. PCR-produced ss probes were superior to other probes used in clinical diagnosis, probably as a result of incorporation of label to high specific activities (10^9 cpm/μg; [35]) and the absence of self-annealing among the probes. Double-stranded probes, synthesized in PCR with 1/3 of the dCTP being radiolabelled were efficient in BCV detection but resulted in strong background after only 8 h of autoradiography [35]. In our studies on detection of TCV, the background was reduced by addition of more "cold" dCTP for ds probe synthesis; in turn, however, fewer numbers of specimens were identified as positive. PCR-produced probes to the BCV N and M genes were, as in the detection of purified BCV [35] and TCV (Fig. 4), also superior in clinical diagnosis of TCV (Fig. 5). In conclusion, BCV or TCV probes may be valuable in molecular hybridization for routine clinical diagnosis of either virus but may be problematic when these two viruses have to be distinguished. Studies are currently in progress to establish the nucleotide sequence of the TCV structural

genes in order to determine the exact degree of genomic relatedness between the two viruses.

Acknowledgements

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