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Genomic relationship of porcine hemagglutinating encephalomyelitis virus to bovine coronavirus and human coronavirus OC43 as studied by the use of bovine coronavirus S gene-specific probes

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Summary. The genomic relationship of porcine hemagglutinating encephalomyelitis virus (HEV) to bovine coronavirus (BCV) and human coronavirus (HCV) strain OC43 was examined by dot blot hybridization assays. Two BCV S gene-specific probes were generated by polymerase chain reaction from the avirulent L9-strain of BCV. Probes were located in the S1 and the S2 region of the peplomeric (S) glycoprotein gene. The S1 probe (726 bp) hybridized with BCV and HCV-OC43, but not with HEV under moderate stringency hybridization conditions (50 °C). Only slight signals were present with mouse hepatitis virus (MHV) and no signals were observed with feline infectious peritonitis virus (FIPV) or canine coronavirus (CCV). At high stringency conditions (60 °C) the S1 probe hybridized with BCV only. Using the S2 probe (680 bp) under moderate strin-gency conditions, hybridization signals were obtained with BCV, HCV-OC43 and HEV (strains 67N, NT9, VW572). The signals obtained by the three HEV strains were altogether weaker than with BCV and HCV-OC43. The S2 probe did not react with MHV, FIPV and CCV. At high stringency the S2-specific probe hybridized with BCV and HCV-OC43 but did not hybridize with HEV. Nucleotide sequence analysis of the region covering the S2 probe in HEV revealed 92.6% nucleotide sequence homology to BCV and 91.9% to HCV-OC43. In contrast, the region covering the S1 probe in HEV could not be amplified using the BCV S1-specific primers. The hybridization and sequencing results thus indicate a closer genomic relationship between BCV and HCV-OC43 than there is between HEV and BCV or HCV-OC43, respectively.

Introduction

Coronaviruses are important pathogens in animals and man. Two major antigenic groups of mammalian coronaviruses are distinguished. Porcine transmissible gastroenteritis virus (TGEV), human respiratory coronavirus 229E (HCV-229E), porcine epidemic diarrhea virus (PEDV), porcine respiratory coronavirus (PRCV),

canine coronavirus (CCV) and feline infectious peritonitis virus (FIPV) are classified into one antigenic group. The second group, characterized by the presence of a hemagglutinin gene, is represented by bovine coronavirus (BCV), human respiratory coronavirus OC43 (HCV-OC43), porcine hemagglutinating encephalomyelitis virus (HEV) and mouse hepatitis virus (MHV) [13].

The coronavirus genome consists of a single-stranded, nonsegmented RNA with positive polarity. Three to four major structural proteins are encoded: the nucleocapsid protein (N), the membrane protein (M) and the spike or peplomer protein (S). Members of the second antigenic group have an additional glycoprotein, the hemagglutinin esterase protein (HE). The S proteins of BCV and MHV are cleaved by host-cell proteases into two subunits of similar size, S1 and S2, respectively [1, 3, 17].

Genome sequences of the hemagglutinating coronaviruses BCV and HCV-OC43, mainly concerning the genes coding for structural proteins, were published [6–8, 10, 11, 19–21]. Homologies between the deduced aminoacid sequences of the BCV-L9 and HCV-OC43 structural proteins revealed the S protein to be less conserved (91.2% homology) than the N (97.5% homology), M (94% homology) and HE (95% homology) protein.

To date, studies on the HEV genome have not been conducted. In order to study the relationship of HEV to BCV and HCV-OC43 two probes were generated from the BCV S gene and investigated for their reactivities with HEV and HCV-OC43 by dot-blot hybridization assays.

Materials and methods

Cells and viruses

The L9 [2] and the LY138 [4] strain of bovine coronavirus, the OC43 strain of human respiratory coronavirus [9] and the 67N- [ATCC VR 7419], VW572- [12] and NT9 [5]- strains of porcine hemagglutinating encephalomyelitis virus were propagated on the human rectal tumor cell line HRT-18 [14]. Canine coronavirus (ATCC VR-809) and feline infectious peritonitis virus (kindly provided by Dr. Frost, Staatl. Veterinäruntersuchungsamt, Frankfurt, Germany) were grown on Crandell feline kidney cells (ATCC CCL-94). The virus suspensions were harvested from the supernatants of infected cells, partially purified from cell debris and concentrated by ultracentrifugation at 70 000× **g** for 1.5 h at 4 °C.

Indirect immunofluorescence assay

An indirect immunofluorescence assay was performed with monoclonal antibodies (Mab) I7, F7 and I16 which were shown to differentiate between BCV, HCV-OC43 and HEV [16]. Virus infected cells were fixed with formaldehyde for 10 min, permeabilized with 0.1% Triton X in PBS for 6 min and incubated each with Mab F7, I7 and I16 at 37 °C for 1 h. After rinsing two times with PBS the slides were stained with an anti mouse IgG-FITC (Sigma, Deisenhofen, Germany) at 37 °C for 1 h.

RNA isolation

RNA was isolated from virus-infected HRT-18 cells by the guanidinium-thiocyanate method using a RNA-isolation-kit (Stratagene, Heidelberg, Germany) according to the manufacturers' instructions.

Primer for cDNA synthesis and polymerase chain reaction

Primers used for cDNA synthesis (RT) and polymerase chain reaction (PCR) were chosen from the BCV-L9 S-gene sequence (EMBL/Gen Bank accession No. M 64667). Primers 3′S1 (5′-CACCATCTTGATTGAAAGCTAG-3′) and 5′ S1 (5′-GCACTGATATTGTCGATGTTAC-3′) were chosen to amplify a fragment of 726 bp located near the 5′ -end of the S1-glycoprotein gene of BCV corresponding to nucleotides 128–853 (Fig. 1). For preparation of the probe located within the S2- glycoprotein gene a nested PCR was performed. The outer primer pair 3′ S2a (5′-AATATATCGTCAGGAGCCAATA-3′)/5′S2a (5′-ACACTACACAGTTGCAAGTAGCT-3′) and the inner primer pair 3′ S2b (5′-ATCACAACAACCACCACATATC-3′)/5′S2b (5′-GGAGAAGGTTAATGAATGTGTCA-3′) were used to amplify a fragment of 680 bp corresponding to the nucleotides 3378–4058 (Fig. 1).

cDNA synthesis and probe preparation

Single-stranded cDNA was synthesized with SuperScript Plus RNase H– reverse transcriptase (GIBCO BRL, Eggenstein, Germany) and primer 3′ S1 or 3′ S2a, respectively, at 47.5 °C for BCV and 42 °C for HEV according to the manufacturers' instructions.

Probes were amplified in a reaction mixture adjusted to 1 × PCR buffer, 200 μM dATP, dGTP, and dCTP each, 160 μM dTTP and 40 μM DIG-11-dUTP (Boehringer, Mannheim, Germany), 400 nM of each primer and 0.5 U Tfl-polymerase (Biozym, Hameln, Germany) in a final volume of 50 μl with annealing temperatures of 58 °C.

Dot blot hybridization

To determine the detection limit of each probe log 2-dilutions of a BCV-L9 suspension as well as a dilution series of homologous DNA in amounts ranging from 100 ng to 40 pg were directly applied to the nylon membrane.

For dot blot hybridization assays log-2 dilutions of each virus starting with 5×10^5 $TCID_{50}$ were adjusted to 1 × SSC (0.015 M NaCitrat, 0.15 M Nacl, pH 7.0) and directly applied to a positively charged nylon membrane (Boehringer, Mannhein, Germany) presoaked with H_2O and equilibrated with 20 × SSC, by means of a dot-blot apparatus (Biorad, München, Germany). Blots were air dried and viral RNA was fixed and denatured by UV-illumination for 5 min (TFL-20M,

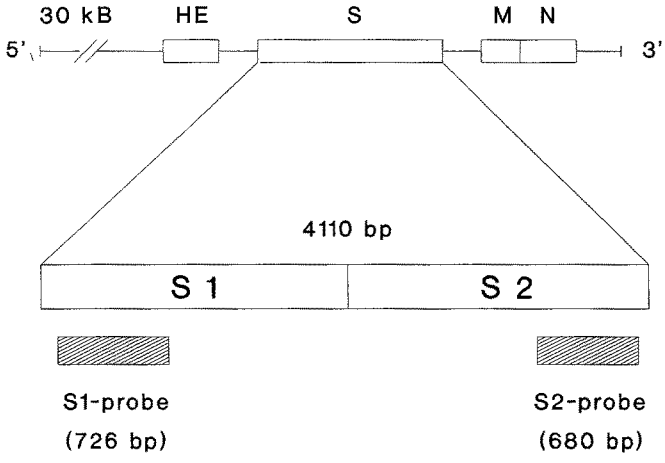

Fig. 1. Location and size of BCV S gene-specific probes

Biometra, Göttingen, Germany). Prehybridization and hybridization were basically done according to the manufacturers instructions (DIG luminescent detection kit for nucleic acids, Boehringer, Mannheim, Germany). Prehybridization was performed at 42 °C for 4 h followed by an incubation with hybridization solution containing 30 ng/ml of freshly denatured Digoxigenin-labeled probe at 42 °C overnight. Membranes were washed twice at room temperature for 5 min in 2 × SSC, 0.1% SDS and then twice for 15 min at moderate (50 °C) or high (60 °C) stringency conditions in 0.1 × SSC, 0.1% SDS. The hybridization signals were immunologically detected according to the manufacturers directions. The blots were exposed to a X-Ray film for 7 h.

DNA sequencing

Non-radioactive sequencing reactions were performed using the prism ready reaction dye-deoxy terminator cycle sequencing kit (ABI, Applied Biosystems Inc., Weiterstadt, Germany) according to the manufacturers instructions. Further analysis was done on the ABI 373A sequencer using standard protocols from ABI. The PCR products were purified for sequencing with the Quiaquick-spin PCR purification kit (Quiagen, Chatsworth, CA). 300 ng purified DNA were used as template for each sequencing reaction. The primers used for sequencing were 3′ S2b and 5′ S2b as well as two additional primers (3′ S3: 5′-TATAGTTAGCAGCACAGGTA-3′; 5′ S3: 5′-GCT-GAACATTTCAACACCC-3′). For detailed DNA sequence analysis the DNASTAR software package (DNASTAR Inc., London, U.K.) was used.

Results

Indirect immunofluorescence assay

The indirect immunofluorescence assay results gained by the use of Mabs, previously published by Vautherot et al. [16], revealed different reaction patterns between the bovine, human and porcine coronavirus strains (Table 1). Mab J7 only

Table 1. Results of the indirect immunofluorescence assay of BCV-L9, HCV-OC43 and HEV-67N, -VW572 and-NT9 with Mab I7, F7 and I16[a] produced against BCV

Virus-strain	Monoclonal antibody I7[b]	F7[c]	I16[d]
BCV-L9	+	+	+
HCV-OC43	–	+	+
HEV-67N	–	–	+
HEV-VW572	–	–	+
HEV-NT9	–	–	+

[a]Vautherot et al. [16]

[b]Specific for the BCV-S glycoprotein, no antigenic cross reactivity with other hemagglutinating coronaviruses

[c]Specific for the BCV-S glycoprotein, antigenic cross reactivity with HCV-OC43

[d]Specific for the BCV-S glycoprotein, antigenic cross reactivity with HCV-OC43 and HEV

reacted with BCV. Mab F7 recognized BCV as well as HCV-OC43 and Mab I16 recognized HEV-67N, -NT9 and -VW572, additionally.

Detection limit of the BCV specific S1 and S2 probe

Using the S1 probe, the minimal detectable amounts of the BCV-L9 virus suspension were 7.8×10^3 and 3.1×10^4 $TCID_{50}$ and the minimal detectable amounts of homologous DNA were 781 pg and 3.12 ng at washing temperatures of 50 °C and 60 °C, respectively. By hybridization with the S2 probe the detection limit was lower. With this probe 3.9×10^3 $TCID_{50}$ of BCV-L9 or 390 pg DNA at 50 °C and 1.5×10^4 $TCID_{50}$ or 781 pg DNA at 60 °C could be detected.

Cross hybridization

The hybridization results are shown in Fig. 2. In general both probes did not react with viruses belonging to the antigenic unrelated group (CCV, FIPV) or with mock infected cells (data not shown). The S1 probe hybridized with BCV and HCV-OC43

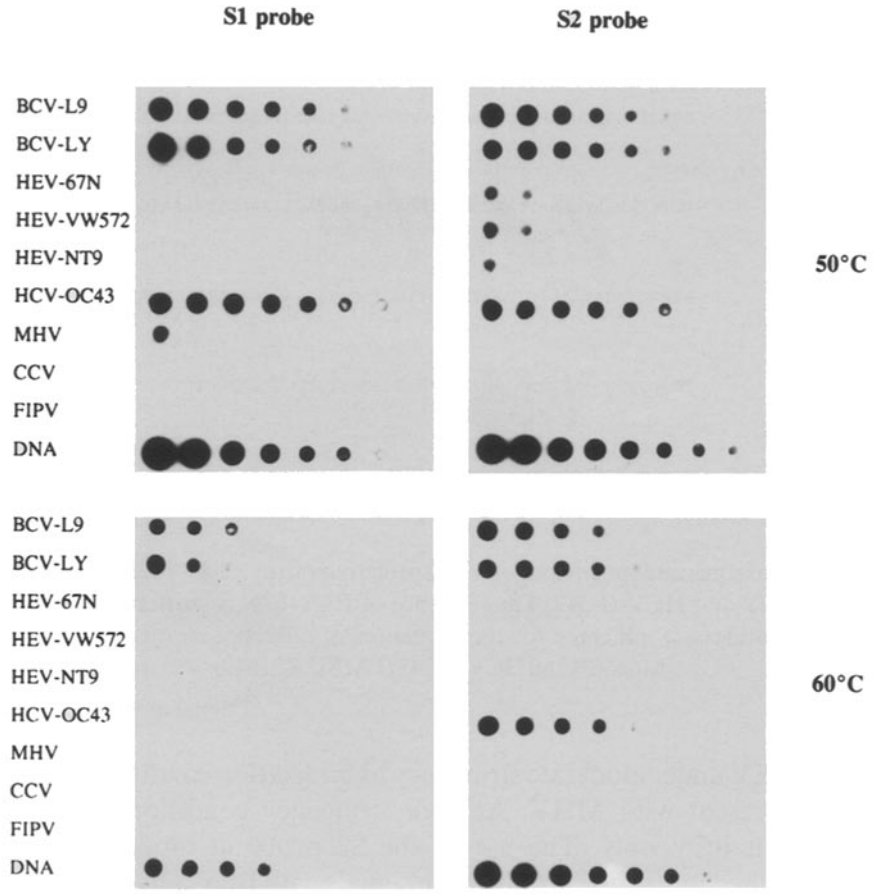

Fig. 2. Dot blot hybridization of Digoxigenin-labeled BCV S1- and S2-gene-specific probes with log-2 dilutions of different coronavirus strains under moderate (50 °C) and high (60 °C) stringency conditions

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HEV-67N      GGTTAATGAATGTGTCAAAAGCCAATCATCTAGGATAAATTTCTGTGGTA      50
BCV-L9                                                    T
HCV-OC43

HEV-67N      ATGGTAATCATATTATATCATTAGTACAGAATGCTCCATATGGTTTGTAT     100
BCV-L9                              G
HCV-OC43                            G

HEV-67N      TTTATCCATTTTAGCTATGTCCCCACCAAGTATGTTACAGCAAAGGTTAG     150
BCV-L9              C             T  T        C  T  G
HCV-OC43            C     T       T  T        C     G

HEV-67N      TCCTGGTTTGTGCATTGCTGGCGATATAGGAATATCGCCTAAGAGTGGTT     200
BCV-L9          C   C             T    G   T   G C
HCV-OC43        C   C             T    G   T   G T

HEV-67N      ATTTTATTAATGTAAATAATTCTTGGATGTTCACTGGTAGTAGCTATTAC     250
BCV-L9            G               A                   G T
HCV-OC43          G               A         A         G T  C

HEV-67N      TACCCTGAACCTATAACCCCAAATAATGTTGTTGTGATGAGAACCTGTGC     300
BCV-L9                  C     TGG               T     T
HCV-OC43           T            TGA               T     T     G

HEV-67N      TGTTAATTATACTAAAGCACCGGATCTAATGCTGAACACATCGACACCCA     350
BCV-L9         C   C           G     G           TT  A
HCV-OC43                       G   T  G             T  A T

HEV-67N      ACCTTCCTGACTTCAAGGAAGAATTGTATCAATGGTTTAAAAACCAATCT     400
BCV-L9           C A   T  T        G   G                   A A
HCV-OC43               T  T        G   G                T   A A

HEV-67N      TCAGTGGCACCAGATTTGTCACTTGATTATATTAATGTTACGTTCTTGGA     450
BCV-L9                                      A        A
HCV-OC43                                    A        A

HEV-67N      CCTACAAGATGAAATGAATAGGTTACAAGAGGCTATAAAAGTTTTAAATC     500
BCV-L9                                   G     A
HCV-OC43           T                     G     A        C

HEV-67N      AGAGCTACATCAATCTCAAGGACATTGGTACATATGAGTATTATGTTAAA     550
BCV-L9                                                    A
HCV-OC43                                          A       A

HEV-67N      TGGCCTTGGTATGTATGGCTTTTAATTGGCCTTGCTGGTGTAGCTATGCT     600
BCV-L9                                    T
HCV-OC43                                CT

HEV-67N      TGTCTTACTATTCTTCATATGCTGCTGTACAGGATGTGGGACTAG          645
BCV-L9          T                   T
HCV-OC43        T                   T
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Fig. 3. Nucleotide sequence comparisons of the S2 probe region (nt 3384 – 4028, EMBL: M64667) of HEV-67N, BCV and HCV-OC43. The sequence of HEV-67N is written in full, while only changes in nucleotides are indicated for the sequences of reference strains BCV-L9 (EMBL: M64667) and HCV-OC43 (EMBL: Z21849)

but not with HEV under moderate stringency hybridization conditions. Only slight signals were present with MHV. At high stringency conditions the S1 probe hybridized with BCV only. The use of the S2 probe at moderate stringency conditions resulted in strong hybridization signals with BCV and HCV-OC43 and less pronounced signals with all three HEV strains. The S2 probe did not react with MHV. At high stringency the S2-specific probe hybridized with BCV and HCV-OC43. It did not hybridize with HEV.

HEV nucleotide sequences in the S2-probe region

A fragment of 680 bp of the HEV S gene was amplified with the BCV-specific primer pair 3'S2 and 5'S2. 645 bases (corresponding to position 3384–4028, EMBL: M64667) of this fragment were sequenced on both strands (Fig. 3).

Fourty-eight mismatches were found in HEV compared to the corresponding BCV-L9 sequence (EMBL: M64667) and 52 compared to HCV-OC43 (EMBL: Z21849). No insertions or deletions occured. The resulting nucleotide sequence homology was 92.6% with BCV-L9 and 91.9% with HCV-OC43.

Discussion

Hybridization and sequencing results presented in this paper indicate a high degree of genomic relationship of HEV to BCV and HCV-OC43. However, the two BCV S gene-specific probes used for cross hybridization experiments were able to differentiate these three hemagglutinating coronaviruses.

The S1 probe failed to detect HCV-OC43 by hybridization under high stringency conditions. This result reflects the nucleotide sequence data. Alignment of the published genome sequences of the region corresponding to the S2 probe in BCV and HCV-OC43 revealed 24 mismatches. In contrast, 41 mismatches as well as 24 insertions into the HCV-OC43 genome occured in the sequence corresponding to the S1 probe [7, 19].

The failure of the S1 probe to hybridize to HEV even under moderate stringency hybridization conditions and the failure of the S2 probe to detect HEV under high stringent hybridization conditions indicated that the S genes of HEV and BCV or HCV-OC43, respectively, were less related. This hypothesis was further confirmed by PCR results utilizing the BCV S1-and S2-specific primers. Both primer pairs produced amplicons of the expected length from HCV-OC43 cDNA. In contrast, a specific amplicon from HEV cDNA could be synthesized only with the S2 primers. The nucleotide sequence analysis of this 680 bp-amplicon (HEV strain 67N) and the alignment with the corresponding sequence in BCV and HCV-OC43 revealed a lesser degree of homology between HEV-67N and BCV-L9 (92.6%) or HCV-OC43 (91.9%) than exists between BCV-L9 and HCV-OC43 (96.3%).

Sequence data of the S genes of BCV and HCV-OC43 revealed a high degree of homology between their deduced aminoacid sequences (91.2% identity). As compared to the S2 protein (95.3% homology) greater sequence divergence occured in the S1 protein (88% homology) [7, 11, 19]. The hybridization results presented in this paper indicate that the S2 region of HEV-67N is similarly conserved, as it is reported for BCV and HCV-OC43 [7, 11, 19].

The failure of the S1 probe to hybridize with HEV and the obvious lack of the BCV S1-specific primers during HEV-PCR are indicative of a higher degree of genotypic divergence between HEV and BCV or HCV-OC43, respectively, than there is between BCV and HCV-OC43.

Similar differences were demonstrated on the phenotypic level. HEV, BCV and HCV-OC43 share common antigenic determinants but they can be differentiated by their reactivities with certain anti-BCV Mabs [15, 16]. Identical results were obtained

for the coronavirus strains used in this study by immunofluorescence assays with Mabs directed against different S1 epitopes, which were published by Vautherot et al. [16].

HEV was recently shown to have a strong tropism to neurons in experimentally infected young mice [18]. In naturally infected newborn pigs the virus spreads to the central nervous system via peripheral nerves. BCV and HCV-OC43, in contrast, have a tropism to the respiratory and enteric tract [17]. It could be hypothesized that divergences in the putative bulbous part (S1) of the S protein are related to tissue tropism. The complete amino acid sequence of the HEV S protein is needed to provide the knowledge for studying its possible role in tissue tropism.

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