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Identifying Bovine Respiratory Syncytial Virus by Reverse Transcription-Polymerase Chain Reaction and Oligonucleotide Hybridizations†

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An assay to identify tissue culture cells infected with bovine respiratory syncytial virus (BRSV) that utilizes reverse transcription (RT), the polymerase chain reaction (PCR), and a synthetic oligonucleotide hybridization probe has been developed. The RT-PCR assay uses a BRSV-specific negative-sense oligonucleotide primer to synthesize cDNA from a BRSV fusion protein mRNA template and another BRSV-specific oligonucleotide primer (positive sense) upstream from the negative-sense primer for PCR amplification. In the presence of mRNA templates of BRSV isolates originating from locations throughout the United States, the BRSV RT-PCR assay resulted in amplified products (381 bp) that were specific to BRSV, as demonstrated in hybridizations with a positive-sense oligonucleotide probe complementary to internal sequences and in sequence comparisons with the F protein of BRSV 391-2. In analysis of the BRSV RT-PCR assay with prototype strains of human RSV subgroups A and B, amplification of a similar 381-bp RT-PCR product was not evident, and no RT-PCR product hybridized with the internal probe. We conclude that the specific ability to amplify DNA sequences of BRSV F protein mRNA by RT-PCR and then to demonstrate the presence of the amplified product with a BRSV-specific oligonucleotide probe will greatly add to the speed, sensitivity, and specificity of BRSV diagnostics.

Bovine respiratory syncytial virus (BRSV), a pneumovirus in the family *Paramyxoviridae*, is an important cause of acute respiratory disease in postweaning calves and feedlot cattle in the United States (1–3, 6). In Europe, BRSV infection is considered one of the most significant causes of bovine respiratory disease (11, 23). Although most infections are inapparent (2, 6, 12), the high prevalence of seropositive cattle in the United States indicates that the rate of infection is high and augments the belief that BRSV is an important contributor to bovine respiratory disease.

A major problem with studying the pathogenesis of BRSV is the difficulty associated with rapidly and accurately identifying the presence of the virus. Successful laboratory diagnosis of BRSV is generally based on one of four criteria: (i) virus isolation, (ii) identification of BRSV antigens in suspected tissues, (iii) indications of BRSV seroconversion, or (iv) histopathology. However, the lack of standardized reagents, the high prevalence of cattle with antibody titers to BRSV (60 to 80% seropositive) (6), and the need for skilled personnel to process and interpret fluorescent-antibody results have hindered development of a routine diagnostic test. Similarly, successful isolations of virus from typical clinical cases of BRSV infection are often unsuccessful and can take from 11 to 21 days (with reports of >45 to 50 days [18, 24]) because of the late appearance of any noticeable cytopathic effect. Because of these difficulties, isolation of BRSV is not recommended as a routine approach to diagnosis (8).

Human RSV (HRSV) is the most important cause of respiratory disease in infants and young children, and the rapid detection of HRSV antigens in clinical specimens has been useful in diagnosing and limiting the nosocomial spread of HRSV associated with bronchiolitis and pneumonia (17, 21). Similarly, synthetic oligonucleotide probes have been used in in situ hybridizations to identify HRSV in nasopharyngeal secretions (7), and cDNA probes and synthetic oligonucleotides have been used to differentiate HRSV subgroups (19, 20). More recently, combined reverse transcription-polymerase chain reactions (RT-PCR) have been used to amplify and detect low levels of HRSV mRNA from nasopharyngeal aspirates (16) and otitis media effusion (15). Similar diagnostic success has not yet been reported for BRSV infections in cattle.

In the following study, we describe a method for amplifying, detecting, and differentiating BRSV fusion (F) protein mRNA from HRSV mRNA by using RT-PCR and an oligonucleotide probe. To evaluate the specificity of the technique, BRSV isolates from different regions of the United States were assessed, as were prototype HRSV strains (subgroups A and B), with the same primers and oligonucleotide probe. The results indicate that a combined BRSV-specific RT-PCR and hybridization procedure with an oligonucleotide probe is capable of amplifying and then differentiating BRSV F protein mRNA from HRSV F protein mRNA.

MATERIALS AND METHODS

Primer selection and synthesis. Sequence data on the F protein to BRSV RB 94 (22) and BRSV 391-2 (14) were

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compared; BRSV 391-2 was obtained from the GenBank genetic sequence data bank (accession number, M58350) (4). Conserved regions were analyzed as potential oligonucleotide primers or internal oligonucleotide probes with software specifically designed for primer selection (Nucit; Compuright, Washington Grove, Md.). The oligonucleotide primer pair and 20-mer internal hybridization probe were synthesized by using standard phosphoramidite chemistry on a DNA synthesizer (model 391 PCR-Mate; Applied Biosystems). Oligonucleotides were removed from columns with 100% ammonium hydroxide and heated at 55°C for 18 h to eliminate protective groups, dried under vacuum, and resuspended in 100 μl of water according to the manufacturer's instructions. Gel filtration on Sephadex G-50 columns (Nick Columns; Pharmacia LKB) were used to purify oligonucleotides.

Viruses. The BRSV strain 375 was the prototype BRSV in the initial testing of the RT-PCR protocol. This is a BRSV strain plaque purified more than three times that was originally obtained from cattle and is bovine viral diarrhea virus free (9, 13). Reference strains and field strains of BRSV and HRSV were purchased from the American Type Culture Collection (ATCC) (HRSV A-2 strain [ATCC VR-1302], HRSV Long strain [ATCC VR-26], HRSV 9320 strain [ATCC VR-955]) or were supplied by other laboratories. Virus isolate BRSV A51908 (ATCC VR-794) was supplied by J. Evermann, Washington Animal Disease Diagnostic Laboratory, Washington State University, Pullman. BRSV isolates 391-2 (originally isolated in North Carolina) and FS1-1 (originally isolated from cattle in Iowa) and HRSV isolates A2 (subgroup A) and B8/60 (subgroup B) were provided by L. Potgieter, University of Tennessee, Knoxville. The HRSV Long strain (designated HRSV Long strain-KSU) was provided by R. M. Phillips, Kansas State University, Manhattan. Viruses supplied by C. Kelling, University of Nebraska, Lincoln, were BRSV 236-652, originally recovered in Nebraska; BRSV 391-2, isolated in North Carolina; BRSV NDKS-7, isolated in North Dakota; BRSV 87-14594, isolated in South Dakota; BRSV 1344R, isolated in Colorado; BRSV 411-727, isolated in New York; HRSV CH18537 (subgroup B); and HRSV WV4843 (subgroup B).

Isolation of RNA. RSV strains were grown in bovine turbinate, Madin-Darby bovine kidney (MDBK), or HEp-2 cells in modified Eagle's minimum essential medium supplemented with 10% fetal calf or horse serum. Viruses were allowed to replicate and form the characteristic syncytia or cytopathic effect, at which point the RNA was isolated (5). Briefly, cells in 75-cm^2 flasks were rinsed with phosphate-buffered saline and then lysed in 1.0 ml of lysis buffer (4 M guanidinium isothiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). To each 500 μl of lysed cell suspension, 50 μl of 2 M sodium acetate (pH 4.0) was added and mixed with 500 μl of a buffered phenol-chloroform-isoamyl alcohol mixture (1:1:0.05). After 15 min on ice, the mixtures were centrifuged at 14,000 × *g* for 20 min, and the top aqueous phase was removed to a new microcentrifuge tube, mixed with 1.0 ml of 100% ethanol, stored at −70°C for 15 to 30 min, and centrifuged at 14,000 × *g* for 20 min. The resulting pellets were dissolved in 300 μl of lysis buffer and mixed with 750 μl of 100% ethanol, stored at −70°C for 15 to 30 min, and recentrifuged at 14,000 × *g* for 20 min. The pellet was washed in 300 μl of 80% ethanol, recentrifuged for 5 min, vacuum dried, and resuspended in diethylpyrocarbonate-treated water; then, the nucleic acid concentration was quantified (A_{260} to A_{280}).

RT-PCR protocol. The RSV mRNA was reverse transcribed to single-stranded cDNA and amplified by PCR as described by the manufacturer (GeneAmp RNA PCR kit; Perkin-Elmer Cetus) with a thermal cycler (GeneAmp PCR System 9600; Perkin-Elmer Cetus). Two micrograms of total nucleic acid from BRSV-infected cells served as the source of mRNA template for RT with 1 μl of the negative-sense primer (1 μM initial concentration). The final volume for the RT reaction mixture was 20 μl. Incubation temperatures and times for RT were 42°C for 3 min and 45 s, 99°C for 1 min and 15 s, and 5°C for 1 min and 15 s. The resulting cDNA was amplified by PCR by adding 1 μl of the positive-sense primer (1 μM initial concentration) and bringing the final volume for the PCR reaction to 100 μl as per kit instructions. Initial denaturing was for 30 s at 95°C, followed by 35 cycles (15 s at 95°C and 15 s at 60°C) and 1 cycle (1 min and 45 s at 60°C).

Electrophoresis and Southern blotting. Electrophoretic analysis was completed by electrophoresing 40 μl of each RT-PCR reaction mixture on polyacrylamide gels (4% stacking and 15% resolving gels) in Tris buffer (0.025 M Tris [pH 8.3], 0.192 M glycine) for 18 h at 60 V. The polyacrylamide gels were transferred to nylon paper (Zeta Probe; Bio-Rad) by electroblotting (Transblot; Bio-Rad) for 2 h at 75 V in 1× TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA [pH 8.0]).

The blots were hybridized overnight with internal 20-mer oligonucleotide probes that had been labeled with [τ-^{32}P]ATP by using T4 polynucleotide kinase. The hybridization buffer consisted of 10% dextran sulfate, 1% sodium dodecyl sulfate, and 1 M NaCl at T_h ($T_h = T_d - 10$°C where T_h is temperature of hybridization and T_d is temperature of denaturation). Washes consisted of 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (twice for 5 min at room temperature and twice for 30 min at T_h). Membranes were exposed to X-ray film at −70°C with intensifying screens.

Sequencing BRSV 375 RT-PCR product. The BRSV (strain 375) RT-PCR-amplified product was ligated into the pT7Blue(R) vector (Novagen, Madison, Wis.) and transformed into NovaBlue cells (Novagen). A colony that contained the amplified BRSV fragment was selected and sequenced as previously described (*fmole* DNA Sequencing System; Promega) with T7 and U19 sequencing primers.

RESULTS

With the aid of primer selection software, a primer pair was selected (positive-sense 5′-ttaccacacccctcagtaca and negative-sense 5′-cattgtgtcacagaacactc) that would encode a 381-bp fragment (nucleotides 741 to 1123) of the F_1 subunit of the BRSV (strain 391-2) F protein mRNA. The following internal oligonucleotide was selected as a probe: BRSV884-903 (positive-sense 5′-gtggtcaaagaagaggtcat; nucleotides 884 to 903; T_h = filter hybridization dissociation temperature of ≈52°C). The primer and probe sequences were identical to sequences previously identified within the BRSV strains RB94 (22), 391-2 (14), and ATCC A51908 (10).

RT-PCR amplification of BRSV (strain 375) mRNA with primers selected from sequence information to the F protein of BRSV 391-2 and RB94 resulted in a discrete fragment of ≈380 bp (Fig. 1a, lane H; Fig. 1b, lane A). When BRSV (strain 375) RT-PCR fragments were blotted and hybridized with the internal probe, a radiographic signal was apparent at the same location (Fig. 1a, lane H [inset]). Subcloning and sequencing the BRSV strain 375 RT-PCR fragment indicated that the insert was 381 bp and contained the same sequence as that described for BRSV 391-2 for nucleotides 741 to 1123 (14). In a sampling of other BRSV strains, including BRSV

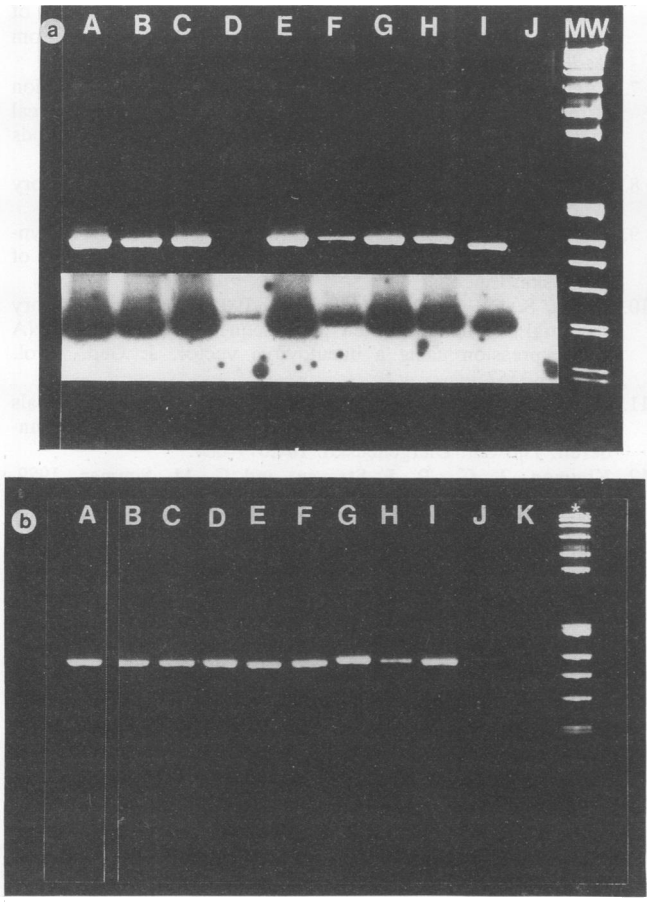

FIG. 1. (a) Comparison of amplification products obtained from RT-PCR of RNA from MDBK cells infected with different BRSV strains isolated from cattle at different times and from locations in the United States. The amplified products were electrophoresed on polyacrylamide gels and stained with ethidium bromide. (Inset) Hybridization of transferred DNA from the same gel with the ^{32}P-labeled BRSV oligonucleotide probe BRSV884-903. Lanes: A, BRSV 391-2; B, BRSV NDKS-7; C, BRSV 87-14594; D, BRSV 1344R; E, BRSV 411-727; F, BRSV isolate 391-2; G, BRSV isolate FS1-1; H, BRSV 375; I, BRSV A51908 (ATCC VR-794); J, uninfected MDBK cells; MW, molecular weight standard (1-kb DNA ladder; GIBCO-BRL, Gaithersburg, Md.). (b) Ethidium bromide-stained polyacrylamide gel. Lanes: A, BRSV 375; B, BRSV 236-652; C and D, BRSV 391-2; E, BRSV 411-727; F, BRSV NDKS-7; G, BRSV 87-14594; H, BRSV 1344R; I, BRSV isolate 391-2; J, BRSV isolate FS1-1; K, uninfected MDBK cells; *, molecular weight standard (1-kb DNA ladder). The figure is a composite of lanes from the same gel.

391-2, RT-PCR with the same primers resulted in the amplification of similar products of ≈380 bp (Fig. 1A and B). Hybridizations with the oligonucleotide probe resulted in a consistent radiographic signal of ≈380 bp in all of the BRSV strains examined (Fig. 1a, inset).

After BRSV RT-PCR on the RNAs from cultures infected with HRSV were completed, no products were evident at ≈380 bp. Reference strains of HRSV group A virus (Long and A2) that were amplified with the primers resulted in amplified products of ≈150 bp (Fig. 2, lanes A to E). Amplification and electrophoresis of HRSV group B (subgroup B1 and subgroup B2) resulted in different profiles. Amplification of subgroup B1 isolates (WV4843, B8/60, and

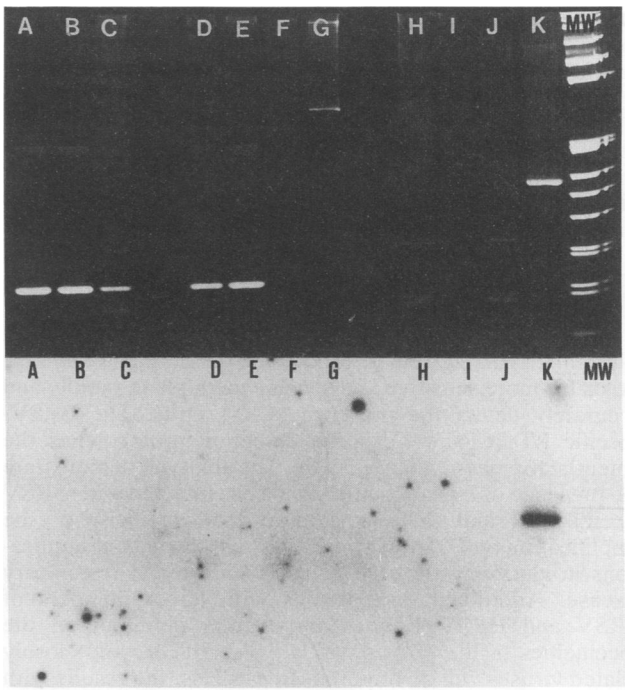

FIG. 2. Results of RT-PCR of RNA from HEp-2 cells infected with prototype strains of HRSV representing subgroups A and B. The amplified products were electrophoresed on polyacrylamide gels and stained with ethidium bromide. (Inset) Hybridization of transferred DNA from the same gel with the ^{32}P-labeled BRSV oligonucleotide probe BRSV884-903. Lanes: A, HRSV Long strain; B, HRSV Long strain; C, HRSV Long strain (ATCC VR-26); D, HRSV A2 strain; E, HRSV A-2 strain (ATCC VR-1302); F, HRSV WV4843; G, HRSV CH18537; H, HRSV B8/60; I, HRSV 9320 strain (ATCC VR-955); J, uninfected HEp-2 cells; K, BRSV A51908 (ATCC VR-794) grown in MDBK cells; MW, molecular weight standards (1-kb DNA ladder).

VR 9320) did not result in major amplification products (Fig. 2, lanes F, H, and I), whereas amplification of subgroup B2 (CH18537) resulted in a primary product of ≈900 bp (Fig. 2, lane G).

Blotting and hybridizing the HRSV RT-PCR products with the BRSV884-903 oligonucleotide probe resulted in no identifiable signal from any HRSV strain at ≈380 bp or elsewhere on Southern blots (Fig. 2, lanes A to J [inset]).

DISCUSSION

Specific RT-PCR primers that encode sequences to the F_1 subunit of HRSV F protein mRNA have been reported elsewhere for HRSV detection (15, 16); however, those primers were upstream from the BRSV primers described here, and comparisons with BRSV isolates were not made. In the present report, we describe the use of BRSV-specific primers in an RT-PCR that can specifically amplify a 381-bp sequence of BRSV F protein mRNA and an oligonucleotide probe (BRSV884-903) that is capable of specifically detecting the amplified cDNA products and distinguishing them from those of HRSV. This allows for the specific identification of BRSV F protein mRNA.

Important observations made from these data are as follows. (i) All BRSV strains studied gave RT-PCR-amplified products with the same size (≈380 bp), as determined by electrophoretic comparison. Sequencing BRSV (strain 375)-

amplified products confirmed the size to be 381 bp. (ii) The specificities of the RT-PCR-amplified products originating from BRSV templates were confirmed in hybridizations with a synthetic oligonucleotide probe complementary to an internal sequence of the predicted negative-sense strand of amplified DNA and by comparing the sequence of the BRSV strain 375 RT-PCR fragment with known sequences to BRSV strain 391-2 F protein mRNA. (iii) None of the HRSV strains that were tested, which included prototype strains of HRSV subgroups A and B, gave any indication of yielding RT-PCR products that would hybridize with the BRSV oligonucleotide probe BRSV884-903.

The confirmation and isolation of BRSV from cattle in naturally occurring field infections are difficult, and the need exists for more sensitive and specific methods to rapidly and accurately detect the presence of the virus. The BRSV-specific RT-PCR-hybridization detection protocol has the potential for meeting those needs. The ability to demonstrate the presence of F protein mRNA in cells infected with BRSV by RT-PCR and then to confirm the specificity of the amplification by DNA hybridizations has significant implications in clarifying the role of BRSV in bovine respiratory disease. Additional comparisons with larger numbers of BRSV and HRSV isolates and further definition of the specificities of the probes with BRSV, HRSV, and closely related viruses will be required. Regardless, the results with the viruses analyzed in this study indicate that a specific primer pair will amplify a BRSV-specific product, and the specificity of the amplification can be confirmed by hybridizing with a synthetic oligonucleotide probe.

An important application of this technology in the future will be the direct testing of nasopharyngeal aspirates, respiratory secretions, or lung tissues from cattle for the presence of BRSV. Nasopharyngeal aspirates were not assayed in this study; however, the screening of cattle for BRSV with sample collection and processing techniques, as recently reported for evaluating nasopharyngeal aspirates from infants and children with HRSV, is being evaluated by the investigators and offers hope for a rapid, yet specific and sensitive, diagnostic protocol for detecting BRSV.

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