Evaluation of a Nested Reverse Transcription-PCR Assay Based on the Nucleoprotein Gene for Diagnosis of Spontaneous and Experimental Bovine Respiratory Syncytial Virus Infections

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The first nested reverse transcription (RT)-PCR based on the nucleoprotein gene (n RT-PCR-N) of the bovine respiratory syncytial virus (BRSV) has been developed and optimized for the detection of BRSV in bronchoalveolar lavage fluid cells of calves. This test is characterized by a low threshold of detection (0.17 PFU/ml), which is 506 times lower than that obtained by an enzyme immunosorbent assay (EIA) test (RSV TESTPACK ABBOTT). During an experimental infection of 17 immunocompetent calves less than 3 months old, BRSV RNA could be detected up to 13 days after the onset of symptoms whereas isolation in cell culture was possible only up to 5 days. Compiling results obtained by conventional techniques (serology, antigen detection, and culture isolation) for 132 field samples collected from calves with acute respiratory signs revealed that n RT-PCR-N showed the highest diagnostic sensitivity and very good specificity. This n RT-PCR-N with its long period of detection during BRSV infection thus provides a valuable tool for diagnostic and epidemiological purposes.

Bovine respiratory syncytial virus (BRSV), like human respiratory syncytial virus (HRSV), is a pneumovirus in the Paramyxoviridae family (7). The epidemiology of BRSV is very similar to that of HRSV (34). Spontaneous infection in young cattle is frequently associated with severe respiratory signs (10, 31, 37), whereas experimental infection generally results in milder disease with slight pathologic changes (4). Diagnosis of BRSV infection is based solely on antigen detection and serology (3). The method of choice in dead cattle consists of the detection of BRSV antigens in pulmonary samples by immunofluorescence (20) or immunoperoxidase (23) technique. BRSV antigen detection is less frequently undertaken in live cattle. Nasal secretion sampling (NS), which is slightly invasive and currently used for human infants, has sometimes been described in calves (3). Bronchoalveolar lavage (BAL) (21, 33) can be more sensitive, since the lung is usually extensively infected in naturally occurring cases (39). BRSV lability makes routine isolation in cell culture laborious and time-consuming (21). To our knowledge, comparison of sampling of the upper respiratory tract and lower respiratory tract has only rarely been conducted in infants (9) and no comparison is available in cattle. As previously demonstrated, in young calves under 3 months, maternally transmitted immunoglobulin (Ig) G1 does not prevent BRSV infection but hinders serological diagnosis (21, 33). However, the combined use of serology, antigen detection, and culture, which could potentially increase the proportion of positive diagnoses, is laborious and expensive.

Reverse transcription (RT)-PCR is potentially useful for improving the sensitivity of RSV detection. Several RT-PCRs for HRSV detection based on the fusion (F) gene (17, 28), the 1B gene (36), the polymerase (L) gene (13), and the nucleoprotein (N) gene (8, 15) have been developed and have been evaluated in studies involving numerous field specimens. The sensitivity results are controversial. Some authors consider RT-PCR less sensitive than expected when compared with conventional techniques of HRSV detection (8, 13, 28), whereas for others, the sensitivity was slightly (36) or more-strongly improved (15, 17). The RT-PCR tests reported for BRSV are directed against F (25, 26, 38) and glycoprotein (G) (38) genes. None based on the N gene from BRSV has been described even though this is one of the most conserved of the pneumovirus genes (19). Furthermore, these BRSV studies involved small numbers of strains, diseased calves, and infected herds (26, 38).

The aims of this work were therefore (i) to evaluate the threshold of detection of a nested RT-PCR developed based on the N gene (n RT-PCR-N), (ii) to determine the duration of presence of the BRSV in BAL fluid during an experimental infection, and (iii) to evaluate the sensitivity and specificity of the RT-PCR obtained during a large field study and compare them with those of currently used laboratory techniques (serology, antigen assays, and isolation in cell culture).

MATERIALS AND METHODS

Animals. (i) Spontaneous infections. Between 1991 and 1998, 111 live calves and 21 dead calves (n = 132; average age, 58 days; minimum age, 6 days; and maximum age, 480 days) from 49 different herds were studied to compare sites of sampling and laboratory techniques used to diagnose BRSV infection. Calves were in cow-calf herds located in Belgium (9 herds) and in central and southwestern France (40 herds). Animals exhibited acute respiratory signs compatible with BRSV infection. Dead calves were necropsied within 36 h of death. (ii) Experimental infection. Seventeen male calves of PrimHolstein breed, with a BRSV-seronegative status, were reared in isolation. When they were 2 to 3 months old, they were used for challenge experiments after their BRSV serological status had been confirmed to be negative. Eleven calves were inoculated with BRSV (BRSV A2 gelfi), and six calves were inoculated with bovine turbinate (BT) cells free of virus (controls), on 3 consecutive days (D0, D1, and D2). Each day, half of a 20-ml portion of inoculum containing no BRSV or 2.5 × 10^7
PFU of BRSV was instilled intranasally and the other half was injected endo-
bronchially. BAL was performed at D2 on all the animals and then every 2 days for five calves from D4 to D18 and every 2 days for six calves from D5 to D19 and for sampling of the animals at D30 and D36 (clinical I). A standard clinical examination was carried out every day from D2 to D36, and the mean clinical score was calculated (12).

**Virral strains.** A field BRSV strain (A2 gelfi) isolated in 1994 during a BRSV outbreak in France involving adults and calves was propagated in BT cells in minimum essential medium with Earle’s salt and glutamine (MEM) (Gibco BRL, Cergy Pontoise, France) supplemented with 5% fetal calf serum (FCS). The sixth passage was aliquoted and stored at −80°C. The titer was 1.25 × 10^9 PFU/ml. This strain has previously provided the challenge inoculum and positive controls for PCR, isolation in cell culture, and antigen detection. The absence of common viral respiratory pathogens, including bovine viral diarrhea virus (BVDV), in the inoculum was checked.

BRSV isolates or strains W6 Toulouse, from France, VF160, 220/69, MRV533, 5761, and RB-94 from Belgium (kindly provided by G. Wellems, Levallois and V347, from The Netherlands (kindly provided by T. J. Kimman and R.S. Schrijver), and 127, from the United Kingdom (kindly provided by T. J. Kimman), were used as strains of BRSV representative of the diverse strains found in Europe (29).

Infectious bovine rhinotracheitis virus (IBRV), parainfluenza virus 3 (PI3), and BVDV strains isolated at the Toulouse Veterinary School were used to check n RT-PCR-N specificity.

**Clinical samples.** Clinical samples included serum, NS, BAL fluid cells, and lung samples. Two blood samples were collected at a 2- to 3-week interval, and the serum was stored at −20°C until analysis. Nasal cells and NS were collected with cotton swabs, placed in storage at 4°C less than 2 h after collection, and stored at −20°C until immunoassay (EIA) and cell cytocentrifugation for indirect immunofluorescence (IIF) were performed just after sampling. Slides for IIF were stored at 20°C until immunoassay.

BRSV isolates or strains were W6 Toulouse, from France, VF160, 220/69, MRV533, 5761, and RB-94 from Belgium (kindly provided by G. Wellems, Levallois and V347, from The Netherlands (kindly provided by T. J. Kimman and R.S. Schrijver), and 127, from the United Kingdom (kindly provided by T. J. Kimman), were used as strains of BRSV representative of the diverse strains found in Europe (29).

**Serology.** Paired serum samples were analyzed with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (LSI/VRS, LSI, L’arbresle, France). The percentage of animals with antibodies against BRSV was calculated.

**Antigen detection.** The detection of BRSV antigen was carried out by IIF (with the same antibodies as described for identification of BRSV on BT cells) or by a commercial EI, EIA RSV TESTPACK ABBOTT (EIA-TP) (Abbott, Rungis, France) which was based on cytopathic effect (CPE) in BS cells and cytoxicity of lung. For cytopin, BAL fluid cells were filtered through gauze, adjusted to a total number of 5 × 10^7, and cytocentrifuged (450 rpm for 8 min) by using a Cytospin 3 (Shandon, Pittsburgh, Pa.). EIA-TP was performed according to the manufacturer’s recommendations on BAL fluid cells contained in 10 ml of BAL fluid, on NS, or on clarified homogenate of lung used for culture isolation.

**RNA extraction.** The procedure for RNA extraction was adapted from a method previously described (6). RNA extractions were performed on BAL fluid cells contained in 10 ml of BAL fluid and on approximately 1 g of crushed lung sample. The RNA sample was first treated with a mixture of guanidine thiocyanate isothiocyanate (TRIZol Reagent; Gibco BRL). It was then thawed and incubated at room temperature for 5 min, and 250 g of chloroform was added to each sample. The tubes were centrifuged at 12,000 × g for 5 min at 4°C after vigorous mixing, and the supernatant was then allowed to stand at room temperature for 5 min. The supernatant was collected, and 1 ml of a more-concentrated solution of phenol and guanidine isothiocyanate (Trizol ± 2 Reagent; Gibco BRL) was added to each tube. After purification by chloroform (vol/vol) and DNA decontamination by isopropanol alcohol (1/10 volume), the RNA was precipitated in isopropanol (vol/vol) with 0.3 M sodium acetate (pH 5.2) (Sigma) and 2 μl of Pellet Paint Co-Precipitant (Novagen, Abingdon, England) overnight at 4°C. The sample was then centrifuged at 12,000 × g for 10 min (4°C), the supernatant was removed, and the pellet was washed in 1 ml of 70% ethanol. The sample was then centrifuged at 7,500 × g for 5 min at 4°C, the supernatant was removed, the pellet was dried for 10 min, and the RNA was resuspended in 36 μl of diethylpyrocarbonate-treated (DEPC) water.

**RT-PCR.** Primers used for n RT-PCR-N were based on a multiple alignment, performed by using the ClustalW 1.6 program (18), of available sequences of N genes of RSVs. These sequences were from the following strains: three BRSV strains Toulouse, from France (isolated in 1970, A51908 isolated in Maryland in 1975, and 391.2 isolated in North Carolina in 1985 (GenBank accession no. L27840, M35076, and S40504, respectively); one ovine RSV (GenBank accession no. U07233); and two HRSV strains representing the HRSV subtypes A and B, strain 18537 (GenBank accession no. M35076, respectively). PRIMER version 0.5 (22) was used for primer selection to ensure an optimal primer length of 20 nucleotides, to be compatible with the annealing temperature, and to have no more than eight bases of self-complementarity or no more than four bases of self-complementarity in the 3′ terminal matching region. Primers were selected according to the consensus sequence of BRSV strain in conserved regions with HRSHV strains and ovine RSV.

For RT, 1 μl (2 pmol) of primer N2.1 (5′-ATGGCTCTGAGCAAGGATCA-3′); positions 19 to 39 of the N coding region of the BRSV genotype 1 strain 9 μl of DEPC-treated water containing RNA was incubated at 68°C for 10 min and then at 58°C for 10 min and finally chilled on ice. Each tube received 4 μl of a solution containing each nucleotide triphosphate (10 mmol), 1 μl of RNAsin (40 U) (Promega, Charbonnières, France), 2 μl of dithiothreitol (0.1 M) (Gibco BRL), 4 μl of SuperScript TM II buffer (250 mM Tris HCl, 375 mM KCl, 15 mM MgCl2) (Gibco BRL), and 1 μl of SuperScript TM II (200 U) (Gibco BRL) and was incubated at 42°C for 50 min. The reverse transcriptase was inactivated by heating at 70°C for 15 min. The RNA-cDNA hybrids were diluted 10 times in DEPC-treated water. Ten microliters of diluted cDNA was mixed in a final volume of 100 μl with 5 μl of primer N2.1 (50 pmol) and 5 μl of primer N2.2 (50 pmol) (5′-TCTAGGTTTCTGTTGATCTCC-3′; positions 1054 to 1013 on the N coding region of the BRSV genome) (30) and used for the first round of PCR, which was carried out in a solution containing 200 μl of each nucleotide triphosphate, 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1 mM MgCl2, and 2.5 IU of AmpliTaq Gold polymerase (Perkin Elmer Cetus, Roissy-Charles de Gaulle, France) and which produced a final product of 1,034 nucleotides (nt), was performed in a GeneAmp PCR System 480 (Perkin Elmer Cetus) by using the following program: 94°C for 12 min followed by 35 cycles of denaturation at 94°C for 60 s, annealing at 58°C for 60 s, and elongation at 72°C for 90 s and ending with a final elongation for 10 min. Ten microliters of the PCR products diluted 10 times was used to perform the second round of PCR with the same mix but containing the internal primers N2.3 (5′-CATCTCAAAATGGTTTGAGTG-3′; positions 127 to 146 of the N coding region of the BRSV genome) and N2.4 (5′-TCTACAACCTGTTCCATTTC-3′; positions 857 to 838 on the N coding region of the BRSV genome) (30). The second round of PCR amplified 731 nt and used the following program: 94°C for 12 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s, and elongation at 72°C for 90 s and ending with a final elongation for 10 min. The PCR products were detected by electrophoresis on a 2% agarose gel containing ethidium bromide (0.1 μg/ml).

**RESULTS**

Threshold of detection of n RT-PCR-N. In order to improve the detection in BAL samples, an n RT-PCR-N was developed to amplify a region of the N gene. Different preliminary tests concerning the number of PCR cycles (it was found that 35 cycles produced better results than 25) and hybridization temperatures (it was found that for the first step 58°C produced better results than 56 and 60°C and for the second step 49°C produced better results than 50 and 52°C) were performed to

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Table 1. Comparison of positive results obtained by n RT-PCR performed by using the N gene, isolation in cell culture, and IIF of BAL cells collected at various times after experimental infection of calves with BRSV

<table>
<thead>
<tr>
<th>Method</th>
<th>0/11</th>
<th>11/11</th>
<th>11/11</th>
<th>11/11</th>
<th>11/11</th>
<th>7/11</th>
<th>7/11</th>
<th>2/11</th>
<th>2/11</th>
<th>0/11</th>
<th>0/5</th>
<th>0/3</th>
</tr>
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<tbody>
<tr>
<td>n RT-PCR</td>
<td>0/6</td>
<td>6/6</td>
<td>6/6</td>
<td>3/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Isolation on cell culture</td>
<td>0/6</td>
<td>11/11</td>
<td>11/11</td>
<td>11/11</td>
<td>4/11</td>
<td>0/11</td>
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<td>0/11</td>
<td>0/5</td>
<td>0/5</td>
<td>0/3</td>
</tr>
<tr>
<td>IIF</td>
<td>0/6</td>
<td>6/6</td>
<td>6/6</td>
<td>3/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>0/6</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

ND, not done.

TABLE 2. Comparison of BRSV diagnostic assays on field samples from calves with clinical signs or lung lesions compatible with BRSV infection

<table>
<thead>
<tr>
<th>Laboratory techniques</th>
<th>Type of sample</th>
<th>No. of animals</th>
<th>No. of samples with indicated result for assay 1/assay 2</th>
<th>Prevalence according to assay 1 (%)</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>PPV (%)</th>
<th>NPV (%)</th>
</tr>
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<tbody>
<tr>
<td>Assay 1</td>
<td>Assay 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>EIA-TP</td>
<td>EIA-TP</td>
<td>BAL fluid cells/NS</td>
<td>83</td>
<td>15</td>
<td>6</td>
<td>0</td>
<td>62</td>
<td>25.3</td>
</tr>
<tr>
<td>EIA-TP</td>
<td>Serologyb</td>
<td>BAL fluid cells/serum</td>
<td>83</td>
<td>8</td>
<td>13</td>
<td>2</td>
<td>60</td>
<td>25.3</td>
</tr>
<tr>
<td>EIA-TP</td>
<td>IIF</td>
<td>BAL fluid cells</td>
<td>83</td>
<td>19</td>
<td>2</td>
<td>1</td>
<td>61</td>
<td>25.3</td>
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<tr>
<td>IIF</td>
<td>Lung</td>
<td>16</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>87.5</td>
<td>100.0</td>
</tr>
<tr>
<td>IIF</td>
<td>Serologyb</td>
<td>BAL fluid cells/serum</td>
<td>83</td>
<td>7</td>
<td>13</td>
<td>3</td>
<td>60</td>
<td>24.1</td>
</tr>
<tr>
<td>IIF</td>
<td>Culture</td>
<td>Lung</td>
<td>13</td>
<td>1</td>
<td>10</td>
<td>0</td>
<td>2</td>
<td>84.6</td>
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<tr>
<td>Culture</td>
<td>EIA-TP</td>
<td>BAL fluid cells</td>
<td>28</td>
<td>19</td>
<td>1</td>
<td>0</td>
<td>8</td>
<td>71.4</td>
</tr>
<tr>
<td>n RT-PCR</td>
<td>EIA-TP</td>
<td>BAL fluid cells</td>
<td>28</td>
<td>19</td>
<td>4</td>
<td>0</td>
<td>5</td>
<td>82.1</td>
</tr>
<tr>
<td>n RT-PCR</td>
<td>Lung</td>
<td>21</td>
<td>17</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>90.5</td>
<td>89.5</td>
</tr>
</tbody>
</table>

a When different samples were used, they are indicated as follows: sample type of assay 1/sample type of assay 2.
b Serology was considered positive when a significant increase of antibodies for the second of two blood samples collected at a 2- to 3-week interval was observed.
c Obtained for assay 2 when assay 1 is taken as the reference.
first serum sample were shown to be positive by EIA-TP or by IIF on BAL fluid cells. The sensitivity of detection of antigen on BAL fluid cells (IIF or EIA) was significantly higher ($P < 0.05, \chi^2$ test) than that of serology (Table 2). Moreover, three calves were positive by serology assay and negative by antigen detection (Table 2). IIF and EIA-TP with BAL fluid cells gave similar results except for three calves (Table 2). In contrast to the calf positive only by IIF, the only two calves that were positive by EIA-TP could be considered true positives according to their serological results or by the detection of BRSV in other calves from the same herd.

For 28 field BAL fluid samples, the comparison of cell culture and EIA-TP gave similar results (Table 2). It might seem that the highest sensitivity was obtained by isolation on cell culture followed by EIA-TP but these results were not significantly different ($\chi^2$ test). The results obtained with necropsy specimens of lungs were slightly different from those obtained during the life of the calves. BRSV detection, with 16 lungs, by IIF and by EIA-TP gave identical results (Table 2). However, isolation on cell culture had a significantly lower sensitivity than IIF and EIA-TP ($P < 0.01, \chi^2$ test) and a low NPV (Table 2).

The nested RT-PCR was then compared to EIA-TP (28 BAL fluid cell specimens and 21 lungs) and to isolation on cell culture (28 BAL fluid cell specimens and 13 lungs) for 49 naturally occurring cases of respiratory disease in young cattle (Table 2). For BAL fluid cells, results were in concordance except for four samples which were positive by n RT-PCR-N and negative by EIA-TP. Three of them were also negative by isolation on cell culture. Nineteen of 21 lungs were found to be positive by n RT-PCR-N; among them 17 were simultaneously identified as positive by EIA-TP.

Isolation on cell culture was also attempted on 13 of the 21 lungs. Eleven of these were simultaneously positive by IIF, EIA-TP, and n RT-PCR-N. However, BRSV was isolated from only one of these lungs. Compared to n RT-PCR-N, the sensitivity of isolation on cell culture was 0.87 for BAL fluid cells and 0.09 for lung.

The spectrum of detection of the n RT-PCR-N was also verified by using nine other field isolates (W6, FY 160, 220/69, MRV533, 5761, BB-94, Lelystad, V347, and 127) originating from three European countries. All of them were correctly amplified. The specificity of this n RT-PCR assay was also tested by using different pathogens (IBRV, PI3, and BVDV) not related to BRSV and associated with common respiratory disorders. The results were negative.

**DISCUSSION**

Differences in prevalence (ranging between 25.3 and 90.5%) were observed in our field study depending on the series of calves studied. These differences can be explained by numerous factors, such as choice of herds and animal inclusion or diagnostic tools.

Nasal swabs and, even more often, nasopharyngeal aspirates rather than BAL fluid are commonly used to detect HRSV in infants (1). However, in BRSV infections (this study) as in HRSV infections (9), the sensitivity of EIA-TP on BAL fluid seems higher. Several hypotheses can be put forward. RSV is highly conserved and sequences differed by at least 1 nucleotide (not shown). This high conservation of the N gene in HRSV (19) and BRSV (2, 30) enabled us to define efficient and conserved primers. We therefore developed and optimized a nested RT-PCR whose target was the N gene to ensure high sensitivity and specificity. The ability of this n RT-PCR-N to detect a very small amount of virus was demonstrated by the very low threshold of detection (0.17 PFU/ml). The duration of the detection of BRSV in BAL fluid cells was investigated during an experimental infection of calves, which developed moderate to severe clinical signs. The duration of shedding obtained by n RT-PCR-N (13 days) was approximately more than twice that by isolation on cell culture or by IIF (5 days). Other authors reported that the detection of BRSV by isolation in cell culture (4, 32, 35) or by n RT-PCR targeting the F gene (11, 38) for more than 6 days was not possible. According to our data, the period of shedding of BRSV in calves is thus similar to that of HRSV in infants (8). Whether BRSV can be detected for as long as HRSV in immunocompromised infants (14, 16) is still open to debate.

The sensitivities and specificities of different laboratory techniques were also analyzed by using a series of clinically and epidemiologically identified field specimens. The sensitivity of n RT-PCR-N was higher than those of all the other techniques tested. We did not find any specimen identified as positive by conventional assay and negative by RT-PCR-N, in contrast to other reports for HRSV (13, 15, 17). These results could be linked to the elimination of potential inhibitory factors or to the fitness of the primers to the RNA template. On the other hand and despite potential RNA degradation, identification of BRSV RNA on necropsied lung samples was possible until 36 h after death. The specificity of n RT-PCR-N was checked. No amplification could be observed when n RT-PCR-N was performed by using control BAL fluid cells in the experimental model or the different common respiratory pathogens. Moreover, the sequences of portions of amplified N, G, and F genes were determined for each of 13 instances of discordance between results of n RT-PCR-N and isolation from BAL fluid cells or lungs. Pairwise comparisons indicated that these sequences differed by at least 1 nucleotide (not shown). This strongly supports the idea that a different BRSV isolate is present in each of the specimens.

In conclusion, the n RT-PCR presented in this study seems to be more sensitive than and at least as specific as IIF, EIA, or isolation in cell culture. It will allow (i) a broadening of the
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REFERENCES


