Short communication

Detection of Brazilian bovine respiratory syncytial virus strain by a reverse transcriptase-nested-polymerase chain reaction in experimentally infected calves

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Abstract

A reverse transcriptase (RT)-nested-polymerase chain reaction (PCR) was standardised to detect bovine respiratory syncytial virus (BRSV), using a Brazilian isolate, in three experimentally infected calves. This followed initial tests in infected chicken embryo related (CER) cells. One animal had lesions, characterized by interstitial multifocal pneumonia, severe interstitial and subpleural emphysema, and lung consolidated areas. Lung and tracheal tissues collected 6 days after infection were analysed by RT-nested-PCR. Primers, specific for the BRSV G and F glycoproteins genes, yielded amplification fragments of 371 and 481 bp, respectively, from the RNA of the cell-propagated virus. Using RNA extracted from organs of infected calves, RT-nested-PCR amplified the fragment of the G gene in all tracheal samples, but in only two of three lung samples analysed. These results suggest that RT-nested-PCR could be a promising assay for diagnosis and epidemiological analysis of BRSV in Brazil.

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1. Introduction

Bovine respiratory syncytial virus (BRSV), which belongs to the *Pneumovirus* genus of the Paramyxoviridae family, is an important pathogen of the respiratory tract in cattle. Laboratory diagnosis of infections, caused by respiratory syncytial viruses, is often difficult owing to their limited growth in cell cultures, and in most experimental animals, and also because of their instability. Currently, the diagnosis of BRSV infections is most commonly done by detecting specific antibodies in serum (Westenbrink et al., 1985; Westenbrink and Kimman, 1987). However, the time needed to obtain the results (Dubovi, 1993), and the fact that some infections are serologically undetectable (Collins et al., 1996, pp. 1313–1351), means that there is need for a quick, sensitive and specific method for the diagnosis of BRSV infections.

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BRSV is widely distributed in many countries (Baker et al., 1986; van der Poel et al., 1993; Paton et al., 1998; Obando et al., 1999; Norström et al., 2000; Sorden et al., 2000; Utenthal et al., 2000; Wolde-meskel et al., 2000), including Brazil, with the first virus detection in 1993, by immunofluorescence test in trachea and lung tissues, and the first virus isolation in 1995, from nasotracheal secretions of calves, from the southern and southeastern provinces of the country (Gonçalves et al., 1993; Arns et al., 2003). Little, however, is known about the role of BRSV in respiratory disorders in Brazilian cattle. Moreover, although serological tests for BRSV show positivity (Gonçalves et al., 1993), not much is known about the real distribution and participation of this virus in production loss in Brazilian herds. In this work, a RT-nested-PCR was developed to detect BRSV in cultured cells and in tissues of experimentally infected calves. This assay may be useful in the diagnosis of BRSV, and in epidemiological studies of this virus in Brazilian cattle.

2. Methods and results

Monolayers of chicken embryo related (CER) cells were inoculated with Brazilian strain BRSV-25-BR (10^4.3 DICC50/ml), isolated in the Laboratório de Virologia Animal of the Instituto de Biologia at UNICAMP (Arns et al., 2003). RNA was extracted from infected cultures after 2 days using phenol guanidine isothiocyanate (TRIzol, Invitrogen™, Carlsbad, CA, USA) and chloroform. Total RNA was precipitated with the same volume of 100% isopropanol, followed by centrifugation. The pellet was washed with 70% ethanol and diluted in 25 µl of water containing 0.1% diethylpyrocarbonate (DEPC; Sigma–Aldrich, St. Louis, MO, USA). Negative controls were mock-infected cells. The RT-nested-PCR was standardized to amplify a 481 bp fragment corresponding to part of the F gene and/or a 371 bp fragment corresponding to part of the G gene of BRSV. The outer and inner primers were those described by Vilcek et al. (1994). cDNA was synthesized using a commercial SuperScript™ II Rnase H Reverse Transcriptase kit (Invitrogen™, CA, USA). The final reaction volume of 50 µl consisted of 5 µl of 10 X amplification buffer, 2 µl of each dNTP (10 mM); 10 pmol of primer B1 (F gene) or B5A (G gene), 10 pmol of primer B2A (F) or B6A (G), 0.6 µl of the enzyme Platinum™ Pfx DNA Polymerase (2.5 U/µl; Invitrogen™, CA, USA), 1 µl of MgSO4 (50 mM); 2 µl of cDNA and water to complete the final volume. After an initial denaturation at 94 °C for 2 min, 25 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min. The second PCR was done using 2 µl of the first PCR product, with the primers now being B3 and B4A (F) or B7A and B8 (G), for 35 cycles that included denaturation at 94 °C for 30 s, annealing at 62 °C for 45 s and elongation at 72 °C for 1 min. A final elongation step of 7 min was done in both PCRs.

To guarantee that the amplicons from the RT-nested-PCR, standardized with the virus multiplied in vitro, corresponded to the F and G genes of BRSV, the fragments were sequenced in an automatic sequencer ABI 377-96 (Perkin-Elmer, CA, USA). The sequences confirmed that the fragments were from the F and G genes.

To assess sensitivity of the RT-nested-PCR, the cDNA synthesized for F and G genes was quantified spectrophotometrically, serially diluted (10^-1 to 10^-3) and then, amplified by RT-nested-PCR. The assay amplified the fragment corresponding to the G gene in both pure material and material diluted 10^-2 (containing 0.024 µg of cDNA), while the fragment corresponding to protein F was only amplified from pure (undiluted) material (2.45 µg; Fig. 1).

Three 5-month-old calves (nos. 139, 151 and 165), seronegative for BRSV, were anaesthetised with 1% acepromazine (0.1 mg/kg) and intratracheally inoculated with 20 ml of viral antigen containing 10^4.3 DICC50/ml of virus. Macroscopic lesions, compatibles with BRSV-infection, were seen in two calves (nos. 139 e 165). The lesions present in calf no. 139 were mild, characterised by interstitial pneumonia and emphysema. Calf no. 165 showed more severe interstitial multifocal pneumonia, severe interstitial and subpleural emphysema, and consolidated lung areas (Fig. 2). Lung and trachea tissues from the calves were analysed by RT-nested-PCR using the same protocol, but only with primers directed to the gene for G protein. All three tracheal samples were successfully amplified, whereas only two lung samples (from calves 139 to 151) were positive (Fig. 3).
3. Discussion

Brazil has the largest commercial bovine herd of the world, with 180 millions of animals, and production of around 7 millions ton of meat (Confederação da Agricultura e Pecuária do Brasil, 2003) and more than 20 million litres of milk per year (Instituto Brasileiro de Geografia e Estatística, 2003). Therefore, there is clear interest on the health of the Brazilian herd. Very little is known, however, about the significance of BRSV infection in Brazil. This is probably because there are very few Brazilian research groups working with this virus, and due to the mistaken belief that losses caused by BRSV are not a significant problem in extensive production. Nevertheless, serological analysis of Brazilian cattle indicated that BRSV has already infected 75% of the bovines on 65 farms in several southern Brazilian provinces (Campalans and Arns, 1997), and >95% of Brazilian cattle ≤3 years-old, with 70% of the calves being infected within the first year of life (Gonçalves et al., 1993). The mortality rate for confirmed acute infections ranged from 5 to 20% (Campalans and Arns, 1997). BRSV was isolated for the first time, in

Fig. 1. Sensitivity of the RT-nested-PCR. Lane 1, pure gene F cDNA (1225 g/ml); lane 2, gene F cDNA diluted 1:10; lane 3, gene F cDNA diluted 1:100; lane 4, gene F cDNA diluted 1:1000; lane M, molecular weight marker; lane 5, pure gene G cDNA (1197.5 g/ml); lane 6, gene G cDNA diluted 1:10; lane 7, gene G cDNA diluted 1:100; lane 8, gene G cDNA diluted 1:1000.

Fig. 2. Macroscopic lesions in experimentally infected calves. (A) Interstitial pneumonia and subpleural emphysematous bullae, (B) areas of emphysema, (C) consolidated areas.
southern Brazil, in 1995 from nasotracheal secretion samples of calves (Arns et al., 2003). So far, only one strain of the virus had been isolated in this country.

As shown here, the experimental infection of calves, done for the first time in Brazil, resulted in BRSV disease in one of three inoculated animals. An RT-nested-PCR, standardised using G gene as the target, was able to detect BRSV in bovine tissue samples. The sensitivity of the RT-nested-PCR was at least 100 times greater in amplifying the G gene fragment compared to the F gene fragment. Although there are no reports of similar results for PCRs used to detect RSV, this phenomenon may reflect inherent properties of the primers, annealing temperature, G–C content of the target sequences, and secondary structures of the RNA, among others. Even so, more studies need to be done. Based on the difference in sensitivity, and on the fact that positive results were obtained only with primers to the G gene in Balb/c mouse tissue (Almeida et al., 2004), only G gene was used as a target for RT-nested-PCR in bovine tissues since the technique was more sensitive and, consequently, more useful as a diagnostic tool.

The RT-nested-PCR amplified a fragment the G gene in all bovine tracheal samples, but in only two of the three lung samples. Interestingly, the animal with a respiratory syndrome, characterized by signs and lesions compatible with a BRSV infection, was the only one in which it was not possible to detect the virus in lung tissue. In some cases, respiratory signs may persist, or there may be serological evidence of the virus without detection of BRSV (Vilcek et al., 1994; Belknap et al., 1995). In addition, BRSV may not be detected by PCR, even when isolated (West et al., 1998). These data suggest that antigen detection may only occur during a short period that coincides with the acute phase of the respiratory disease (Vilcek et al., 1994; Belknap et al., 1995). Valarcher et al. (1999) detected BRSV by an RT-nested-PCR for the N gene, up to 13 days post-infection, in bronchoalveolar lavage fluid of experimentally infected calves with moderate to severe clinical signs. Similarly, West et al. (1998) detected the virus up to the 8th day post-infection in nasal swabs from experimentally infected calves, and found the PCR to be more sensitive later in the infection (6–8 days post-infection), showing the same clinical situation with RT–PCR for the F gene. In contrast, others have been unable to detect BRSV by RT–PCR of the F gene for more than 6 days after infection (Elvander, 1996). Moreover, respiratory disease caused by BRSV enhances bacterial colonization and adherence, and alters the specific and non-specific defence mechanisms of the respiratory tract (Larsen, 2000). Thus, an animal with intense signs of respiratory disease may have secondary infections by bacteria, the presence of which may inhibit the PCR reaction and make it difficult to detect BRSV in lung tissue. Another explanation could be virus lability,
since the tissues travelled 24 h in regular ice before arriving in our laboratory.

In conclusion, an RT-nested-PCR was standardised for the detection of BRSV in tracheal and lung tissues of experimentally infected calves. This technique may be promising for the detection and diagnosis of natural BRSV infections in cattle, as well as for epidemiological studies. The information, thus, gained will increase the knowledge of the distribution of this virus in the Brazilian bovine herd.

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References


