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Original article

Detection of bovine respiratory syncytial virus in experimentally infected balb/c mice

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Abstract – The present study used an RT-nested-PCR and an immunohistochemistry assay to detect bovine respiratory syncytial virus in tissues from experimentally infected balb/c mice. As a first step, Chicken Embryo Related (CER) cell monolayers infected with the BRSV-25-BR strain isolated in Brazil were used for antigen production. Then, the infected lung and tracheal tissues of female balb/c mice were collected on 3, 5, 7 and 10 days post-infection and submitted to both techniques. Primers specific to F and G genes that amplify fragments of 481 bp and 371 bp, respectively, were used. The BRSV detection was not successful in all of the animals tested. The genomic fragment of the G gene from the organs of some infected mice on all analyzed post-infection days was amplified. However, in the RT-nested-PCR corresponding to the F gene, it was not possible to observe any amplified fragment. This was probably due to the higher sensitivity of the developed technique to amplify the fragment corresponding to the G gene compared to the F gene. Moreover, only three of the lungs collected five days post-infection were positive by immunohistochemistry. To the author's knowledge, this is the first study reporting bovine respiratory syncytial virus detection in balb/c mice after experimental inoculation.

bovine respiratory syncytial virus / RT-nested-PCR / immunohistochemistry / mice / experimental infection

1. INTRODUCTION

The bovine respiratory syncytial virus (BRSV) is an agent that belongs to the Paramyxoviridae family, the Pneumovirus genus, being one of the pathogens of higher importance in respiratory diseases [23], and responsible for significant economic losses in commercial cattle production [7]. Specific antibodies for the BRSV are more frequently detected in animals up to six months of age [18]. In individuals of two weeks to three months of age, the clinical manifestation of the illness is inversely related to the level of maternal specific serum antibodies conferred to these animals [16]. These maternal antibodies also interfere with the serological diagnosis and with the response to vaccination.

Laboratory diagnosis of BRSV is generally based on criteria such as virus isolation, BRSV antigen detection in the suspected

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tissues, serologic methods or histopathological examination. There are however, frequent difficulties related to this due to its limited growth in cell cultures or in the majority of experimental animals, and to the lability of the virus. Hence, the isolation of the virus in cell cultures is a procedure less indicated for the diagnosis of BRSV infections, even in clinical samples containing high virus concentration.

Currently, the diagnosis of BRSV infections is more commonly performed through the detection of specific antibodies by sero-diagnostic methods such as the serum neutralization test, complement fixation test, indirect immunofluorescence test and ELISA [29, 30]. Although the serum neutralization test is the standard gold test for the detection of specific antibodies against BRSV, difficulties arise regarding the time needed to obtain the results, which takes an average of 5 to 7 days [12]. Furthermore, there are reports of asymptomatic infections by the Human Respiratory Syncytial Virus (HRSV), which are serologically undetectable [10]. In analogy to the HRSV, since BRSV and HRSV are antigenetically related of the up to 80% in proteins N, M, M2 and F [31], is possible to speculate that part of the infections by BRSV cannot be diagnosed through the detection of specific serum antibodies. This probably occurs due to the simple fact that these animals do not develop antibodies in levels high enough to be detected by conventional techniques. In addition, there is evidence indicating the existence of latent infections, since the virus has been isolated from asymptomatic animals, as well as from animals infected seven months before the appearance of the symptoms [1].

There is an urgent need, therefore, for a quick, sensitive and specific method for the diagnosis of infections by the BRSV virus. Several authors have been working on the development of techniques to simplify and to increase the sensitivity of BRSV infection diagnosis. The reverse transcription-PCR assay has shown high speed, sensitivity and specificity in great parts of these works [20–22, 27, 28], providing a valuable tool for diagnostic and epidemiological purposes.

Experimental animals represent a useful alternative for experiments with viruses and other microorganisms, mainly when it is not possible to work with their natural host. Collins et al. [10] have pointed out that a wide range of animals, including marmosets, nonhuman primates, ferrets, mink, chinchillas, guinea pigs, cotton rats and mice, could be experimentally infected when RSV is inoculated directly into the respiratory tract. Small experimental animals have been used as models of lower respiratory tract infection with RSV [5, 8, 9, 15, 17, 19, 24, 25]. The present study detected BRSV in experimentally infected balb/c mice by RT-nested-PCR and immunohistochemistry.

2. MATERIALS AND METHODS

2.1. Virus

The Brazilian BRSV-25-BR strain, isolated in the Laboratorio de Virologia Animal do Institute de Biologia da Universidade Estadual de Campinas (UNICAMP) [2], was used in the present study. This strain was isolated from samples of nasotracheal secretions collected from live animals from the Southern and Southeastern Brazilian States, inoculated successively in MDBK (Madin Darby Bovine Kidney) and CER (Chicken Embryo Related) cells.

2.2. Cells

The cell cultures used were from the continuous cell line of CER. The cells were cultivated in bottles of 75 cm^2, with 1.5×10^5 cells/mL (initial concentration) in minimal essential Eagle medium (MEME) supplemented with 10% BRSV-free fetal calf serum (FCS) and kept at 37 °C.

2.3. Antigen production

For the production of the viral antigen used in the mice infection and submitted to the extraction of the viral RNA for RT-nested-PCR, cellular monolayers, were inoculated with $10^{4.3}$ $DICC_{50}$/mL of the BRSV-25-BR strain. The bottles were incubated at 37 °C and the cells were observed daily under an inverted light microscope (Axiovert 100, Carl Zeiss, Oberkochen, Germany) in order to detect a cytopathic effect (CPE). Then, the infected cells were submitted to RNA extraction or scraped and the supernatant was clarified through centrifugation, aliquoted and stored at –70 °C until use in the mice infection.

2.4. Animal infection

During the experiment, all mice were kept in the laboratory animal house of the Departamento de Microbiologia e Immunologia do Instituto de Biologia da UNICAMP. The infected animals were installed separately from negative control animals.

Four-week-old specific-pathogen-free (SPF) female balb/c mice, purchased from the Centro Multidisciplinar de Investigação Biológica of UNICAMP (CEMIB-UNICAMP), kept behind barriers, were used in this study. The animals were distributed into two groups, one containing 16 animals and the other, a negative control, containing 8 animals. All animals of the first group were intranasally inoculated with 100 µL of the viral antigen with an infectious dose of $10^{4.3}$ $DICC_{50}$/mL, using a micropipette. The negative control animals received an amount of supernatant CER cells free of virus. The animals were observed daily for clinical signs. The samples of lung and tracheal tissues of the infected (4 animals) and control group (2 animals), collected 3, 5, 7 and 10 days post-infection (dpi), were submitted to immunohistochemistry and RT-nested-PCR assays. These inoculations were performed in three repetitions, in different periods of time, resulting in a total of 48 virus-infected and 24 mock-infected animals.

2.5. Histopathology

Tissue samples were fixed in 4% paraformaldehyde (Sigma-Aldrish Co., Missouri, USA), embedded in paraffin wax, cut in 4 µm thick sections, and mounted on microscope slides. For a routine histological examination, the sections were stained with haematoxylin and eosin (HE). The same protocol was used for section preparation for the immunohistochemistry assay.

2.6. Immunohistochemistry (IHC)

Immunohistochemistry was carried out at the Royal Veterinary and Agricultural University, Copenhagen, Denmark. Briefly, the sections were de-paraffinated by heating up to 70 °C for 10–15 min and baths of xylene and ethanol. After a pre-treatment with 0.018% protease for 5 min at room temperature, the sections were blocked with 5% normal pig serum and incubated overnight at 4 °C with a polyclonal bovine-serum against BRSV in a 1:600 dilution (primary antibody). After this, the sections were incubated with 0.9% streptavidin (DAKO, Glostrup, Denmark) and following with 0.9% biotinylated alkaline phosphatase for 30 min at 37 °C (DAKO). Fast red (KemEnTec, Copenhagen, Denmark) was used for 10–15 min to visualize the positive reactions. The sections were counter-stained with Harris haematoxylin and mounted in Glycergel (DAKO) for light microscopy.

2.7. RNA extraction

The RNA extraction from approximately 100 mg of tissue samples was carried out using phenol guanidine isothiocyanate (TRIzol, Invitrogen^{TM}, Carlsbad, California, USA) and chloroform. The precipitation of the total RNA was performed with 100% ethanol followed by centrifugation. The pellet was washed with 70% ethanol and diluted in 25 µL of water containing 0.1% of diethyl pyrocarbonate (DEPC, Sigma-Aldrish Co., Missouri, USA).

Positive controls have been processed using the virus multiplied in CER cell cultures. The RNA extracted from non-infected mice tissue was used as the negative control.

2.8. RT-nested-PCR

The technique was standardized to amplify a fragment of 481 bp corresponding to a part of the F gene and 371 bp corresponding to a part of the G gene of the BRSV, using outer and inner primers described by Vilcek et al. [28].

The different RT-nested-PCR steps, from RNA extraction until the second amplification round, have been processed in different rooms to avoid contamination problems.

2.8.1. cDNA synthesis

For the synthesis of the cDNA, the commercial kit, SuperScript™ II Rnase H Reverse Transcriptase (Invitrogen™, California, USA) was used according to the manufacturers' recommendations and primers B2A (F gene) or B6A (G gene) designed by Vilcek et al. [28].

2.8.2. PCR reactions

For a final volume of 50 µL, the following reagents were added in a PCR tube: 5 µL 10× amplification buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl]; 2 µL dNTP 10 mM; 1 µL primer B1 (F gene; 10 pmoles) or B5A (G gene; 10 pmoles); 1 µL primer B2A (F) or B6A (G); 0.6 µL of the enzyme Platinum™ *Pfx* DNA Polymerase 2.5 U/µL (Invitrogen™, California, USA); 1 µL of $MgSO_4$ (50 mM); 2 µL cDNA and sterilized distilled water with DEPC until reaching the final volume. The mixture was heated for 2 min at 94 °C for the initial denaturation. The amplification was carried out in a thermal cycler PCR System 9700 (Gene Amp, Applied Biosystems, Perkin-Elmer, California, USA). The cycling program consisted of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and elongation at 72 °C for 1 min. Twenty-five cycles of amplification were carried out. The second PCR was done using 2 µL of the first PCR product, substituting the primers for B3 and B4A (F gene) or B7A and B8 (G gene). After initial denaturation at 94 °C for 2 min, the thermal cycle consisted of denaturation at 94 °C for 30 s, annealing at 62 °C for 45 s and elongation at 72 °C for 1 min. Thirty-five cycles of amplification were carried out. The last cycle of elongation was prolonged for 7 min in both PCR reactions.

RT-nested-PCR reactions were submitted to electrophoresis in 0.7% agarose gel containing 0.5 mg of ethidium bromide/mL, run at 110 V for 60 min. The molecular sizes of the products were compared with those of a 50 bp ladder (Invitrogen™, California, USA). The ethidium bromide-stained bands were visualized by UV light and recorded with the equipment Image Master VDS (Amersham Biosciences, Buckinghamshire, England).

2.9. RT-nested-PCR sensitivity analysis

The cDNA synthesized from viral RNA extracted from infected CER cells, both for the F gene and G gene, was quantified by spectrophotometry, submitted to serial dilutions (10^{-1} to 10^{-3}) and amplified by RT-nested-PCR.

3. RESULTS

3.1. Animal infections

The balb/c infected mice did not present apparent clinical signs during this experiment. However, macroscopic lesions were observed in the pulmonary tissue of one of the infected and sacrificed animals with 10 dpi, characterized for intense hemorrhage. Histopathological analysis demonstrated infiltration of mononuclear cells in

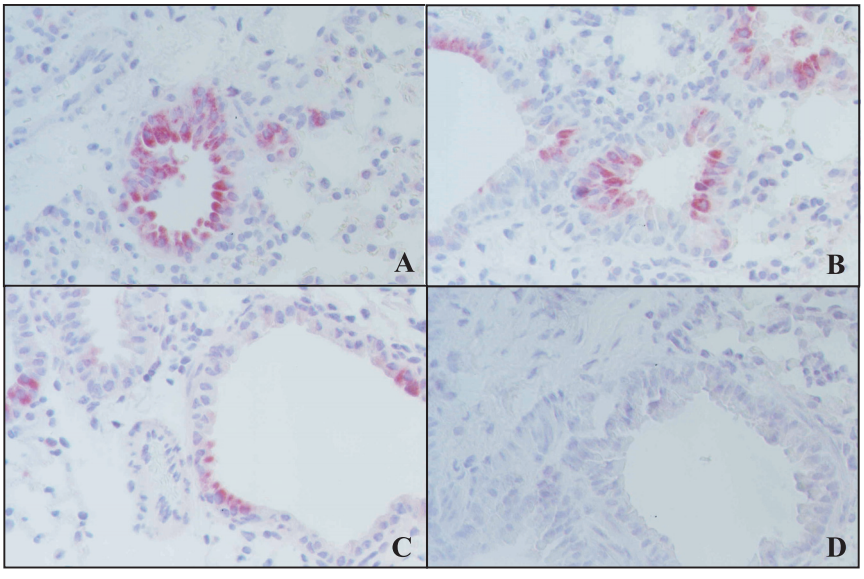


Figure 1. A, B and C: Immunohistochemistry of 3 infected mice lungs sacrificed 5 days post-infection. The red coloration represents the infected cells (see www.edpsciences.org/vetres for a colour version of this figure). **D:** negative control (magnified 20×).

the lungs with intense thickening of alveolar walls and mucosal oedema, periarterial oedema, mononuclear infiltrate in lamina propria, multifocal proliferative tracheitis and hyperplasia of the mucus glands in the tracheae (data not shown).

3.2. Immunohistochemistry

A positive result was detected only in three lung samples extracted from mice five days post-infection. The positive reaction was present in bronchial epithelial cells (Fig. 1).

3.3. RT-nested-PCR

Positive results were obtained with the amplifications of the fragment corresponding to a part of the G gene, from RNA extracted from 2 lungs and 2 tracheae collected on 3 dpi; 2 lungs and 1 trachea collected on 5 dpi; 1 lung and 1 trachea collected on 7 dpi and 2 lungs and 2 tracheae collected on 10 dpi. In the RT-nested-PCR corresponding to the F gene, however, it was not possible to observe any amplified fragment (Tab. I and Fig. 2).

3.4. Nested-PCR sensitivity analysis

The technique amplified the fragment corresponding to the G gene of the material diluted up to 10^{-2} (containing 1225 µg/mL of cDNA), while the fragment corresponding to the F protein could only be amplified from pure material (1 197.5 µg/mL; Fig. 3).

4. DISCUSSION

Several small experimental animals have been used as models of infections with RSV. In this study, balb/c mice were elected to be infected with the BRSV-25-BR strain due to the impossibility of maintaining large experimental animals – such as calves – in our institution. Several authors have used this lineage mice during experiments carried out with HRSV [4, 11, 17, 24, 25]

Table I. The results of RT-nested-PCR of BRSV F and G genes from the tracheae and lungs of experimentally infected balb/c mice.

Days post-inoculation (dpi)	No. F gene amplified/ total animals		No. G gene amplified/ total animals		Positive total
	Trachea	Lung	Trachea	Lung	
3 days	0/12	0/12	2/12	2/12	4
5 days	0/12	0/12	1/12	2/12	3
7 days	0/12	0/12	1/12	1/12	2
10 days	0/12	0/12	2/12	2/12	4
Positive total	0	0	6	7	13

and BRSV [4]. According to Kumar et al. [17], balb/c mice provide a good model for the study of infection by HRSV, in which the infection duration and the pulmonary pathology resembles those in human beings. Viral replication was detected in balb/c mice experimentally infected with RSVs through tests of immunofluorescence [24], in situ hybridization [11] and ELISA [4, 17]. Bastien et al. [3, 4] obtained virus titles in balb/c mice challenged with HRSV and BRSV during experiments with synthetic peptides to confer protection against these viruses. Notwithstanding, some authors mentioned the great difficulty to get BRSV detection after the infection of mice. They comment that some reasons for this phenomenon may exist, since many strains do not grow well in mice and this would seriously reduce the possibility of viral nucleic acid detection. However, intranasal inoculation of balb/c mice with live BRSV induces similar levels of serum antibodies as those detected in mice infected with HRSV (G. Taylor, Institute for Animal Health, Compton, UK, personal communication) and BRSV could undergo an abortive infection in the lungs of mice (A. Easton, University of Warwick, Coventry, UK, personal communication). Taylor et al. [24] have also concluded that the growth of the HRSV strain in different cell lines affects its infectivity for mice.

In the present study, no clinical signs were observed in the animals, in agreement with the findings of some authors [4, 11, 17, 24]. However, it was possible to detect BRSV in mice tissue by RT-nested-PCR and immunohistochemistry which, to the author's knowledge, was the first time that this virus was detected in balb/c mice. The F gene was chosen as the target for the RT-nested-PCR because it is one of the most conservative RSV genes and, consequently, represents a good alternative to be used for virus detection in calves with unknown history about this virus. The G gene, however, is described as the more variable portion of the RSV genome, and can be used in the future in Brazilian field sample characterization. Therefore, these genes are good choices to study the BRSV in our country. The RT-nested-PCR was able to amplify a fragment corresponding to a part of the G gene from the RNA extracted from the lungs and tracheae collected 3, 5, 7 and 10 dpi but only from one or two infected mice each day. However, in the amplifications corresponding to the F gene, it was not possible to observe any amplified fragment. The RT-nested-PCR sensitivity, up to one hundred times superior in the amplification of the G gene fragment as compared to the F gene fragment, may explain the amplification of only the first one of the infected mice tissues. Probably, the viral RNA extracted from this material was enough only to allow the amplification of the G gene and not the F gene. Although there are no reports of similar results in the literature, this phenomenon may have occurred due to inherent conditions of the primers. Even so,

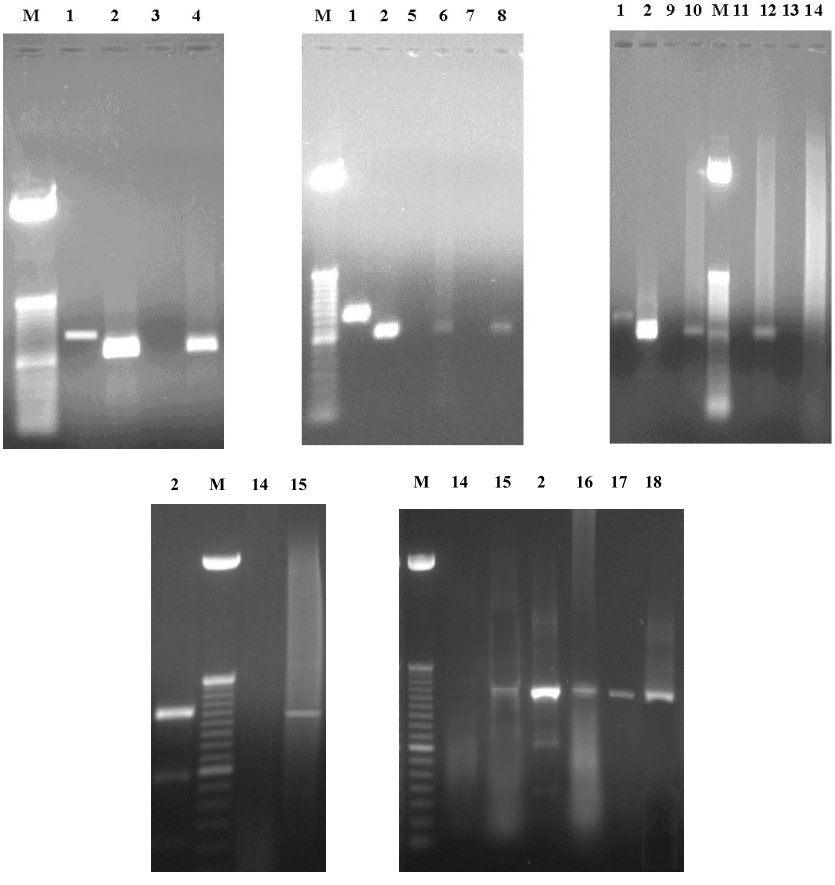


Figure 2. RT-nested-PCR for the detection of a part of the G genes. M: Molecular weight marker. 1: positive control of the F gene (fragment of 481 bp). 2: positive control of the G gene (fragment of 371 pb). 3, 5, 7, 9: F gene from the lungs of infected mice on 3, 5, 7 and 10 dpi, respectively. 4, 6, 8, 10: G gene from the lungs of infected mice on 3, 5, 7 and 10 dpi, respectively. 11: F gene from the tracheae of infected mice on 10 dpi. 12, 18: G gene from the tracheae of infected mice on 10 dpi. 15, 16, 17: G gene from the tracheae of infected mice on 3, 5, and 7 dpi. 13, 14: negative controls.

more studies to analyze this must be performed. Furthermore, only lungs five days post-infection were positive by immunohistochemistry. This can be explained by the higher sensitivity normally shown in PCR reactions when compared to immunohistochemistry, as observed by other authors in the detection of several infectious agents, including viruses [6, 13, 14, 26]. By immunohistochemistry the presence of the BRSV antigen in bronchial epithelial cells, as detected by Prince et al. [22] in cotton rats infected with HRSV, was verified. Differently, Taylor et al. [25] found positive immunohistochemistry reactions in the small scattered foci in cells of alveolar walls using a reference strain of HRSV for inoculation in balb/c mice. In this study, nevertheless, we also had considerable difficulty in recovering BRSV from balb/c tissues, and it was necessary to use a great number of animals to obtain the results shown here.

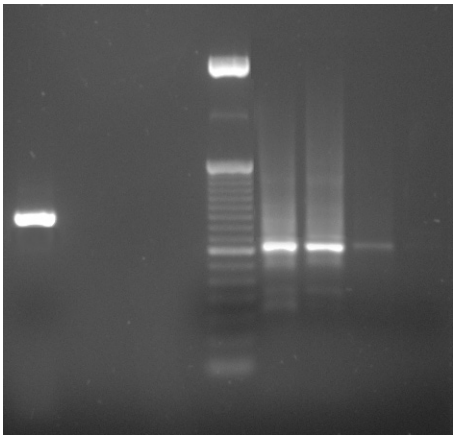


Figure 3. Analysis of the sensitivity of RT-nested-PCR. 1: pure cDNA of the F gene (1225 µg/mL). 2: cDNA 1:10 dilution of the F gene. 3: cDNA 1:100 dilution of the F gene. 4: cDNA 1:1000 dilution of the F gene. M: molecular weight marker. 5: pure cDNA of the G gene (1197.5 µg/mL). 6: cDNA 1:10 dilution of the G gene. 7: cDNA 1:100 dilution of the G gene. 8: cDNA 1:1000 dilution of the G gene.

Although the present study permitted the detection of BRSV in experimentally infected balb/c mice, this procedure showed difficulties. The results here obtained demonstrate that the use of balb/c mice without any artifice can be laborious and often unsuccessful. The use of immunodepressed mice could probably represent a good alternative in order to obtain better results with experimental models to BRSV. Moreover, in our study, only one cell line type and virus strain were tested. It is important to emphasize that studies using more cell lines and other virus strains to complement our unedited data, should be carried to analyze the real application condition of this particular experimental animal.

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