Development of a real time reverse transcriptase polymerase chain reaction for the detection of bovine respiratory syncytial virus in clinical samples and its comparison with immunohistochemistry and immunofluorescence antibody testing

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Title: Development of a real time reverse transcriptase polymerase chain reaction for the
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Abstract

Bovine respiratory syncytial virus is an agent involved in calf pneumonia complex, a disease of significant economic importance. Accurate diagnosis of the agents involved on farm premises is important when formulating disease control measures, including vaccination. We have developed a real time reverse transcriptase polymerase chain reaction (rRT-PCR) and compared it with the diagnostic tests currently available in the United Kingdom: immunohistochemistry (IHC) and immunofluorescence antibody test (IFAT). The rRT-PCR had a detection limit of ten gene copies and was 96% efficient. Recent UK isolates and clinical samples were tested; the rRT-PCR was more sensitive than both conventional tests.

Keywords

Bovine Respiratory Syncytial Virus, BRSV, diagnosis, real time RT-PCR, immunohistochemistry, immunofluorescence.

Introduction

Bovine respiratory syncytial virus (BRSV) is an important respiratory pathogen of calves, causing morbidity and mortality worldwide and significant economic losses to the cattle industry (Stott and Taylor, 1985). A member of the Pneumovirus genus of the subfamily Pneumovirinae in the family Paramyxoviridae (Van Regenmortel, 2000), BRSV is closely related to human respiratory syncytial virus (HRSV). These enveloped viruses have a negative sense, non-segmented ~15Kb RNA genome, encoding 11 proteins, including the nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G) and fusion (F) proteins(Collins et al, 2001). Similar to HRSV, BRSV demonstrates a seasonal incidence of disease, most cases occurring in late autumn and winter (Van der Poel et al, 1994). Clinical disease usually affects calves less than six months of age; disease is only rarely reported in adult BRSV naive cattle (Elvander, 1996). Transmission is by contact with infectious respiratory secretions and/or aerosols (Baker et al, 1997). Accurate diagnosis of the agents...
present is important when designing a vaccination regime for calf pneumonia. Virus isolation for BRSV lacks sensitivity due to the highly membrane bound and labile nature of the virus or low viral titre (Kimman et al, 1986; Baker et al, 1997; Collins et al, 2001) and diagnosis in the UK is most often performed using immunofluorescence antibody test (IFAT) on lung impression smears or bronchoalveolar lavage (BAL) fluids (Thomas and Stott, 1981; Baker et al, 1997; Larsen, 2000). Alternatively, antigen detection can be performed on fixed tissues using immunohistochemistry (IHC) (Haines et al, 1989; Masot et al, 1993, 2000). These methods are time consuming and suffer from low sensitivity; the use of the reverse transcriptase polymerase chain reaction has been demonstrated to be more sensitive than IFAT for HRSV detection (Kuypers et al, 2006). A number of RT-PCR tests have been developed to detect BRSV F, G or N genes (Vilcek et al 1994; Larsen et al, 1999; Valarcher et al, 1999; Valentova et al, 2003, 2005); more recently real time RT-PCR techniques have also been described directed at the F gene (Achenbach, 2004; Hakhverdyan et al, 2005) and the N gene (Boxus et al, 2005) for use in experimental infections. We describe here the development of a one step real time RT-PCR targeting the N protein of BRSV and compare this assay with the conventional detection methods on field material.

Materials and Methods

Real time RT-PCR primers, probes and reaction conditions

Real Time RT-PCR was carried out using primers and a minor groove binding (MGB) probe targeting the N gene of BRSV. This region was selected as it is well conserved (Valarcher et al, 2000) and should have a high number of transcripts, as the promoter-proximal 3’ end of the genome is transcribed more efficiently then the promoter-distal 5’end due to the sequential stop-restart manner of transcription employed by the paramyxoviruses (Lamb and Kolakofsky, 2001). A MGB probe was chosen to allow design of shorter probes. Oligonucleotides were designed using Primer Express (Applied Biosystems), based on a
multiple sequence alignment (MegAlign, DNAStar) of BRSV sequences available in Genbank. Primers used were BRSVnF (5’ ggtcaaatgacacactttcaacag-3’), BRSVnR (5’-agcatacctacatgagatg-3’) and the probe was BRSVnMGB (5’-FAM-tagttcagttgacacattg-NFQ-MGB-3’). A proprietary one-step real time RT-PCR reagent set was used (SuperScript™ III Platinum® One-Step real time RT-PCR Kit, Invitrogen) for all reactions. All reactions were performed in quadruplicate using 2µl RNA per reaction in a 25µl volume at 600nM each primer and 100nM BRSVnMGB probe, in the presence of 3mM MgSO₄ and 500nM ROX in an ABI Prism 7000 (Applied Biosystems) using the thermal cycling profile of one cycle 50°C for 15 minutes, one cycle 95°C for 2 minutes, 50 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Data was analysed as per the manufacturers’ instructions (Applied Biosystems). All samples giving a negative result were further tested using an endogenous β-actin control primer/probe set (Willoughby, data not shown) to ensure that negative results were not due to failure of RNA isolation or RT-PCR inhibition.

Viruses

A vaccine isolate (Rispoval RS, Pfizer Ltd) was used for assay optimisation and eight BRSV isolates (table 1) were used for preliminary assessment on different BRSV isolates.

Preparation of RNA controls

A plasmid containing the RT-PCR target sequence was produced (TOPO TA cloning kit, Invitrogen) and RNA transcribed in vitro (Riboprobe system, Promega) using the manufacturers’ protocols and standard methods as previously described (Willoughby et al 2006). RNAs were quantified on a RNA Nano labchip on an Agilent 2100 Bioanalyser (Agilent technologies) and used to construct standard curves from 10⁰–10¹⁰ copies.

Real time RT-PCR specificity

The specificity of the real time RT-PCR was determined by testing viral nucleic acid extracted from a range of veterinary viruses, maintained in cell culture and validated in other
diagnostic tests, comprising border disease virus, bovine viral diarrhoea virus, bovine adenovirus, ovine adenovirus, canine distemper virus, bovine herpesvirus 1, 2 and 4, alcelaphine herpesvirus 1, ovine and bovine parainfluenza virus, ovine parvovirus, ovine reovirus, Semliki forest virus, and orf virus. Additionally, four viruses closely related to BRSV were tested; avian metapneumovirus (a gift from Prof R.C Jones, University of Liverpool) and three human RSV isolates (RNA gifted by Prof C.A.Hart, University of Liverpool).

**Clinical samples**

Clinical samples from calves with respiratory disease or presenting at necropsy with pneumonia were collected by the Scottish Agricultural College (SAC) Disease Surveillance Centres in the winter of 2004/2005. There were one hundred and thirty one samples in total, comprising 82 lung tissues, 29 bronchoalveolar lavages (BAL) and 20 respiratory tract swabs (three bronchial, eleven nasal and six from an unspecified site) in virus transport medium (VTM). All lung tissues were taken from the margin of an antero-ventral consolidated area of the right lung, using strict precautions to prevent RT-PCR contamination, and placed in VTM. On arrival, VTM was aspirated and the tissue stored at -70°C until RNA isolation. An immediately adjacent piece of lung was taken into neutral buffered formalin (NBF) and submitted for immunohistochemistry. For BALs and swabs in VTM, samples were prepared for IFAT testing as per a standard operating procedure (SOP) in place at SAC and remaining cellular deposit and supernatant were stored at -70°C until RNA extraction and RT-PCR testing. All tests (IFAT, IHC and real time RT-PCR) were carried out blind to each other.

**Immunohistochemistry**

Lung samples were either directly immersed in 10% NBF or snap frozen and thawed in 10% NBF, fixed for 4-29 days at room temperature, and processed to wax by routine histological
procedures. Sections cut at 3µm were collected onto polysine-coated slides, dewaxed and immersed in 0.01 M citrate buffer pH 6.0 at 97°C for 10 minutes. The following steps and the intervening washes were performed in a robotic immunostainer (Dakocytomation) at room temperature: 2 hour immersion in monoclonal antibody 18B2 (Pothier et al, 1985) (Argene Biosoft, France) directed against the fusion protein of HRSV which cross-reacts with BRSV (Haines et al, 1989; Masot et al, 1993) blocking of endogenous peroxidase activity and detection of sites of primary antibody binding by the polymer-based immunoperoxidase EnVision system (Dakocytomation). Sections were counterstained with Mayer’s haematoxylin, cover-slipped, and examined using an Olympus BX50 microscope.

IFAT testing
The IFAT test (Thomas and Stott, 1981) was performed on lung impression smears and cellular deposits from BAL samples by standard procedures following the SOP in place at SAC.

Results
Real time RT-PCR specificity and sensitivity
Specific positive signals were not detected for any of the other viruses tested. Using titrated virus, the real time assay was able to detect 0.2 TCID₅₀ BRSV/ml, presumed due to detection of non-infectious RNA due to the viral transcription strategy. Using in vitro transcribed RNA the limit of linear detection was 10 genome copies, single copy detection was possible in two of four replicates and no detection was observed at less than one copy (figure 1). The reaction was 96% efficient. A threshold of CT ≤ 37 1(~10 genome copies or 1 TCID₅₀/ml) was considered positive for clinical tissues. All real time curves were examined to ensure they were of the correct logarithmic shape confirming exponential amplification.

BRSV isolates and clinical samples
All BRSV isolates were detected by the real time RT-PCR (CTs of 15-25). Of the 82 lung samples, 11 were positive on IFAT, 12 were positive on IHC and 23 were positive on real time RT-PCR (Table 2a). When the results on IFAT and IHC were compared to the CT values of the real time RT-PCR, all samples positive by either IHC or IFAT were found in the lower CT range (Figure 2), which correlates with higher virus titre, demonstrating the greater sensitivity of the real time RT-PCR test.

Of the 29 BALs, seven were positive on IFAT and on real time RT-PCR. Of the 20 respiratory tract swabs, all were negative on IFAT and seven were positive by real time RT-PCR (table 2b). Three samples were bronchial swabs from animals where there was a lung sample also; the lung samples were also real time RT-PCR positive. The remaining four real time RT-PCR positive samples were from one farm. There was a history of respiratory disease on this farm and other bovine respiratory pathogens had been identified, but BRSV had not previously been detected.

**Discussion**

The real time RT-PCR assay was more sensitive than the other techniques when used on lung samples and respiratory swabs. The real time RT-PCR gave concordant results to IFAT on BALs. The accuracy of diagnosis in BAL samples likely reflects the stage in the disease at which the sample was taken; as all lung tissues were from calves dying of respiratory disease, it is possible that IFAT/IHC detection may be masked by the development of antibody at this stage of disease (Larsen, 2000).

The sensitivity of this assay is 10 copies; previously reported real time RT-PCR assays have sensitivities of 171 copies (Achenbach, *et al*, 2004) and 1000 copies (Boxus *et al*, 2005). The assay of Hakhverdyan (2005) does not cite copy number detection, but is sensitive to 3.16TCID$_{50}$/ml, approximately 10 fold less sensitive than our assay (0.2 TCID$_{50}$/ml).
Technical differences (target choice, one-step or two-step RT format and use of TAMRA quenched probes) may have influenced this sensitivity.

As real time RT-PCR detects virus genome rather than virus antigen, detection of low levels of virus genome may represent a sub-clinical infection or detection of a primary pathogen where the animal has developed secondary disease. The lung samples were taken from animals which were culled, or died of, ‘calf pneumonia’ and these are likely to be in the later stages of infection (days 5-15), where the level of BRSV is lower than in the early (days 3-6) stages of infection (Larsen, 2000). A further speculation is the detection of animals persistently infected with BRSV. The presence of persistently infected animals has been previously proposed (Thomas et al, 1980; Van der Poel et al, 1993; De Jong et al, 1996) but there is to date limited experimental evidence for virus persistence (Van der Poel et al, 1997, Valarcher et al, 2001). Study of virus persistence was outside the scope of our study; further work on this aspect of BRSV infection is required.

We have looked at eight clinical isolates and 131 clinical samples from calves from farms suffering outbreaks of respiratory disease. The results suggest that the real time RT-PCR can be successfully utilised, especially where the on-farm conditions do not favour the collection or rapid submission of BALs, on nasopharyngeal swabs or necropsy material to improve diagnostic sensitivity and allow better design of vaccination programs for individual premises. Additionally, the sensitivity of this assay may prove useful in future studies of BRSV persistence.

Acknowledgements

We are grateful to colleagues in the SAC Disease Surveillance Centres for supplying clinical specimens and performing IFAT testing and to the staff of the histology laboratory VLA Lasswade for excellent technical assistance. The Moredun Research Institute receives financial support from the Scottish Executive Environmental Rural Affairs Department.
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References


Figure 1: Standard curve on 10 fold dilutions of (a) in vitro transcribed RNA and (b) titrated virus

(a) in vitro transcribed RNA demonstrating linearity of detection down to 10 copies and detection of single copy in 2/4 replicates. The slope value is -3.424, intercept 40.49 and $R^2$ 0.994. Efficiency is 96% (Efficiency = $(10^{-1/slope \text{ value}})-1 \times 100\%$).

(b) Titrated virus demonstrating linearity of detection down to 0.2TCID$_{50}$/ml and detection of 0.022TCID$_{50}$/ml in 1/4 replicates. The slope value is -3.383, intercept 36.5 and $R^2$ 0.981. Efficiency is 97.5%.
**Figure 2**: Comparison of IFAT and IHC result with real time RT-PCR CT for all 23 lung samples giving a positive result with any test, arranged in order of increasing CT.
Table 1. Isolates used in this study. These isolates have been previously characterised by Nettleton et al (2003)

<table>
<thead>
<tr>
<th>Isolate name</th>
<th>Year of isolation</th>
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<tbody>
<tr>
<td>R4017/5</td>
<td>1991</td>
</tr>
<tr>
<td>A58/19</td>
<td>1997</td>
</tr>
<tr>
<td>A4644/3</td>
<td>1997</td>
</tr>
<tr>
<td>B4332/1</td>
<td>1998</td>
</tr>
<tr>
<td>B4332/2</td>
<td>1998</td>
</tr>
<tr>
<td>B4446/A</td>
<td>1998</td>
</tr>
<tr>
<td>D4658/7</td>
<td>1999</td>
</tr>
<tr>
<td>D4636/1</td>
<td>1999</td>
</tr>
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</table>
**Table 2**: Comparison of results on lung samples between real time RT-PCR, IFAT and IHC on (a) lung samples and (b) Swabs and BALs

(a) lung samples

<table>
<thead>
<tr>
<th>rt-RT-PCR</th>
<th>IFAT</th>
<th>IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>positive (n=23)</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>negative (n=59)</td>
<td>0</td>
<td>59</td>
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</tbody>
</table>

(b) swabs and BALs

<table>
<thead>
<tr>
<th>rt-RT-PCR</th>
<th>IFAT (Respiratory swabs)</th>
<th>IFAT (BALs)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>positive</td>
<td>negative</td>
</tr>
<tr>
<td>positive (n=14)</td>
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</tr>
<tr>
<td>negative (n=33)</td>
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