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Development of a real-time RT-PCR for quantification of bovine TLR4 mRNA and evaluation of its use during a BRSV vaccine challenge

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SUMMARY

The Bovine respiratory syncytial virus (BRSV) causes bronchiolitis and interstitial pneumonia, predominantly in calves, and is a major cause of bovine respiratory disease worldwide. In humans, BRSV is paralleled by the closely related Human respiratory syncytial virus (HRSV), an important cause of respiratory disease, most severe in infants.

The clinical signs and pathology during RSV infection is caused, not only by the direct effects of viral replication, but also by the response of the host immune system. The immunopathology of RSV has long obfuscated our understanding of the disease, and development of effective treatment and vaccines will be very difficult until greater knowledge is gained.

One of the components of the immune system that has come into focus in RSV research the last few years, is the Toll-like receptor 4 (TLR4). The TLR4 receptor is well known as the receptor that binds lipopolysaccaride (LPS), and initiates the host response to bacterial infection. Recently, it has been shown that the fusion protein of RSV also interacts with, and up-regulates the expression of, the TLR4 receptor. Whether this has a predominantly protective effect, as would be expected from an immune response, or if it is mainly detrimental to the host, remains to be determined.

The objective of this work was to develop an assay for quantification of TLR4 mRNA in clinical samples and to determine if TLR4 mRNA in bronchoalveolar lavage (BAL) cell samples could be used as a marker of protection during experimental BRSV infection.

Two one-step quantitative real-time PCR systems were developed and optimized in this work. One for detection of TLR4 mRNA, and the other for detection of the housekeeping gene product 28S rRNA. The assays showed good efficiency as well as intra- and inter-assay reproducibility. Furthermore, BAL cell samples collected during an experimental vaccinal challenge were used to evaluate the level of TLR4 mRNA expression in relation to detected BRSV RNA. When the results were analyzed, it appeared that TLR4 mRNA quantification can not be used as a marker of protection against BRSV infection after previous vaccination.

SAMMANFATTNING

Bovint respiratoriskt syncytialt virus (BRSV) orsakar bronkiolit och interstitiell pneumoni, framförallt hos kalvar, och är en betydande orsak till respiratoriskt lidande hos nötkreatur över hela världen. Lika betydelsefullt på humansidan är det nära besläktade Humant respiratoriskt syncytialt virus (HRSV), som orsakar sjukdom liknande BRSV, framförallt hos spädbarn.

Kliniska symtom och patologi vid RSV-infektion är inte bara en direkt följd av virusets replikation, utan även en effekt av värdens immunsvar. Denna immunopatologi har länge höljt patogenesen för RSV i ett dunkel, och utvecklandet av effektiva behandlingar och vacciner kommer att bli svårt tills en djupare förståelse erhållits.

En av de komponenter i immunförsvaret som kommit i blickfånget de senaste åren inom RSV-forskning, är Toll-like receptor 4 (TLR4). TLR4 är välkänd för att binda lipopolysackarid (LPS) från Gram-negativa bakterier, och därmed initiera immunförsvaret vid bakteriella infektioner. Nya studier har visat att även RS-virusets fusionsprotein interagerar med TLR4, och uppreglerar uttrycket av denna receptor på infekterade cellers yta. Hurvida detta har övervägande skyddande effekt, som man skulle förvänta sig av ett immunsvar, eller om denna uppreglering har huvudsakligen negativa effekter för värden, är inte klarlagt.

Syftet med denna studie var att utveckla en analysmetod för att kvantifiera uttrycket av TLR4 i kliniska prover, samt att avgöra om nivån av TLR4 mRNA i cellprover från bronchoalveolar lavage (BAL) kunde användas som en markör för hur väl skyddat djuret är mot experimentell BRSV infektion.

Två kvantitativa realtids PCR-metoder (qPCR) utvecklades och optimerades i detta projekt. En av dessa qPCR-metoder kvantifierar uttrycket av TLR4 mRNA, den andra 28S rRNA. De uppmätta värdena av 28S rRNA användes för att standardisera resultaten för TLR4 mRNA och BRSV-titer. Båda dessa qPCR-metoder uppvisade god effektivitet och reproducerbarhet. Dessutom analyserades BAL-prover, som samlats in från kalvar som deltog i ett vaccinförsök, för att utvärdera uttrycket av TLR4 mRNA i förhållande till BRSV-titer. När dessa resultat analyserades verkade det som om nivån TLR4 mRNA inte utgör någon bra markör för graden av skydd mot BRSV-infektion efter tidigare vaccination.

ACKNOWLEDGEMENTS

INTRODUCTION

The Bovine respiratory syncytial virus (BRSV) is an enveloped, negative sense single-strand RNA virus, classed in the family Paramyxoviridae, subfamily Pneumovirinae, genus *Pneumovirus*. Also in the genus *Pneumovirus* are the Human respiratory syncytial virus (HRSV), the Ovine respiratory syncytial virus (ORSV) and the Caprine respiratory syncytial virus (CRSV) (Fauquet et al. 2005). Although they are all closely related, BRSV, ORSV and CRSV are more closely related, while HRSV is more distantly so (Fauquet et al. 2005; Trudel et al. 1989). The caprine RSV might even be considered a strain of BRSV (Trudel et al. 1989).

The human respiratory syncytial virus, HRSV, was first isolated from a chimpanzee in 1956, then from infants in 1957. In the late 1960's suspicions were raised that cattle too could be infected with HRSV, as antibodies against RSV had been found in cattle. Then, in 1970, the bovine respiratory syncytial virus was isolated as a separate virus (Stott & G Taylor 1985). Since then, BRSV has been further characterized and 3 antigenic subgroups have been described, along with 6 phylogenetic branches, all belonging to the same serotype (Valarcher & Taylor 2007).

Respiratory disease in cattle, and especially in calves and young stock, constitute a large proportion of total morbidity. Worldwide, it causes extensive costs for the farmer in way of decreased production, and increased prophylactic and veterinary expenses (Fulton 2009; Van der Poel et al. 1994). The specific cause of respiratory disease in cattle is often left undiagnosed and is often multifactorial, but it has been shown that BRSV plays a major role in acute bovine respiratory disease (Stott et al. 1980). In a similar way, human infection with HRSV is a substantial source of morbidity globally (Eidelman et al. 2009; Tregoning & Schwarze 2010; Van der Poel et al. 1994). Infant, elderly and immunosuppressed patients are especially susceptible (Tregoning & Schwarze 2010; Hashem & Hall 2003). Apart from acute respiratory disease, HRSV has been associated to long-term complications in humans, such as wheezing and asthma (Tregoning & Schwarze 2010). In addition to individual suffering, the health care costs of HRSV infection constitute a great burden to society (Eidelman et al. 2009; Forbes et al. 2010; Palmer et al. 2010; Hashem & Hall 2003).

RSV causes bronchiolitis and interstitial pneumonia. The clinical signs and pathology during a natural RSV infection is thought to derive from three principal mechanisms: direct viral syncytial and cytolytic effects; inflammatory response by the host; and effects of secondary bacterial infection. The respective significance and exact linkages of these mechanisms are still to be determined (Valarcher & Taylor 2007). However, the virus infects both the upper and lower airways, where it replicates in epithelial and alveolar cells and is found most abundant close to the lumen. During viral replication syncytia form and lysis of many infected cells occur, which has a structurally detrimental effect on alveoli, leading to alveolar collapse. This collapse of lung tissue, combined with an impaired bronchiolar ciliary function, due to RSV infection, predisposes the host to secondary bacterial infection (Viuff et al. 1996). In parallel, RSV induces an immune response in the host, which is believed to be responsible for much of the clinical signs (Valarcher & Taylor 2007; Tregoning & Schwarze 2010). This immune response is not fully understood, but an array of inflammatory mediators has been implicated (Valarcher & Taylor 2007). Furthermore, for BRSV, considerable variability in

apparent pathogenesis during infection has been observed, and it has been proposed that this could be explained by differences in virus-host interaction between separate BRSV strains (Valarcher & Taylor 2007).

One of the many components of the immune system that has been implicated in the pathogenesis of RSV is the Toll-like receptor 4 (TLR4) (Lizundia et al. 2008; Hunninghake & Schwartz 2002; Rudd et al. 2005; Haynes et al. 2001). The Toll-like family of receptors are evolutionarily highly conserved molecules in eucaryotic cells. Their function is to recognize and signal the presence of equally conserved pathogenic antigens. These antigens, conserved in certain microbes, are collectively called pathogen-associated molecular patterns (PAMPs). Lipopolysaccharide (LPS), found in the cell wall of Gram-negative bacteria, is an example of a PAMP. LPS binds to, and activates the TLR4 receptor (Means et al. 2000). TLR4 is a transmembrane receptor, and upon activation it signals the presence of LPS into the cell, thus starting a signaling cascade which activates NF-κB. NF-κB is a complex of proteins that regulates the expression of genes involved in inflammation and the innate immune system. This results in increased expression of certain proinflammatory mediators, such as IL-1, IL-6 and IL-8 (Poltorak et al. 1998; Medzhitov et al. 1997). The signaling pathway of the TLR4 receptor is the same in cattle and humans (Arsenault et al. 2009). Apart from LPS, studies have shown that the F protein of RSV also activates the TLR4 receptor, and thereby triggers an innate immune response (Lizundia et al. 2008; Kurt-Jones et al. 2000; Haynes et al. 2001). The implications of this, with regard to the pathogenesis of RSV, is still unclear. However, mice with TLR4 gene deletions that were inoculated with HRSV, had delayed virus clearance, compared to wild-type TLR4 mice (Kurt-Jones et al. 2000; Haynes et al. 2001). Furthermore, TLR4 will become more abundant on the surface of airway epithelial cells during HRSV infection, thereby causing the airway epithelium to become hypersensitive to normal levels of endotoxin (Monick et al. 2003). Thus, it is apparent that TLR4 plays a role in the pathogenesis and the immune response during RSV infection. Whether this has a detrimental or benign effect on the host still needs to be determined. Either way, further knowledge about the interaction between RSV and TLR4 will undoubtedly be helpful in understanding the disease, and conceivably help develop new treatments and vaccines against RSV infection.

Partially attributable to the complexity of the immunopathology of HRSV, there is currently no commercially available vaccine for humans (van Drunen Littel-van den Hurk et al. 2007). Although there are commercially available vaccines against BRSV, there are contradictory reports as to their efficacy and duration of protection against disease (Larsen et al. 2001; Ellis et al. 2005; Ellis et al. 2001; Patel & Didlick 2004; Ellis et al. 2007). For a vaccination of calves against BRSV to be beneficial, it needs to be performed well before the age of 2 months, as the highest incidence of BRSV disease in calves is between 2 to 6 months of age (Valarcher & Taylor 2007). However, at this young age, calves often have high titers of maternal BRSV-specific antibodies, since the seroprevalence of BRSV in adult cattle is often over 70% (Van der Poel et al. 1994), and BRSV-specific maternal antibodies are passed in colostrum. Not only do maternal antibodies give an incomplete protection against RSV infection (Van der Poel et al. 1994), they may also neutralize the vaccine, and inhibit its immunostimulatory effects (Valarcher & Taylor 2007). Successful BRSV vaccine candidates need to overcome this challenge.

Other obstacles for BRSV vaccine development are issues of safety. There are indications that previous BRSV vaccination of calves, with certain vaccines, can cause more aggressive pathogenesis and higher mortality rates in natural outbreaks (Schreiber et al. 2000; Larsen et al. 2001). This phenomena has been called a vaccine-induced immunopathology. The effects of this phenomena first became apparent in HRSV and humans following clinical trials of a HRSV vaccine in the 1960's. The vaccinated children got more severe lower respiratory symptoms after natural infection with HRSV, than did the control children. After these catastrophic trials, it has been difficult to develop and launch new vaccines against HRSV (Kapikian et al. 1969).

In light of the similarities between BRSV and HRSV, further knowledge about BRSV, and the development of a satisfactory vaccine for cattle, would most likely benefit HRSV research a great deal. Studies in cattle offer a good complement as model of HRSV in human, since cattle is a natural host for BRSV and clinical signs of disease can be reproduced. Furthermore, since much of the clinical signs and pathology during HRSV infection, and probably during BRSV infection, is derived from the host response, focus must fall on studying the immunopathology of the two diseases. In this scenario, the TLR4 receptor is emerging as an interesting target of BRSV research.

The purpose of the present study was to see whether the level of expression of TLR4 in BAL cell samples could be used as a marker of protection against BRSV infection. If so, TLR4 expression would parallel the titers of BRSV in BAL cell samples. This study was carried out in the context of a larger project, studying the protective effect of a vaccine against BRSV infection.

MATERIAL AND METHODS

Vaccine

An experimental BRSV vaccine, BRSV ISCOMs, and a protein control vaccine without adjuvant were produced outside the EEF project, as described previously (Hägglund et al. 2004). Briefly, BRSV (no 9402022, Denmark) was propagated in vero cell culture, was purified by ultracentrifugation through sucrose and solubilized by detergent. The solubilized virus was additionally ultracentrifuged through sucrose. A fraction of proteins was either mixed with Quil A, cholesterol and phospholipids (for BRSV-ISCOMs) or not (for BRSV proteins). After dialysis against ammonium acetate the BRSV-ISCOMs and the BRSV proteins were purified by ultracentrifugation through sucrose and passed through a 0,45µm filter.

Animals and immunization

Outside the EEF project, four groups of five 3-8 week old conventional calves, allocated into groups according to age and levels of BRSV-specific maternal antibodies, were immunized subcutaneously twice at a three weeks interval with i) BRSV-ISCOMs (containing 190 µg BRSV proteins), ii) BRSV proteins (190 ug), iii) adjuvant , or iv) PBS.

Challenge and sampling

Two weeks after second vaccination all calves were challenged with the homologous BRSV though administration of 3 ml virus infected turbinate cell lysate via 10 min aerosol inhalation, as described earlier (Hägglund et al. 2004). All animals underwent daily clinical examinations and nasal swabs (Sigma, virocult®) were collected daily, along with repeated blood sampling. Six days after challenge all calves were euthanized by exsanguinations under general anesthesia overdose (5 mg/kg ketamine followed by 15 mg/kg pentobarbital sodium). The EEF project started on this day.

Shortly after the calves were euthanized, their lungs were removed for investigations of gross and histopathology and for virus detection in lung tissue. Moreover, bronchoalveolar lavage (BAL) was performed using 500 ml sterile PBS. The recovered volume (200 ml) was kept on ice, was centrifuged during 15 min at 300 x *g* and a cell pellet originating from half of this volume was dissolved in 3 ml RTL buffer (Quiagen) and stored in -70C°. The cell pellet from the other half of the volume was used for virus isolation, as part of the larger project.

RNA extraction and standardization according to RNA mass

Total RNA was extracted from 350 µl of the BAL samples using the RNeasy Mini Kit (Qiagen), following the manufacturers instructions (Animal Cells Spin). To standardize the quantitative PCR (qPCR) reactions for each sample the RNA concentrations of the BAL RNA samples were measured using spectrophotometry (Picodrop Microliter UV/Vis Spectrophotometer, Picodrop Limited). The RNA purity was verified at OD 260/280. The BAL RNA samples were then diluted to 20 ng/µl each. These diluted samples were used in all described qPCR assays.

Immediately after extraction and between each use the diluted BAL RNA samples were stored in a -70°C freezer.

BRSV RNA quantification

BRSV RNA was detected in the diluted BAL RNA by quantitative real-time PCR (qPCR), and by using previously published primers and probes (Hakhverdyan et al. 2005) at final concentrations of 300 nM for forward and reverse primers and 100 nM for the probe. The iScript One-Step RT-PCR Kit for Probes (Bio-Rad) and the following thermodynamic profile was used in an IQ5 Real Time PCR machine (Bio-Rad): after 10 minutes at 50°C for cDNA synthesis, the reverse transcriptase was inactivated at 95°C for 5 minutes; then, for 40 cycles, cDNA templates were amplified at 95°C for 15 seconds, followed by 60°C for 30 seconds. Probe fluorescence was detected at the end of each cycle. The calculations used to arrive at 28S standardized BRSV results is detailed under the heading Calculations and statistical analysis.

TLR4 mRNA quantification

To quantify the TLR4 gene expression in BAL sample cells, a quantitative real-time PCR (qPCR) assay was developed. The TLR4 qPCR assay uses a TaqMan probe to quantify the copies of bovine TLR4 mRNA. Developing the assay also included establishing a plasmid-based standard dilution series to quantify unknown samples.

Primer and probe design

The sequence for bovine Toll-like receptor 4 (TLR4) was retrieved from NCBI Entrez Nucleotide, accession number DQ839567.1. Large amplicon PCR primers, TaqMan primers and TaqMan probe were designed using the online tool Primer3 (Rozen & Skaletsky, 2000). The large amplicon primers were designed to create a plasmid-insert that contains the sequence of the TaqMan primers and TaqMan probe. Theoretically, there are four PCR products of different length, when applying the large amplicon primers on bovine genomic DNA and total RNA. The lengths of these theoretical PCR products are 7798 bp (both introns included), 5049 bp (first intron included), 3250 bp (second intron included) and 501 bp (messenger RNA, without introns). The 501 bp PCR product was isolated for plasmid insertion, as described below.

The TaqMan primers were designed to straddle a boundary between two exons in the TLR4 mRNA sequence, thereby ensuring only TLR4 mRNA is amplified in the qPCR reaction (see Figure 1).

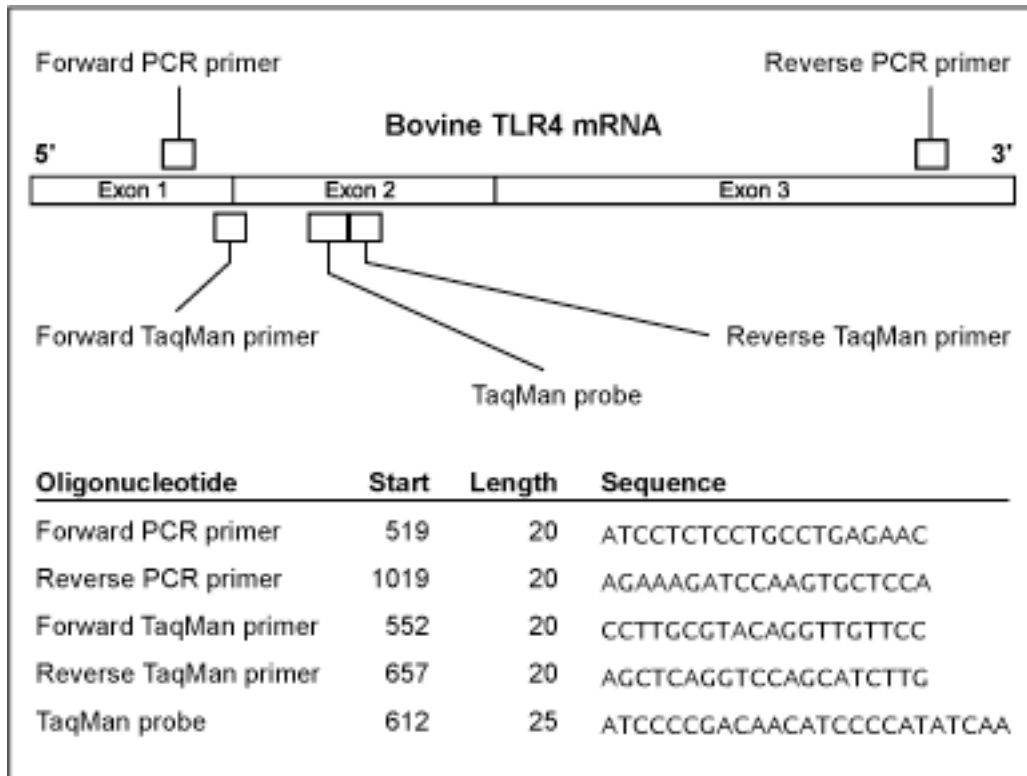| Oligonucleotide	Start	Length	Sequence
Forward PCR primer	519	20	ATCCTCTCCTGCCTGAGAAC
Reverse PCR primer	1019	20	AGAAAGATCCAAGTGCTCCA
Forward TaqMan primer	552	20	CCTTGCGTACAGGTTGTTCC
Reverse TaqMan primer	657	20	AGCTCAGGTCCAGCATCTTG
TaqMan probe	612	25	ATCCCCGACAACATCCCCATATCAA

Figure 1: Illustration showing the bovine TLR4 mRNA and the placement and sequences of the large amplicon (PCR) primers, the TaqMan primers and the TaqMan probe. Start, is the starting position from the 5' end, in the mRNA sequence. Length, is the number of bases in the oligonucleotide.

Creating a standard series for TLR4 mRNA qPCR

A plasmid containing a 501 bp segment of the bovine TLR4 mRNA nucleotide sequence was created, purified and serial diluted, to act as a positive standard in the TLR4 qPCR. To achieve this, total RNA was extracted from a suspension of bovine PBMC's stimulated with LPS. The total bovine RNA was converted to complementary DNA (cDNA), using Oligo(dt) and reverse transcription with murine leukaemia virus reverse transcriptase (MuLV RT). Using the cDNA sample, a segment of TLR4 mRNA (converted into cDNA) was amplified using classic PCR and the large TLR4 amplicon primers described in Figure 1. The length of the PCR product was verified using agarose gel electrophoresis. From the agarose gel, a single band of 501 bp was cut out and DNA was purified using PureLink Quick Gel Extraction Kit (Invitrogen).

The verified and purified PCR product, was inserted into a plasmid vector (pcDNA™ 3.1/V5-His TOPO®), using pcDNA™ 3.1/V5-His TOPO® TA Expression Kit (Invitrogen) by following the manufacturers instructions. Using the same kit, the recombinant vectors were transformed into chemically competent TOP10 *E. coli*. Plasmid-carrying *E. coli* were isolated by utilizing their vector-given resistance to ampicillin, and were further propagated by overnight culture. Plasmids from the isolated *E. coli* were extracted and purified using the E.Z.N.A Plasmid Miniprep Kit I (Omega Bio-Tek), by following the manufacturer's instructions. Inclusion of the TLR4 insert into the plasmid of

isolated plasmid-carrying *E. coli* was verified by classic PCR, using the large amplicon primers, and agarose gel electrophoresis (where purity of samples could also be ascertained).

The DNA concentrations of the isolated and purified plasmid samples were measured using spectrophotometry (Picodrop Microliter UV/Vis Spectrophotometer, Picodrop Limited). The DNA purity was verified at OD 260/280. One of the plasmid samples were chosen for further processing. From this sample, a plasmid dilution series (dilution factor 50) was prepared to act as a positive standard series for TLR4 mRNA qPCR.

Verification and optimization of the TLR4 mRNA qPCR assay

Using the TLR4 standard dilution series, known TLR4 positive RNA samples, and negative control samples, the TaqMan primers and TaqMan probe (presented in Figure 1) were tested using the iScript One-Step RT-PCR Kit for Probes (Bio-Rad) in an IQ5 Real Time PCR machine (Bio-Rad). After confirmation that the primers and probe could detect TLR4, the qPCR assay was optimized with regard to annealing temperature and primers and probe concentrations.

In addition to analyzing the BAL cell samples presented here, four additional batches of samples were analyzed using the TLR4 qPCR assay. Total samples analyzed included: 86 unknown samples in triplicates (total RNA extracted from bovine lung tissue and bovine cell cultures); 25 TLR4 standard samples in triplicates; and 15 negative controls.

Quantification of TLR4 mRNA expression in BAL cell samples

The amount of TLR4 mRNA was quantified, in triplicates, in the diluted BAL cell RNA samples, using the optimized TLR4 qPCR assay described above. The calculations used to arrive at 28S standardized TLR4 values is detailed under the heading Calculations and statistical analysis.

Ribosomal 28S RNA quantification

Although the RNA concentration in BAL RNA samples was determined and samples diluted to a common RNA concentration before qPCR, the TLR4 and BRSV qPCR results was further standardized using a so-called house-keeping gene. A house-keeping gene is a gene that tends to be constantly expressed in the relevant cells. Ribosomal 28S RNA is the product of such a gene (Xue et al. 2010), and in this study a qPCR assay quantifying expression of the ribosomal 28S RNA was developed and used to standardize results.

Development of the 28S qPCR assay, and quantification of 28S rRNA was executed in the same way as described for TLR4, except for the design and use of primers and probe (described below). When 28S rRNA was quantified in the 28S qPCR assay, samples were analyzed in duplicates. Further differences are outlined below.

In addition to analyzing the BAL cell samples presented here, three additional batches of samples were analyzed using the 28S qPCR assay. Total samples analyzed included: 82 unknown samples in triplicates or duplicates (total RNA extracted from bovine lung tissue and bovine cell cultures); 20 standard samples in triplicates or duplicates; and 14 negative controls.

Primer and probe design

The sequence for bovine 28S ribosomal RNA was retrieved from NCBI Entrez Nucleotide, accession number NR_036644.1. Primers and TaqMan probe were designed using the online tool Primer3 (Rozen & Skaletsky, 2000) and are presented in Figure 2. The primers that were used to create an insert (114 bp) for a plasmid, were also used as TaqMan primers in the qPCR reaction. Because the 28S gene doesn't have any introns, PCR amplification of genomic DNA (as opposed to 28S rRNA) in unknown samples cannot be avoided. However, the number of genomic 28S gene copies, compared to the number of ribosomal RNA 28S copies was considered negligble and non-biasing.
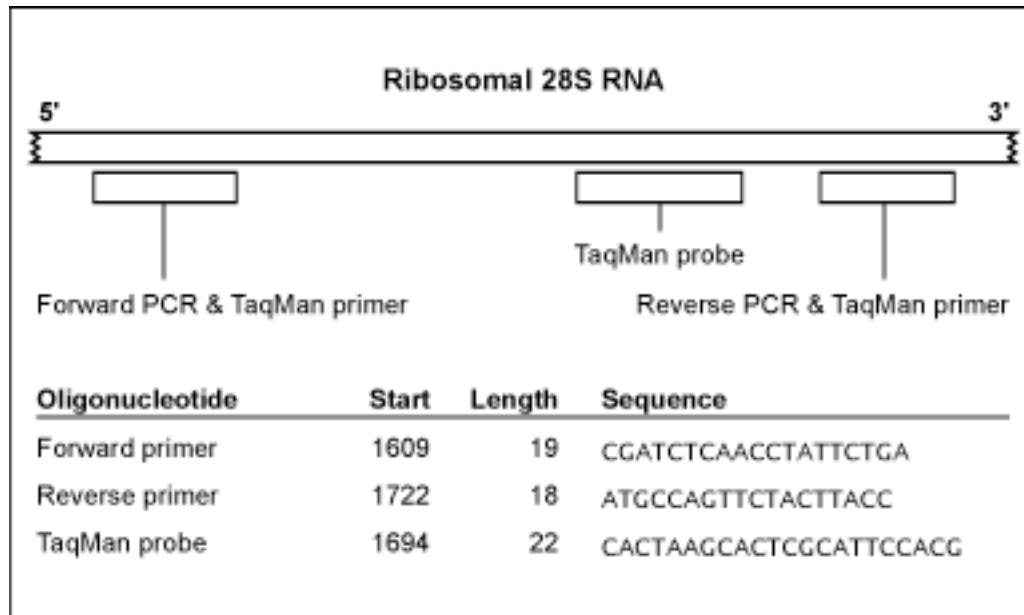

Figure 2: Illustration showing the bovine ribosomal 28S RNA and the placement and sequences of the primers and TaqMan probe for the 28S assay. The primers that were used to create an insert for a plasmid (PCR primers), were also used as TaqMan primers in the qPCR reaction. Start, is the starting position from the 5' end of the RNA sequence. Length, is the number of bases in the oligonucleotide.

Calculations and statistical analysis

Estimation of qPCR performance and quantification of RNA in BAL cell samples

In each cycle of the qPCR reaction, the TaqMan probe emits fluorescence that is proportional to the amount of PCR product. The starting quantity of each sample being analyzed is calculated from the number of cycles required to reach a set threshold of fluorescence. In each of the qPCR assays used in this study, the fluorescence threshold were set manually, in a range where the amplification of all standard samples appeared exponential.

For the plasmid-based standards used in this study (TLR4 and 28S), by knowing the length of the vector used, and the length of the plasmid-insert, the molecular weight of the plasmid could be calculated. By also knowing the DNA concentration of each dilution in the standard series, the plasmid copy number per sample could be calculated.

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For the BRSV qPCR assay, which have a BRSV RNA-based standard, starting quantity was given as relative units to the TCID50 value of the extracted BRSV infected cell culture (100 000 TCID50 ml^{-1}). The BRSV standard dilution samples were each assigned a TCID50 equivalent value, in accordance with their dilution. Standard sample 1 is 100 000 TCID50 equivalent, and the dilution was ten-fold so standard sample 2 is 10 000 TCID50 equivalent, and so on for six standard dilutions. However, only the odd numbered dilutions were used in this study. The qPCR results for the BAL cell samples were thus quantified relatively to the standard and expressed in TCID50 equivalent units.

By plotting the threshold cycle values of each of the standard samples on a logarithmic scale, against the known starting quantity of each standard sample, standard curves were constructed, one for each assay (Figures 3, 4 and 5). To verify the standard curves, the correlations between known starting quantities and threshold cycle values were calculated using Pearson's coefficient (r), then squared into the coefficient of determination (r^2). Values for r^2 over 0,995 are generally considered good when calculating standard curves for qPCR.

In the qPCR methods described above, the standard dilution series and the BAL cell samples were analyzed in triplicates (duplicates for 28S qPCR). Using the sample triplicate/duplicate mean threshold cycle value, the starting quantity was calculated using the related standard curve. Also, the standard deviation (unit is amplification cycles) of each triplicate/duplicate were calculated and used to estimate the intra- and inter-assay reproducibility.

Standardization of TLR4 and BRSV qPCR results, using 28S qPCR results, was achieved by division, applying these formulas on each sample results:

$$\text{TLR4 copies / 28S copies} = \frac{\text{TLR4 copies / sample}}{\text{28S copies / sample}}$$

$$\text{BRSV TCID50 eq. / 28S copies} = \frac{\text{BRSV TCID50 eq. / sample}}{\text{28S copies / sample}}$$

In each qPCR assay, at least two negative control samples were included as sentinels for contamination and cross-contamination between wells. Negative samples were considered negative after qPCR if the fluorescence in these wells did not reach the set fluorescence threshold.

The efficiency (E) of the qPCR assay corresponds to the DNA amplification per cycle, and is calculated from the slope of the standard curve, using this formula:

$$E = 10^{-1/slope}$$

The efficiency in percent is calculated using this formula:

$$\text{Efficiency (\%)} = (E - 1) \times 100$$

Sample exclusion

Out of 20 BAL samples from 20 calves, 3 samples were excluded from final analysis: 1 sample was excluded because it could not be standardized using 28S, since there was insufficient RNA mass to include it in the 28S qPCR assay; 1 sample was excluded after 28S qPCR, because of an extreme 28S value (3 copies of 28S rRNA, average of included samples was 474429 copies of 28S / sample); 1 sample was excluded after 28S standardization, because of an extreme TLR4/28S value (485 copies of TLR4 / 28S copy, average of included samples was 4 copies of TLR4 / 28S copies).

Testing for significant differences in TLR4 expression between subsets of animals

To test for significant differences in TLR4 expression between subsets of animals, a two-sided Student's T-test, assuming equal variance, was used (Microsoft Excel 2004 for Mac). T-test results of $p < 0,05$ were considered significant.

Correlation of BRSV and TLR4 results

To calculate the degree of linear dependence between TLR4 expression and concentration of BRSV RNA in the BAL cell samples, the Pearson product-moment correlation coefficient was calculated, and referred to as r. Calculations were done using Microsoft Excel 2004 for Mac, on all the samples and subsets of samples.

qPCR assay development and verification

TLR4 qPCR

Optimization-runs of the TLR4 qPCR yielded optimal concentrations of the TaqMan primers and TaqMan probe (presented in Figure 1) of 100 nM, and an optimal annealing temperature of 57°C.

When the BAL cell samples where analyzed for TLR4 mRNA, using the qPCR method described above, an efficiency of 97% was achieved. When constructing the standard curve (Figure 3), used to determine the starting quantity of TLR4 in the BAL cell samples, a coefficient of determination (r^2) of 0,997 was attained. The standard deviation within triplicates of the standards threshold cycle values were 0,09-0,39 amplification cycles (ac).

Figure 3: TLR4 qPCR standard curve calculated from the threshold cycle values and the known starting quantity in each TLR4 plasmid standard samples.

The mean efficiency of all the TLR4 qPCR assays, that were run in the course of developing this method, was 92% (SD 3,2%), and the mean coefficient of determination (r^2) 0,998 (SD 0,0004).

The mean of threshold cycle standard deviations within the triplicates of 83 unknown samples that were analyzed using the TLR4 assay, was 0,18 amplification cycles.

The mean calculated from the means of threshold cycle standard deviations for each of the five standard dilutions that were used in all the TLR4 assays, was 0,71 amplification cycles.

28S qPCR

Optimization-runs of the 28S qPCR yielded optimal concentrations of the primers of 200 nM, a concentration of the TaqMan probe of 100 nM, and an optimal annealing temperature of 57°C.

When the BAL cell samples where analyzed for 28S rRNA, using the qPCR method described above, an efficiency of 104% was achieved. When constructing the standard curve (Figure 4), used to determine the starting quantity of 28S in the BAL cell samples, a coefficient of determination (r^2) of 0,997 was attained. The standard deviation within triplicates of the standards threshold cycle values were 0,09-0,34 amplification cycles (ac).
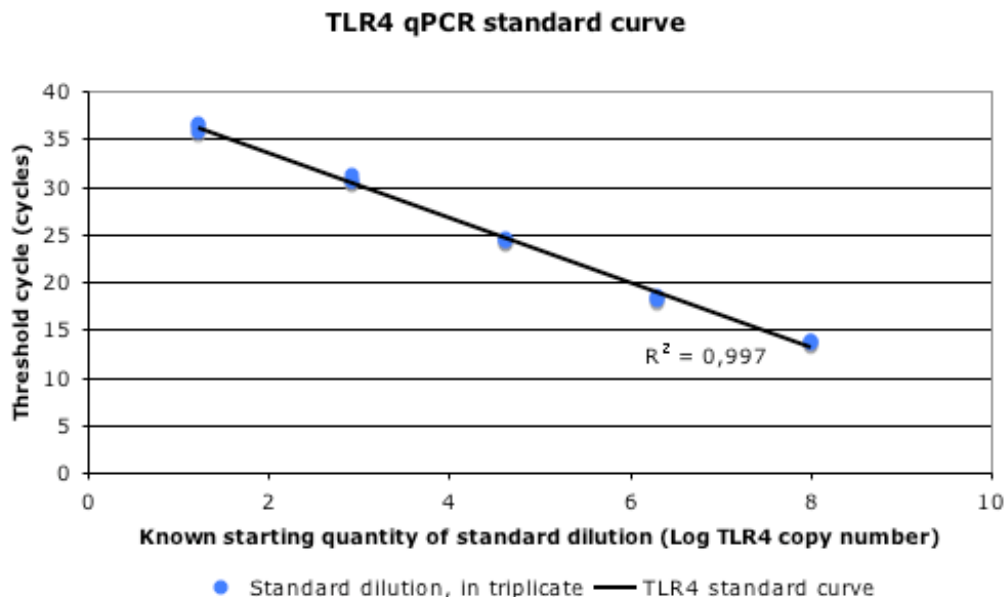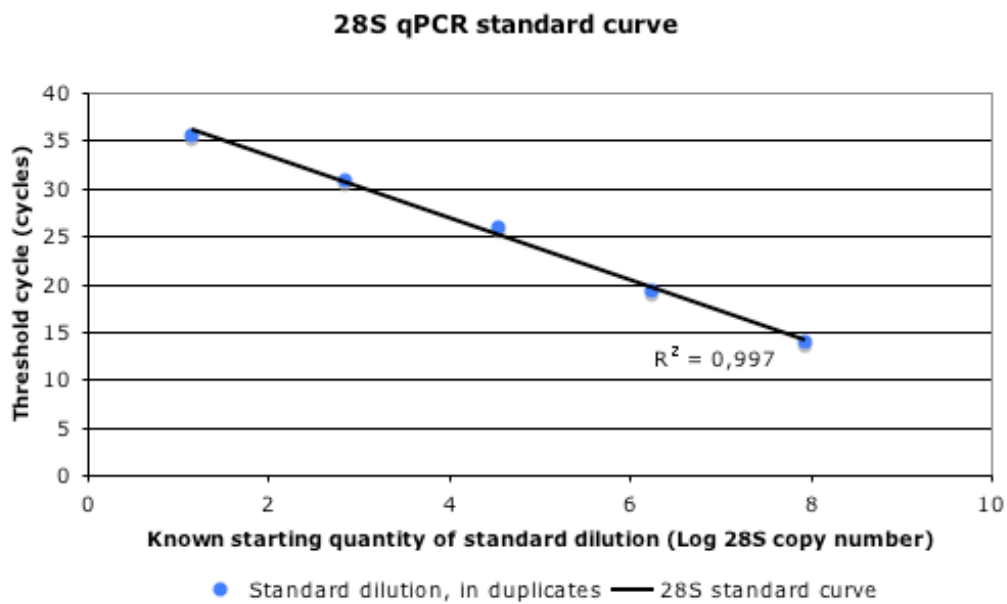

Figure 4: 28S qPCR standard curve calculated from the threshold cycle values and the known starting quantity in each 28S plasmid standard samples.

The mean efficiency of all the 28S qPCR assays, that were run in the course of developing this method, was 108% (SD 5,5%), and the mean coefficient of determination (r^2) 0,998 (SD 0,001).

The mean of threshold cycle standard deviations within the replicates of 82 unknown samples that were analyzed using the 28S assay, was 0,34 amplification cycles.

The mean calculated from the means of threshold cycle standard deviations for each of the five standard dilutions that were used in all the 28S assays, was 0,38 amplification cycles.

BRSV qPCR

The usage of previously published primers and probe worked well under the new thermoprofile and mastermix conditions. When analyzing the BAL cell samples using the BRSV qPCR assay, a correlation between known starting TCID50 equivalents in standard BRSV RNA dilutions and threshold cycles in the qPCR reaction was achieved, with a coefficient of determination (r^2) of 1,000 (see Figure 5). The standard deviation within duplicates of the standard threshold cycle values were 0,04-0,34 cycles, and an efficiency of 96 % was attained.
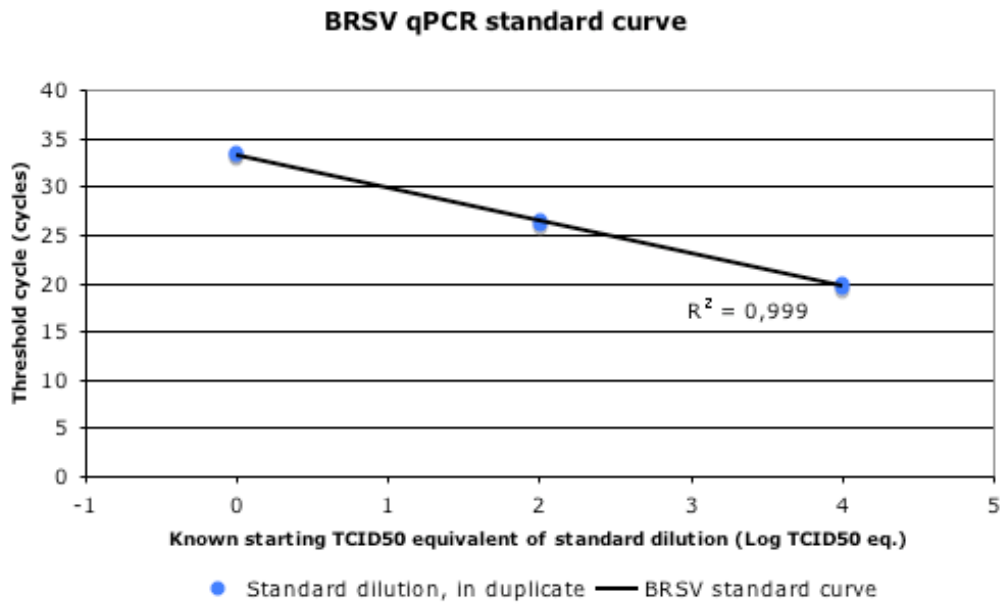
BRSV qPCR standard curve

Figure 5: Standard curve calculated from the threshold cycle values of the standard dilutions of the BRSV RNA.

TLR4 in experimentally infected animals

No significant difference in BAL cell TLR4 expression could be observed between calves with low and high levels of detectable BRSV RNA in BAL cells after BRSV challenge. (see Table 1). Nor could significant differences in TLR4 expression be observed between any combination of calf groups with regard to previous immunization.

BRSV in experimentally infected animals

A strong reduction of BRSV RNA was found in BAL cells of the calves immunized with BRSV-ISCOMs compared to controls. On day 6 after challenge, generally considered as the peak day of BRSV replication, none of the BRSV-ISCOM vaccinated animals had more than 6 TCID50 eq. units / million 28S rRNA copies in the qPCR from BAL (see Table 1). This agreed with results from the larger project, in which three of five calves were tested completely BRSV RNA negative in nasal swabs on day 6, and the remaining two calves had ≤ 1 TCID50 eq. unit. Furthermore, the calves immunized with BRSV-ISCOMs were all negative on first passage virus isolation from BAL and only one calf was positive in the second passage (Hägglund et al, manuscript in preparation). The BAL from this animal group were thus considered as weakly BRSV RNA positive in the further analysis of TLR4 expression (Figure 6).

In contrast to the BRSV-ISCOM immunized animals, all controls had more than 3900 TCID50 equivalent units / million 28S rRNA copies in the BRSV qPCR (see Table 1), and were positive on first passage virus isolation. They were thus considered as strongly BRSV positive in the further analysis of TLR4 expression (Figure 6).

Table 1: Results for BAL cell samples from TLR4 qPCR and BRSV qPCR, standardized with the results of 28S qPCR

Immunization group	Calf ID	TLR4 copies / 28S copy	BRSV TCID50 eq. / 10^6 28S copies
BRSV-ISCOM	1	0,5	0,1
	2	0,8	0,3
	3	0,5	5,6
	11	1,4	4,7
	12	0,1	0,1
BRSV-protein	6	0,7	35865,7
	7	0,2	21081,6
	8	0,4	6999,3
	16	0,6	23179,1
Adjuvant	9	0,8	22742,3
	10	3,5	76943,8
	18	0,9	17098,3
	20	0,7	17153,0
PBS	4	0,6	30209,1
	5	0,6	23435,9
	13	0,2	3921,2
	15	4,4	200490,3

Correlation between BRSV and TLR4

Among the calves considered to be strongly BRSV positive, a correlation was observed (Pearson's r of 0,92) between the BRSV qPCR results and the TLR4 qPCR results, both standardized using 28S. In Figure 6, these results, and those of the calves considered as weakly BRSV positive, are presented logarithmitized using base 10.
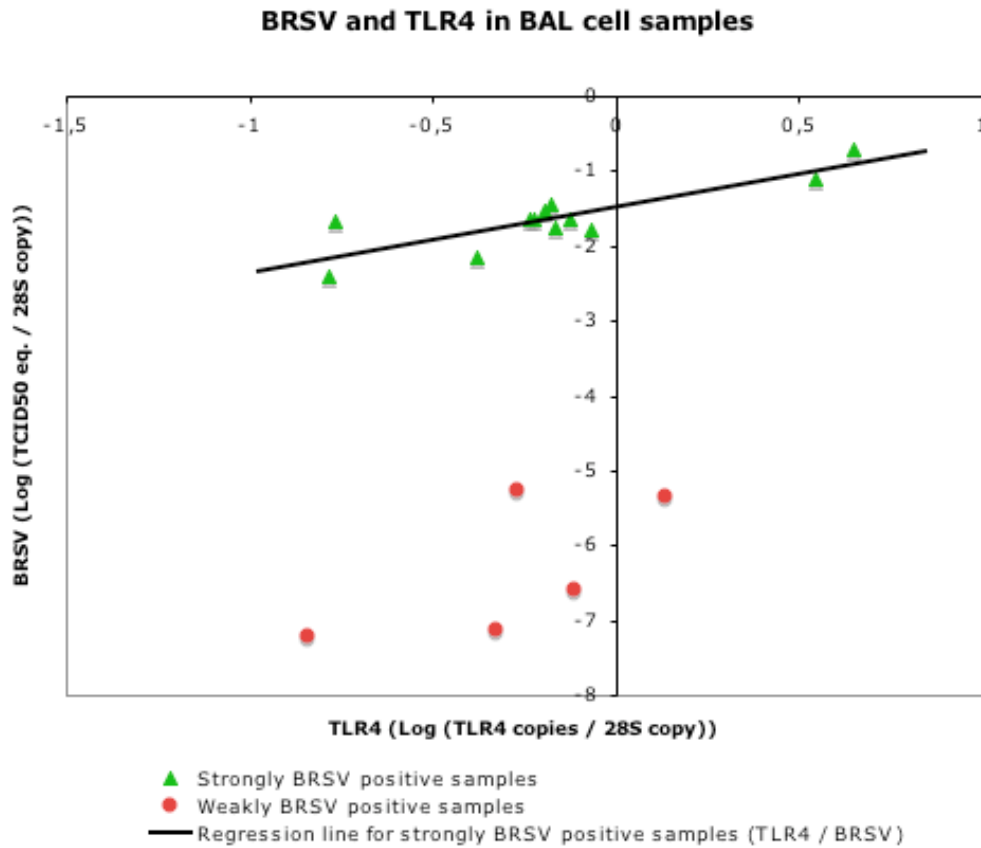

Figure 6: BAL sample BRSV qPCR results plotted against TLR4 qPCR results, both standardized using 28S qPCR results. All values are shown logarithmitized using base 10.

DISCUSSION

Evaluation of the TLR4 and the 28S qPCR methods

When evaluating a TaqMan-based quantitative real-time PCR (qPCR) method, such as the assays developed and employed in this study, there are a couple of criteria that can be measured: the specificity and efficiency of target amplification and detection; the fit of the constructed standard curve to the results of the standards; and the intra- and inter-assay reproducibility.

The specificity and efficiency of target amplification and detection is commonly measured by calculating efficiency from the slope of the standard curve for each qPCR assay. An ideal efficiency is 100%, meaning that the target sequence doubles each cycle. If the efficiency is more than 100%, this can indicate some degree of unspecific amplification. Commonly acceptable values for efficiency is 90-110%. The TLR4 qPCR had a mean of 92%, and the 28S qPCR had a mean efficiency of 108%.

Since linear dilution series are used for positive standards in qPCR, linearity is sought in the threshold cycle values of the standard samples. The degree of this linearity is calculated from the fit of the constructed standard curve to the results of the standards, as r^2. A generally acceptable limit for qPCR standard curve fit is $r^2 \geq 0,995$. The mean r^2 values of the TLR4 and 28S qPCR assays were both 0,998.

The intra-assay reproducibility of a qPCR assay is generally measured by the spread of threshold cycle values within replicates of the same sample. Both the TLR4 and the 28S qPCR assays had very low degrees of variation within replicates, and thus a high level of intra-assay reproducibility.

The inter-assay reproducibility of the two qPCR methods was estimated by the variability in threshold cycle of the standard dilutions. Here both the TLR4 and the 28S qPCR showed low variability and good reproducibility, although in this study the fluorescence thresholds was set manually to different values each time an assay was performed.

Since all the negative controls were clearly negative, in all the assays performed, the risk for interference of results by contamination or cross-contamination between wells, can be considered minimal.

In summary, both the TLR4 and the 28S qPCR assays performed very well in this study, with the described reagents and protocols.

TLR4 and BRSV

Although a correlation was observed between TLR4 expression and BRSV titers in the poorly protected calves after BRSV inoculation, the hypothesis that TLR4 can be used as a marker of protection against BRSV, could not be proven through these experiments. The reason being that no significant difference in TLR4 expression was observed between the strongly BRSV positive animals, and the weakly BRSV positive animals. Furthermore, it could not be determined whether TLR4 expression was increased after BRSV infection in all the animals (as have been reported during BRSV infection in other studies), including those animals that were only weakly BRSV positive. To determine this, a baseline of BAL cell

TLR4 expression in healthy non-infected calves would have to be established for comparison. This could be done by sampling the animals before BRSV inoculation, in addition to post-mortem sampling. However, pre-inoculation bronchoalveolar lavage may interfere with the natural course of respiratory disease being studied. A more general BAL cell TLR4 baseline for healthy calves could be established by performing post-mortem BAL-sampling of healthy non-infected calves at slaughterhouses.

Conclusions

This study demonstrated the design of quantitative real-time PCR assays for detection of TLR4 and 28S mRNA in clinical samples, with good efficiency as well as intra- and inter-assay reproducibility.

Furthermore it indicated that TLR4 cannot be used as a marker of protection against experimental BRSV infection of vaccinated animals, since all animals had equivalent levels of TLR4 expression, regardless of BRSV titers.

Further studies, regarding the BRSV and TLR4 interaction, is clearly needed to fully understand the role that the TLR4 receptor plays in the pathogenesis and immunopathology of BRSV. Hopefully, this knowledge will be helpful in the development of treatment during BRSV infection, or even an effective vaccine.

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