Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.

SAUNDERS

Research in Veterinary Science 74 (2003) 55–65

RVS

www.elsevier.com/locate/rvsc

An experimental infection model for reproduction of calf pneumonia with bovine respiratory syncytial virus (BRSV) based on one combined exposure of calves

K. Tjørnehøj[a,d,*], Å. Uttenthal[a], B. Viuff[c], L.E. Larsen[b], C. Røntved[e], L. Rønsholt[a]

[a] Danish Veterinary Institute, Lindholm, DK-4771 Kalvehave, Denmark
[b] Danish Veterinary Institute, Bülowsvej 27, DK-1790 Copenhagen V, Denmark
[c] Department of Pharmacology and Pathobiology, The Royal Veterinary and Agricultural University, Ridebanerej 3, DK-1870 Frederiksberg C, Denmark
[d] Department of Veterinary and Microbiology, Laboratory of Virology and Immunology, The Royal Veterinary and Agricultural University, Stigbøjlen 7, DK-1870, Frederiksberg C, Denmark
[e] Danish Institute of Agricultural Sciences, Research Centre Foulum, DK-8830 Tjele, Denmark

Accepted 21 August 2002

Abstract

Bovine respiratory syncytial virus (BRSV) has been recognised as an important pathogen in calf pneumonia for 30 years, but surprisingly few effective infection models for studies of the immune response and the pathogenesis in the natural host have been established. We present a reproducible experimental infection model for BRSV in 2–5-month-old, conventionally reared Jersey calves. Thirty-four colostrum-fed calves were inoculated once by aerosol and intratracheal injection with BRSV. Respiratory disease was recorded in 91% of the BRSV-inoculated calves, 72% had an accompanying rise in rectal temperature and 83% exhibited >5% consolidation of the lung tissue. The disease closely resembled natural outbreaks of BRSV-related pneumonia, and detection of BRSV in nasal secretions and lung tissues confirmed the primary role of BRSV. Nine mock-inoculated control calves failed to develop respiratory disease. This model is a valuable tool for the study of the pathogenesis of BRSV and for vaccine efficacy studies.
© 2002 Elsevier Science Ltd. All rights reserved.

Keywords: BRSV; Respiratory syncytial virus; Bovine; Pneumonia; Calves; Infection model

1. Introduction

Bovine respiratory syncytial virus (BRSV) was first reported in 1970 in Switzerland (Paccaud and Jacquier, 1970) and has subsequently been identified worldwide as an important pathogen in acute respiratory disease in calves (Baker and Velicer, 1991; Bryson et al., 1978; Larsen, 2000; Uttenthal et al., 1996; Verhoeff and van Nieuwstadt, 1984).

Early attempts to experimentally reproduce pneumonia with BRSV had limited success, and the role of BRSV in calf pneumonia could therefore not be verified (Belknap et al., 1995). Thus, Smith et al. (1975) and Castleman et al. (1985) obtained mild clinical disease but very little postmortem evidence of pneumonia following single inoculations of conventionally reared calves, while others only obtained postmortem evidence of BRSV-pneumonia with little or no clinical disease in gnotobiotic calves (Gaddum et al., 1996; Thomas et al., 1984). When inoculating intratracheally and intranasally for four consecutive days, some authors (Ciszewski et al., 1991; Bryson et al., 1983) reproduced moderate to severe signs of respiratory disease as well as consolidated lung tissue, while others only achieved mild signs of BRSV-infection (Kimman et al., 1987; Otto et al., 1996). Thus, even when using this rather heavy inoculation regimen, reproducibility of the disease has been inconsistent. However, disease has recently been reproduced in 2–6-month-old calves by single aerosol inoculations with North-American field isolates of BRSV (West et al., 1999; Woolums et al., 1999).

* Corresponding author. Fax: +45-55-86-97-00.
E-mail address: kt@vetinst.dk (K. Tjørnehøj).

0034-5288/02/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PII: S0034-5288(02)00154-6

In this study we describe a reliable experimental infection model for BRSV in 2–5-month-old conventionally reared Jersey calves using a European field isolate of BRSV. The results presented in the present paper have been accumulated over several years and support the papers published by West et al. (1999) and Woolums et al. (1999).

2. Materials and methods

2.1. Calves

Five colostrum-deprived (<6 h old) and 41 colostrum-fed (4–14 days old) Jersey bull calves were obtained from two closed dairy herds and transferred to isolation units at Lindholm. Colostrum-deprived calves were fed 175 ml serum from BRSV-antibody-free cattle in an electrolyte solution for 3 days and treated with amoxicillin (Synulox, Pfizer) on day 2 and 3 after birth. From day 4, all calves were fed milk replacer ("Sundkalv, Type 1," DLG, Denmark) and reared according to normal management procedures for dairy calves.

The calves were tested weekly for BRSV-specific IgM and IgG1 antibodies (Uttenthal et al., 2000) to rule out natural BRSV-infection during rearing. They were 7–22 weeks old at inoculation (median value 14 weeks, interquartil range 11–16 weeks), and were free from persistent infection with bovine virus diarrhoea virus (BVDV) (Rønsholt et al., 1996).

2.2. Experimental design

Eight experiments were conducted during 24 months (Table 1), one in colostrum-deprived (exp. III) and seven in colostrum-fed (exp. IV, Va, Vb, VI, VIII, IX, and XI) calves. All calves were inoculated on day 0 PI in each individual experiment. A total of 37 calves were inoculated with BRSV and killed on day 2, 4, 6, 7, 8, 15, or 30 post-inoculation (PI), while nine mock-inoculated control calves were killed on day 2, 4, 6, 8, or 15 PI (Table 1).

The four calves in exp. XI were treated with 2.5 mg enrofloxacin per kg (Baytril, Bayer) from the day before infection until euthanasia on day 6 PI.

2.3. Inocula

A 1-month-old, colostrum-deprived calf (calf #2031) was killed on day 6 after inoculation with lung lavage fluid from a naturally BRSV-infected calf with severe pneumonia (Viuff et al., 1996, calf #3). The left lung of calf #2031 was lavaged with 100 ml of Eagles' minimal essential medium (Glasgow modification) without antibiotics (MEM−), and this lung lavage fluid inoculum (BRSVLLF) was snapfrozen in liquid nitrogen in aliquots of 4 ml and kept at −80 °C.

Filtered (0.2 μm) extract of lung tissue from calf #2031was cultured in low passage, semi-primary foetal bovine lung cells (FBL) grown in MEM− with 5% foetal bovine serum, and harvested with sterile glass beads between passages. First, third and fourth cell culture passages (BRSVCCP1, 3a, 3b, and 4) were frozen at −80 °C in aliquots of 4 ml and used as inoculum (Table 1).

Control inocula consisted of lung lavage fluid from healthy, young calves (CTRLLFa and b) or uninfected FBL cells (CTRCCP1).

Co-infection of inocula with bacteria and mycoplasmas was excluded by conventional isolation methods. The inocula were cultured on monolayers of calf kidney cells (CK), which were examined for cytopathogenic effect (CPE), and screened for infection with bovine entero virus (BEV), bovine herpes virus 1 (BoHV-1), parainfluenza 3 virus (PI-3V), bovine adeno virus (BAV), mammalian REO viruses (REO), and BVDV by indirect immunofluorescence test (IFT) using pooled virus-specific hyperimmune rabbit- and swine-sera and anti-species conjugates (Dako, Glostrup, Denmark). The presence of bovine corona virus (BCV) was investigated by ELISA (Uttenthal et al., 1996).

2.4. Inoculation procedure

The calves were inoculated once by a combination of 10 min aerosol exposure, mostly containing droplets less than 3 μm (Waechtomat inhalator VM 82, Kruuse, Denmark), followed by one intratracheal injection. The aerosol inoculum was diluted to a total of 5 ml in phosphate-buffered saline (PBS) and administered through a mask covering nostrils and mouth. The intratracheal inoculum was diluted to 20 ml in PBS and injected into trachea during provoked heavy inhalation. A dose of $10^{4.6}$–$10^{5.2}$ $TCID_{50}$ of BRSV was administered each way to each of the 37 BRSV-infected calves, while the nine control calves received BRSV-negative inoculum. Any possible loss of infectivity during aerosol creation was not measured.

2.5. Clinical monitoring and sampling

Groups of control calves and BRSV-infected calves were kept in separated isolation rooms after inoculation, hence, clinical assessment could not be double blind. Respiratory rates, rectal temperatures, and other clinical signs were recorded, and serum samples and nasal swabs collected, daily from the day of inoculation (day 0 PI) until slaughter. Sampling was reduced to nasal swabs and serum samples three times weekly after day 15 PI. Nasal swabs were collected in 0.8 ml MEM including 1 million IU penicillin and 1 g streptomycin per liter (MEM+).

The calves were anaesthetised with 30% pentobarbital (1 ml per 6 kg bodyweight) and bled out through Aa.

and Vv. axillaris and iliaca externa. The lungs were immediately removed and photographs taken, from which the extent of macroscopic lesions was rated with a score between 0 and 5 according to Fig. 1. The gross appearance of the pathological changes of the lung tissue, and the amount and nature of the exudate, were evaluated during the postmortem procedure for the two lungs.

The left lung was lavaged as described above, and lung tissue samples (2 g) were recovered from nine predetermined sites representing the dorsal, medial, and ventral part of the three lobes, for detection of BRSV antigen by ELISA. Lung lavage fluid and lung tissue from the right lung, preferably from consolidated areas, were examined for BRSV by isolation on FBL cell culture. Growth of BRSV was demonstrated by IFT using guinea pig anti-BRSV serum and anti-guinea pig conjugate (Dako, Glostrup, Denmark).

Tissue samples from eight predetermined sites in the right lung, trachea, nasal epithelium, ln. tracheobronchalis, tonsil, and spleen were fixed in 10% neutral-buffered formalin. The tissue samples were examined by conventional histological techniques, and the presence of BRSV-antigen was investigated by immunohistochemistry as previously described (Viuff et al., 1996).

2.6. BRSV antigen ELISA

Nasal swabs were pre-treated with 5% Triton X-100 (Serva) for 30 min, while lung tissue samples were triturated (Stomacher) in equal amounts of ELISA buffer (0.015 M Na_2HPO_4, 0.005 M KH_2PO_4, 0.5 M NaCl, and 0.05% Tween 20, pH 6.8) and centrifuged at 1600 G for 10 min. One standardised positive control was included for every nine samples.

Microplates with 96 wells (Maxisorp U96 immunoplate, Nunc, Denmark) were coated with rabbit anti-BRSV hyperimmune serum (1:800) in carbonate buffer (pH 9.6) for 1 h at 37 °C. Between each step, all wells were rinsed three times in ELISA buffer and left for five minutes with a fourth rinse.

The steps were duplicate twofold dilution series (100 μl) of the samples (undiluted—1:128), followed first by normal or anti-BRSV guinea pig serum in alternating columns, then by peroxidase-conjugated rabbit anti-guinea pig immunoglobulins (Dako, Glostrup, Denmark), and finally by OPD substrate. The titrated

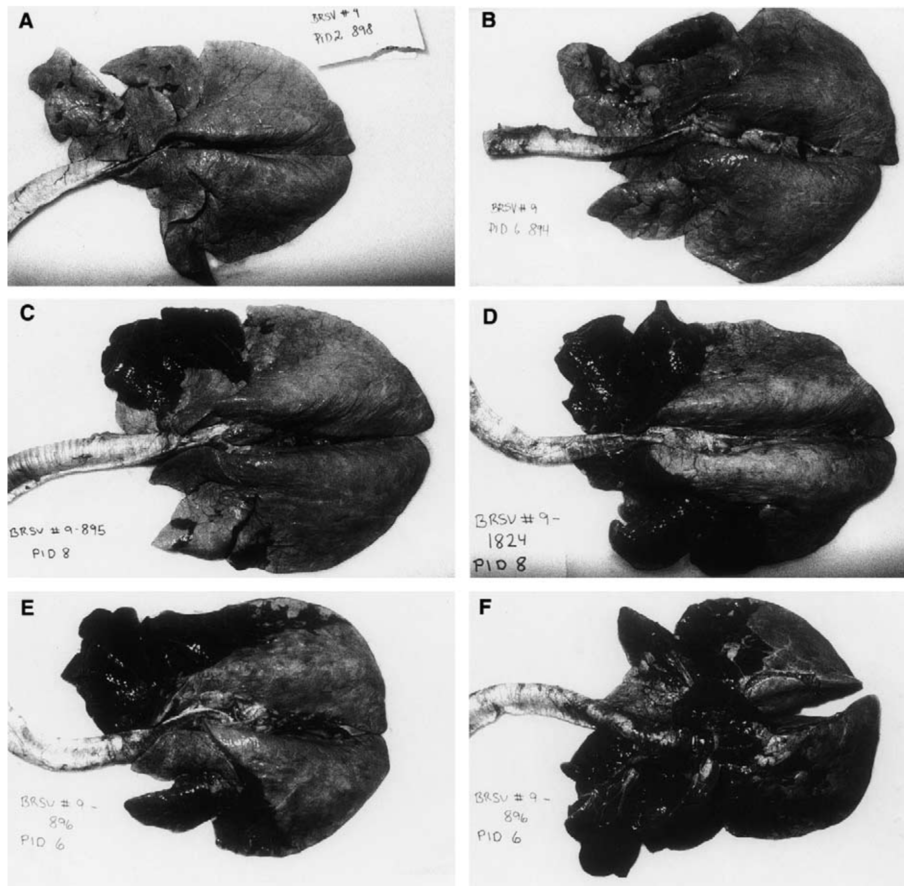

Fig. 1. Basis for scoring of extent of lung damage. Score 0 is given to a lung without pathological lesions. (A) Score 1 (1–5%); (B) score 2 (5–15%); (C) score 3 (15–30%); (D) score 4 (30–50%); (E) score 5 (>50%), dorsal aspect; (F) score 5 (>50%), ventral aspect.

samples were left overnight at 5 °C, while the guinea pig sera (1/200 with 5% normal rabbit serum) and conjugate (1:700) were incubated for 30 min at 37 °C. The substrate reaction was stopped after 10 min at room temperature by adding 1.0 M H_2SO_4, and the plates were read at OD 492 nm.

The titre of a sample was expressed as the highest positive dilution. Dilutions were considered positive if the OD value of the well added anti-BRSV guinea pig serum was >0.100, and the ratio between the OD values of this well and the corresponding negative well added normal guinea pig serum (P/N ratio) was 3 or above.

2.7. Serology

Sera from the day of inoculation and the day of slaughter were tested for antibodies against PI-3, BAV, and BCV by ELISA (Uttenthal et al., 1996), as well as for neutralising antibodies against a Danish BRSV field strain. The neutralising BRSV titre was based on neutralisation peroxidase labelling assay (NPLA), and calculated as a 50% endpoint titre ($NPLA_{50}$).

2.8. Other infectious agents

Supernatant of triturated lung tissue was inoculated into CK monolayers, which were examined for CPE during two cell culture passages, followed by IFT for specific viruses as described for inocula. In addition, tissue from the right lung was tested for the presence of PI-3, BAV, and BCV antigen by ELISA (Uttenthal et al., 1996). Moreover, lung-, spleen-, and liver-samples were investigated for aerobic and anaerobic bacteria, and bronchial swabs for mycoplasmas, by conventional isolation methods.

2.9. Statistical analysis

Statistical analysis was performed using GraphPad InStat tm (GraphPad Software, San Diego, CA, USA). The differences between the BRSV-inoculated and mock-inoculated calves in Table 4 were tested for statistical significance using Fisher's exact test, while Spearman's correlation coefficient was used to investigate correlations between neutralising antibodies and clinical parameters, as well as in-between clinical parameters (Table 3).

3. Results

Baselines for recording of clinical signs and pathological changes were determined from the 6–21-week-long monitoring of the 46 calves before inoculation, and from the findings in the nine control calves after mock-inoculation, respectively. The baselines were set at a respiratory rate of 40 per minute, a rectal temperature of 39.3 °C and 5% consolidation of the lung tissue.

Number of days with clinical signs and nasal excretion of BRSV between day 0 and 8 PI are summarised in Table 1.

3.1. Mock-inoculated control calves

Except for a febrile reaction and a lung score of 2 in one control calf in exp. III and one control calf with a respiratory rate of 52 on day 1 PI in exp. IV, there were no signs of disease in the mock-inoculated control animals (Table 1). Daily nasal swabs were free from BRSV antigen, and lung tissue samples were free from BRSV antigen and live BRSV.

3.2. BRSV-inoculated calves

Respiratory disease was induced in all of the eight experiments (Table 1), as the majority of the BRSV-infected animals in each experiment (67–100%) developed respiratory rates above 40 min^{-1} for 1–5 days between day 4 and 8 PI, which in 67–100% of the sick animals was accompanied by febrile reactions. BRSV excretion, demonstrated as antigen in nasal swabs, was found in 67–100% of the BRSV-infected calves in each of the experiments (Table 1).

Clinical signs and virus shedding were accompanied by enhanced vesicular or bronchial respiration, nasal discharge, coughing, and varying degrees of anorexia and depression (data not shown).

Temporal development in average respiratory rates and in average rectal temperatures from inoculation to slaughter are shown for calves inoculated with lung lavage fluid based inocula in Fig. 2I and for calves inoculated with cell culture passaged inocula in Fig. 2II. For clarity, data have been pooled for experiments using BRSVCCP3 (Va, VI, VIII, and IX). Both lung lavage BRSV-inoculum and cell culture passaged BRSV-inocula induced increases in average respiratory rates and average rectal temperatures on day 4–6 PI; accordingly the two BRSV-inoculated calves killed on day 2 PI did not show any signs of respiratory disease. Average rectal temperatures returned to normal levels around day 8–9 PI, while average respiratory rate remained high until day 13 PI (based on five calves).

After day 4 PI, the average rectal temperature in BRSVLLF-inoculated calves (Figs. 2I and B) was higher in the remaining colostrum-deprived calf than in the colostrum-fed calves (seven calves); however, two of the colostrum-fed calves had as severe febrile reactions as the colostrum-deprived calf. This situation illustrates the effect of the variation in time of onset (1–2 days) and in severity of clinical disease between individual calves on the average values. As a consequence we have also considered the individual peak respiratory rate, peak rectal

Table 1
Summary of eight BRSV-infection experiments[A]

Inoculum[i]	Exp. no.	Day of kill	No. of calves	Number of animals with respiratory disease	Clinical signs (number of days)		Number of days with excretion of BRSV	Results from necropsy	
					Respiratory rate >40 min^{-1}	Rectal temperature $>39.3\,^{\circ}$C		Extent of lung consolidation (scores 0–5[a])	Detection of BRSV antigen (no. of nine sites)
*brsv*LLF	III[b]	4	2	1/2	1, 0	1, 0	1, 1	2, 1	1[c] 1[c]
		8	1	1/1	4	6	5	5	7
*ctr*LLFa		4	1	0/1	0	0	0	0	0[c]
		8	1	0/1	0	2	0	2	0[c]
*brsv*LLF	IV	4	2	2/2	1, 1	1, 0	2, 0	3, 2	6, 5
		8	2	2/2	5, 4	3, 4	5, 5	4, 4	6, 0
		15	2	2/2	4, 4	4, 3	5, 1	4, 1	0, 0
		30	3	3/3	5, 3, 3	4, 0, 3	6, 4, 6	2[e], 1[e], 1[e]	0, 0, 0
*ctr*LLFb		4	1	0/1	0	0	0	1	0[d]
		8	1	0/1	1[f]	0	0	0	0[d]
		15	1	0/1	0	0	0	1	0[d]
*brsv*CCP1	Vb	6	3	3/3	4, 3, 2	1, 1, 1	1, 1, 0	3, 2, 3	5, 6, 6
*brsv*CCP3	Va	6	3	3/3	2, 2, 1	2, 2, 0	2, 0, 2	3, 3, 2	7, 5, 4
*brsv*CCP3	VI	7	1	1/1	2	3	5	3	4
*brsv*CCP3	VIII	7	6	6/6	4, 2, 3, 4, 3, 4	3, 0, 1, 3, 2, 4	4, 4, 4, 4, 3, 4	5, 3, 3, 3, 2, 4	6, 3, 2, 5, 6, 6
*brsv*CCP3	IX[h]	2	2	0/2	0, 0	0, 0	0, 0	1, 1	0, 0
		4	2	0/2	0, 0	0, 0	1, 1	1, 1	8, 7
		6	2	2/2	2, 2	1, 2	3, 3	2, 5	6, 8
		8	2	2/2	2, 6	0, 3	3, 4	3, 4	0, 2
*ctr*CCP1		2	1	0/1	0	0	0	0	0
		4	1	0/1	0	0	0	1	0
		6	1	0/1	0	0	0	1	0
		8	1	0/1	0	0	0	1	0
*brsv*CCP4	XI[g]	6	4	3/4	2, 1, 3, 0	2, 1, 0, 0	3, 3, 3, 3	4, 1, 2, 0	7, 7, 7, 0

Recordings from seven experiments: clinical signs and BRSV excretion from the nose from day 0 to day 8 PI, and extent of gross pathological changes and BRSV antigen in the lungs at sacrifice. Clinical signs and BRSV excretion are expressed as duration. Detection of antigen displays number of BRSV-positive of nine predetermined sites in the left lung.

[A] Clinical signs and excretion of BRSV from day 0 to day 8 PI and results from necropsy.
[a] According to Fig. 1.
[b] Colostrum-deprived calves.
[c] Only one sample tested.
[d] Four or six samples tested.
[e] Calves killed on day 30 PI, hence, not comparable with other calves, because of unknown degree of repair processes.
[f] This control calf had elevated respiratory rate on day 1 PI, presumably as a result of the inoculation procedure.
[g] Enrofloxacin-treated calves.
[h] Respiratory rates were not measured on day 3 PI in this experiment.
[i] Inoculum consisting of lung lavage fluid (LLF) or first, third, or fourth cell culture passage (CCP), *brsv* for BRSV and *ctr* for control.

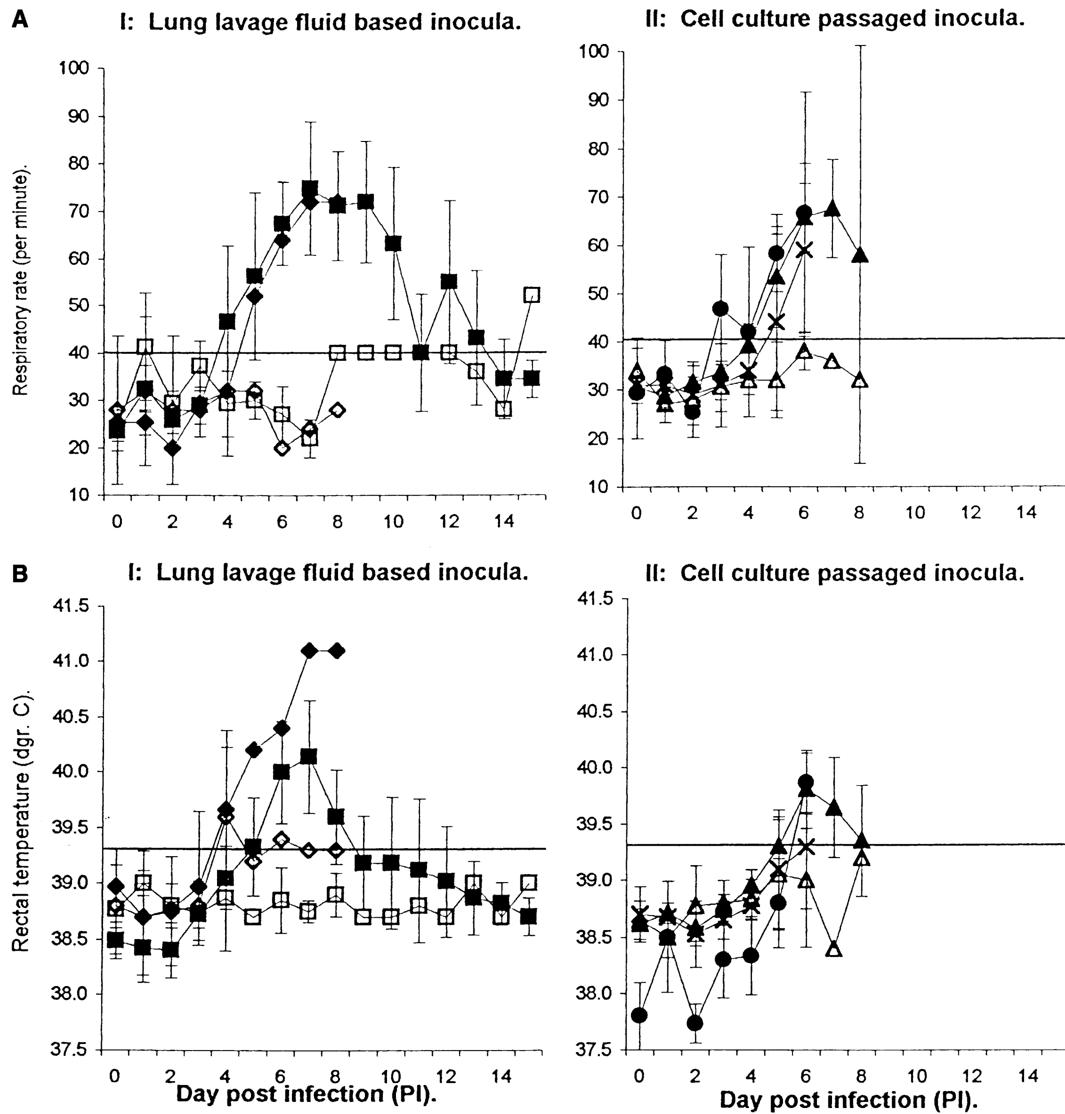

Fig. 2. Temporal development in average respiratory rate (per minute) and average rectal temperature (°C) after inoculation of calves with lung lavage fluid-based (I) or cell culture passaged (II) BRSV- or mock-inoculum. Graphs display 95%-confidence-intervals. 95%-confidence-intervals are not applicable in cases with one animal per group. Baselines indicated in solid at 40 respirations/min and 39.3 °C, respectively. (I) Colostrum-deprived calves (exp. III) inoculated with BRSVLLF (◆) or CTRLLF (◇). Colostrum-fed calves (exp. IV) inoculated with BRSVLLF (■) or CTRLLF (□). (II) Colostrum-fed calves inoculated with BRSVCCP1 (exp. Vb) (●), BRSVCCP3 (exp. Va, VI, VIII, and IX) (▲), BRSVCCP4 (exp. XI) (×), or CTRCCP1 (exp. IX) (△).

temperature and peak titre of BRSV in nasal swabs in the 35 BRSV-infected calves which were killed on day 4 PI and later (Table 2). Median values were 72 min^{-1} for peak respiratory rate, 39.9 °C for peak rectal temperature, and 64 (2^6) for peak titre of BRSV in nasal swabs. Peak rectal temperature correlated with peak respiratory rate, peak titre in nasal swabs and lung score, and peak respiratory rate correlated with peak titre in nasal swabs and lung score, while peak titre in nasal swabs did not correlate significantly with lung score (Table 3).

Substantial pneumonic lesions, manifested as lobular, dark red, consolidated areas with mucopurulent exudate

Table 2
Peak values for signs of respiratory disease in the 35 BRSV-infected calves which were killed on day 4 PI or later

Sign of	Range	Median value	Interquartil range
Peak respiratory rate	24–104 min^{-1}	72 min^{-1}	54–84 min^{-1}
Peak rectal temperature	38.5–41.1 °C	39.9 °C	39.2–40.4 °C
Peak titre in nasal swab	<1 (2^0)–128 (2^7)	64 (2^6)	8–128 (2^3–2^7)

Table 3
Correlations in-between lung score and peak values for respiratory rate, rectal temperature, and titre in nasal swabs in the 35 BRSV-infected calves which were killed on day 4 PI or later

Sign 1	Sign 2	r	95%-confidence-interval	P
Peak rectal temperature	Peak respiratory rate	0.6419	0.3738–0.8110	<0.0001
Peak rectal temperature	Peak titre in nasal swabs	0.4739	0.1393–0.7113	0.0061
Peak rectal temperature	Lung score	0.6327	0.3706–0.8015	<0.0001
Peak respiratory rate	Peak titre in nasal swabs	0.5347	0.2054–0.7553	0.0023
Peak respiratory rate	Lung score	0.4701	0.1408–0.705	0.0058
Peak titre in nasal swabs	Lung score	0.3284	−0.03377–0.6143	0.0665

in the bronchi, were typically detected in the cranioventral parts of the lungs from day 4 PI in BRSV-inoculated calves (Table 1). Thus, moderate to severe lung consolidation (lung scores 2 or above) was present in 0/2 calves killed on day 2 PI (0%), 3/6 killed on day 4 PI (50%), 22/24 killed on days 6–8 PI (92%), 1/2 killed on day 15 PI (50%), and 1/3 killed on day 30 PI (33%). Interstitial oedema was seen in most BRSV-infected calves, whereas emphysema was seen in animals killed on day 6 and 8 PI. The emphysema was especially pronounced in the caudal lobes, and less frequently seen in the medial and accessory lobes. The incidence of emphysema rose with increasing lung score; lung score 0–2: 0/18 (0%), lung score 3: 2/10 (20%), lung score 4: 3/6 (50%), and lung score 5: 3/3 (100%) (data not shown).

A detailed description of the replication and clearance of BRSV in these calves is given elsewhere (Viuff et al., accepted). Immunohistochemistry revealed BRSV antigen in a few scattered bronchial epithelial cells in calves killed on day 2 PI, and in a high number of bronchial epithelial cells on day 4 PI. Large amounts of BRSV antigen were found in bronchiolar and alveolar epithelial cells on day 6–8 PI (Fig. 3). The pathological lesions were clearly associated with the presence of BRSV as a shift in the main lesions from bronchitis to bronchiolitis and alveolitis was observed corresponding to the sites where BRSV was detected. BRSV antigen was not detected in lung tissue samples from calves killed on days 15 and 30 PI or from mock-inoculated control calves.

The microscopical lesions were characteristic for BRSV-related pneumonia featuring formation of syncytial cells, hyperplasia of bronchial epithelium with loss of cilia, influx of neutrophils, plugging of bronchi, bronchioles, and alveoli, collapse of alveoli, interstitial oedema, and emphysema (Fig. 3). From day 15 PI the overall impression was repair.

The antigen ELISA detected BRSV in lung tissue from 24/25 animals killed between day 4 and 7 PI and from 3/5 calves killed on day 8 PI, while lung tissue samples from calves killed on days 2, 15, and 30 PI were negative (Table 1). Antigen titres in the 143 BRSV-positive lung samples (Table 1) ranged between 2 and >128 (2^1 to $> 2^7$) (median value 64 (2^6), interquartil range 32–128 (2^5–2^7)). Detection of BRSV by isolation on FBL cells preceded detection of antigen by ELISA, while the ELISA demonstrated BRSV later in the course of the infection.

The results from the 39 colostrum-fed calves, which were killed on day 4 PI or later, have been summarised in Table 4. A total of 91% of BRSV-inoculated calves (29/32) developed respiratory rates above 40 min^{-1}, 72% (23/32) had febrile reactions and 91% (29/32) excreted BRSV. Animals killed on day 30 PI have been excluded from lung score due to unknown degree of repair, and animals killed on day 15 and 30 PI from BRSV in lung tissue due to disappearance of antigen after day 8 PI. Thus 83% (24/29) had lung scores above 1 and 89% (24/27) contained BRSV antigen in the lung tissue at slaughter. In contrast, none of the mock-inoculated calves exhibited signs of disease after inoculation. Using Fisher's exact test all five parameters were significantly more prevalent in the BRSV-inoculated calves as compared to the mock-inoculated calves (two-tailed P-value < 0.005).

3.3. Serology

The colostrum-deprived calves were free from neutralising BRSV-specific antibodies at the time of inoculation, while the colostrum-fed calves had varying levels of maternally derived neutralising antibodies (range <2–362 $NPLA_{50}$, median value 91 $NPLA_{50}$, interquartil range 10–256 $NPLA_{50}$). However, the neutralising antibody titre at inoculation did not correlate with peak respiratory rate ($r = -0.002765$, 95%-confidence-interval: −0.4000–0.3953, two-tailed P-value = 0.6060), peak rectal temperature ($r = 0.1475$, 95%-confidence-interval: −0.2497–0.5022, two-tailed P-value = 0.4537) or lung score ($r = 0.08926$, 95%-confidence-interval: −0.2904–0.4446, two-tailed P-value = 0.6390) in BRSV-inoculated, colostrum-fed calves.

Low neutralising titres tended to rise from day 6 PI, while calves with a higher pre-infection titre generally showed a decrease in titre. The neutralising antibody response appeared to develop slightly earlier (Tjørnehøj, 2000) than the IgG1 response, which is described by Uttenthal et al. (2000).

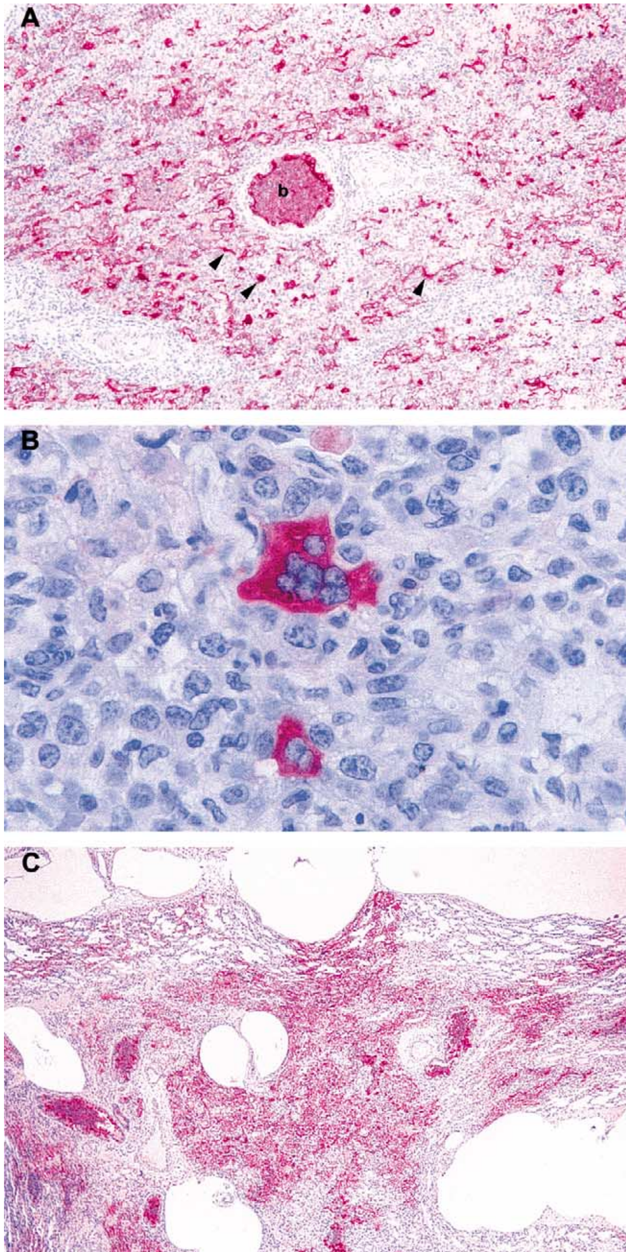

Fig. 3. BRSV antigen demonstrated by immunohistochemistry in a BRSV infected calf killed PID 6 (antigen positive cells are red). (A) High amount of BRSV antigen is detected in alveolar epithelial cells (exemplified by arrowheads) and in the epithelial cells of a bronchiole (b). Also the cellular exudate in the bronchiole is containing BRSV antigen. (B) A high number of BRSV antigen containing syncytial cells is demonstrated PID 6. (C) Emphysema located in an area with BRSV antigen.

3.4. Other infectious agents

All cell culture based inocula (exp. V–XI) were free from interfering bovine pathogens.

In contrast, the lung lavage fluid inocula (BRSVLLF, CTRLLFa, and CTRLLFb) were infected with *Mycoplasma dispar* (exp. III and IV), and CTRLLFa was infected with BCV (exp. III).

At postmortem, bronchial swabs from 67% of the control calves and from 78% of the BRSV-inoculated calves contained *M. dispar*, while the corresponding figures for *Ureaplasma* spp. were 33% and 38%. *Mycoplasma bovirhinis* was recovered from one control and two BRSV-inoculated calves, while three BRSV-infected calves had *Pasteurella multocida,* one *Pasteurella avium*, and two *Haemophilus somnus* in the lung tissue. In experiment III, all five calves excreted BCV, which was also detected in lung tissue from calves killed on day 4 PI.

Sero-conversions to PI-3, BCV, or BAV were not detected in any of the calves.

4. Discussion

In this study, moderate to severe BRSV-induced pneumonia was reproduced in seven experiments in colostrum-fed calves and in one experiment in colostrum-deprived calves. Nasal shedding of BRSV and recovery of BRSV from the lungs at necropsy, in particular the constant demonstration of BRSV antigen in affected lung tissue from BRSV-infected calves by immunohistochemistry, confirmed that this virus caused the disease. The described experimental BRSV-infections were confined to the respiratory system as demonstrated in a previously published systematic screening of other tissues for BRSV by RT-PCR, however, BRSV-specific RNA was detected in the tracheobronchal lymph node from some of the calves killed between day 2 and 6 PI (Larsen et al., 1999).

The severity of the elicited parameters of respiratory disease varied amongst individual calves, but the peak values of respiratory rate, rectal temperature, and titre of BRSV in nasal swabs were significantly correlated with each other, and the peak respiratory rate and rectal temperature correlated significantly with lung score. Likewise, emphysema, a sign of severe respiratory distress, was only noted in severely affected calves. Thus, it is verified that the measured parameters of respiratory disease were associated with each other and caused by the BRSV-infection. The variation amongst individual animals was comparable between our individual experiments.

This disease pattern, the clinical signs and the distribution and character of lung lesions in the BRSV-inoculated calves equalled descriptions of BRSV-related pneumonia in naturally infected calves (Belknap, 1993; Bryson, 1993; Kimman et al., 1989; Pirie et al., 1981; Verhoeff and van Nieuwstadt, 1984; Verhoeff et al., 1984; Viuff et al., 1996). The severity of elicited pneumonia did not correlate to the level of maternally derived neutralising antibodies at inoculation; and after the successful inoculation of colostrum-fed calves in experiment IV, we decided to focus on this type of calves to establish a more natural experimental model.

Table 4
Prevalence of signs of respiratory disease in colostrum-fed calves which were killed on day 4 PI or later

	Number of animals		Percent	
	BRSV-inoculated	Mock-inoculated	BRSV-inoculated (%)	Mock-inoculated (%)
Respiratory rates above 40 min^{-1} [c]	29/32	0/6	91	0
Rectal temperatures above 39.3 °C[d]	23/32	0/6	72	0
Excretion of BRSV[c]	29/32	0/6	91	0
Lung score above 1[e]	24/29[a]	0/6	83	0
BRSV in lung tissue[e]	24/27[b]	0/5[b]	89	0

[a] The three BRSV-inoculated calves killed on day 30 PI have been excluded due to unknown degree of repair in the lung tissue.
[b] The five BRSV-inoculated calves and the one mock-inoculated calf killed on days 15 and 30 PI have been excluded due to disappearance of BRSV from the lung tissue after day 8 PI.
[c] Two-tailed P-value < 0.0001.
[d] Two-tailed P-value $= 0.0018$.
[e] Two-tailed P-value $= 0.0003$.

Only 2/6 BRSV-inoculated calves killed on day 4 PI had signs of disease, but, since lung tissue samples of all six calves were positive for BRSV by both isolation and antigen ELISA, they were probably incubating the disease. Consistently, the two healthy BRSV-inoculated calves killed on day 2 PI had a few BRSV-infected bronchial epithelial cells.

The acute phase of the infection, manifested by nasal virus excretion, febrile reaction, and presence of viral antigen in the lungs, had almost ended by day 8 PI, while nasal discharge, coughing and consolidation of lung tissue persisted until day 15 and 30 PI in some animals, indicating a prolonged reparatory state in the lung tissue. According to the average respiratory rates presented in Fig. 2, enhanced respiratory rates seemed to cease around day 13–15 PI. However, one of the three calves killed on day 30 PI maintained enhanced respiratory rate until this day, and more recent experimental BRSV-infections using our model has confirmed long-term respiratory distress in some of the infected animals (unpublished data).

The degree of respiratory disease achieved in these experiments was more distinct than in most previous studies using single inoculation of conventionally reared calves (Belknap et al., 1995). However, Elazhary et al. (1980), West et al. (1999), and Woolums et al. (1999) have obtained comparable levels of clinical signs with one inoculation. One common factor between these three successful studies and the present model is the exposure to BRSV in an aerosol delivered by a nebulizer over an extended period of time, and we believe this has been crucial for the induction of disease.

The exact mode of transmission during a natural BRSV-infection has not yet been unambiguously defined. It has been demonstrated that the human RSV virus is probably very commonly transferred by contact spread of large droplets between high-risk patients in hospitals (Hall and Douglas, 1981), while Mars et al. (1999) recently demonstrated experimentally that BRSV can be transmitted by air between groups of calves in adjacent rooms. The pathological lesions seen after the combined aerosol and intratracheal exposure in the present study closely resembled the pathological lesions in acute BRSV-related pneumonia in the field (Bryson, 1993; Kimman et al., 1989; Pirie et al., 1981; Viuff et al., 1996); moreover, the infection progressed from the upper to the lower respiratory tract during 4–6 days (Viuff et al., accepted). Taken together, these findings indicate that the inoculation with aerosol for 10 min is probably quite close to the field exposure, where calves due to the close housing are continuously exposed to BRSV during an outbreak.

In experiment III, mock-inoculated and BRSV-inoculated calves experienced a co-infection with BCV, which presumably originated from the BCV-infected mock-inoculum and reached the BRSV-inoculated calves through the contaminated inhalator. This infection probably caused the slightly higher rectal temperature in the colostrum-deprived control calf on day 4–8 PI, and it may also have influenced the rectal temperature of the colostrum-deprived, BRSV-inoculated calf (Figs. 2I and B). However, due to the limited number of animals in this experiment, this is merely a speculation.

Pasteurella multocida, *P. avium*, and *H. somnus* were infrequently recovered from BRSV-inoculated calves, while mycoplasmas were isolated from most BRSV-inoculated calves as well as from control calves in the present study, indicating that the calves were harbouring mycoplasmas at the time of infection. However, there was no clear correlation between degree of respiratory disease and recovery of bacteria, as bacteria could be found in both animals with widespread and limited extent of lung consolidation, and some calves without bacterial infection displayed serious lung lesions. Moreover, respiratory disease was also induced in the enrofloxacin-treated calves in exp. XI.

The interfering bacterial and mycoplasmal airway pathogens in this study are also commonly recovered from the respiratory system of healthy calves (Thomas and Smith, 1972; Woldehiwet et al., 1990a; Woldehiwet

et al., 1990b); essentially Woldehiwet et al. (1990b) were unable to associate isolation rates of mycoplasmas with respiratory disease in calves. Besides, the character of the induced pneumonias in the present study differ from those recorded in experimental infection studies with *H. somnus* (Jackson et al., 1987), *P. multocida* (Gourlay et al., 1989), and mycoplasmas (Friis, 1980; Friis, 1981; Howard et al., 1976; Tanskanen, 1984).

Hence, though it cannot be ruled out that bacteria or mycoplasmas may have played a contributing role in the clinical course of the disease, the combination of cell culture based inoculum and thorough examination of inocula as well as experimental calves for other bovine pathogens made it possible to exclude that they played a primary etiological role in the present study. Thus, we have by consistent experimental reproduction of moderate to severe disease clearly demonstrated the capability of BRSV as a primary etiologic agent of acute pneumonia in calves.

On day 2 PI, BRSV could only be detected in the lung tissue by immunohistochemistry, PCR (Larsen et al., 1999) and isolation, indicating that these methods were more sensitive than the presented antigen ELISA. However, the ELISA detected BRSV later in the course of infection than the isolation method, in lung tissue as well as in nasal swabs (Tjørnehøj, 2000). Whether this was caused by the presence of neutralising antibodies or other inhibiting immunological factors, or by the die off of the BRSV particles, leaving antigen for a few more days, is not known.

In conclusion, we have established a reproducible experimental model for BRSV-infection using a Danish field strain of BRSV. This model was capable of inducing clinical signs resembling natural cases of BRSV-related pneumonia in 80% of the infected conventionally reared calves, and hence mimics the field conditions closely. Consequently, it may be a valuable tool for studying the pathogenesis of BRSV-infection and for vaccine efficacy studies. Studies on these issues have been published elsewhere (Heegaard et al., 2000; Larsen et al., 1998; Larsen et al., 1999; Røntved et al., 2000; Uttenthal et al., 2000; Viuff et al., accepted).

Acknowledgments

The enthusiastic participation of the animal attendants and the laboratory personnel at the three involved institutions is highly acknowledged. The authors thank Dr. N.F. Friis and Dr. R. Hoff-Jørgensen, the Danish Veterinary Laboratory, Copenhagen, and cand. Scient. T. Grubbe for helpful advice. The study was supported by grants from the Danish Agricultural and Veterinary Research Council, the Danish Ministry of Food, Agriculture, and Fisheries, and the Research Centre for the Management of Animal Production and Helath.

References

Baker, J.C., Velicer, L.F., 1991. Bovine respiratory syncytial virus vaccination: current status and future vaccine development. Compendium on Continuing Education for the Practicing Veterinarian 13, 1323–1333.

Belknap, E.B., 1993. Recognizing the clinical signs of BRSV infection. In: Baker, J.C. (Ed.), Symposium on BRSV infection. Veterinary Medicine 88, 886–887.

Belknap, E.B., Ciszewski, D.K., Baker, J.C., 1995. Experimental respiratory syncytial virus infection in calves and lambs. Journal of Veterinary Diagnostic Investigation 7, 285–298.

Bryson, D.G., McFerran, J.B., Ball, H.J., Neill, S.D., 1978. Observations on outbreaks of respiratory disease in housed calves—(1) Epidemiological, clinical, and microbiological findings. Veterinary Record 103, 485–489.

Bryson, D.G., McNulty, M.S., Logan, E.F., Cush, P.F., 1983. Respiratory syncytial virus pneumonia in young calves: clinical and pathologic findings. American Journal of Veterinary Research 44, 1648–1655.

Bryson, D.G., 1993. Necropsy findings associated with BRSV pneumonia. In: Baker, J.C. (Ed.), Symposium on BRSV infection. Veterinary Medicine 88, 894–899.

Castleman, W.L., Lay, J.C., Dubovi, E.J., Slauson, D.O., 1985. Experimental bovine respiratory syncytial virus infection in conventional calves: light microscopic lesions, microbiology, and studies on lavaged lung cells. American Journal of Veterinary Research 46, 547–553.

Ciszewski, D.K., Baker, J.C., Slocombe, R.F., Reindel, J.F., Haines, D.M., Clark, E.G., 1991. Experimental reproduction of respiratory tract disease with bovine respiratory syncytial virus. Veterinary Microbiology 28, 39–60.

Elazhary, M.A.S.Y., Galina, M., Roy, R.S., Fontaine, M., Lamothe, P., 1980. Experimental infection of calves with bovine respiratory syncytial virus (Quebec strain). Canadian Journal of Comparative Medicine 44, 390–395.

Friis, N.F., 1980. *Mycoplasma dispar* as a causative agent in pneumonia of calves. Acta Veterinaria Scandinavica 21, 34–42.

Friis, N.F., 1981. Failure to produce pneumonia in calves by inhalation of *Mycoplasma bovirhinis* and *Ureaplasma* aerosols. Acta Veterinaria Scandinavica 22, 283–285.

Gaddum, R.M., Cook, R.S., Thomas, L.H., Taylor, G., 1996. Primary cytotoxic T-cell responses to bovine respiratory syncytial virus in calves. Immunology 88, 421–427.

Gourlay, R.N., Thomas, L.H., Wyld, S.G., 1989. Experimental *Pasteurella multocida* pneumonia in calves. Research in Veterinary Science 47, 185–189.

Hall, C.B., Douglas, R.G., 1981. Modes of transmission of respiratory syncytial virus. Journal of Pediatrics 99, 100–103.

Heegaard, P., Godson, D.L., Toussaint, M.J.M., Tjørnehøj, K., Larsen, L.E., Viuff, B., Rønsholt, L., 2000. The acute phase response of haptoglobin and serum amyloid (SSA) in cattle undergoing experimental infection with bovine respiratory syncytial virus. Veterinary Immunology and Immunopathology 77, 151–159.

Howard, C.J., Gourlay, R.N., Thomas, L.H., Stott, E.J., 1976. Induction of pneumonia in gnotobiotic calves following inoculation of Mycoplasma dispar and ureaplasmas (T-mycoplasmas). Research in Veterinary Science 21, 227–231.

Jackson, J.A., Andrews, J.J., Hargis, J.W., 1987. Experimental *Haemophilus somnus* pneumonia in calves. Veterinary Pathology 24, 129–134.

Kimman, T.G., Straver, P.J., Zimmer, G.M., 1989. Pathogenesis of naturally acquired bovine respiratory syncytial virus infection in calves: Morphologic and serologic findings. American Journal of Veterinary Research 50, 684–693.

Kimman, T.G., Westenbrink, F., Schreuder, B.E.C., Straver, P.J., 1987. Local and systemic antibody response to bovine respiratory syncytial virus infection and reinfection in calves with and without maternal antibodies. Journal of Clinical Microbiology 25, 1097–1106.

Larsen, L.E., 2000. Bovine respiratory syncytial virus (BRSV): a review. Acta Veterinaria Scandinavica 41, 1–24.

Larsen, L.E., Jensen, N.E., Tjørnehøj, K., Viuff, B., Uttenthal, Å., 1999. Diagnosis of enzootic pneumonia in Danish cattle: reverse transcription-polymerase chain reaction assay for detection of bovine respiratory syncytial virus in naturally and experimentally infected cattle. Journal of Veterinary Investigation 11, 416–422.

Larsen, L.E., Uttenthal, Å., Tegtmeier, C., Arctander, P., Tjørnehøj, K., Viuff, B., Røntved, C., Rønsholt, L., Alexandersen, S., Blixenkrone-Møller, M., 1998. Serological and genetic characterisation of bovine respiratory syncytial virus (BRSV) indicates that Danish isolates belong to the intermediate subgroup: no evidence of a selective effect on the variability of G protein nucleotide sequence by prior cell culture adaption and passages in cell culture or calves. Veterinary Microbiology 62, 265–279.

Mars, M.H., Bruschke, C.J., van Oirschot, J.T., 1999. Airborne transmission of BHV1, BRSV, and BVDV among cattle is possible under experimental conditions. Veterinary Microbiology 66, 197–207.

Otto, P., Elschner, M., Reinhold, P., Köhler, H., Streckert, H.J., Phillippou, S., Werchau, H., Morgenroth, K., 1996. A model for respiratory syncytial virus (RSV) infection based on experimental aerosol exposure with bovine RSV in calves. Compendium on Immunology and Microbiology in Infectious Disease 19, 85–97.

Paccaud, M.F., Jacquier, C., 1970. A respiratory syncytial virus of bovine origin. Archives von Gesamte Virusforschung 30, 327–342.

Pirie, H.M., Petrie, I., Pringle, C.R., Allan, E.M., Kennedy, G.J., 1981. Acute fatal pneumonia in calves due to respiratory syncytial virus. Veterinary Record 108, 411–416.

Rønsholt, L., Nylin, B., Bitsch, V., 1996. A BVDV antigen- and antibody blocking ELISA (DVIV) system used in a Danish voluntary eradication program. In: Proceedings of the Third ESVV Symposium on Pestivirus Infections, Lelystad, The Netherlands, pp. 150–153.

Røntved, C.M., Tjørnehøj, K., Viuff, B., Larsen, L.E., Godson, D.L., Rønsholt, L., Alexandersen, S., 2000. Increased pulmonary secretion of tumor necrosis factor-α in calves experimentally infected with bovine respiratory syncytial virus. Veterinary Immunology and Immunopathology 76, 199–214.

Smith, M.H., Frey, M.L., Dierks, R.E., 1975. Isolation, characterization, and pathogenicity studies of a bovine respiratory syncytial virus. Archives of Virology 47, 237–247.

Tanskanen, R., 1984. Colonisation pattern of the respiratory tract of calves by *Mycoplasma dispar*. Acta Veterinaria Scandinavica 25, 577–592.

Thomas, L.H., Smith, G.S., 1972. Distribution of mycoplasmas in the non-pneumonic bovine respiratory tract. Journal of Comparative Medicine 82, 1–4.

Thomas, L.H., Stott, E.J., Collins, A.P., Jebbett, J., 1984. Experimental pneumonia in gnotobiotic calves produced by respiratory syncytial virus. British Journal of Experimental Pathology 65, 19–28.

Tjørnehøj, K., 2000. Development of a model for experimental infection of calves with bovine respiratory syncytial virus, and use of the model in vaccine efficacy studies. Ph.D. Thesis, The Royal Veterinary and Agricultural University, Copenhagen, Denmark.

Uttenthal, Å., Jensen, N.P.B., Blom, J.Y., 1996. Viral aetiology of enzootic pneumonia in Danish dairy herds: diagnostic tools and epidemiology. Veterinary Record 139, 114–117.

Uttenthal, Å., Larsen, L.E., Philipsen, J.S., Tjørnehøj, K., Viuff, B., Nielsen, K.H., Nielsen, T.K., 2000. Antibody dynamics in BRSV-infected Danish dairy herds as determined by isotype specific immunoglobulins. Veterinary Microbiology 76, 329–341.

Verhoeff, J., van Nieuwstadt, A.P.K.M.I., 1984. BRS virus, PI3 virus, and BHV1 infections of young stock on self-contained dairy farms: epidemiological and clinical findings. Veterinary Record 114, 288–293.

Verhoeff, J., Van der Ban, M., van Nieuwstadt, A.P.K.M.I., 1984. Bovine respiratory syncytial virus infections in young cattle: clinical and haematological findings. Veterinary Record 114, 9–12.

Viuff, B., Tjørnehøj, K., Larsen, L.E., Røntved, C.M., Uttenthal, A., Rønsholt, L., Alexandersen, S., accepted. Replication and clearance of respiratory syncytial virus: apoptosis is an important pathway of virus clearance after experimental infection with bovine respiratory syncytial virus. The American Journal of Pathology.

Viuff, B., Uttenthal, Å., Tegtmeier, C., Alexandersen, S., 1996. Sites of replication of bovine respiratory syncytial virus in naturally infected calves as determined by in situ hybridization. Veterinary Pathology 33, 383–390.

West, K., Petrie, L., Haines, D.M., Konoby, C., Clark, E.G., Martin, K., Ellis, J.A., 1999. The effect of formalin-inactivated vaccine on respiratory disease associated with bovine respiratory syncytial virus infection in calves. Vaccine 17, 809–820.

Woldehiwet, Z., Mamache, B., Rowan, T.G., 1990a. The effects of environmental temperature and relative humidity on the bacterial flora of the upper respiratory tract in calves. British Veterinary Journal 146, 211–218.

Woldehiwet, Z., Mamache, B., Rowan, T.G., 1990b. Effects of age, environmental temperature and relative humidity on the colonization of the nose and trachea of calves by *Mycoplasma* spp. British Veterinary Journal 146, 419–424.

Woolums, A.R., Anderson, M.L., Gunther, R.A., Schelegle, E.S., LaRochelle, D.R., Singer, R.S., Boyle, G.A., Friebertshauser, K.E., Gershwin, L.J., 1999. Evaluation of severe disease induced by aerosol inoculation of calves with bovine respiratory syncytial virus. American Journal of Veterinary Research 60, 473–480.