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Serological indication for persistence of bovine respiratory syncytial virus in cattle and attempts to detect the virus

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Summary. To identify putative persistent bovine respiratory syncytial virus (BRSV) infections in cattle, seven cattle that had experienced BRSV infections were treated with corticosteroids for two periods of 5 days. During the 5-day periods and the 3 weeks after treatment, attempts were made to isolate BRSV from lung lavage fluid and nasal swab specimens. Fluorescent antibody tests were used to detect BRSV antigen in lung lavage cells. A BRSV specific polymerase chain reaction (PCR) assay was developed, and was performed on lung lavage samples of all seven cattle as well as on various tissues of five of the cattle. In addition, nasal swabs of 74 over-one-year-old cattle, in a closed dairy herd were also assayed by PCR. The virus or its RNA was not detected in putative carriers, by any of the methods used, whereas all positive controls were positive. After corticosteroid treatment, three of the seven cattle showed a four-fold rise in antibody titre, suggesting induction of virus replication. BRSV-seronegative sentinel calves, that were housed together with each corticosteroid-treated animal, did not develop antibodies to BRSV indicating that BRSV was not shed by corticosteroid-treated cattle, or was shed at a very low level. In addition BRSV was not detected in seropositive cattle in a closed farm in summer. Although we consider the rises in antibody titres against BRSV an indication for persistence of BRSV in cattle, BRSV or its RNA was not detected in infected cattle.

Introduction

Respiratory syncytial virus (RSV) is a member of the genus *Pneumovirus*, subfamily *Pneumovirinae* of the family *Paramyxoviridae*. Isolation of RSV

from human beings was first reported in 1957 [7], whereas the virus was first isolated in cattle in 1970 [20]. Human RSV and bovine RSV are closely related and both viruses cause respiratory disease and show seasonal periodicity in their natural host [28]. Therefore knowledge about the mechanism of BRSV periodicity may contribute to the study of these issues of HRSV.

BRSV infections and reinfections commonly occur worldwide, and can cause severe respiratory symptoms in calves [3, 5], almost exclusively during autumn and winter. Although BRSV infections seem absent during summer, these infections and BRSV-associated disease annually recur in a herd, even when the herd is closed and reintroduction of the virus is unlikely [29]. A previous study showed that subclinical reinfections did take place in spring and summer, and the results also suggested that BRSV is more likely to persist in a herd in individual seropositive cattle than by continuous circulation [9, 29]. It may be hypothesized that such persistent BRSV infections are reactivated in autumn or winter and act as the source of a new outbreak. Therefore we tried to induce virus replication by corticosteroid treatment of infected cattle and attempted to demonstrate this replication by employing virus isolation, virus antigen detection, polymerase chain reaction (PCR) and antibody tests on samples of experimentally and naturally infected cattle.

Materials and methods

Cattle

Seven cattle were subjected to the experimental procedure in three groups. Group 1, consisting of four Holstein Friesian heifers (Nos. 2 003, 2 004, 2 005 and 2 006), had experienced an outbreak of BRSV infections with respiratory disease in the previous winter, at about 5 months of age [29]. Group 2, consisting of two adult dairy cattle cross breds (Nos. 2 705 and 2 706) of about 4.5 years old, had experienced a fourfold rise in BRSV specific antibody titre, just before the BRSV infection season. These two cattle were bovine herpesvirus 1 (BHV1) antibody positive. Group 3 consisted of a 4-month-old Friesian calf (3 000) (Table 1), that was an immunotolerant carrier of bovine virus diarrhea virus (BVDV). It was raised specific pathogen free and was experimentally infected with the BRSV Odijk strain. This strain was isolated in December 1991, in Odijk, the Netherlands, from a calf showing acute respiratory disease. For experimental infection the Odijk strain was not passaged in cell culture but in spf-calves (methods described by Van der Poel et al. [30]). After inoculation by intranasal (5 ml) and intratracheal route (5 ml), the calf had shown respiratory symptoms.

During the study, which included a quarantine period of 3 weeks, all seven cattle were housed separately in an isolation room with positive air pressure at the Institute for Animal Science and Health (ID-DLO) in Lelystad. After the quarantine period, a BRSV-seronegative sentinel calf was housed together with each of the seven cattle. In addition, two BRSV antibody seronegative calves, housed in a separate isolation room, were inoculated intranasally (5 ml) and intratracheally (5 ml) 3 times a week with 10 ml of lung lavage fluid collected from the cows of group 2.

In order to detect virus shedding by seropositive cattle during summer, in July nasal swabs were collected of all of 74 over-1-year-old cattle, in a closed dairy herd with annual BRSV outbreaks in the Netherlands.

Table 1. Ages of the animals (in weeks) at the time of infections, during corticosteroid treatment and at necropsy

Animal	Age at the time of			
	primary infection	reinfection	corticosteroid treatment	necropsy
2 003	23	–	64–68	72
2 004	21	–	62–66	70
2 005	22	–	63–67	–
2 006	22	–	63–67	–
2 705	*	123	229–233	251
2 706	*	131	237–241	259
3 000	8	–	14–18	23
3 052	31	–	–	32

Group 1: 2 003, 2 004, 2 005 and 2 006
Group 2: 2 705 and 2 706
Group 3: 3 000
Control: 3 052
* Unknown

Corticosteroid treatment

All seven cattle that had experienced a BRSV infection earlier in life, were intramuscularly injected with dexamethasone (Dexadreson, Intervet, Boxmeer, The Netherlands) 0.5 mg/kg a day, during five consecutive days. This treatment was repeated three weeks after the start of the first treatment. Cows in group 2 underwent additional stress treatments: both cows were dried off directly after the quarantine period and during four days, eight hours a day, water was flushed underneath the two cows.

Sampling procedures

Lung lavage samples were collected three times a week, with 60 ml of Hank's minimal essential medium (HMEM), according to the method described by Fogarty et al. [10]. Usually 30 to 50 ml of fluid was recovered. Samples were collected from all seven cattle, before and during corticosteroid treatment and for three weeks after treatment. After collection, samples were aliquoted. One part was immediately processed for virus isolation. The second part was centrifuged (800 rpm, 300 × **g**, 10 min) and the supernatant was stored until assayed for BRSV specific antibodies; cell pellets were suspended in 1 to 5 ml HMEM. About 0.5 ml of the suspension was used to make cell sedimentations on slides for fluorescent antibody tests; the remainder of the cell suspension was directly stored at −70 °C until assayed by PCR. Ten ml of lavage fluids of cows of group 2 were used to inoculate two BRSV antibody seronegative calves. The inoculum was administered intratracheally and intranasally, three times a week, during three weeks, directly after lavage fluids had been collected.

Blood samples and nasal swabs were obtained from all seven corticosteroid-treated cattle, at least three times a week during the whole experiment. All seven sentinel calves, and the two calves which were inoculated with lavage fluid from cattle of group 2, were blood sampled three times a week. Nasal swabs specimens were suspended in 4 ml of

ELISA buffer (see below). Sera and processed nasal swabs were stored at −20 °C until ELISA was performed.

At the end of the experiment, white blood cells were obtained from all seven corticosteroid-treated cattle. Erythrocytes were lysed by adding 2 volumes of 0.83% NH_4Cl. After centrifuge (200–600 × **g**) white blood cell sediments were washed and resuspended in 2 ml HMEM and stored at −70 °C. Five of the seven cattle were euthanised for necropsy. Mucosae of pharynx and trachea, lungs, bronchial and retropharyngeal lymph nodes, and spleen, liver, kidney and bone tissues were collected and also immediately stored at −70 °C, until analysed by fluorescent antibody test and PCR.

Nasal swabs collected from 74, over-1-year-old cattle, were directly placed in 1 ml denaturation buffer and processed for PCR.

Virus isolation

Nasal swab specimens were suspended in 2 ml Hanks' minimal essential medium (HMEM) with 2% fetal bovine serum (FBS) and antibiotics (penicillin 500 U/ml; streptomycin 0.5 mg/ml; kanamycin 0.13 mg/ml; nystatin 70 U/ml). Fetal bovine serum and antibiotics were added to lung lavage samples in the same concentration. Bovine herpesvirus 1 hyperimmune serum, that did not contain BRSV antibodies, was added (12.5%) to specimens of cattle of group 2 in order to neutralize possible reactivated BHV1. After incubation at room temperature for half an hour, nasal swab specimens and lung lavage samples were centrifuged (800 × **g**). Two ml supernatant of nasal swab samples and 5 ml supernatant of lung lavage samples were added to confluent monolayers of a fetal bovine trachea cells or monolayers of fetal bovine diploid lung cells (cattle group 2), grown in 25-cm^2 disposable polystyrene flasks (Costar Europe Ltd.). After 2 to 3 h of preincubation, monolayers were washed once with HMEM (2% FBS, antibiotics). The cultures were incubated at 37 °C for at least 1 week. After incubation, cells were removed from the flasks by trypsinization and after fixation, examined by fluorescent antibody test (see below).

Virus isolation was also performed on tracheal and pharyngeal mucosa, lung, bronchial and retropharyngeal lymph nodes, tonsil, spleen, liver and kidney, of all six necropsied animals (Table 1). For virus isolation, 1 gram of each organ was crushed in a mortar and suspended in 10 ml HMEM (2% FBS, antibiotics). After centrifuging (800 × **g**) 1 ml of the supernatant was incubated onto cell cultures as described above. The cultures were incubated at 37 °C for 1 week. After incubation, cell cultures were tested by immunoperoxidase monolayer assay (IPMA): After discarding the growth medium and washing once with PBS, cells were dried for 45 min at 37 °C and frozen for 45 min at −20 °C. The monolayers were fixed with 4% (w/v) cold paraformaldehyde in PBS for 10 min. After fixation the plates were washed once in PBS 0.5% Tween-20 and incubated for 1 hour at 37 °C with a mixture of 2 anti-BRSV monoclonal antibodies (MAbs) directed against the F-protein (ID-DLO, Lelystad, The Netherlands) diluted 1:30 in 0.5 M NaCl, 1% Tween-80 (pH 7.6), 0.1% NaN_3 in volumes of 50 µl. Rabbit-anti-mouse peroxidase (Dakopatts, Denmark) diluted 1:100 in the same buffer without NaN_3, was used in the second incubation for 1 h at 37 °C. After each incubation plates were washed 5 times in PBS 0.5% Tween 20 and "knocked dry" on a towel. Staining was performed according to Jensen [12] with 3-amino-9-ethyl carbazole (AEC) (Sigma) as substrate.

Antigen detection by fluorescent antibody test (FAT)

Indirect FAT was performed on lung lavage cells immediately after lung lavage samples were obtained. Lavage cell sedimentations on slides were prepared by air drying droplets of

resuspended lavage cell pellets on slides. Cell sedimentations on slides were fixed in acetone for 10 min at room temperature. Cells were indirectly stained with monoclonal antibodies directed against the F-protein of BRSV (ID-DLO, Lelystad) (30 min at 37 °C) and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulins prepared in rabbits (Dakopatts, Denmark) (30 min at 37 °C). Fluorescent antibody test were also performed on white blood cells and tissues collected at necropsy. White blood cell sedimentations on slides were prepared in the same way as lavage cell sedimentations. Sections of frozen tissues were obtained with a microtome and fixed to slides in acetone for 10 min at room temperature before assayed.

To serve as a positive control in FAT and RT-PCR, an 8 month old calf (3 052) (Table 1) was inoculated intratracheally and intranasally with BRSV: the third cell culture passage of the 'Odijk' strain, 10 ml, 10^3 $TCID_{50}$/ml. After inoculation the calf was lung lavaged daily, and necropsied 6 days after inoculation.

Isolation of RNA from test samples

RNA was isolated from lung lavage cells, nasal fluids, white blood cells and postmortem collected tissues, using a cold lysis and RNA extraction procedure that was developed by combining the methods described by Cane et al. [6], and by Chomczynski et al. [8]. The detailed protocol can be obtained from the authors on application.

Oligonucleotide primers and probe

Primers were chosen to selectively amplify BRSV genetical material. Primers were selected in a region which was homologous for all BRSV strains and different from HRSV strains [13, 32].

The sequences of the two primers used were 5′-GTGCATTAAGAACTGGATGG-3′ (P751) and 5′-GCAAAAAGAGGGATACCAGAGT-3′ (P752). These primers define a 204bp segment of the BRSV genome in the region encoding the fusion(F)-protein (Fig. 1). Primer P751 was used in reverse transcription reactions as well as in PCR reactions. Primer P752 was only used in PCR reactions. Besides a 100% primability match, primer P752 shows a second match in the BRSV-F genome with an 88% primability. This second match lies 70 base pairs downstream from its first match, resulting in a second defined segment of the BRSV-F genome of 274 nucleotides (Fig. 1). This BRSV-F specific 274 bp segment is an expedient to judge specificity and PCR reaction conditions.

An 144-mer oligonucleotide of the defined 204-bp segment, located directly beside the P751 match (Fig. 1), was labelled with [gamma^{32}P]ATP using T4 polynucleotide kinase [22] and used as a probe for identification on BRSV specific amplification products (Fig. 1).

Reverse transcription, polymerase chain reaction, gel electrophoresis and southern blotting

Reverse transcription was performed directly after RNA isolation. PCR amplification was performed in a thermocycler (Omnigene, Hybaid, U.K.). Each amplification cycle consisted of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min. In each PCR analysis thirty-eight cycles were performed.

Gel electrophoresis and southern blotting were performed according to standard procedures [22]. The probe was a 144-mer oligonucleotide of the defined 204-bp segment, and is identical to the sense strand of BRSV-F nucleotides 170 to 314 (Fig. 1).

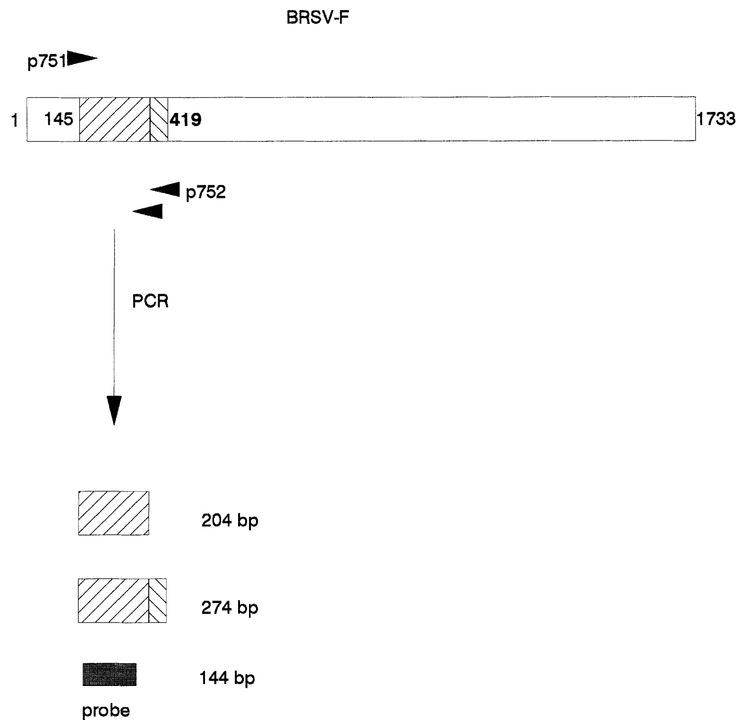

Fig. 1. Localization of the PCR primers and probe in the genomic region encoding the fusion protein (F) of BRSV. PCR primers P751 and P752 are indicated by arrowheads and define a 204 nucleotide segment of the genome. In addition to the 204 nucleotide product, the PCR may result in a relatively small amount of 274 nucleotide product, because P752 shows a second match, which has an incomplete primability

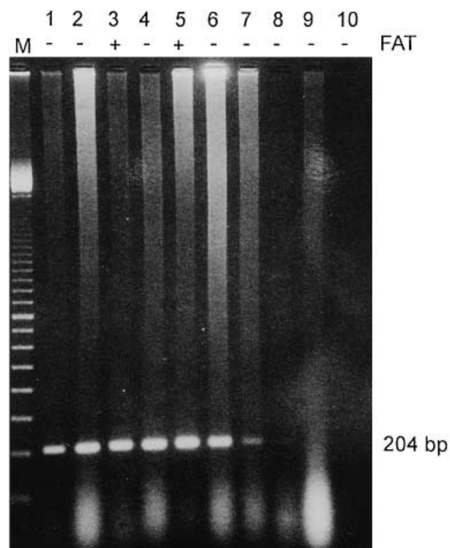

Fig. 2. Results of fluorescent antibody test (FAT) (on top of figure) and RT-PCR analysis (gel electrophoresis) of tissues of a BRSV infected calf, necropsied 6 days post inoculation. Tissues: pharyngeal mucosa (*1*), tracheal mucosa (*2*), lung (*3*), bronchial lymph node (*4*), retropharyngeal lymphnode (*5*), tonsil (*6*), spleen (*7*), kidney (*8*), liver (*9*), bone (osteoclasts) (*10*). Positive FAT reactions were found in lung and retropharyngeal lymph node. Positive PCR reactions were found in all examined tissues except liver and bone tissue

The detailed RT-PCR and southern blotting protocols can be obtained from the authors on application.

Specificity, sensitivity and positive controls of RT-PCR

The detection limit of the PCR was determined by analysing a 10-fold dilution series of molecules of a plasmid that contained a DNA insert encoding the BRSV fusion protein (BRSV-F). For background DNA, 0.1 µg/ml salmon sperm DNA was added. The detection limit of the RT-PCR was determined by analysing 10-fold dilutions of lysed lavage cells (obtained from specific pathogen free (spf) calves) which were spiked with 50 $TCID_{50}$ BRSV Lelystad strain [17] per reaction volume (500 µl). To examine the specificity of the RT-PCR, we tested 3 HRSV strains including both major subgroups A and B: HRSV A A2, HRSV A Long and HRSV B 9320, and 6 BRSV strains: Rispoval, Lelystad, Odijk, Waiboerhoeve, Snook and Compton 127. These 6 BRSV strains have showed diversity in antigenic analyses [11].

Tissues and lavage cells of an experimentally infected calf (Table 1, animal 3052) were used for positive controls in the RT-PCR. Sensitivity of RT-PCR and FAT were compared by analysing tissues of the positive control calf in these two assays. Performing RT-PCR, one of each two samples was always spiked with 10 $TCID_{50}$ BRSV Odijk strain, resulting in a positive control for each reverse transcription and PCR reaction. Nasal swabs of clinically BRSV infected calves were positive controls for RT-PCR sampling procedures.

Bovine respiratory syncytial virus specific immunoglobulin measurements

BRSV specific immunoglobulins in sera of all 7 corticosteroid-treated cattle as well as sentinel cattle and lavage fluid inoculated cattle, were measured by ELISAs. BRSV specific IgM and IgA antibody ELISAs were also performed on nasal swab specimens and lung lavage fluids. BRSV specific antibodies in serum were determined by a double antibody sandwich assay as described by Westenbrink et al. [33]. This ELISA (F-ELISA) detects antibodies directed against the fusion protein of BRSV (BRSV-F). Serial twofold dilutions of sera were tested starting at 1:80. BRSV specific IgM and IgA antibodies by isotype specific antibody capture assay (ACA), as described by Kimman et al. [16]. For IgM detection, sera, lavage supernatants and nasal secretion samples were tested at 1:10 and 1:20 dilutions. For IgA detection, sera, lavage supernatants and nasal secretion fluids were tested at 1:2 and 1:4 dilutions.

In addition, BRSV antibody rises were determined by serum neutralisation tests [33] and antibody titres against the G-protein of BRSV (BRSV-G) were determined in sera of corticosteroid-treated cattle using a peptide blocking ELISA [18]. In short, microtiter plates were coated with 3 µg of a synthetic protein corresponding to a part of the attachment protein G of BRSV and subsequently incubated with test serum, conjugate and substrate solution. Between incubations, plates were rinsed 6 times with deionised water containing 0.05 per cent Tween 80. Dilutions were made in ELISA-buffer (PBS, pH 7.2, 1 mM EDTA, 4% horse serum, 0.35 M sodium chloride, 0.05 per cent [w/v] Tween 80). 1:2 dilutions of each serum sample were incubated for 1 hour at 37 °C. The conjugate was a 1:15 000 dilution of a horseradish peroxidase conjugated monoclonal antibody (BRSV-MAb "20", ID-DLO, Lelystad) directed against the attachment protein (G) of BRSV and was incubated during 1 h at 37 °C. The substrate solution consisted of 10 mmol/l Na-phosphate buffer (pH 6.8), 0.1 mmol/l EDTA, 0.1% 5-aminosalicylic acid, and freshly added 0.005% H_2O_2. Incubation with substrate solution was performed overnight at 4 °C. Colour development was measured at 450 nm (Titertek Multiscan). Sera with a blocking percentage of 40% or

more were scored positive. In animals of group 1, G-ELISA antibody titres were only determined in sera taken before the first and after the last experiment (day −20 and day 28).

In sera of cattle of group 1, specific antibodies directed against bovine virus diarrhea virus (BVDV), parainfluenza 3 (P13) virus were also determined. Bovine virus diarrhea virus antibodies were determined using a blocking ElISA [34] and parainfluenza 3 virus specific antibodies were determined using haemagglutination inhibition assay (HI) [1]. In addition, total bovine immunoglobulin concentrations were determined as described previously [31]. To determine (BHV1) antibody titres in cattle of group 2, their sentinel cattle and the lavage-fluid-inoculated cattle, serum neutralisation tests were used [14].

Results

Virus isolations and FAT

Bovine respiratory syncytial virus was isolated from lung tissue, retropharyngeal lymph node and tonsil of the experimentally infected control animal (animal 3 052), but was not isolated from the rest of the organs. The virus was also isolated from lung lavage fluid. Lung lavage cells of the positive control animal, were positive in FAT, 3 to 6 days after inoculation. BRSV antigen was demonstrated in retropharyngeal lymph nodes and lung tissue of this animal, but not in mucosae of pharynx and trachea, bronchial lymph nodes, tonsils, spleen, kidney, liver and bone tissue (Fig. 2). All other samples in this study collected for virus isolation and FAT were negative for BRSV or BRSV antigen (Table 2).

Specificity and sensitivity of RT-PCR

The RT-PCR assay was specific, because all six BRSV strains were detected in the RT-PCR, whereas HRSV strains of both major subgroups A and B were not detected.

The detection limit of the PCR was about 100 DNA molecules, as evidenced by analysing a 10-fold dilution series of molecules of a plasmid that contained a DNA insert encoding BRSV-F. A detection limit of 0.5 $TCID_{50}$ BRSV was found for the RT-PCR by analysing a 10-fold dilution series of spikes with 50 $TCID_{50}$ BRSV Lelystad strain per reaction volume (500 µl). The RT-PCR was more sensitive in detecting BRSV than virus isolation, because in lavage samples and nasal fluid samples of experimentally BRSV infected calves, BRSV RNA was often detected by RT-PCR, whereas at the same time virus isolation was negative. RT-PCR analysis in tissues was also more sensitive than FAT or virus isolation: BRSV antigen was only detected in lung and retropharyngeal lymph nodes of the control animal, and infectious BRSV was detected in lung, retropharyngeal lymph nodes and tonsil by virus isolation, whereas, by RT-PCR, BRSV RNA was detected in 8 organs of the same animal (Fig. 2). The RT-PCR detected BRSV RNA in nasal swabs of experimentally BRSV-infected calves and of diseased BRSV infected cattle from the field.

Table 2. Results of BRSV detection, and detection of specific BRSV antibody rises (only four-fold antibody rises and antibody seroconversions are listed), after corticosteroid treatment of BRSV-seropositive cattle

Animal	Virus isolation		Virus transm.	Antigen detection (FAT)		RNA detection (PCR)		F-ELISA	G-ELISA	SN-test
	lung lavage	organs		lung lavage	organs	lung lavage	organs			
2 003	–	–	–	–	–	–	–	+	–	+
2 004	–	–	–	–	–	–	–	+	–	+
2 005	–	N.D.	–	–	N.D.	–	N.D.	–	+	–
2 006	–	N.D.	–	–	N.D.	–	N.D.	–	+	–
2 705	–	–	–	–	–	–	–	–	–	–
2 706	–	–	–	–	–	–	–	–	–	–
3 000	–	–	–	–	–	–	–	–	+	+
3 052	+	+	N.D.	+	+	+	+	N.D.	N.D.	N.D.

Animal 3 052 was positive control, necropsied 6 days after BRSV inoculation

RT-PCR analyses

Reverse transcription PCR detected BRSV RNA in all examined respiratory and lymphatic organs and in kidney of the experimentally inoculated control animal. BRSV RNA was not detected by RT-PCR in liver and bone tissues of this animal (Fig. 2).

In none of the lavage fluid samples, nasal swabs and postmortem collected tissues of corticosteroid-treated cattle, BRSV RNA was detected by RT-PCR. In nasal fluid samples, collected in July, from 74 over-1-year-old BRSV antibody positive cattle, BRSV RNA was not detected. In samples spiked with BRSV, BRSV RNA was always detected (positive controls) (Table 2).

BRSV specific antibody responses

Six of the seven corticosteroid-treated cattle, except cow 2 005 (group 1) showed an antibody rise in serum in the F-ELISA: in 2 animals (2 003 and 2 004) (group 1) this was a fourfold rise (Fig. 3). All antibody rises occurred in week 2: after the first corticosteroid treatment. At the same time, cattle 2 003 and 2 004 had four-fold rises in the BRSV neutralisation test (Fig. 4), and no rises in antibody titres against BVDV and P13 virus and in total immunoglobulin concentration (data not shown).

In none of the blood samples, BRSV specific IgM was detected. BRSV specific IgA was only detected in serum of calf 3 000 (group 3). This IgA level showed a twofold rise in titre after corticosteroid treatment. BRSV specific antibodies (IgG, IgM, IgA) were never detected in serum of sentinel calves or the 2 lavage-fluid-inoculated calves. In nasal secretion samples and lavage supernatants, IgM was never detected; IgA was only detected in nasal secretions of calf 3 000 and during corticosteroid treatment a twofold rise in antibody titre was detected (isotype-specific F-ELISA).

Antibody development against the G-protein of BRSV was detected in 2 cattle of group 1: 2 005 and 2 006 (data not shown), and also in animal 3 000 (group 3) (Fig. 4). In animal 3 000 this anti BRSV-G antibody development occurred after the second corticosteroid treatment and was a rise in antibody titre from to < 5 to 40 (4 two-fold dilution steps). In cattle 2 005 and 2 006 (group 1), this rise in antibody titre was a rise from < 5 to 5 (one dilution step), and the time of anti BRSV-G antibody development could not be determined because there was not enough serum available to determine these antibody levels during corticosteroid treatments; antibody titres were only determined in sera obtained before the first and after the last corticosteroid treatment. Cattle of group 2 had detectable BRSV-G antibodies but did not show antibody rises.

Neutralising antibodies against BRSV were found in sera of all 7 cattle. Cattle 2 003, 2 004 (group 1) showed a consistent fourfold antibody rise in the serum neutralisation test (Table 2); this antibody rise occurred after the first corticosteroid treatment. Cattle of group 2 showed fourfold rises in BHV1 serum neutralisation antibody titre. Their sentinel cattle as well as lavage-fluid-inoculated cattle developed antibodies against BHV1.

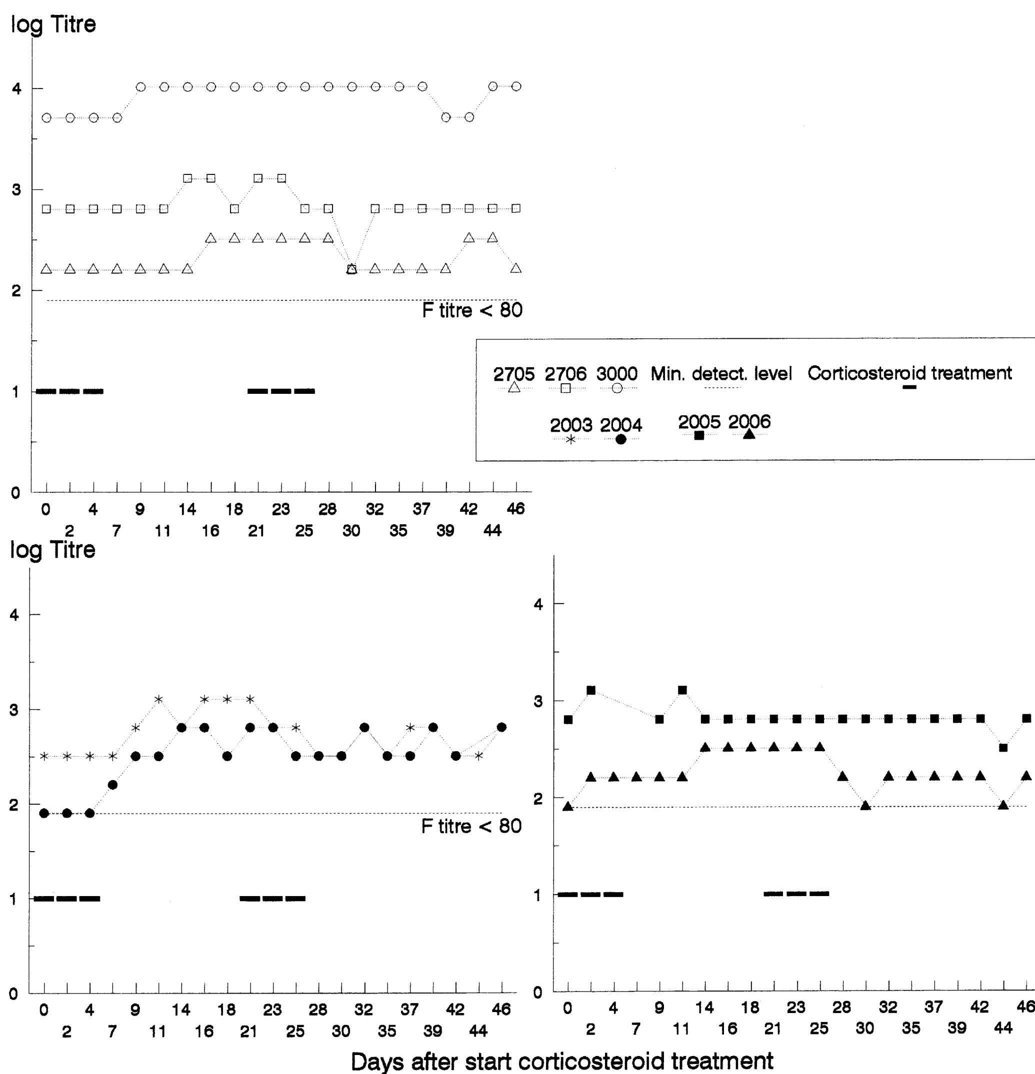

Fig. 3. BRSV specific antibody levels (F-ELISA) in 7 cattle during the corticosteroid treatment experiment. Cattle were injected with dexamethasone 0.5 mg/kg, day 0 to 4 and day 21 to 25 (dashes)

Discussion

To identify putative carriers of BRSV, we attempted to induce replication of BRSV by treating seven BRSV infected cattle twice with corticosteroids. During and after these treatments numerous specimens were tested for the presence of infectious BRSV, BRSV antigen, BRSV RNA, and for significant rises in BRSV specific antibodies. In addition, nasal swabs collected in the field were examined for BRSV RNA.

In none of the corticosteroid-treated cattle in the experiment, BRSV could be detected by virus isolation in cell culture, by inoculation of naive calves, by

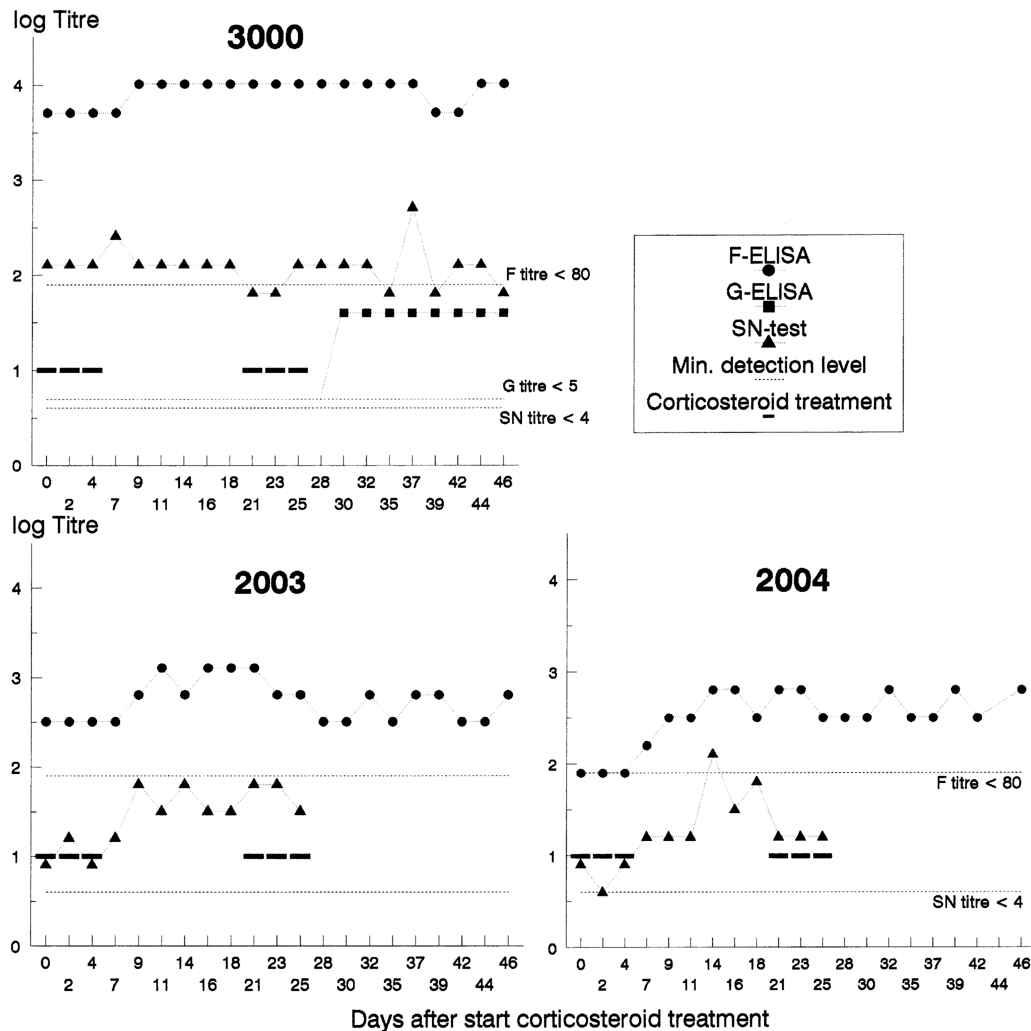

Fig. 4. BRSV antibody titres of animals showing a significant antibody response after corticosteroid treatment. Antibody developments determined by F-ELISA, G-ELISA and serum neutralisation test

antigen or RNA detection, or indirectly by the use of sentinel cattle. In addition, BRSV RNA could not be demonstrated in nasal swabs of all over-1-years-old cattle of a closed dairy herd. In all applied tests positive controls were included and all these controls indeed reacted positively, indicating that methodological problems were not the reason for the failure to detect BRSV. The two adult cattle (group 2) which were latently infected with BHV1 indeed showed recrudescence of BHV1 after corticosteroid treatment, indicating that the treatment worked.

In sera of 3 of the 7 corticosteroid-treated animals, a fourfold (or higher) rise in BRSV antibody titre was detected (animals 2 003, 2 004 and 3 000). In two of these animals (2 003 and 2 004) significant antibody rise in the serum

neutralisation test was detected at the same time as it was detected by the F-ELISA, whereas antibody rises against other viruses and increase in total Ig concentration were not detected. These two animals did not show differences in G antibody levels and after corticosteroid treatment, but they may have responded with a G antibody rise. The G antibody levels decline more rapidly than the F antibodies [23] and the G antibody developments may have been missed by just testing paired serum samples. In three animals (2 005, 2 006 and 3 000) significant BRSV antibody development was only observed in the G-ELISA. Examples of BRSV reinfected animals showing a significant antibody in the G-ELISA and not in the F-ELISA have been observed before by Schrijver et al. [23]. The relatively high starting off levels in the F-ELISA and the SN-test may be the reason that these animals did not show a fourfold rise in these two assays. Two-fold antibody rises may be within the experimental error of the used assays but the detection of a fourfold antibody response in animals 2 004 and 2 005 in both the F-ELISA and the SN-tests, without showing antibody responses to other viruses than BRSV, strongly suggests that these antibody responses were due to specific reactions.

Increasing levels of immunoglobulins after corticosteroid treatment have been reported [24], but in cattle corticosteroid therapy normally results in a steady or decreasing level of immunoglobulins [4, 19]. Therefore, the BRSV specific antibody responses we found after corticosteroid treatment, strongly suggest replication of BRSV.

It is very unlikely that the rises in antibody titres were caused by introduction of BRSV into the isolation room by feed or an animal worker, because all feeds had been sterilised and animal workers had put on clean overalls and sterile gloves, surgical masks and caps every time they entered one of the rooms. Moreover seronegative sentinel calves that were housed together with each corticosteroid-treated animal did not show any antibody response to BRSV.

The increase in BRSV antibody titre was specific and must have been the consequence of virus replication or was caused by a hitherto unknown mechanism. Although a wide array of tests was used to detect infectious virus, virus antigen or RNA in various samples, replication of BRSV could not be detected. Hence, it may have taken place in organs or tissues that were not assayed, or in restricted sites in the assayed organs. BRSV detection methods in the described study were focussed on respiratory organs, lymphatic organs, liver, kidney and bone tissue. Presence of persistent BRSV or BRSV-genome would be most likely in these organs, because epithelial cells of the respiratory tract are target cells for BRSV infections, and after putative induction of replication, BRSV might pass the lymphatic system and liver or kidney. Because detection of paramyxoviridae in osteoclasts has repeatedly been described [21], bone tissue was also examined for BRSV. It can be speculated that paramyxovirus persistence may be achieved by maintenance of virus genetic material in the absence of infectious virus replication [21]. To detect virus genomes, and to use a very sensitive technique, RT-PCR was developed. The RT-PCR indeed proved to be more sensitive than virus isolation, but BRSV-

RNA was still not detected. This may be explained by the fact that only small pieces of tissue (30 mm^3) could be assayed in the RT-PCR, and BRSV RNA concentrations in these small pieces may be under the detection limit of the used technique.

Stress was induced by drying off, flushing of water and corticosteroid treatment. Bovine herpesvirus 1 can easily be reactivated by corticosteroid treatment [26], and BHV1 isolation from BHV1 antibody positive cattle in the experiment, proved that the intended induction of stress was achieved. However, either the animals were not persistently infected or the corticosteroid treatment was not the adequate trigger for overt replication and shedding of BRSV. Whether natural environmental changes in temperature or humidity during autumn or winter can provoke replication and shedding in putative persistently infected cattle should be elucidated.

Persistence of BRSV or HRSV in a population may be based on maintenance of a reservoir of infectious virus through a continuous low rate of reinfections throughout the year or on virus shedding from persistently infected 'carriers'. In the first scenario seropositive individuals excrete infectious virus for a long period to maintain this population persistence [9]. In the second scenario induction of RSV replication must occur in some 'carriers' before every infection season. Persistent RSV infections have been described in guinea pigs [25] but to our knowledge HRSV- or BRSV-'carriers' have never been detected in humans or cattle, respectively.

If BRSV carriers play a key-role in the yearly return of BRSV outbreaks in cattle herds, these carriers must be present in many cattle herds. Nevertheless, BRSV carriers have never been identified. The 7 cattle in the study described were selected on BRSV infection history: disease after infection with virulent virus or recurrent fourfold antibody rises in a preceding year. It might be possible that persistently infected, virus shedding cattle should have been selected on other criterions. Other research workers have suggested recurrent IgA development as a possible indication for BRSV recrudescence [15] and infection during maternal immunity as a putative cause for BRSV persistence [2, 27], but BRSV persistence was never proven.

Although the serological findings in this study are indicative for persistence of BRSV in some infected cattle, we could not detect BRSV or components thereof after stress-simulating treatment of these putative 'carriers'.

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