Comparative Immunology, Microbiology and Infectious Diseases 65 (2019) 213–218

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Human respiratory syncytial virus infection in the pre-clinical calf model

B. Cortjens[a,*], R. de Jong[b], J.G. Bonsing[b], J.B.M. van Woensel[a], R.A. Bem[a], A.F.G. Antonis[b]

[a] Pediatric Intensive Care Unit, Emma Children's Hospital, Amsterdam, the Netherlands
[b] Wageningen University and Research, Bioveterinary Research, Lelystad, the Netherlands

ARTICLE INFO

Keywords:
Respiratory syncytial virus
Animal model
Bronchiolitis
Upper and lower respiratory tract infections
Calves
Neutrophil

ABSTRACT

Human respiratory syncytial virus (hRSV) is the most important respiratory pathogen in young children worldwide. Experimental modelling of hRSV disease by bovine RSV (bRSV) infection in calves provides an important tool for developing new strategies for prevention and treatment. Depending on the scientific hypothesis under investigation, this cognate host-virus model might have the disadvantage of using a highly related but not genetically identical virus. In this study, we aim to describe viral kinetics and (clinical) disease characteristics in calves inoculated with hRSV. Our results show that hRSV infects the upper and, to a lesser extent, the lower respiratory tract of calves. Infection causes upper airway clinical disease symptoms and neutrophilic infiltration of the lower airways. We conclude that a hRSV model in calves may aid future research involving distinct scientific questions related to hRSV disease in children.

1. Introduction

Human respiratory syncytial virus (hRSV) is one of the most important respiratory pathogens in children worldwide [1]. In low and middle income countries, hRSV is responsible for almost a third of the community-acquired pneumonias in young children, making hRSV a global health issue with tremendous socio-economic impact [2]. Acquiring insight into the key processes that influence hRSV disease is essential to develop new strategies for prevention and treatment.

Animal models are a valuable tool to study hRSV disease. They form the bridge between tissue culture experiments and human randomized controlled trials. They also serve to evaluate the efficacy and safety of new vaccine candidates and new therapeutic intervention strategies. Different animal models are available to mimic hRSV disease and each model has its distinct advantages and disadvantages (reviewed in [3]). One of the models used to study hRSV disease is the bovine respiratory syncytial virus (bRSV) infection model in calves [4]. This natural-host model has distinct advantages over models that are based on a non-natural host-virus interaction e.g. the heterologous hRSV mouse model [3,5]. There is a striking similarity in age-dependency between bRSV disease in cattle and hRSV disease in humans [3,6,7] and unlike heterologous models, bRSV infection in calves manifests with overt clinical symptoms, such as wheezing, coughing, and tachy- and dyspnoea [6,8], similar to hRSV infections in young infants. Importantly, these clinical signs of bRSV LRTD are accompanied by histopathological evidence of bronchiolitis and (interstitial) pneumonia, including prominent neutrophilic inflammation in the airways and lungs, thereby closely mimicking findings in children [3,6,9–11].

Despite the similarities between hRSV disease in humans and bRSV disease in calves, differences inevitably remain. The most important one being that bRSV and hRSV are not identical viruses, although both are members of the same family (*Pneumoviridae*) and genus (*Orthopneumovirus*) thus phylogenetically strongly related [12]. Differences in the genetic composition of viral surface glycoproteins are illustrated by a 38–41% and 81% protein sequence identity between the bRSV and the hRSV G protein and F protein, respectively [13,14], but also the SH, NS1 and M2-2 proteins differ between species [15,16]. The use of the bRSV calf model for preclinical testing of specific vaccines and antivirals may be hampered by these inherent genetic differences between bRSV and hRSV.

To overcome the potential drawback of studying different viruses without losing major advantages of the large animal bovine model, we were interested to explore viral dynamics and disease characteristics of experimental hRSV infection in calves. Although it has been shown that bovine cell culture-adapted hRSV can infect calves decades ago [17], this report has offered very limited information on viral dynamics and specific disease characteristics. Here, we aimed to further explore and compare experimental hRSV infection in calves and thereby discuss its potential use in future hRSV research.

* Corresponding author at: Meibergdreef 9, 1105 AZ, Amsterdam, the Netherlands.
E-mail address: b.cortjens@amc.nl (B. Cortjens).

https://doi.org/10.1016/j.cimid.2019.04.006
Received 17 February 2018; Received in revised form 19 January 2019; Accepted 8 April 2019

2. Methods

We performed an experimental observational pilot study of human-RSV infection in 4 caesarean-derived maternal derived antibodies (MDA)-deficient 5 week old calves (3 males and 1 female). Pre-infection data collected in other experiments with MDA-deficient 5–6 week old calves (N = 16) served as baseline uninfected controls. All animals were raised in an isolated environment from birth until the start of the study. All calves were tested free of antibodies against natural occurring bRSV before start of the study by ELISA (Priocheck bRSV, Thermo Scientific, Rockford, USA). All animal experiments were authorized by the Animal Ethics Committee of the Animal Sciences Group, part of Wageningen University (2011088.b; 2013125.c) or under legislation of the Dutch Central Authority for Scientific procedures on Animals and after approval of the Animal Welfare Body of Wageningen University and Research (2016004.6) and conducted in accordance with the Dutch law for animal experimentations.

2.1. Virus inoculation

Animals received 10^5 $TCID_{50}$ in 2 mL of hRSV clinical isolate Memphis 37 (EV9508, Meridian LifeScience, Memphis, USA, subtype A, seventh *in-vitro* passage on Vero cells, N = 4), based on back-titration on HEP2 cells. Intranasal inoculation of virus was performed on day zero using an air-jet nebulizer, as described before [4], with a jet stream of 0.2 mm, producing 10% droplets < 26 μm in section, 50% droplets < 50 μm in section, and 90% droplets < 99 μm in section.

2.2. Sample procedures

For assessment of infection of the upper respiratory tract (URT), nasopharyngeal (NP) samples were collected on study days 0–9 after inoculation using sterile nylon bristle brushes (MW126, Medical Wire and Equipment Co. Ltd, Corsham, UK). Following sampling, the brushes were agitated in 3 mL tissue culture medium (EMEM), 2% antibiotic/antimycotic, 2% foetal calf serum. For assessment of infection of the lower respiratory tract (LRT), broncho-alveolar lavages (BAL) were collected on study days 5–9 after inoculation. BAL was performed by blind catheter placement according to the method described by Fogarty et al. [18], using 100 mL of D-PBS (GIBCO) instillation. After centrifugation, the supernatant of NP and BAL samples were stored at −80 °C.

2.3. Clinical monitoring

Clinical observations for hRSV-related signs of illness were performed on study days -2 to 12 by a bio technician. Clinical observations were performed according to the scoring system outlined in Table 1. Rectal temperature measurements were performed simultaneously.

2.4. Viral load detection and virus isolation

Viral load in NP and BAL supernatant samples were measured by RT-PCR. From 200 μL BAL or NP, total nucleic acid was isolated using the MagNA Pure LC Total Nucleic Acid Isolation Kit (Hoffmann-La Roche, Basel, Switzerland). The hRSV N-gene was amplified (45 cycli) as described before [19]. Data are expressed as ΔCt (total number of PCR cycles minus threshold cycle). Normalization was done by correcting for the amount (in millilitres) of BAL retrieval. Infectious virus in NP and BAL supernatants was detected by virus isolation on HEP2 cells. Duplicate samples (200 μL) were added to a suspension of HEP2 cells on duplicate 24-well plates. The plates were incubated for six days at 37 ± 2 °C and 5 ± 1%CO_2. An IPMA was performed to detect virus infected cells by use of an anti-hRSV polyclonal antibody (ABIN238061, Antibodies-Online, Aachen, Germany). Subsequently, 50% endpoint titers were determined by inoculating 10-fold serial dilutions of samples on duplicate 96-well plates (50 μL sample). Endpoint titres (50% infected cell cultures) were estimated according to Reed and Munch, resulting in a detection limit of 1.3 $\log_{10}$ $TCID_{50}$.

2.5. Inflammatory cell responses

Total white blood cell counts in BAL samples were performed using a Coulter Counter (Beckman Coulter, Brea, USA) on days 6 and 9. Cells were centrifuged to a slide in the Shandon cytospin 3 (Thermo Scientific) and stained with a modified Wrights staining for differential counts (400 cells counted) by microscopic evaluation. For an indication of neutrophil degranulation, we determined myeloperoxidase concentrations (MPO) in BAL supernatant by ELISA (BM0039, Neobiolab, Woburn, USA) following the manufacturer's instructions.

2.6. Histopathology

On day 12 (N = 2) and day 16 (N = 2) post virus inoculation, calves were euthanized with an overdose of Pentobarbital followed by exsanguination. The macroscopic consolidated lung area was calculated (Image Pro Plus) and the extent of consolidation was rated as a percentage of the total lung tissue area [20]. Lung tissue samples were collected from 10 pre-determined sites and stored in 10% neutral buffered formalin, followed by microscopic examination of each hematoxylin and eosin stained lung tissue section.

2.7. Statistics

Data were analyzed in time (repeated measures, non-parametrical) by the Friedman's test. For data between groups (non-parametrical) we

Table 1
Clinical scoring system for disease severity.

Score	Upper Respiratory Tract Disease	Lower Respiratory Tract Disease
0	No nasal discharge	Normal respiration
(absent)	No coughing	RR < 50 (breathings per minute)
	No ocular discharge	
1	Nasal or ocular discharge	Abnormal respiration
(mild)	Intermittent watery – mucus	RR 51-70 (breathings per minute)
	Occasional spontaneous dry cough – induced cough (unproductive) present	
2	Increased nasal or ocular discharge	Abnormal respiration – obvious abdominal breathing
(moderate)	Persistent mucoid – mucopurulent discharge	RR 71-100 (breathings per minute)
	Frequent spontaneous productive cough – induced productive cough present	
3	Severe nasal or ocular discharge	Abnormal respiration – severe abdominal breathing,
(severe)	Persistent purulent – haemorrhagic discharge	Dyspnoeic (e.g. mouth breathing / frothing)
	Frequent spontaneous productive cough – induced productive cough present, prolonged when induced	RR > 100 (breathings per minute)

A score is met when one or more of the symptoms from that category apply.

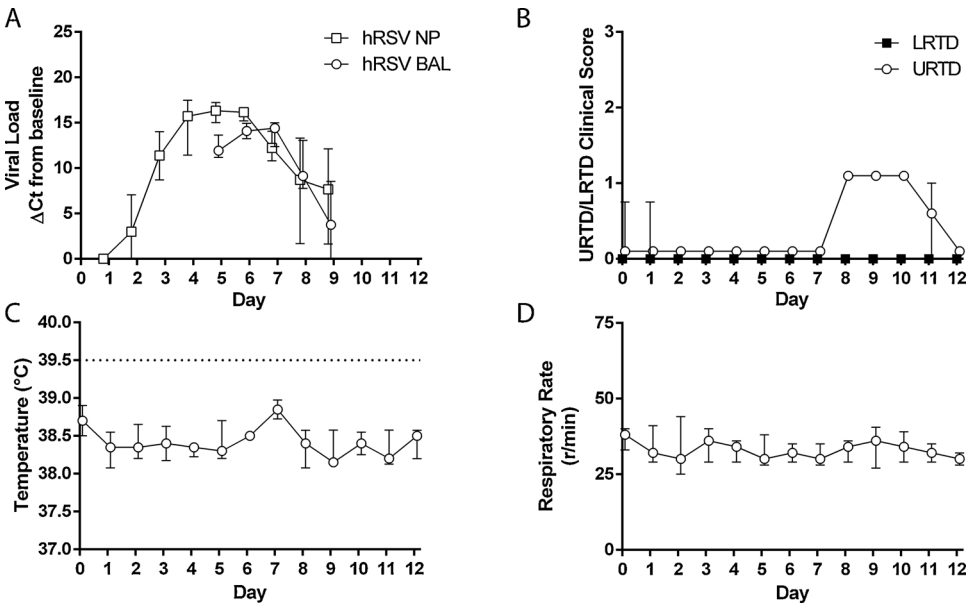

Fig. 1. Parameters of disease in hRSV infected calves.
(A) Viral load detected by PCR in nasopharyngeal brush samples (NP, squares) and broncho-alveolar lavage samples (BAL, circles) in hRSV infected calves (N = 4). Expressed as ΔCt values (total number of PCR cycles minus threshold cycle) (NP: $P < 0.001$ and BAL: $P < 0.01$). **(B)** Upper respiratory tract disease (URTD) score of hRSV infected calves (white circles, $P < 0.0001$) and lower respiratory tract disease (LRTD) score in hRSV infected calves (black squares, $P = 0.45$). **(C)** Respiratory rate (breathings per minute) in hRSV infected calves ($P = 0.78$). **(D)** Rectal temperature in hRSV infected calves ($P = 0.05$). Data are represented as median ± IQR.

performed Mann-Whitney tests using Graphpad Prism 6 (Graphpad Software Inc, La Jolla, USA). Results are presented as median with interquartile range (IQR). A two-sided P value of < 0.05 was considered significant.

3. Results

3.1. Viral replication

First, we aimed to explore if hRSV inoculation in calves results in virus replication in the upper and lower respiratory tract. We therefore compared the dynamics of viral loads as detected by PCR in NP and BAL respectively. There was a significant rise in hRSV load in NP during infection (Fig. 1A, $P < 0.001$). HRSV was first detected at day 2 in NP samples of the upper respiratory tract and viral loads peaked on day 5. HRSV was also detected in BAL samples collected from the lower respiratory tract (Fig. 1A, $P < 0.01$), although less pronounced and with a delay in the peak viral load (day 7) as compared to the upper airways. Viral isolation showed no infectious virus in BAL, but showed positive results in NP in 3 out of 4 calves between days 4–6 (data not shown). However, virus titers could not be determined as the end-point titration results were at or below the detection limit (data not shown). At necropsy on day 12 or 16 after inoculation (after recovery of the clinical disease), no calf had detectable viral antigen by immunohistochemistry on lung sections (data not shown).

3.2. Clinical disease

HRSV infected calves developed upper respiratory tract disease (URTD) between day 8–10 (Fig. 1B, $P < 0.0001$). URTD symptoms consisted mainly of nasal discharge and occasional coughing. None of the hRSV infected calves showed lower respiratory tract disease (LRTD) symptoms (Fig. 1B, $P = 0.45$), fever (Fig. 1C, $P = 0.05$) or increase in respiratory rate throughout the course of disease (Fig. 1D, $P = 0.78$).

3.3. Lung inflammatory cell responses

To assess the (innate) pro-inflammatory responses in the lungs of hRSV inoculated calves we determined the total and differential white blood cell counts in BAL samples prior to inoculation and on two time points after hRSV inoculation (days 6 and 9). BAL cell concentrations peaked at day 6 and were significantly higher compared to uninfected calves (hRSV day 6: 0.5: $0.3-0.9 \times 10^6$/mL versus uninfected: 0.15: $0.1-0.3 \times 10^6$/mL, $P = 0.02$, Fig. 2A). Interestingly, hRSV infection resulted in the neutrophilic infiltration of the lower airways, significantly higher compared to uninfected calves (hRSV day 9: 25%: 20–37%, versus uninfected: 5%: 0.5–12%, $P < 0.01$, Fig. 2B). The remaining BAL cells were almost exclusively macrophages (Fig. 2C), with very few lymphocytes appearing at day 9 in hRSV infected calves (data not shown). We evaluated BAL MPO content as a marker of neutrophil activation and found a significant increase of MPO content during disease in hRSV infected calves ($P = 0.03$, Fig. 2D). Viral neutralization assay did not show neutralizing titers on day 12 and 16 (data not shown).

3.4. Lung pathology after recovery from hRSV or bRSV disease

Lung histopathology data upon sacrifice (day 12 N = 2, day 16 N = 2) were only available after full clinical recovery from hRSV disease. Macroscopic lung consolidation was not observed. However, microscopically changes consisting of mild bronchiolitis and bronchopneumonia were visible (Fig. 3).

4. Discussion

The goal of this experimental pilot study was to describe the viral kinetics and clinical disease characteristics of experimental hRSV infection in MDA-deficient calves. Our results show that hRSV inoculation in calves results in viral infection in the URT, and to a lesser extent in the LRT. Infection resulted in minor URTD symptoms and no LRTD symptoms, despite influx of neutrophils in the LRT. Infection of calves with hRSV may aid future research related to hRSV disease in children.

Since its discovery in 1970 [21], bRSV has been recognized as a leading pathogen in outbreaks of respiratory disease in dairy cattle [7]. The calf has been used to study bRSV disease epidemiology, pathogenesis and prevention in an experimental setting [6,22]. More recently bRSV-infected calves have also been exploited as an age-relevant model of hRSV infection in infants [23], owing to its striking similarities with human disease [3]. In the present work, we increase our current understanding of RSV animal models, as it is the first structural description of experimental hRSV infection in calves. One report from the 1980's showed viral shedding between day 3 and 11 after inoculation of hRSV A2 in 2–7 week old calves [17], which is comparable with our results. However, the animals used were of a different age and were

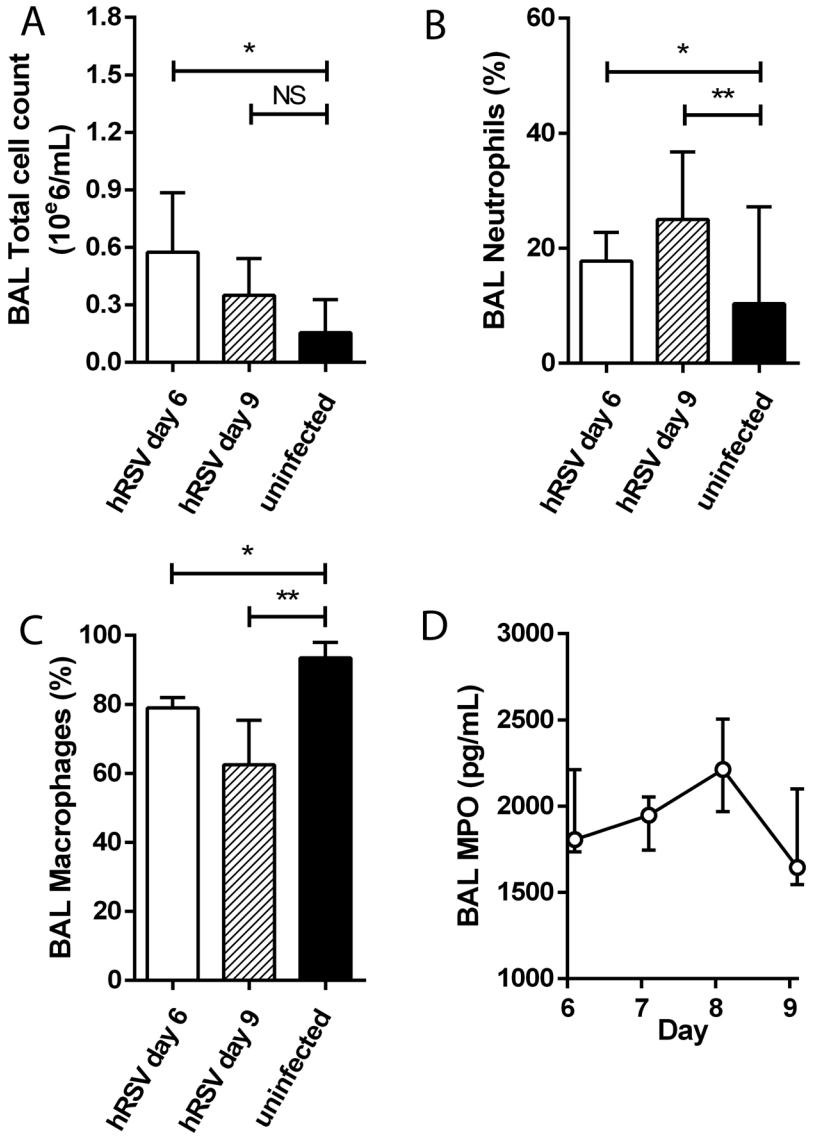

Fig. 2. Inflammatory response during hRSV infection in calves.
(A) Broncho-alveolar lavage (BAL) total cells during hRSV infection (white bars) on day 6 and 9 after infection compared to uninfected calves (day 6: * $P = 0.02$, day 9: not significant $P = 0.15$). **(B)** BAL neutrophil percentages (day 6: * $P = 0.02$, day 9: ** $P < 0.01$). **(C)** BAL macrophage percentages (day 6: * $P = 0.03$, day 9: ** $P < 0.01$). **(D)** BAL MPO concentration from day 6 until day 9 ($P = 0.03$). Data are presented as median ± IQR. NS; not significant.

sacrificed at different time points. Furthermore, their viral inoculum was highly passaged in several human and bovine cell cultures prior to inoculation and none of the calves developed any clinical signs of infection. We have elaborated on these experiments and showed hRSV replication in the upper and to a lesser extent in the lower airways, peaking on day 6, which is similar to that of experimental hRSV infection in human adults (day 4 after infection) [24] and combined with clinical symptoms of the upper respiratory tract and inflammatory responses in the lower respiratory tract. This is in contrast to the high disease severity, involving the LRT, after bRSV infection [6]. A possible (partial) explanation for the difference in pathogenicity, is the difference between in vivo passaged bRSV versus in vitro passaged hRSV, as in vivo passaged strains retain more virulence [25]. Besides we used a vero-passaged Memphis 37 strain of RSV in this pilot, while it has been described that HEP2-passaged Memphis 37 resulted in more severe disease compared to VERO passaged Memphis 37 in experimental infection in lambs [26]. It's unknown if other RSV strains commonly used in rodent models (e.g. RSV-long, RSV-A2) would result in a different outcome. As we aimed to investigate the dynamics of viral replication and disease characteristics over the whole course of experimental hRSV infection, we only studied lung histopathology after full recovery of the animals. Still, there were appreciable mild histopathological changes in hRSV infected animals, consistent with local inflammation by hRSV.

In this study the peak viral load in NP and BAL samples (day 6–7) slightly preceded the peak in clinical symptoms (day 8), which is comparable with data from hRSV infected children [27] and bRSV infected calves [6]. We used RT-PCR CT values to investigate dynamics of viral replication, which represents the amount of virions found in a particular volume with high sensitivity. We could only find infectious titers in the NP samples and not in the BAL samples, indicating less efficient replication in the LRT after inoculation with 10^5 $TCID_{50}$ of hRSV in comparison to inoculation of 10^3 $TCID_{50}$ of bRSV which resulted in recovery of infectious virus in both NP and BAL samples [4]. Whether hRSV is able to replicate in the LRT when using higher doses of inoculum is yet unknown. Future studies utilizing different doses or different RSV-strains are needed to refine our current setup.

Despite the fact that LRTD symptoms and neutrophilic infiltration are less in hRSV infected calves compared to hRSV infection in humans and bRSV infection in calves, which seem to hamper the detailed study of hRSV pathogenesis, a hRSV model in calves may still be beneficial

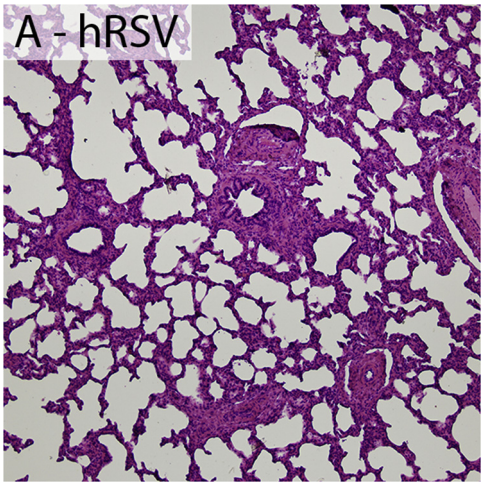

Fig. 3. Histopathological lung injury after clinical recovery from hRSV infection.
Representative lung tissue section with haematoxylin and eosin staining, showing mild peri-bronchiolar and alveolar cellular infiltrates with areas of alveolar wall thickening, adjacent to areas with normal alveolar architecture in hRSV infected animals, on day 12 after virus inoculation. Magnification 40×.

over other heterologous RSV models (e.g. mice, cotton rats, ferrets, lambs) depending on the research question under investigation. For example, contrast to the relatively high inoculation doses needed in other semi-permissive hRSV models (lambs [28] or mice [29–31]), the dose of the hRSV inoculum in calves was in the range of the viral inoculum needed to establish productive infection in humans [24]. Furthermore, the age-dependency of hRSV is less present in mice and mice are less susceptible to hRSV re-infection [32], which contrasts the frequent re-infection of calves and humans by bRSV and hRSV respectively [33]. Additionally, anatomical differences between rodents and children are significant [3]. The calf model is better suited to overcome these differences and is further useful for its ability to deprive animals from colostrum, which contains all maternal antibodies as there is no trans-placental transfer of antibodies in the bovine species. Depriving new born calves from colostrum results in maternally derived antibody (MDA)-deficient calves, which allows for controlled vaccine studies without interference of the maternal immune system. We have utilized colostrum-deprived calves in this study to optimise chances for active hRSV infection in a small group of animals, when properly established, further testing in common (colostrum receiving) calves could further refine hRSV infection to mimic a more natural clinical situation. The hRSV model in calves may also be advantageous in testing therapeutics that require specific hRSV epitopes (e.g. vaccines or monoclonal antibodies). For example, mucosal hRSV vaccination has recently received great interest and showed preliminary efficacy in mouse and cotton rat models [34–36]. However, rodents are difficult to sample longitudinally, lack mucosal immune reactive areas (e.g. tonsils), have less submucosal glands and have large areas of the airways which are not lined with ciliated cells [33,37]. Similar to humans, calves have tonsils and submucosal glands, which play key roles in the regulation of immune responses to respiratory pathogens [38–41]. Furthermore, this large animal model allows in-depth longitudinal analysis of the immunological pathways involved in vaccination response and efficacy at the mucosal level.

In conclusion, in this study we explored the viral kinetics during primary hRSV infection in MDA-deficient calves. HRSV infection in calves was associated with clinical symptoms and viral replication in the URT and a neutrophilic inflammatory response in the lung. Experimental infection of calves with hRSV may serve as a tool for tailored research questions, in particular related to the evaluation of (mucosal) vaccines and antiviral therapies against hRSV disease in humans that are not cross-reacting with bRSV.

Conflict of interest

None.

Acknowledgements

We would like to thank Frank Coenjaerts for his help with the hRSV viral load detection. This work was supported by Steun Emma Children's Hospital [CC200001], the Netherlands and the Amsterdam Economic Board [EZ1311 ALOHA RSV].

References

[1] H.C. Meissner, Viral bronchiolitis in children, N. Engl. J. Med. 374 (2016) 62–72.
[2] I. Rudan, K.L. O'Brien, H. Nair, L. Liu, E. Theodoratou, S. Qazi, I. Luksic, C.L. Fischer Walker, R.E. Black, H. Campbell, Child Health Epidemiology Reference, G, Epidemiology and etiology of childhood pneumonia in 2010: estimates of incidence, severe morbidity, mortality, underlying risk factors and causative pathogens for 192 countries, J. Glob. Health 3 (2013) 010401.
[3] R.A. Bem, J.B. Domachowske, H.F. Rosenberg, Animal models of human respiratory syncytial virus disease, Am. J. Physiol. Lung Cell Mol. Physiol. 301 (2011) L148–156.
[4] A.F. Antonis, R.S. Schrijver, F. Daus, P.J. Steverink, N. Stockhofe, E.J. Hensen, J.P. Langedijk, R.G. van der Most, Vaccine-induced immunopathology during bovine respiratory syncytial virus infection: exploring the parameters of pathogenesis, J. Virol. 77 (2003) 12067–12073.
[5] G. Taylor, Animal models of respiratory syncytial virus infection, Vaccine 35 (2017) 469–480.
[6] A.F. Antonis, M.C. de Jong, W.H. van der Poel, R.G. van der Most, N. Stockhofe-Zurwieden, T. Kimman, R.S. Schrijver, Age-dependent differences in the pathogenesis of bovine respiratory syncytial virus infections related to the development of natural immunocompetence, J. Gen. Virol. 91 (2010) 2497–2506.
[7] W.H. Van der Poel, A. Brand, J.A. Kramps, J.T. Van Oirschot, Respiratory syncytial virus infections in human beings and in cattle, J. Infect. 29 (1994) 215–228.
[8] S. Philippou, P. Otto, P. Reinhold, M. Elschner, H.J. Streckert, Respiratory syncytial virus-induced chronic bronchiolitis in experimentally infected calves, Virchows Arch. 436 (2000) 617–621.
[9] B. Cortjens, O.J. de Boer, R. de Jong, A.F. Antonis, Y.S. Sabogal Pineros, R. Lutter, J.B. van Woensel, R.A. Bem, Neutrophil extracellular traps cause airway obstruction during respiratory syncytial virus disease, J. Pathol. 238 (2016) 401–411.
[10] M.L. Everard, A. Swarbrick, M. Wrightham, J. McIntyre, C. Dunkley, P.D. James, H.F. Sewell, A.D. Milner, Analysis of cells obtained by bronchial lavage of infants with respiratory syncytial virus infection, Arch. Dis. Child. 71 (1994) 428–432.
[11] P.S. McNamara, P. Ritson, A. Selby, C.A. Hart, R.L. Smyth, Bronchoalveolar lavage cellularity in infants with severe respiratory syncytial virus bronchiolitis, Arch. Dis. Child. 88 (2003) 922–926.
[12] C.L. Afonso, G.K. Amarasinghe, K. Banyai, Y. Bao, C.F. Basler, S. Bavari, N. Bejerman, K.R. Blasdell, F.X. Briand, T. Briese, A. Bukreyev, C.H. Calisher, K. Chandran, J. Cheng, A.N. Clawson, P.L. Collins, R.G. Dietzgen, O. Dolnik, L.L. Domier, R. Durrwald, J.M. Dye, A.J. Easton, H. Ebihara, S.L. Farkas, J. Freitas-Astua, P. Formenty, R.A. Fouchier, Y. Fu, E. Ghedin, M.M. Goodin, R. Hewson, M. Horie, T.H. Hyndman, D. Jiang, E.W. Kitajima, G.P. Kobinger, H. Kondo, G. Kurath, R.A. Lamb, S. Lenardon, E.M. Leroy, C.X. Li, X.D. Lin, L. Liu, B. Longdon, S. Marton, A. Maisner, E. Muhlberger, S.V. Netesov, N. Nowotny, J.L. Patterson, S.L. Payne, J.T. Paweska, R.E. Randall, B.K. Rima, P. Rota, D. Rubbenstroth, M. Schwemmle, M. Shi, S.J. Smither, M.D. Stenglein, D.M. Stone, A. Takada, C. Terregino, R.B. Tesh, J.H. Tian, K. Tomonaga, N. Tordo, J.S. Towner, N. Vasilakis, M. Verbeek, V.E. Volchkov, V. Wahl-Jensen, J.A. Walsh, P.J. Walker, D. Wang, L.F. Wang, T. Wetzel, A.E. Whitfield, J.T. Xie, K.Y. Yuen, Y.Z. Zhang, J.H. Kuhn, Taxonomy of the order Mononegavirales: update 2016, Arch. Virol. 161 (2016) 2351–2360.
[13] M. Elvander, S. Vilcek, C. Baule, A. Uttenthal, A. Ballagi-Pordany, S. Belak, Genetic and antigenic analysis of the G attachment protein of bovine respiratory syncytial virus strains, J. Gen. Virol. 79 (Pt 12) (1998) 2939–2946.
[14] R.A. Lerch, K. Anderson, V.L. Amann, G.W. Wertz, Nucleotide sequence analysis of the bovine respiratory syncytial virus fusion protein mRNA and expression from a recombinant vaccinia virus, Virology 181 (1991) 118–131.
[15] B. Bossert, K.K. Conzelmann, Respiratory syncytial virus (RSV) nonstructural (NS) proteins as host range determinants: a chimeric bovine RSV with NS genes from human RSV is attenuated in interferon-competent bovine cells, J. Virol. 76 (2002) 4287–4293.
[16] J.F. Valarcher, G. Taylor, Bovine respiratory syncytial virus infection, Vet. Res. 38 (2007) 153–180.
[17] L.H. Thomas, E.J. Stott, A.P. Collins, S. Crouch, J. Jebbett, Infection of gnotobiotic calves with a bovine and human isolate of respiratory syncytial virus. Modification of the response by dexamethasone, Arch. Virol. 79 (1984) 67–77.
[18] U. Fogarty, Evaluation of a bronchoalveolar lavage technique, Equine Vet. J. 22 (1990) 174–176.
[19] L. Tan, P. Lemey, L. Houspie, M.C. Viveen, N.J. Jansen, A.M. van Loon, E. Wiertz,

G.M. van Bleek, D.P. Martin, F.E. Coenjaerts, Genetic variability among complete human respiratory syncytial virus subgroup A genomes: bridging molecular evolutionary dynamics and epidemiology, PLoS One 7 (2012) e51439.
[20] B. Viuff, K. Tjornehoj, L.E. Larsen, C.M. Rontved, A. Uttenthal, L. Ronsholt, S. Alexandersen, Replication and clearance of respiratory syncytial virus: apoptosis is an important pathway of virus clearance after experimental infection with bovine respiratory syncytial virus, Am. J. Pathol. 161 (2002) 2195–2207.
[21] M.F. Paccaud, C. Jacquier, A respiratory syncytial virus of bovine origin, Arch. Gesamte Virusforsch. 30 (1970) 327–342.
[22] E.B. Belknap, D.K. Ciszewski, J.C. Baker, Experimental respiratory syncytial virus infection in calves and lambs, J. Vet. Diagn. Invest. 7 (1995) 285–298.
[23] P. Otto, M. Elschner, P. Reinhold, H. Kohler, H.J. Streckert, S. Philippou, H. Werchau, K. Morgenroth, A model for respiratory syncytial virus (RSV) infection based on experimental aerosol exposure with bovine RSV in calves, Comp. Immunol. Microbiol. Infect. Dis. 19 (1996) 85–97.
[24] J.P. DeVincenzo, R.J. Whitley, R.L. Mackman, C. Scaglioni-Weinlich, L. Harrison, E. Farrell, S. McBride, R. Lambkin-Williams, R. Jordan, Y. Xin, S. Ramanathan, T. O'Riordan, S.A. Lewis, X. Li, S.L. Toback, S.L. Lin, J.W. Chien, Oral GS-5806 activity in a respiratory syncytial virus challenge study, N. Engl. J. Med. 371 (2014) 711–722.
[25] K. Blodorn, S. Hagglund, D. Gavier-Widen, J.F. Eleouet, S. Riffault, J. Pringle, G. Taylor, J.F. Valarcher, A bovine respiratory syncytial virus model with high clinical expression in calves with specific passive immunity, BMC Vet. Res. 11 (2015) 76.
[26] R.J. Derscheid, A. van Geelen, J.L. McGill, J.M. Gallup, T. Cihlar, R.E. Sacco, M.R. Ackermann, Human respiratory syncytial virus Memphis 37 grown in HEp-2 cells causes more severe disease in lambs than virus grown in Vero cells, Viruses 5 (2013) 2881–2897.
[27] P.F. Wright, W.C. Gruber, M. Peters, G. Reed, Y. Zhu, F. Robinson, S. Coleman-Dockery, B.S. Graham, Illness severity, viral shedding, and antibody responses in infants hospitalized with bronchiolitis caused by respiratory syncytial virus, J. Infect. Dis. 185 (2002) 1011–1018.
[28] A. Larios Mora, L. Detalle, A. Van Geelen, M.S. Davis, T. Stohr, J.M. Gallup, M.R. Ackermann, Kinetics of respiratory syncytial virus (RSV) Memphis strain 37 (M37) infection in the respiratory tract of newborn lambs as an RSV infection model for human infants, PLoS One 10 (2015) e0143580.
[29] S.M. Johnson, B.A. McNally, I. Ioannidis, E. Flano, M.N. Teng, A.G. Oomens, E.E. Walsh, M.E. Peeples, Respiratory syncytial virus uses CX3CR1 as a receptor on primary human airway epithelial cultures, PLoS Pathog. 11 (2015) e1005318.
[30] A. Sharma, W. Wu, B. Sung, J. Huang, T. Tsao, X. Li, R. Gomi, M. Tsuji, S. Worgall, Respiratory syncytial virus (RSV) pulmonary infection in humanized mice induces human Anti-RSV immune responses and pathology, J. Virol. 90 (2016) 5068–5074.
[31] M.T. Stier, M.H. Bloodworth, S. Toki, D.C. Newcomb, K. Goleniewska, K.L. Boyd, M. Quitalig, A.L. Hotard, M.L. Moore, T.V. Hartert, B. Zhou, A.N. McKenzie, R.S. Peebles Jr., Respiratory syncytial virus infection activates IL-13-producing group 2 innate lymphoid cells through thymic stromal lymphopoietin, J. Allergy Clin. Immunol. 138 (814-824) (2016) e811.
[32] B.S. Graham, L.A. Bunton, P.F. Wright, D.T. Karzon, Reinfection of mice with respiratory syncytial virus, J. Med. Virol. 34 (1991) 7–13.
[33] R.E. Sacco, R.K. Durbin, J.E. Durbin, Animal models of respiratory syncytial virus infection and disease, Curr. Opin. Virol. 13 (2015) 117–122.
[34] H. Gu, T. Li, L. Han, P. Zhu, P. Zhang, S. Zhang, S. Sun, Y. Duan, L. Xing, Z. Zhao, C. Lai, B. Wen, X. Wang, P. Yang, Protection conferred by virus-like particle vaccines against respiratory syncytial virus infection in mice by intranasal vaccination, Hum. Vaccin. Immunother. 11 (2015) 1057–1064.
[35] K.M. Morabito, T.R. Ruckwardt, A.J. Redwood, S.M. Moin, D.A. Price, B.S. Graham, Intranasal administration of RSV antigen-expressing MCMV elicits robust tissue-resident effector and effector memory CD8+ T cells in the lung, Mucosal Immunol. (2016).
[36] A. Pierantoni, M.L. Esposito, V. Ammendola, F. Napolitano, F. Grazioli, A. Abbate, M. Del Sorbo, L. Siani, A.M. D'Alise, A. Taglioni, G. Perretta, A. Siccardi, E. Soprana, M. Panigada, M. Thom, E. Scarselli, A. Folgori, S. Colloca, G. Taylor, R. Cortese, A. Nicosia, S. Capone, A. Vitelli, Mucosal delivery of a vectored RSV vaccine is safe and elicits protective immunity in rodents and nonhuman primates, Mol. Ther. Methods Clin. Dev. 2 (2015) 15018.
[37] C.G. Irvin, J.H. Bates, Measuring the lung function in the mouse: the challenge of size, Respir. Res. 4 (2003) 4.
[38] P. Brandtzaeg, Immune functions of nasopharyngeal lymphoid tissue, Adv. Otorhinolaryngol. 72 (2011) 20–24.
[39] C. Casteleyn, S. Breugelmans, P. Simoens, W. Van den Broeck, The tonsils revisited: review of the anatomical localization and histological characteristics of the tonsils of domestic and laboratory animals, Clin. Dev. Immunol. 2011 (2011) 472460.
[40] H.K. Choi, W.E. Finkbeiner, J.H. Widdicombe, A comparative study of mammalian tracheal mucous glands, J. Anat. 197 (Pt 3) (2000) 361–372.
[41] M.C. Rebelatto, C. Mead, H. HogenEsch, Lymphocyte populations and adhesion molecule expression in bovine tonsils, Vet. Immunol. Immunopathol. 73 (2000) 15–29.