

**Determination of the viral kinetics of human RSV M37 strain in newborn lambs for use in
therapeutic testing and characterization of clinical signs and sickness behavior**

by

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This dissertation is dedicated to:

Rafael Mora Mora
My family
Bruce and Barbara Munson
Collegiate United Methodist Church
Carolyn Tatum
Lupe Gutierrez
The lambs

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ABSTRACT

Respiratory Syncytial Virus (RSV) is an enveloped single stranded RNA pneumovirus in the pneumoviridae family that causes bronchiolitis and pneumonia in preterm and term newborns and elderly adults resulting in 75,000 to 125,000 hospitalizations of children below 1 year of age in the US each year.

Currently, there are no approved vaccines or effective therapeutic drugs for RSV infection, with treatment being solely limited to supportive care. One of the hurdles for developing vaccines or effective therapeutic drugs for RSV infection is a clinically relevant animal model. Animal models developed to study RSV infection include mice, cotton rats, ferrets, non-human primates, cattle, and lambs. Lambs have several biological features that closely mimic RSV infection in human infants such as: similarities to human infants in lung development, lung structure and airway branching, cellular composition and immune responses, and susceptibility to various strains of RSV, including human strains (Long, A2, and Memphis Strain 37).

To utilize the neonatal lamb model for therapeutic and vaccine studies, the viral kinetics of human M37 RSV strain was characterized to follow the progressive development of clinical signs, lung pathology and inflammatory/immune responses over time that closely resembled those of human infants. Once the viral kinetics of M37 were established, ALX-0171, a trivalent nanobody targeting the RSV F-protein was evaluated for its therapeutic potential, safety, and dose levels in newborn lambs infected with RSV M37. ALX-0171 was well-tolerated in RSV infected newborn lambs and exerted a positive effect on RSV-induced lung lesions while reducing symptoms of illness.

To determine the clinical response(s) of the therapeutic drugs being tested, the use of video recording and surveillance was implemented to better define and correlate clinical signs and sickness behavior of RSV to severity of disease in the neonatal lamb model. Overall, clinical signs and sickness behavior were confounded by non-RSV clinical signs of tremors and lameness in both RSV infected and non-infected groups, as well as bacterial pneumonias in the non-infected group. Future lamb studies should make an effort to solely demonstrate RSV infection in lambs by identifying and preventing secondary or opportunistic pathogens.

CHAPTER 1. GENERAL INTRODUCTION

Statement of the Problem

Human Respiratory syncytial virus (hRSV) is a common cause of acute lower respiratory disease in infants and young children worldwide. It is the most important viral pathogen causing acute lower respiratory tract infections (ALRI) in infants younger than 5 years old and it is estimated to have resulted in ~3.4 million hospitalizations and ~200,000 deaths worldwide in 2005 [1]. It is estimated that in the U.S., the cost associated with the hospitalization of RSV infection in children under five is about 394 million U.S. dollars [2].

Currently, there are no approved vaccines or effective therapeutic drugs for RSV infection, with treatment being solely limited to supportive care. There exists an inhaled nucleoside analog (Ribavirin) that is approved for therapeutic use, but which has limited treatment efficacy, as well as a monoclonal antibody (Synagis®, palivizumab), but whose use is limited to prophylactic application in high risk infants [3]. One of the hurdles for developing vaccines or effective therapeutic drugs for RSV infection is a clinically relevant animal model.

Lambs have several biological features that closely mimic RSV infection in human infants, and when infected with human RSV strains, lambs develop mild clinical symptoms including fever, tachypnea, and increased expiratory effort and wheeze, as well as mild to moderate gross and histologic lesions [4]. These similarities make neonatal lambs a relevant animal model to study human RSV and for the utilization of therapeutic and vaccine studies.

Specific Aims

The goal of these studies was to establish the viral kinetics of human RSV Memphis Strain 37 (M37) in the lamb model of RSV infection (Chapter 2). This set a baseline of more

distinct and precise clinical, viral, physiological, and immunological parameters, as well as the progression of pathology and lesions in the lamb model and these parameters that closely resembles those of that of human infants. Once the viral kinetics of M37 were established, therapeutic compounds such as ALX-0171 were tested for their safety, efficacy, and dose levels in the challenged RSV neonatal lamb model (Chapter 3). To determine the clinical response(s) of the therapeutic drugs being tested, the use of video recording and surveillance was implemented to better define and correlate clinical signs and sickness behavior of RSV to severity of disease in the neonatal lamb model (Chapter 4).

Dissertation Organization

The dissertation is organized into five Chapters, with the first Chapter being a general introduction and literature review. The second Chapter, 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, was published in the journal *PLoS One*. The third Chapter, Delivery of ALX-0171 by Inhalation Greatly Reduces Respiratory Syncytial Virus (RSV) Disease in Newborn Lambs, will be submitted to the Journal of *Monoclonal Antibodies*. The fourth Chapter, Determining Clinical Signs and Sickness Behavior in a Human RSV Neonatal Infection Lamb Model, will be submitted to the journal *PLoS One*. Finally, Chapter 5 is the conclusion and summary of the future of the lamb model.

Literature Review

Respiratory Syncytial Virus Classification and Morphology

Human respiratory syncytial virus (hRSV) is classified in the genus Orthopneumovirus of the newly created Pneumoviridae family within the order Mononegavirales, standing apart from the original Paramyxoviridae family member [5]. In addition, RSV has an envelope with a linear

single stranded, non-segmented RNA molecule of negative-polarity of approximately 15.2 kb, which has 10 genes that encodes for 11 proteins [6, 7].

Viral Genome and Protein Function

The two major antigenic surface glycoproteins of RSV include the G-protein and F-protein (fusion) that have a crucial role in viral replication as they are responsible for viral binding to the target cell and virus-cell membrane fusion (syncytia formation), respectively [8]. The small hydrophobic (SH) protein is the third surface protein of RSV and is believed to be involved in the formation of ion channels which increase RSV virulence degree and permeability to small compounds [9]. The non-structural proteins, NS1 and NS2, are virulence factors that impair interferon secretion-signaling, inhibit apoptosis of infected cells, modulate innate immune response and induce shedding of infected cells into large airways of the lungs, respectively [10, 11, 12]. The structural proteins that wrap around the RSV genome include nucleoprotein (N), phosphoprotein (P), and the large RNA-dependent RNA polymerase (L) that is involved in genomic replication [13]. The matrix (M) protein is necessary for the assembly of RSV [14, 15]. The M2.1 and M2.2 proteins are responsible for the assembly of the nucleocapsid and modulation of the synthesis of genome and anti-genome, respectively [16].

The F protein is the most highly conserved and is the major antigenic protein target for the neutralizing epitope, which has four antigenic sites (I, II, IV, and site zero (0)) [17]. The F protein is synthesized as a precursor F_0 that is cleaved by furin like protease that leads to F_1 and F_2 subunits, with the mature protein containing three copies of F_1 and F_2 polypeptides. The F protein also has two conformations, metastable and stable, with the metastable protein having the prefusion conformation (PrF) and the stable protein having the postfusion conformation (PoF) after infection. Both metastable and stable conformations of the F protein are present in the

surface of RSV; however, neutralizing epitope sites targeted with antibodies differ between both conformations [18].

Viral Phylogenetics and hRSV Strains

There are two major antigenic groups of human RSV, Type A and B, with the difference between both groups being mostly at the G-protein. Type A has been reported to be the most virulent and causing severe clinical disease in the hospital setting [8, 9]. However, there are also reports that both types A and B are equally as virulent and that both are able to remain genetically distinct by infecting slightly different niches within a population, which alternate between seasons of RSV Types A and B [17, 19].

Two relevant human RSV strains used to study RSV are M37 and A2. M37 RSV is a wild type RSV-A, first isolated from a 4 month old infant [20] and used in human clinical studies [21–23]. A2, also belongs to group Type A, and is extensively used in research to study the immune response to RSV in numerous cell lines and animal models [24-26].

Epidemiology and Susceptibility of hRSV

Human Respiratory Syncytial Virus (hRSV) is a major cause of respiratory disease in infants and young children in the United States and worldwide [1]. This lower respiratory tract infection results in hospitalization in about 3% of RSV-infected infants less than 1 year old, and in about 0.5% of RSV-infected children aged between 1 and 2 years [27]. Each year, it is estimated that RSV causes at least 3.4 million lower respiratory tract infections requiring hospitalization and up to 200,000 deaths worldwide in infants less than 5 years of age [1] and in approximately 177,000 hospital admissions and 10,000–14,000 deaths per year in the United States in the elderly.

Children under the age of 1 are the most susceptible population to RSV. Predisposing factors that are associated with the increased risk to severe RSV disease include: premature infants, congenital heart disease, chronic lung disease, cystic fibrosis, neurological conditions, congenital malformations, Down's syndrome, and immunocompromised children. It has also been reported that pre-existing disease was a predisposing factor for RSV mortality [28].

Seasonality, Transmission, and Reinfection of hRSV

RSV infection is seasonal and annual epidemics occur mostly in the winter months in temperate climates, with substantial morbidity and low mortality [29]. RSV is transmitted by direct and indirect contact with nasal or oral secretions from an infected individual [30]. Most children are infected with RSV by the age of two at least once, and reinfection can be repetitive, even within the same outbreak season. Reinfection has been attributed to RSV promoting an inefficient, short lasting adaptive immunity [31].

Clinical Signs of hRSV

As a respiratory virus, RSV infection may present as an upper respiratory tract infection (including rhinitis, otitis media and pharyngitis), or as a lower respiratory tract infection (including acute bronchiolitis or pneumonia) in vulnerable populations such as infants, elderly and immunocompromised adults [32]. Clinical signs in infants and in children develop four to six days after infection with RSV, and usually subside after one to two weeks [33]. These signs vary with severity of disease and range from mild flu-like symptoms (coughing, sneezing, fever, and loss of appetite) in 25% to 40% of first-time exposed infants to severe bronchiolitis with or without pneumonia (rapid breathing, difficulty breathing, and wheezing) necessitating hospitalization in 0.5% to 2% of infants [34]. In very young infants, irritability, decreased

activity, and apnea may be the only symptoms of infection. These clinical symptoms have been attributed to both the immune response to RSV, as well as the direct damage to RSV-infected bronchiolar epithelium [35, 36].

Treatment and Prevention of hRSV

Currently, there are no approved vaccines or effective therapeutic drugs for RSV infection, with treatment being solely limited to supportive care. In severe RSV infections, the only approved antiviral treatment is the nucleoside analog Virazole (ribavirin), which is delivered by inhalation. However, due to concerns for potential teratogenicity and minimal evidence of benefit it is not recommended for routine use in infants, but may be considered for use in select patients with documented, potentially life-threatening RSV infection [37]. In the prevention of RSV infections, Palivizumab (Synagis®, MedImmune), a humanized monoclonal antibody (IgG) against the F protein of RSV, has been approved for use in high risk infants; however, due to high costs associated with palivizumab prophylaxis limitations to its use have been introduced by national boards although cost-effectiveness has been proven in certain high-risk populations [38].

Host Innate Immune Response to hRSV

The innate immune system is the first line of defense against RSV. However, before the innate immune system is activated, there are physical barriers to the virus, such as the mucociliary clearance system, which helps remove dust particles and microbial agents out of the respiratory system through sneezing and coughing. Also, the microbiota that inhabits the mucosal surfaces prevents colonization of harmful bacteria [39].

Physical barriers are not enough to keep persistent microbial invaders from infecting the respiratory tract; therefore, the innate immune system responds through non-specific chemical

and cellular defense mechanisms. The chemical defense mechanisms against microbial invaders include antimicrobial peptides and digestive enzymes produced by the innate immune cells as well as the enzymes that compose the complement system [39].

Collectins are soluble pattern recognition molecules, which recognize pathogen associated molecular patterns (PAMPs) on the surface of microbial agents. Their function is to help eliminate microbial invaders by aggregation, opsonization, complement activation, phagocytosis, and inhibition of microbial growth. In the lungs there are specific collectins called surfactant protein-A (SP-A) and surfactant protein-D (SP-D) that are produced and secreted by type II epithelial cells, and to lesser extent Club (Clara cells) [40]. These collectins are reportedly reduced during RSV infection, as well as the production of surfactant. SP-A enhances viral clearance by binding to the F protein, and both SP-A and SP-D regulate alveolar macrophage function and inflammation [41, 42]. Genetic polymorphisms of SP-A and SP-D are associated with severity of RSV disease [43].

Defensins are another class of antimicrobial peptides that are produced by respiratory epithelium, submucosal glands, and inflammatory cells. Humans have two classes of defensins, α and β , which neutralize viruses by targeting viral envelopes, glycoproteins, capsids, and inhibit viral fusion and post-entry. Defensins also inhibit viral replication and act as chemokines to alter the immune response [44, 45]. In RSV infection of human lung epithelial A549 cells by A2 RSV strain, Human β defensin 2 (HBD2) was reported to block viral entry by destabilizing the viral envelope [46].

In humans and animals there is also an oxidative host defense system at the mucosal surface level that has microbicidal activity against bacteria and viruses [47, 48]. This oxidative host defense system is composed of a dual oxidase (Duox)/lactoperoxidase (LPO) reaction [H_2O_2

+ SCN⁻ → OSCN⁻] that leads to the production of oxythiocyanate [OSCN⁻]. Respiratory epithelial cells produce thiocyanate (SCN⁻), as well as hydrogen peroxide (H₂O₂) via dual oxidases, that are transported to the epithelial surface (air surface liquid of the mucus layer) where they are catalyzed by LPO, which is produced by submucosal glands, to produce OSCN⁻ [49,-50]. When SCN⁻ is substituted by Iodide (I⁻) in the Duox/LPO reaction, hypoiodite (OI⁻) is produced. Unlike OSCN⁻, OI⁻ has *in vitro* and *in vivo* anti-RSV activity [51-52]. In lambs challenged with RSV M37 strain, daily administration of potassium iodide (KI) led to decreased RSV disease severity [53].

As indicated, Club (Clara) cells are nonciliated bronchiolar epithelial cells that have several roles that include secretion of immunomodulatory substances (CC10), biotransformation of xenobiotics, and are progenitor cells for type II pneumocytes and Club (Clara) cells [54, 55]. This population of cells is vulnerable in neonates and chronic smokers [56, 57]. Club (Clara) cells secrete Clara cell secretory protein (CC10) (also known as CC16, or uteroglobin), which in CC10 knock out mice infected with RSV had increased viral persistence and lung inflammation, with Th2 cytokines and neutrophil chemokines being elevated, when compared to CC10 wild type mice. The role of CC10 regulating the lung inflammatory response was confirmed with the restoration of CC10 in knockout mice that had decreased viral persistence, lung inflammation, and airway reactivity [54]. CC10 has been reported to be decreased in patients with chronic asthma, chronic obstructive pulmonary disease (COPD), and bronchopulmonary dysplasia [54-57]. On the one hand, CC10 increases in bronchoalveolar lavage fluid and serum during acute lung injury, such as smoke inhalation and with pneumotoxins (naphthalene, 4 ipomeanol, chloroethylene) [55]. As indicated, Club (Clara) cells secrete SP-A and SP-D, but also secrete β

defensins, β galactoside binding protein, and RSV receptors (e.g. retinoic acid inducible gene-1) that influences the inflammatory response [45].

If the physical and chemical barriers of the innate immune system do not remove the infectious agent, then target cells (epithelial ciliated cells, macrophages, dendritic cells, basal cells infected by hRSV) will initiate an inflammation response upon infection by recognizing pattern associated molecular patterns (PAMPs) on the surface of microbial agents through pattern recognition receptors such as toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD)-like receptors, and retinoic acid-inducible gene I (RIG1)-like receptors [58, 59, 60]. Pattern recognition receptors that interact with RSV include TLR-2, TLR-3, TLR-4, TLR-6, and TLR-7, as well as RIG I-like receptors, and NOD-like receptor protein 3/apoptosis-associated speck-like protein containing a caspase recruitment domain (NLRP3/ASC) through indirect activation of TLR-2 first. [61-64].

These pattern recognition receptors are the first line of defense, and when activated lead to an intracellular cascade that activates type I interferons (Type I IFNs), proinflammatory cytokines (IL-1, IL-6, IL-18) through NF- κ B pathway, and the indirect activation of the inflammasome (NLRP3/ASC) through TLR2 activation [61]. The Fusion protein of RSV also stimulates the release of NF- κ B to activate IL-1 β and IL-6 [65]. However, as previously mentioned this initial inflammatory response is disrupted by RSV virulence factors, NS1 and NS2 proteins that interfere with the secretion of Type III IFNs [10, 11, 12, 66, 67]. Also, the nucleoprotein of RSV can act as a virulence factor by locating within the membrane of infected cells, which has been reported to inhibit T-cell activation [68, 69]. The infection of macrophages and dendritic cells can also disrupt the formation of an effective immunological synapse to activate T cells [13, 60]. These changes together result in a weak T cell immune response that

leads to reduced cytotoxic activity by CD8⁺ T cells, and a decrease in RSV clearance [66, 67]. The humoral response to RSV in infants leads to the production of IgG1 and IgG3 antibodies isotypes, which are protective, but not optimal for viral clearance. These antibody isotypes are associated with a Th2 immune response [70, 71].

Overall, the immune response to human RSV is characterized by an exacerbated inflammation response in the airways that is driven by a Th2 (IL-4, IL-5, IL-10, IL-13) and Th17 (IL-17, IL-21) and a mild Th1 (INF gamma, IL-12) immune response [72]. This inflammation response varies with severity as previously mentioned, but ultimately leads to the infiltration of bronchioles by neutrophils, eosinophils, and monocytes that release their contents and cytokines and chemokines (IL-6, IL-8, TNF- α , CXCL2, CXCL10, IL-17, etc.) to recruit macrophages and dendritic cells, that all together cause damage to lower airways (bronchiolitis) [72]. The bronchiolitis along with the increase production of mucous plugs in the airways, results in the clinical symptomology described above.

Besides causing bronchiolitis, hRSV infection has been associated with arrhythmias, myocardial failure, hepatitis in children, and encephalopathies [73-76]. Long term, severe hRSV disease in infancy has been reported to be a high risk factor for the development of asthma [77, 78].

Advances in Vaccine Development and Therapeutics for hRSV

The search for an effective RSV vaccine was initially held back by the formalin inactivated RSV vaccine in the 1960's that led to enhanced disease and the death of two infants [79]. Since then, the development of vaccines have focused on live virus, vectored virus, attenuated and inactivated subunits of RSV, as well as specific RSV proteins incorporated into nanoparticles and bacterial/virus/plant vectors [55, 80]. These new efforts in vaccine

development have primarily focused on the subunits of the F protein of RSV, specifically the Ø epitope region of the pre fusion conformation, which is the most significant target for antibody recognition that has resulted in high levels of neutralizing antibodies [81, 82, 83]. Using the PrF F protein for vaccine development has its limitations with this conformation being unstable in the presence of trypsin; therefore, the generation of recombinant PrF F proteins has been investigated in cotton rats for vaccine development. Cotton rats had no detectable virus loads with significant levels of neutralizing antibodies for the PrF F protein when compared to the PoF F protein vaccine [82, 83]. Virus vectors such as influenza have also been used to express chimeric proteins containing the F protein, which in challenged mice not only provided protection from RSV through neutralizing antibodies, but also decreased lung damage and secreted antiviral cytokines [84]. Another example of a recombinant prototype vaccine is the mycobacterium bovis BCG (rBCG-N-hRSV), which is used as a vector to express the nucleoprotein [13]. In mice, the rBCG-N-hRSV vaccine led neutralizing antibodies, Th1 like profile, and a decreased RSV pathology. A new approach to vaccines is the use of Virus like-Particles (VLPs). There is two prototypes for hRSV using VLPs, one for the F and the second for the M protein, both of which in mice have shown high neutralizing antibodies and an effective antiviral cytokine response [85].

There have been new advancements made in the anti-RSV therapeutic field since the release of Virazole (ribavirin) and Palivizumab to treat and prevent hRSV in high risk infants, respectively. ALX-0171 is a novel biotherapeutic by Ablynx NV that is composed of a trivalent Nanobody that targets the antigenic site II of the F protein of RSV. Antiviral efficacy of ALX-0171 has been demonstrated in challenged hRSV cotton rats and recently in newborn lambs,

were ALX-0171 exerted a positive effect on RSV induced lung lesions and inflammation and reduced symptoms of illness [86]. Currently, ALX-0171 is in phase IIb of human clinical trials.

Viral fusion inhibitors are another area of antiviral-RSV biotherapeutics that has seen a large growth. JNJ-53718678 by Johnson & Johnson is a small molecule fusion inhibitor that binds to the F protein in the prefusion conformation. In neonatal lambs, JNJ-53718678 demonstrated antiviral efficacy and reduced lung inflammation [87]. Currently, a clinical trial with JNJ-53718678 is being planned to treat hospitalized infants infected with RSV infection [88]. JNJ-53718678 is just one example of many viral fusion inhibitors in the pipeline. There are also many viral inhibitors for the SH, N, L, and M2-1 proteins [89]. Many other laboratories have ongoing therapeutic compounds often aimed toward inhibiting viral entry and replication such as RNA polymerase inhibitors and Fusion inhibitors.

Animal Models of RSV Infection

Different animal models for RSV infection, including non-human primates, calves, lambs, mice, guinea pigs, ferrets, hamsters and cotton rats have been described [90, 91]. The cotton rat (*Sigmodon hispidus*) RSV infection model has been successfully used in the development of both RSV-IgIV (RespiGam®, MedImmune, Inc., Gaithersburg, MD, USA) and palivizumab and findings in the cotton rat for those agents translated well to the clinical setting [92, 93]. The cotton rat model also serves as the primary model for the determination of vaccine safety as they develop vaccine-enhanced pulmonary disease that reflects what is seen in humans and non-human primates [18]. However, and although susceptible to RSV infection, cotton rats do not exhibit any clinical signs of upper and lower respiratory tract disease or any age-related susceptibility to hRSV in contrast to what is seen for human infants [91].

Lamb Model for RSV Infection

Colostrum-deprived neonatal lambs are highly relevant for the study of RSV infection and may serve as a model of RSV infection in human infants due to the natural susceptibility of lambs to ovine, bovine and human strains of RSV as well as similarities in disease pathogenesis and anatomical, physiological and developmental similarities to that of human infants [94-96]. Experimentally, it has been shown that lambs and other ruminants can be infected with human or bovine RSV strains and that the infection induces lung lesions that resemble those observed in human RSV pathology such as bronchiolitis with epithelial cell necrosis, syncytial cell formation, hyperplasia of nearby epithelium and infiltrates of neutrophils with occasional macrophages [51, 97-103]. Moreover, RSV infected lambs develop mild to moderate clinical symptoms such as expiratory effort, fever, tachypnea, wheeze, malaise and listlessness [97, 51, 98, 99, 102] and formalin-inactivated RSV vaccination in lambs induces enhanced lesions upon RSV infection [104] as observed in infants [79, 105].

Clinical Signs and Sickness Behavior in Lambs

Clinical signs in lambs infected with RSV can be variable and unpredictable, and there interpretation can be confounded by many variables that include: strain of hRSV, experience of the interpreter observing clinical signs, handling of lambs, sampling schedule, experience in getting respiration and heart rate, unknown side effects of therapeutic drugs, administration of anesthetics, and secondary bacterial infections. To add to this list, lambs are herd animals and don't express clear signs of sickness until severe disease is present or near death.

Sickness behavior is an adaptive organized immune response that leads to physiological and behavioral changes that are manifested in animals commonly as fever, depression, decreased lipido, adipisia, anorexia, and decreased grooming and social interaction due to potentially

injurious stimuli, such as microbial agents (e.g. virus, bacteria, fungal, parasites) [106-108]. This evolved behavioral response helps to conserve energy through lethargy and fatigue and fight bacterial and viral infections by increasing body temperature through fever [108].

Important immune mediators that contribute to the sickness response include proinflammatory cytokines IL-1, IL-6, and tumor necrosis factor [106-108]. Together these cytokines mediate local, systemic, and central nervous responses during acute and chronic infection that leads to sickness behavior [106]. However, with fever and decreased appetite, the immune response can be metabolically expensive, and if prolonged animals may succumb to the disease, or become susceptible to predation [108].

Sickness behavior can be easily induced and studied in animals with exposure to lipopolysaccharide (LPS) endotoxin. For example in rodents, fever, anorexia, and decreased activity and grooming are at their highest at increased lipopolysaccharide (LPS) endotoxin doses [109]. Therefore, sickness behavior varies with disease severity. This can also be seen in cattle with mastitis and bovine respiratory disease, where the sickness behavior was greater for severe disease when compared to mild mastitis and bovine respiratory disease [110, 111]. Sickness behavior can be used to detect infected animals; however, identifying animals with mild disease and subclinical signs may be more challenging [112].

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CHAPTER 2. 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.

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Abstract

Rationale

Respiratory syncytial virus (RSV) infection in preterm and newborn infants can result in severe bronchiolitis and hospitalization. The lamb lung has several key features conducive to modeling RSV infection in human infants, including susceptibility to human strains of RSV such as the A2, Long, and Memphis Strain 37 (M37). In this study, the kinetics of M37 infection was investigated in newborn lambs in order to better define clinical, viral, physiological, and immunological parameters as well as the pathology and lesions.

Methods

Newborn lambs were nebulized with M37 hRSV (6 mL of 1.27×10^7 FFU/mL), monitored daily for clinical responses, and respiratory tissues were collected from groups of lambs at days 1, 3, 4, 6, and 8 post-inoculation for the assessment of viral replication parameters, lesions and also cellular, immunologic and inflammatory responses.

Results

Lambs had increased expiratory effort (forced expiration) at days 4, 6, and 8 post-inoculation. Nasal wash lacked RSV titers at day 1, but titers were present at low levels at days 3 (peak), 4, and 8. Viral titers in bronchoalveolar lavage fluid (BALF) reached a plateau at day 3 (4.6 Log₁₀ FFU/mL), which was maintained until day 6 (4.83 Log₁₀ FFU/mL), and were markedly reduced or absent at day 8. Viral RNA levels (detected by RT-qPCR) in BALF were indistinguishable at days 3 (6.22 ± 0.08 Log₁₀ M37 RNA copies/mL; mean \pm se) and 4 (6.20 ± 0.16 Log₁₀ M37 RNA copies/mL; mean \pm se) and increased slightly on day 6 (7.15 ± 0.2 Log₁₀ M37 RNA copies/mL; mean \pm se). Viral antigen in lung tissue as detected by immunohistochemistry was not seen at day 1, was present at days 3 and 4 before reaching a peak by day 6, and was markedly reduced by day 8. Viral antigen was mainly present in airways (bronchi, bronchioles) at day 3 and was increasingly present in alveolar cells at days 4 and 6, with reduction at day 8. Histopathologic lesions such as bronchitis/bronchiolitis, epithelial necrosis and hyperplasia, peribronchial lymphocyte infiltration, and syncytial cells, were consistent with those described previously for lambs and infants.

Conclusion

This work demonstrates that M37 hRSV replication in the lower airways of newborn lambs is robust with peak replication on day 3 and sustained until day 6. These findings, along with the similarities of lamb lung to those of infants in terms of alveolar development, airway branching and epithelium, susceptibility to human RSV strains, lesion characteristics (bronchiolitis), lung size, clinical parameters, and immunity, further establish the neonatal lamb as a model with key features that mimic RSV infection in infants.

Introduction

Human Respiratory Syncytial Virus (hRSV) is an enveloped, non-segmented, single stranded negative sense RNA pneumovirus of the paramyxoviridae family that causes lower airway respiratory disease in preterm newborns, term newborns, and elderly adults [1, 2]. It is the most important viral pathogen causing acute lower respiratory tract infections (ALRI) in infants younger than 5 years old and it is estimated to have resulted in ~3.4 million hospitalizations and ~200,000 deaths worldwide in 2005 [3]. RSV is transmitted by direct and indirect contact of nasal or oral secretions from an infected individual and primarily targets the lower airway respiratory epithelium (bronchioles) [4]. Clinical signs in infants and in children develop four to six days after infection with RSV, and usually subside after one to two weeks [5]. These signs vary with severity of disease and range from mild flu-like symptoms (coughing, sneezing, fever, and loss of appetite) in 25% to 40% of first-time exposed infants to severe bronchiolitis with or without pneumonia (rapid breathing, difficulty breathing, and wheezing) necessitating hospitalization in 0.5% to 2% of infants[6]. In very young infants, irritability, decreased activity, and apnea may be the only symptoms of infection. These clinical symptoms have been attributed

to both the immune response to RSV, as well as the direct damage to RSV-infected bronchiolar epithelium [7, 8].

Current treatment of RSV infection is limited to supportive care. There exists an inhaled nucleoside analog (Ribavirin) that is approved for therapeutic use but which has limited treatment efficacy, as well as a monoclonal antibody (Synagis®, palivizumab), but whose use is limited to prophylactic application in high risk infants [9]. Two major hurdles in the development of preventative and therapeutic regimens are (i) the safety considerations following vaccination in young infants exemplified by the disastrous initial formalin-inactivated vaccine clinical trials where vaccination potentiated the disease rather than being protective and (ii) the lack of an available, clinically relevant model of RSV infection [10].

Animal models developed to study RSV infection include mice, cotton rats, ferrets, non-human primates, cattle, and lambs [9]. Lambs have several biological features that closely mimic RSV infection in human infants such as: similarities to human infants in lung development, lung structure and airway branching, cellular composition and immune responses, survival after late-preterm birth, susceptibility to various strains of RSV including human strains (Long, A2, and Memphis Strain 37), similar histologic lesions and lung size to human infants, and the ability to obtain lambs lacking maternal antibodies [11–15]. Despite these advantages and the many previous and on-going studies of RSV infection in lambs, the progressive development of clinical signs, lung pathology and inflammatory/immune responses over time after inoculation with a human strain of RSV have not been fully characterized. Thus, the aim of this study was to gain further insight and understanding of the effects of RSV infection in the neonatal lamb model throughout infection (days 1, 3, 4, 6 and 8 after inoculation) with the human RSV Memphis

Strain 37. This kinetic information is needed in order to more fully characterize and utilize the lamb model for therapeutic and vaccine studies.

Material and Methods

Experimental Design

Colostrum-deprived neonatal lambs (2–7 days of age) received daily antibiotics (Ceftiofur, Pfizer, New York, NY; 1–2 mg/kg, intramuscular) to prevent secondary bacterial infections. They were randomly assigned to five M37 hRSV-infected groups, of 3 lambs ($n = 3$). Each lamb received three 2-mL installments of 1.27×10^7 FFU/mL in DMEM over a 23-minute period using a PARI LC Sprint™ nebulizer (PARI Respiratory Equipment, Inc., Lancaster, PA, USA) at 4L/min at 16 PSI (Philips Respironics Air Compressor, Andover, MA, USA) attached to a conical mask fitted with a round rubber diaphragm with a pre-cut center hole through which the nose and mouth of the lamb was inserted (MidWest Veterinary Supply, Inc., Burnsville, MN). Following infection, lambs were euthanized by sodium pentobarbital overdose and necropsied at different days post viral infection (p.i.): days 1, 3, 4, 6, and 8 p.i. After euthanasia the thorax was opened, lungs were removed, and RSV gross lesions (not including bacterial pneumonia lesions) were scored and photographed ex vivo. Tissue samples were collected from each lung lobe of all animals in the same manner, with uniform sampling of each lobe, and avoiding areas of bacterial pneumonia. Before lung dissection, bronchoalveolar lavage fluid (BALF) was collected from the right caudal lung lobe for infectious Focus-Forming Unit (FFU) assay and RT-qPCR for M37 hRSV total nucleoprotein RNA and accessory lobe, for cytology (total and differential cell counts), as described below. Three samples from each lobe were snap frozen in liquid nitrogen for reverse transcription quantitative polymerase chain reaction (RT-qPCR), and two samples

from each lobe were placed in tissue cassettes and put in 10% neutral-buffered formalin for histological and immunohistochemical analyses.

Due to limitations in housing and number of lambs that can be handled in one study, the day 6 assessments were derived from an additional group of 3 lambs that was infected shortly after the necropsy of the other groups. The procedures, viral stock used and animal handling were identical to the viral kinetic (VK) study. The only difference was that the day 6 animals were slightly younger and lighter than the VK study animals as these came from another supplier (mean bodyweights of 3.4 ± 0.17 vs 6.3 ± 0.24 for the VK study on day 0; mean \pm se). In accordance to the 3R-principles, control lambs were not included in this study as previous work in our lab showed that non-infected lambs entirely lacked evidence of clinical illness, lung pathology, or immune and inflammatory changes that are consistent with M37 hRSV infection [1, 16-20]. Animal use and experimental procedures were approved by Iowa State University's Animal Care and Use Committee (IACUC).

Virus

Memphis 37 (M37) RSV is a wild type RSV-A, first isolated from a 4 month old infant [21] and used in human clinical studies [22–24]. The Memphis 37 RSV strain used in this study was passaged 6 times on Vero cells then twice on HEp-2 cells. Sucrose was added to 20% and the virus stock was frozen at -80°C and titered for infectivity on HEp-2 cells as we have characterized previously in this model [20].

Monitoring of Clinical Signs

Lambs were monitored daily for body weights, rectal temperatures, heart rate and percent blood oxygenation measurements (PalmSAT® 2500A VET pulse oximeter, Nonin Medical Inc.,

Plymouth, MN, USA), and manual heart and respiratory rates (by auscultation). Increased expiratory effort (forced expiration) was scored daily as were animal “wheeze” scores (Table 1).

Table 1. Scoring criteria for lung function by auscultation

Score	Expiratory efforts	Wheezing (High-pitched whistling sound made while breathing)
0	No expiratory effort	No wheeze
1	Earliest detection of increased expiratory effort	Earliest detectable wheeze by auscultation
2	Moderate expiratory effort (>1 sec) observed with some abdominal effort	Audible wheeze in all lung fields by auscultation
3	Expiratory effort (>3 sec) with hard abdominal effort	Wheeze audible without stethoscope

Collection of Nasal Wash Fluid (NWF)

Just before euthanasia the nasal cavity of each lamb was washed with double-modified Iscove’s medium (DMIM) containing 42.5% Iscove's modified Dulbecco's medium, 7.5% glycerol, 1% heat-inactivated FBS, 49% Dulbecco’s Modified Eagle medium (DMEM), and 5 µg/mL kanamycin sulfate. Using a 6-mL syringe fitted with a mucosal atomization device (MAD) conical foam end-piece (Intranasal Mucosal Atomization Device, Wolfe Tory Medical, Inc., Salt Lake City, UT, USA) a single 5 mL aliquot of DMIM was instilled into the right nare, and then (while still preserving a good seal between the nare and the MAD-device conical end-piece), any out-coming fluid was extracted fairly quickly, in one motion, back into the delivery

syringe to collect (1.3–2.5 mL) NWF; which was dispensed into a 15 mL conical tube and placed on ice.

Collection of Bronchoalveolar Lavage Fluid (BALF)

Following euthanasia the lungs of each lamb were removed and each left and right lung was separated and weighed. The excised right caudal lung lobe was instilled with 5 mL of cold DMIM (42.5% Iscove's modified Dulbecco's medium, 7.5% glycerol, 1% heat-inactivated FBS, 49% DMEM, and 5 µg/ml kanamycin sulfate) after which 1 mL of the resulting BAL fluid was placed on ice and spun down for 5 minutes in a centrifuge at 3,000 x g to pellet large debris. Approximately 800–850 µL of each supernatant was collected and then spun through 850 µL-capacity 0.45 µm Costar SPIN-X filter (microcentrifuge 15,600 x g) for 5 minutes before being used in the standard infectious focus forming unit assay (FFU).

Gross Lesions Evaluation and Scoring

Following euthanasia, the thorax was opened and the heart and esophagus were removed from the lungs. The percentage parenchymal involvement of gross RSV lesions was scored for each individual lung lobe, and if present, the area and amount of lung lobes affected by bacterial pneumonia was also recorded. The percentage of a specific lobe tissue that was affected by RSV in relation to the overall lobe tissue being scored was estimated based on a score as done previously [1]. Mean percentage averages per lobe were calculated for each day of necropsy.

Histologic Evaluation and Scoring

A histologic score was given by determining percent involvement followed by conversion to an additional integer-based consolidation scale used by our laboratory previously [1] wherein: 0% consolidation = 0; 1%-9% consolidation = 1; 10%-39% consolidation = 2; 40%-

69% consolidation = 3; 70%-100% consolidation = 4. In total, multiple fields from 4 slides per animal were scored for the lesions. Each slide contained 2 different sections from the same lobe. Histologic score for each animal was the mean of all 4 slides and group averages were calculated for the alveolar consolidation score. In addition to the consolidation score, bronchitis/bronchiolitis, neutrophil infiltration, peribronchiolar and perivascular infiltration of lymphocytes, syncytial cell formation, and epithelial alterations were also scored according to criteria indicated in Table 2.

Table 2. Histologic lung lesion scoring criteria

Score	Bronchitis	Bronchiolitis	Syncytial cells	Epithelial necrosis (bronchi or bronchioles)	Epithelial hyperplasia (bronchi, bronchioles)	Neutrophil (in bronchi, bronchioles or alveoli)
0	no remarkable lesions	no remarkable lesions	none	none	none	none
1	minimal detectable lymphoplasmacytic infiltrates in lamina propria and adventitia	minimal detectable lesions (epithelial degeneration) in one or a few bronchioles per 20x field	one distinct syncytial cell	minimally detectable in one or a few per 20x field	minimally detectable in one or a few airways per 20x field	minimally detectable
2	segmental to circumferential infiltrates	epithelial degeneration involving less than 10% of the airway lumen; minimal neutrophils, cell debris; adventitial lymphocytes in multiple bronchioles	up to three in three 20x fields	10% in multiple airways per 20x field	10% of airway per 20x field	10 or less neutrophils in one or a few airways/alveoli
3	dense infiltrates	epithelial degeneration involving >10-50% of the airway lumen with cell debris, neutrophils; adventitial lymphocytes; multiple bronchioles	more than three in three 20x fields	10-50% in multiple airways per 20x field	10-50% in multiple airways per 20x field	10 or more neutrophils in several airways/alveoli
4	infiltrates with nodular aggregates	circumferential bronchiolitis with dense adventitial lymphocytes; multiple bronchioles	Numerous in three 20x fields	circumferential in multiple airways	circumferential	10 or more involving many/most airways/alveoli

Table 2. (continued)

Score	Bi nodules (these are peribronchiolar lymphocytic infiltrates)	Vessel nodules (lymphocytic infiltrates around blood vessels)	Eosinophilic infiltrates in bronchi or bronchioles, alveoli	Eosinophilic infiltrates in vessels	Eosinophils in vessel lumens
0	none	none	none	none	none
1	earliest detectable lymphocytic infiltrates in the adventitia	earliest detectable lymphocytic infiltrates in the adventitia	minimally detectable	minimally detectable	minimally detectable
2	segmental to circumferential infiltrates	segmental to circumferential infiltrates	up to five eosinophils in one to several airways/alveoli per 20x field	up to five eosinophils associated with a vessel	up to five in one or a few vessels
3	circumferential infiltrates that expand more than three cells wide	circumferential infiltrates that expand more than three cells wide	over five in over five airways per 20x field	> five in over five vessels per 20x field	> five in over five vessels
4	circumferential infiltrates that form nodules	circumferential infiltrates with nodules	dense accumulations of eosinophils	dense accumulations of eosinophils	dense accumulations

Immunohistochemistry for Viral Antigen Detection

Immunohistochemistry for the detection of RSV antigen was performed on 5 µm-thick formalin-fixed paraffin-embedded (FFPE) lamb lung tissue sections taken from the right and left cranial, left middle, and left caudal lung lobes of each animal in accordance with methods published previously [17, 25], but with the following variations: instead of Pronase E antigen retrieval, heated buffer antigen retrieval was performed in TRIS-EDTA-0.05% Tween 20, pH 9.0 in a pressure cooking device (Decloaking ChamberTM Plus, Biocare Medical, Concord, CA) using the factory default 40-minute program (125°C reached in 18 minutes and cooling to 80°C in another 22 minutes). Primary polyclonal goat anti-RSV (all antigens) antibody (EMD Millipore Corporation, Billerica, MA, USA) was applied for 90 minutes at room temperature diluted 1:500 in TBS-tween containing 10% NSS and 3% BSA. After rinsing with TBS-tween, biotinylated

rabbit anti-goat secondary antibody (Kirkegaard-Perry Labs, Gaithersburg, MD, USA) diluted 1:300 in TBS-tween containing 10% NSS and 3% BSA was applied for 45 minutes, after which slides were rinsed with TBS-tween, treated with 3% H_2O_2 in TBS-tween for 25 minutes, rinsed and then incubated with streptavidin-conjugated HRP (Invitrogen) diluted 1:200 in TBS-tween for 30 minutes. Development of the color was performed in custom 12-slide plastic containers (Antibody AmplifierTM containers, ProHisto, LLC, Columbia, SC, USA) by applying Nova Red (Vector Laboratories, Inc.) for about 90 seconds followed by copious rinses with ddH₂O, counterstaining with Harris' hematoxylin (for 2 minutes), bluing with alkaline Scott's water (for 1 minute), dehydration and coverslipping with Permount mounting medium (Sigma, St. Louis, MO, USA). 20 unique 10X fields on each slide (containing two lung sections each) were assessed for RSV antigen staining by counting positively-stained cells within bronchioles and alveoli. The mean number of stained bronchi/bronchioles and alveoli per field were counted for each day of necropsy.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) Assessment of RSV and Chemokine Gene mRNA Expression Levels in Lamb Lung

For each animal, tissue samples from right and left cranial, left middle and left caudal lung lobes (0.3–0.4 g of each lobe) were homogenized for total RNA isolation in TRIzol (Invitrogen) and previously described methods [1]. RNA was assessed for quantity and purity by spectrometry (Beckmann DU® 640B, Beckmann Coulter Inc., Brea, CA, USA) and all OD_{260nm/280nm} values measured between 1.96 and 2.12. Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) analysis of RNA prior to DNase treatment consistently yielded RIN values ≥ 8.0 for all lamb lung RNA samples isolated this way [16]. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed using One-Step

Fast qRT-PCR Kit master mix (Quanta, BioScience, Gaithersburg, MD, USA) in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) and PREXCEL-Q for all set up calculations [26, 27]. Primer and probe sequences for all targets were designed with ABI Primer Express 2.0, and have been used previously [1, 17, 28]. Primers and hydrolysis probe for targeting M37 hRSV NP RNA were designed using ABI Primer Express version 2.0 based on RSV accession number M74568. Thermocycling conditions were 5 minutes at 50°C; 30 seconds at 95°C; and 45 cycles of 3 seconds at 95°C and 30 seconds at 60°C. Samples and standards were assessed in duplicate, and each target gene quantification cycle (Cq) value was converted to a relative quantity (Qr) based on each target's standard curve using: $Qr = E^{AMP(b-Cq)}$, wherein "b" and "EAMP" are the y-intercept and exponential PCR amplification value, respectively. EAMP values were obtained from the slope (m) of each target standard curve by: $EAMP = 10(-1/m)$, and all Qr values interpolated from standard curves were normalized to total lung RNA per RT-qPCR (0.784 ng RNA/μL for all reactions). No-RT control (NRC) reactions gave either no signal or generated Cq values greater than 13 cycles later than those in the corresponding RT-qPCR target reactions.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) for RSV in Nasal Wash Fluid and Bronchoalveolar Lavage Fluid

Viral RNA was quantified by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in NWF and BALF obtained from each animal at necropsy. NWF was obtained from the right nasal cavity and BALF from the right caudal lung lobe of each animal (see section on NWF and BALF collection). Briefly, 100 μL of each collected fluid sample was pipetted directly into 1 mL of TRIzol (Invitrogen/Life Technologies, Carlsbad, CA, USA) on ice, inverted to mix, and then transferred to -80°C for storage until RNA isolation and subsequent

RT-qPCR. Upon thawing, each 1.1 mL sample was vortexed for 10 seconds and allowed to sit at room temperature for 10 minutes. RNA isolation from NWF and BALF samples continued as per manufacturer's instructions. The resulting (non-visible) RNA pellets, were each dissolved in 100 μ L of nuclease-free water (Invitrogen/Life Technologies), vortexed thoroughly, microfuged briefly, warmed to 60°C for 3 minutes, vortexed for 5 seconds, microfuged briefly, then 80 μ L of each was diluted 1:10 with a combination of 10 μ L RNaseOUT™ (to 0.5 Units/ μ L), and 710 μ L nuclease-free water, then stored at 4°C prior to RT-qPCR. RT-qPCR for RSV was then carried out as described above in the section: "Reverse transcription quantitative polymerase chain reaction (RT-qPCR) assessment of RSV and chemokine gene mRNA expression levels in lamb lung".

Focus-Forming Unit (FFU) Assay

Viral titers in both nasal wash fluid (NWF) and bronchoalveolar lavage fluid (BALF) from the right lung caudal lobe were determined using an infectious focus assay (FFU). In brief, 200 μ L of serially-diluted NWF or BALF samples were applied to HEp-2 cells grown to 70% confluence in 12-well culture plates (Fisher Scientific, Hanover Park, IL) in DMEM media (Mediatech, Inc., Manassas, VA) supplemented to 10% with heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA) and 50 μ g/mL kanamycin sulfate (Invitrogen/Life Technologies). Each sample was analyzed undiluted and at four additional serial-dilutions of 1:10, 1:100, 1:1,000 and 1:10,000 in duplicate. Following a 48-hour incubation at 37°C, 5% CO₂, the cells were fixed with cold 60% acetone/40% methanol solution for 1 minute. Overnight primary polyclonal goat anti-RSV (all antigens) antibody (EMD Millipore Corporation, Billerica, MA, USA) incubation was followed by washing and secondary antibody (Alexa Fluor® 488 F(ab')₂ fragment of rabbit anti-goat IgG (H+L), Molecular Probes/Life Technologies) incubation

for 30 minutes. Plates were rinsed and inspected for the presence of fluorescing foci of infection using the FITC/GFP filter on an inverted fluorescence microscope (Olympus CKX41, Center Valley, PA). Five or more fluorescing cells were counted as single focal events. An average of 40 counts in a 1:10-diluted (duplicate) sample indicated an original NWF or BALF sample “titer” of 2,000 $[40 \text{ counts} \times \text{dilution of } 10 \times 1,000 \mu\text{L/mL}] / 200 \mu\text{L assessed} = 2,000 \text{ infectious focus-forming units/mL (FFU/mL)}$.

Results

Clinical Findings

Following RSV-infection, there were no differences in weight gain, body temperature, heart rate, and percent blood oxygen saturation when compared to control lambs from previous studies in which the procedures, facilities, animal handling, and source and age of lambs were similar [1, 16–20]. Despite a small drop in mean blood oxygen saturation levels on day 6 ($92.4\% \pm 2\%$; mean \pm SD) when respiratory distress was present in most lambs, these remained above the 90% limit. Respiratory rates (not shown) were variable and non-predictable for each day of the study and were likely confounded by the heavy sampling schedule and the resultant stress level of the newborn lambs. Increased expiratory efforts and wheezing were the only noteworthy clinical features observed in RSV-infected lambs in this study. Following inoculation with M37 hRSV, expiratory efforts became apparent on day 3 in 4 out of 12 lambs (33%). On day 5 this proportion increased to 4 out of 6 lambs (66%) and on day 6 to 5 out of 6 lambs (83%). By day 7 all lambs (100%) had increased expiratory efforts, but on day 8 this proportion dropped to 2 lambs out of 3 (66%). The severity of expiratory efforts increased from day 3 to day 7 as shown by the mean expiratory effort score (Fig 1A). Similarly, wheezing was apparent on day 3

although only in 1 out of 12 lambs (8.3%). The proportion of lambs that developed wheeze gradually increased to reach a peak on day 5 (83.3%) and decreased on day 8 (33%). The mean wheeze score followed a similar time profile (Fig 1B).

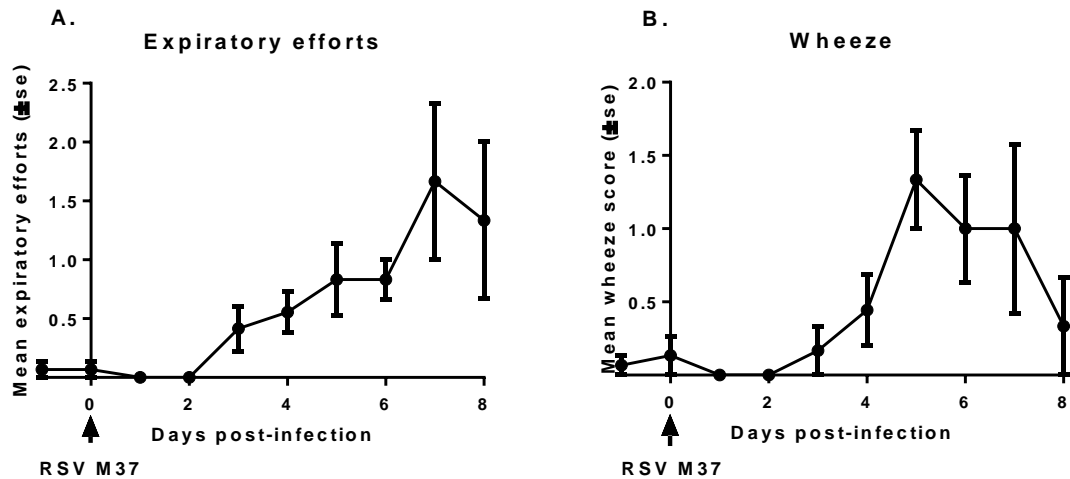


Figure 1. Respiratory distress score of lambs inoculated with human respiratory syncytial virus (hRSV) strain Memphis 37 (M37). Respiratory distress score was assessed daily for each lamb by auscultation or visual observation, and was categorized by expiratory effort (A) and wheezing (B). Result are shown as mean \pm standard error.

Gross and Microscopic Lung Lesions

Following necropsy, gross examination of the lungs determined the percent of each lobe that was covered with typical RSV-induced lesions. In some cases, areas of lung with lesions suggestive of bacterial pneumonia were also present along with RSV-induced lesions, and lung lobes with lesions suggestive of bacterial pneumonia were recorded, but not scored as RSV lesions. RSV lesions were bilateral, evenly-distributed and characterized by multifocal to locally extensive dark plum-red, well-demarcated foci of pulmonary consolidation which varied from mild to severe (Fig 2A); consistent with RSV infection in lambs as reported previously [1, 17, 29]. In contrast to RSV lesions, lung lobes affected by bacterial pneumonias were mild,

unilateral, and cranial ventral (right and middle lung lobes being mostly affected), and were characterized by multifocal, locally extensive, firm, red-brown areas. Following RSV-infection, gross RSV viral lesions were already detectable on day 1, and further increased by day 3, to reach maximal levels by day 6 of around 40% and decreased thereafter (Fig 2B). For the lambs used in this study, only the last group of lambs (10 and 12) necropsied at day 8, had gross lesions of bacterial pneumonia affecting the right middle lung lobe. For day 6 lambs, lamb 14 had bacterial pneumonia affecting the right cranial and middle lung lobes. Again, these lung regions bearing non-RSV-induced lesions were not sampled for attaining experimental endpoints.

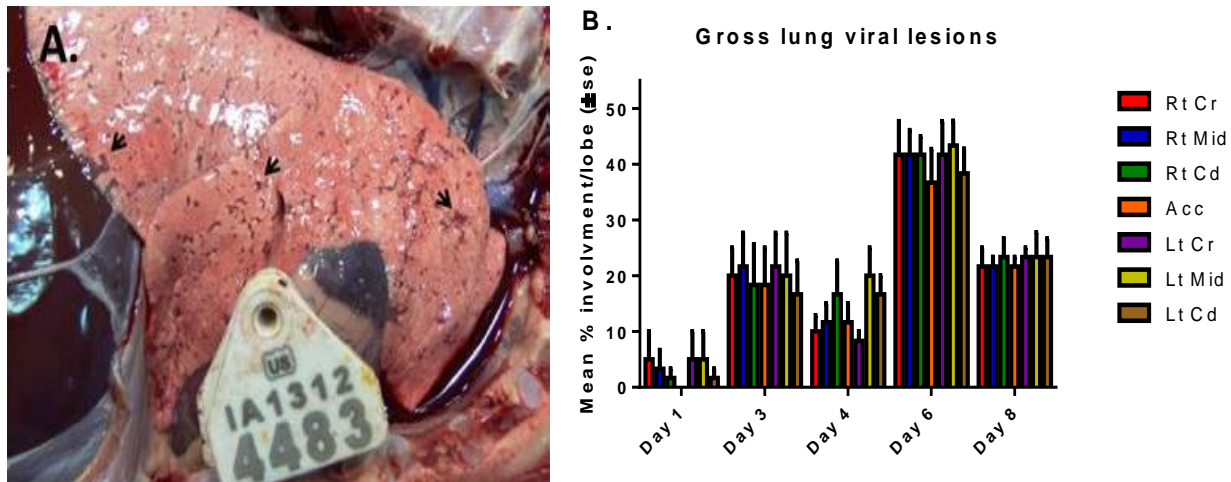


Figure 2. Scoring of gross lesions caused by M37 hRSV in lambs. Viral gross lesions caused by M37 hRSV infection in neonatal lambs. (A) Picture of a lung on day 6 post-infection. Dark plum-red, well-demarcated foci of pulmonary consolidation are indicated by arrowheads. (B) Percentage parenchymal involvement was estimated for each lung lobe and mean percentage averages per lobe were calculated for each day of necropsy (\pm standard error). Legend: Rt Cr = Right cranial lobe; Rt Mid = Right Middle lobe; Rt Cd = Right Caudal lobe; Acc = Accessory lobe; Lt Cr = Left Cranial lobe; Lt Mid = Left Middle lobe; Lt Cd = Left Caudal lobe.

Microscopically, lungs of infected lambs had multifocal to coalescent foci of an inflammatory infiltrate that partially filled the lumen of bronchi/bronchioles, alveolar spaces, and alveolar septa. The airway lumen was also partially occluded by seroproteinaceous fluid and cell

debris intermixed with mucin. These lesions increased progressively with time and were similar to those described previously with M37 hRSV and with hRSV A2 strains [1, 17, 18, 25]. On day 1 p.i., small numbers of neutrophils were present within the lumen of bronchioles and occasional bronchi. On day 3, microscopic lung lesions were characterized by mild to moderate infiltrates of neutrophils in bronchiolar lumens with small amounts of seroproteinaceous fluid and mucin. A mild but detectable infiltration of lymphocytes in the tunica adventitia of bronchioles and nearby blood vessels was also present. There was degeneration (cells with rounded cell borders and basophilic/pyknotic nuclei) of epithelial cells in bronchioles (Fig 3A). The intensity of the lesions were further increased on day 4 and characterized by the neutrophil infiltration with sloughed, necrotic epithelial cells, seroproteineous fluid and small amounts of mucin in bronchioles and bronchi along with occasional macrophages and the formation of occasional syncytial cells in bronchio-alveolar spaces. The alveolar septa were mildly to moderately thickened by hyperplasia of type II pneumocytes and the bronchioles were surrounded by moderate to mild numbers of lymphocytes and plasma cells; a few lymphocytes were present within the alveolar septa. By day 6, all observed lesions present on day 4 peaked, with the notable exception of lymphocytic infiltration in the peribronchiolar region and blood vessels. Neutrophils were prominent on day 6 p.i. but reduced/absent on day 8 p.i. which is consistent with our previous studies in the lamb model [17, 28]. Peribronchiolar and perivascular lymphocyte infiltration was increased on day 8 while all other parameters were reduced (Fig 3B).

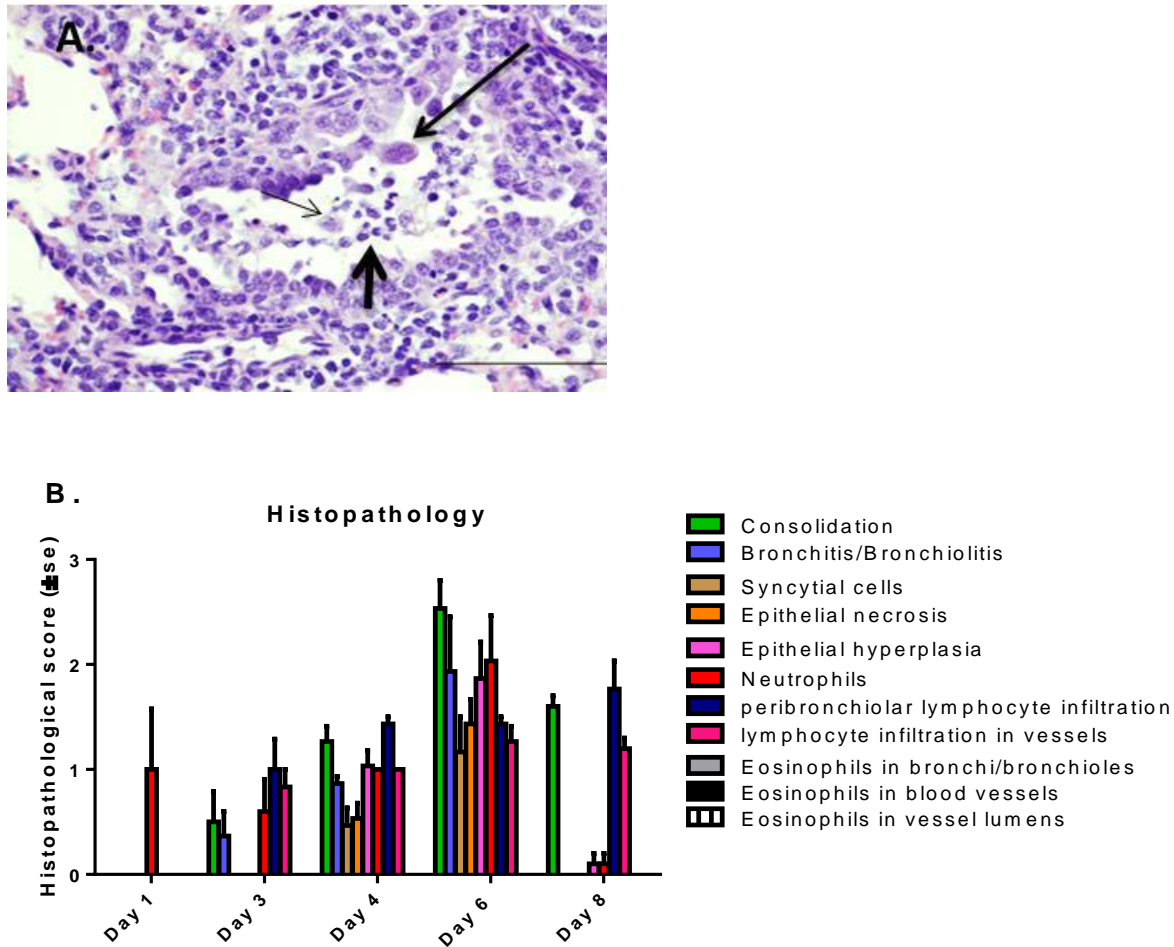


Figure 3. Microscopic lung lesions severity score in M37 hRSV infected neonatal lambs. (A) Histopathologic lesions included bronchiolitis with degenerate/necrotic individual epithelial cells (thin arrow), occasional syncytial cells (long arrow), accumulation of degenerate neutrophils (short arrow), and occasional macrophages. H&E Bar = 50 µm. (B) A histologic score was given by determining percent consolidation followed by conversion to an integer-based consolidation scale used by our laboratory previously [1]: 0% consolidation = 0; 1%-9% consolidation = 1; 10%-39% consolidation = 2; 40%-69% consolidation = 3; 70%-100% consolidation = 4. Group averages were calculated for alveolar and bronchiolar consolidation scores. In addition to the consolidation score, bronchitis, bronchiolitis, neutrophil infiltration, peribronchiolar and perivascular infiltration of lymphocytes, syncytial cell formation, and epithelial alterations were also scored. Results are indicated as mean \pm standard error for each scored parameter.

Viral Titers, Viral RNA Levels and Viral Antigen Expression

Levels of M37 hRSV total nucleoprotein RNA in lung tissue, BALF, and NWF were measured by RT-qPCR, while cultivatable virus was quantified by infectious focus (FFU) assay in NWF and BALF collected on each day of necropsy. Viral titers and viral RNA in BALF and lung tissue increased progressively from day 1 to day 3 and were sustained or increased slightly until day 6. On day 8, viral titers and RNA decreased substantially as observed for all other virology endpoints (viral antigen expression, gross lung lesions, and microscopic lesions). In contrast, viral titers and viral RNA levels in NWF were more variable when comparing them to levels in BALF and lung tissue. Viral titers in NWF were highest at day 3 (1.7 log₁₀ FFU/mL), whereas this was the case on day 6 (2.9 log₁₀ RNA copies/mL) for viral RNA. Overall, intranasal viral replication was substantially lower than viral replication in the lung (Table 3) and may be due to the administration of virus by nebulization which may bypass the nose to some extent or be indicative of a lower permissiveness of lamb nasal epithelial cells for RSV replication.

With immunohistochemistry, RSV antigen was present in areas with microscopic lesions. Within these areas, RSV antigen was present in the entire cytoplasm of epithelial cells lining bronchi and bronchioles, alveoli, and the cytoplasm of occasional macrophages (Fig 4A). On day 1 p.i. no viral antigen expression was detected in the lungs of infected lambs and was only apparent on day 3 predominantly in the epithelial cells of bronchi and bronchioles when compared to the alveoli. There was an increasing progression of viral antigen expression in both bronchi/bronchioles and alveoli, which reached a peak on day 6 with a marked shift in viral antigen expression occurring in the alveoli. Degenerate and necrotic epithelial cells within lumens of bronchioles also contained viral antigen at days 3, 4, and 6. On day 8, viral antigen

expression in lung tissue had decreased substantially (Fig 4B) consistent with a decline in RSV titers in lung (Table 3).

Table 3. Quantification of RSV replication via RT-qPCR and infectious focus assay in lambs inoculated with M37 hRSV

Parameter		Day 1	Day 3	Day 4	Day 6	Day 8
Viral load (Nasal Washes)*	Viral culture (Log ₁₀ FFU/mL ± se)	BDL (0.7)	1.7 ± 0.4	0.6 ± 0.1	0.99 ± 0.3	0.8 ± 0.1
	RT-qPCR (Log ₁₀ M37 RNA copies/mL ± se)	1.44 ± 1.1	1.68 ± 1.4	0.42 ± 0.1	2.89 ± 0.34	1.46 ± 1.2
Viral load (BALF)*	Viral culture (Log ₁₀ FFU/mL ± se)	2.53 ± 0.09	4.60 ± 0.32	3.94 ± 0.25	4.83 ± 0.04	1.02 ± 0.32
	RT-qPCR (Log ₁₀ M37 RNA copies/mL ± se)	3.80 ± 0.03	6.22 ± 0.08	6.20 ± 0.16	7.15 ± 0.2	4.70 ± 0.38
Viral transcripts (Lung tissue)	RT-qPCR (Log ₁₀ M37 RNA copies/mg lung tissue ± se)	4.81 ± 0.15	6.51 ± 0.08	6.67 ± 0.25	7.63 ± 0.07	5.24 ± 0.26

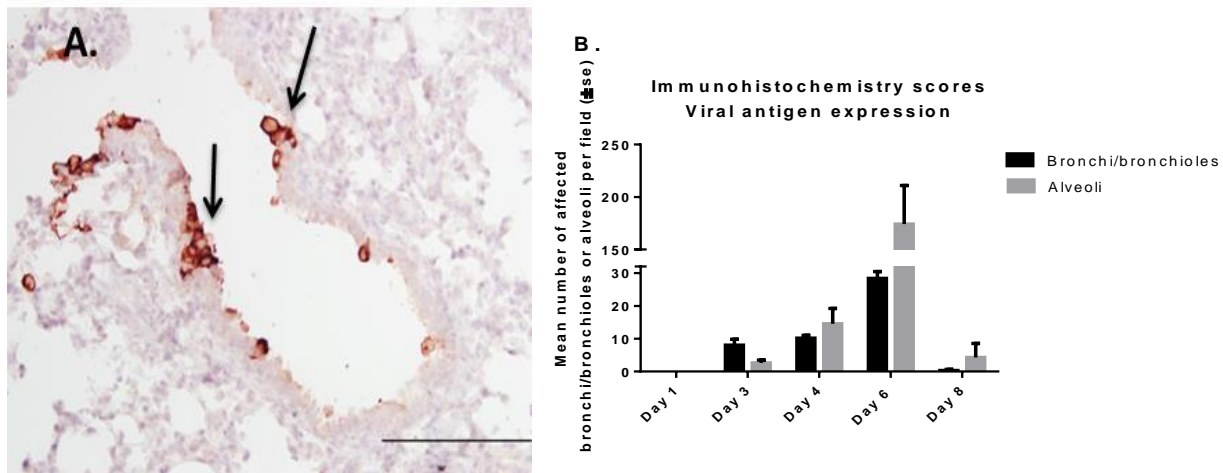


Figure. 4. Immunohistochemistry and scoring of RSV antigen expression in lambs inoculated with M37 hRSV. Immunohistochemistry was used to detect viral antigen using an all-antigens polyclonal antibody for RSV. RSV immunoreactivity is shown within epithelial cells lining the bronchioles (brown cells). The mean number of virally-infected bronchi/bronchioles and alveoli per field was counted for each day of necropsy. Bar = 50 μ m.

Chemokine and Cytokine Expression in Lung Tissue

Lung cytokine mRNA expression levels were quantified by RT-qPCR and demonstrated varying patterns of expression in M37 hRSV-inoculated lambs throughout infection. While IL-10 expression levels were highest on day 3 post-infection other chemokines and cytokines had maximal expression at later timepoints (e.g. TGF- β on day 4; IL-8, RANTES and MCP-1 α on day 6 and IFN- γ on day 8) (Fig 5). Similarly, previous results from our laboratory have shown that MCP-1 α , MIP-1 α , RANTES, IFN- γ , and IL-8 were increased upon RSV-infection in neonatal lambs on day 6 [1], whereas the anti-inflammatory mediator, IL-10, was down regulated at day 6 post-infection, but increased on day 3 post-infection [28].

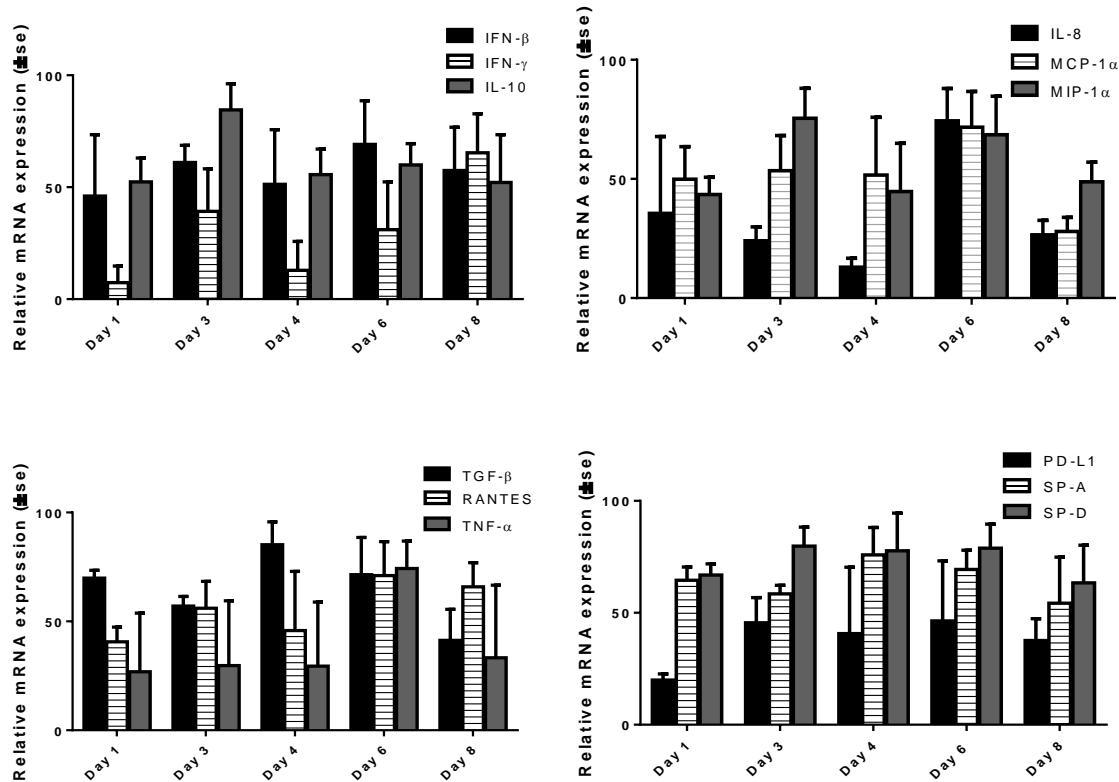


Figure 5. Lung chemokine and cytokine mRNA expression by RT-qPCR in lambs inoculated with M37 hRSV.

Figure 5. (continued) Lung tissue obtained from each animal was evaluated for the following mRNA targets: surfactant protein A (SP-A), surfactant protein D (SP-D), interleukin 8 (IL-8), interleukin (IL-10), macrophage inflammatory protein 1 alpha (MIP-1 α), monocyte chemotactic protein 1 alpha (MCP-1 α), tumor necrosis factor alpha (TNF- α), transforming growth factor beta (TGF- β), interferon beta (IFN- β), interferon gamma (IFN- γ), programmed cell death 1 ligand 1 (PD-L1), and regulated on activation normal T-cell expressed and secreted (RANTES). Mean relative mRNA expression was calculated for each target with respect to each day of necropsy. Relative mRNA expression means: relative to the total amount of RNA loaded per reaction (which is kept constant) and relative to the values established by the standard curves for each target.

Discussion

This study aimed to determine the time course of M37 hRSV replication in neonatal lambs and the corresponding pathophysiology i.e. clinical signs (wheezing, respiratory distress), airway inflammation, and lung histopathology. Such a time course is difficult to assess in infants since the diagnosis is often only done at the time of or near peak viral titers.

Colostrum-deprived neonatal lambs are highly relevant for the study of RSV infection and may serve as a model of RSV infection in human infants due to the natural susceptibility of lambs to ovine, bovine and human strains of RSV as well as similarities in disease pathogenesis and anatomical, physiological and developmental similarities to that of human infants [13–15]. Experimentally, it has been shown that lambs and other ruminants can be infected with human or bovine RSV strains and that the infection induces lung lesions that resemble those observed in human RSV pathology such as bronchiolitis with epithelial cell necrosis, syncytial cell formation, hyperplasia of nearby epithelium and infiltrates of neutrophils with occasional macrophages [1, 11, 17, 29–33]. Moreover, RSV infected lambs develop mild to moderate clinical symptoms such as expiratory effort, fever, tachypnea, wheeze, malaise and listlessness [1, 11, 17, 29, 32] and formalin-inactivated RSV vaccination in lambs induces enhanced lesions

upon RSV infection [19] as observed in infants [34, 35]. To date, and to our knowledge, no study has yet specifically addressed the time course of viral replication, histopathology, airway inflammation, and associated clinical symptomatology (wheezing, respiratory distress) in neonatal lambs following infection by nebulized inhalation of the M37 hRSV strain. The purpose of this study, therefore, was to define the time course of Memphis 37 RSV replication in the lungs of neonatal lambs and the corresponding clinical features and pathophysiology.

Following RSV infection, this study demonstrates that there is robust viral replication as determined by titers and mRNA levels in the lungs, which peaked on day 3, were sustained until day 6 and decreased by day 8. Viral replication was present in the nasal cavity with maximal titers detected at days 3 (cultivable virus) and 6 (mRNA) post inoculation, but this replication was less robust compared to lung, as viral titers were ~3 Log lower in the nasal wash fluid than in lung on day 3. Such differences in NWF and BALF titers may be related to the administration route which partly bypasses the nasal meatus and also because the sampling of nasal cavity (washes of a cavernous space) differs from solid lung tissue and BALF (taken from a bronchus directly at post mortem). The kinetics of RSV RNA expression levels in BALF and lung tissue displayed a similar profile. Viral antigen expression followed a similar time-profile as titers and mRNA levels, albeit with a delay, and was detected in intact and degenerate/necrotic bronchiolar epithelial cells and ciliated airway epithelial cells in bronchi; cell types that were shown to be permissive to RSV infection [36–41], as well as in the cytoplasm of occasional macrophages, consistent with the distribution of virus-infected cells in fatal cases of RSV-infection [33]. Viral antigen expression in bronchi/bronchioles and alveoli for each day post-inoculation followed the progression/severity of microscopic lung lesions, gross lung lesions, and respiratory distress.

Microscopically, the bronchiolar epithelia had evidence of necrosis on days 3 and 4 and epithelial hyperplasia, which increased until day 6. The infected bronchiolar cells can, as a result, become degenerate and necrotic and contribute to the cell debris entering and partially occluding the airway lumen. In addition, the necrotic areas and the inflammatory mediators released in this process facilitate neutrophil infiltration and accumulation of seroproteinaceous fluid and mucin, all of which can further occlude the airway lumen. Lymphocytes were first observed by day 3 and 4 and appeared to achieve peak levels by day 6 to 8. Immune cell infiltrates accumulated in the tunica adventitia of bronchi, bronchioles and small blood vessels, and slightly more macrophages were observed in the alveolar and bronchiolar lumens. On day 8 post-infection, airway lumens were observed to contain only occasional neutrophils and macrophages, while infiltrates of lymphocytes and plasma cells remained present in the adventitia, reflecting a change from neutrophilic to lymphocytic and plasmacytic inflammation. The role of lymphocytes in RSV clearance and convalescence has previously been evidenced in human and mouse studies [42–44] and the failure to develop an adaptive cytotoxic T lymphocyte response has been proposed to be related to the pathogenesis of RSV infection of the lower respiratory tract [45]. Conversely, neutrophils are the most abundant cell type recovered from the respiratory tract in infants-hospitalized for RSV disease [46–48] and it has been suggested that they can contribute significantly to epithelial cell damage and to disease severity [49, 50].

In addition to inflammatory cell influx in the lungs, it has been shown that chemokines and cytokines, such as IL-8, RANTES, MIP-1 α , IL-6 and IL-10 are increased in RSV-infected infants [49, 51–57] and likely to promote the intense inflammatory process present in the airways of these infants. For this reason, we sought to investigate the time course of chemokine expression in lung tissue from RSV-infected lambs. Expression of proinflammatory chemokines

and cytokines such as IL-8, IFN- γ , IFN- β , MCP-1 α , MIP-1 α and TNF- α or anti-inflammatory cytokines such as IL-10 was detected and was consistent with previously published data [1, 28]. Peak expression of IL-8 was present at day 6 post-infection and coincided with peak neutrophil influx in the lungs of RSV-infected lambs which indicates that the increased chemokine expression contributed to neutrophil infiltration into the site of intense RSV infection, in accordance with the role of IL-8 in neutrophil chemotaxis [7]. In contrast, IFN- γ expression, a cytokine mainly produced by NK cells and activated CD4+ and CD8+ T cells that promotes cell-mediated immune responses to intracellular pathogens, was maximal at day 8 post-infection at a time when peak lymphocyte lung infiltration was noted. Taken together, this data suggests that RSV infection in neonatal lambs results in an initial innate inflammatory response, the peak of which coincides to that of peak disease, and is characterised by IL-8 secretion and neutrophil influx. During the transition from the initial inflammatory response, there is infiltration of lymphocytes and, as the disease resolves, the inflammatory response is characterised by IFN- γ secretion and continued lymphocyte influx. Interestingly, a suppressed lymphocyte function and increased plasma IL-8 levels were shown to be markers of severe disease in RSV bronchiolitis [58].

The most interesting results, however, obtained in the current study were those related to the clinical parameters. Infants with RSV-disease typically develop clinical manifestations such as bronchiolitis and pneumonia symptoms of which include wheezing, crackles, rhonchi, tachypnea, nasal flaring, and intercostal muscle retractions [59, 60]. In our study, the neonatal lambs developed respiratory distress (forced expiration, abdominal breathing and wheeze) following RSV-infection consistent with previous reports [1, 18]. The progression of respiratory distress coincided with that of viral replication (titers, viral RNA and viral antigen expression),

lung gross viral lesions, and histopathological changes. The resultant partial occlusion of the small airways by sloughed epithelial cells, inflammatory cells, respiratory secretions and mucous plugs as observed microscopically may lead to air trapping, lung hyperexpansion and increased airway resistance causing respiratory distress [61]. The deterioration of lung function and clinical symptomatology thus appears to be a direct effect of viral replication and its induction of bronchiolar/lung pathology. In experimentally-infected adults, a similar close temporal association between onset, peak, and clearance of viral replication, and the onset, peak, and resolution of the disease, has been described as well. However, viral replication and disease are limited to the upper airways in this human model [22].

This viral kinetic study of M37 hRSV in newborn lambs establishes a baseline of clinical features and pathology in a model where RSV infection and the consequences thereof resembles that of human infant RSV disease in the lower airways and may serve as a valuable tool to assess vaccine and antiviral drug safety and efficacy.

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CHAPTER 3. DELIVERY OF ALX-0171 BY INHALATION GREATLY REDUCES RESPIRATORY SYNCYTIAL VIRUS (RSV) DISEASE IN NEWBORN LAMBS

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Abstract

Rationale

Rationale: Respiratory syncytial virus (RSV) is a common cause of acute lower respiratory disease in infants and young children worldwide. Currently, treatment is limited to symptomatic treatment, with no vaccines available. The use of neonatal lambs to model RSV infection in human infants may provide a valuable tool to assess safety and efficacy of vaccines and antiviral drugs. Nanobodies® are therapeutic proteins based on the smallest functional fragments of naturally occurring heavy chain antibodies. ALX-0171 is a trivalent Nanobody targeting the RSV fusion (F) protein. Its therapeutic potential was evaluated in newborn lambs infected with a human strain of RSV following daily ALX-0171 nebulization for 3 or 5 consecutive days.

Methods

Colostrum-deprived newborn lambs were nebulized with RSV-M37 (~3.5 x 10⁷ FFU/lamb) on day 0, and were subsequently treated by daily nebulization after RSV-infection with either ALX-0171 or placebo. Two different treatment regimens were examined: day 1-5 or

day 3-5 post-infection. Lambs were monitored daily for general well-being and clinical parameters. Respiratory tissues and bronchoalveolar lavage (BAL) fluid were collected at day 6 post-inoculation for the quantification of viral lesions, lung viral titers, viral antigen and lung histopathology.

Results

On day 3 post-infection, the general well-being of the placebo-treated lambs declined as was reflected by inactivity, lethargy, weakness, drooping of ears, and lack of appetite. A pilot study previously indicated that this is also the time of peak viral loads. Both viral RNA and cultivatable virus was consistently present in all lung lobes of these lambs on day 6. In addition, gross lung examination revealed extensive viral lesions involving ~40% of all lung lobes which were correlated to viral antigen expression in the bronchioli/alveoli. Histologically, the lesions were consistent with those described previously for lambs and infants after RSV infection. Following treatment with ALX-0171, treated lambs had a >10,000-fold decline in cultivatable virus, markedly reduced lung viral antigen expression, reduced lung viral lesions and reduced histological changes on day 6. ALX-0171 treatment exerted a positive effect on clinical parameters of the lambs and was well-tolerated.

Conclusion

Administration by inhalation of ALX-0171 was well-tolerated in RSV infected newborn lambs. Robust antiviral effects and positive effects on RSV-induced lung lesions and reduction in symptoms of illness were noted. These effects were still apparent when treatment start was delayed and coincided with peak viral loads (day 3 post-infection) and at a time point when signs of respiratory syncytial virus disease were apparent. The latter design is expected to have high

translational value for planned clinical trials. These results are indicative of the therapeutic potential of ALX-0171 in infants.

Introduction

Human respiratory syncytial virus (hRSV) is classified in the genus Orthopneumovirus of the newly created Pneumoviridae family within the order Mononegavirales, standing apart from the original Paramyxoviridae family member [1]. As such, it encodes two major surface glycoproteins termed G-protein and F-protein. These two glycoproteins play a crucial role in viral replication as they are responsible for viral binding to the target cell and virus-cell membrane fusion, respectively. In addition, RSV has a linear single stranded, non-segmented RNA molecule of negative-polarity of approximately 15kb.

As a respiratory virus, RSV infection may present as an upper respiratory tract infection (including rhinitis, otitis media and pharyngitis), or as a lower respiratory tract infection (including acute bronchiolitis or pneumonia) in vulnerable populations such as infants, elderly and immunocompromised adults [2]. This lower respiratory tract infection results in hospitalization in about 3% of RSV-infected infants less than 1 year old, and in about 0.5% of RSV-infected children aged between 1 and 2 years [3]. Each year, it is estimated that RSV causes at least 3.4 million lower respiratory tract infections requiring hospitalization and up to 200,000 deaths worldwide in infants less than 5 years of age [4] and in approximately 177,000 hospital admissions and 10,000–14,000 deaths per year in the United States in the elderly.

Currently, there are no approved vaccines or effective therapeutic drugs specifically for RSV infection, with treatment being limited to supportive care. In severe RSV infections, the only approved antiviral treatment is the nucleoside analog Virazole (ribavirin), which is

delivered by inhalation. However, due to concerns for potential teratogenicity and minimal evidence of benefit it is not recommended for routine use in infants but may be considered for use in select patients with documented, potentially life-threatening RSV infection [5]. In the prevention of RSV infections, Palivizumab (Synagis®, MedImmune), a humanized monoclonal antibody (IgG) against the F-protein of RSV, has been approved for use in high risk infants; however, due to high costs associated with palivizumab prophylaxis, use is restricted to certain high-risk pediatric populations [6].

There is thus a need to develop new therapeutic treatment options for RSV infection. Different animal models for RSV infection, including non-human primates, calves, lambs, mice, guinea pigs, ferrets, hamsters and cotton rats have been described [7, 8]. The cotton rat (*Sigmodon hispidus*) RSV infection model has been successfully used in the development of both RSV-IgIV (RespiGam®, MedImmune, Inc., Gaithersburg, MD, USA) and palivizumab and findings in the cotton rat for those agents translated well to the clinical setting [9, 10]. The cotton rat model also serves as the primary model for the determination of vaccine safety as they develop vaccine-enhanced pulmonary disease that reflects what is seen in humans and non-human primates [11]. However, and although susceptible to RSV infection, cotton rats do not exhibit any clinical signs of upper and lower respiratory tract disease or any age-related susceptibility to hRSV in contrast to what is seen for human infants [8].

Similarly to humans, lambs exhibit several key features of RSV vaccine enhanced disease [13]; anatomically, the respiratory tract of sheep and humans share many structural features, such as the size of the nasal cavity and airways [14] as well as lung development where alveolarization starts pre term [15]. When experimentally infected with either bovine (bRSV) or human (hRSV) strains of RSV, neonatal lambs develop mild clinical symptoms including fever,

tachypnea or increased expiratory effort (wheeze) and malaise, as well as mild to moderate gross and histologic lesions [12].

ALX-0171 is a novel inhaled biotherapeutic in development for the treatment of RSV infections in infants [16]. Local pulmonary administration of ALX-0171 was considered optimal for this indication as it enables targeted delivery straight to the site of infection, with a potentially more rapid onset of action while using lower doses compared to systemic administration [17]. Preclinical evaluation of ALX-0171 using a face-mask, which is highly relevant for the intended clinical use, would thus require a larger animal species than rodents. In addition to the pathophysiological similarities of RSV disease with human infants, lambs also have advantages in terms of drug delivery by inhalation. Indeed, drug deposition in the lung is affected by the respiratory rates and breathing volumes, both parameters being dependent on body size [18], and lung anatomy which are similar to infants. Another important parameter that affects drug deposition is the breathing maneuver. Lambs are nasal breathers, although oral inhalation is possible when the nasal airways are obstructed, which is also the case for human infants [19]. For the reasons stated above, the neonatal lamb model was selected for the evaluation of ALX-0171 efficacy and safety in a neonatal setting.

Material and Methods

Animals

Male and female Suffolk, Polypay or Dorsett cross colostrum-deprived neonatal lambs (1-3 days of age) were obtained from local farms (Lester, Iowa, USA). Lambs were fed lamb milk replacer (Milk Products Inc., Chilton, WI, USA) that lacked supplemental iodide as of birth and were given Naxcel (Ceftiofur sodium, Pfizer) 1–2 mg/kg subcutaneously once daily to

reduce/prevent secondary bacterial infections. The animals had not been subjected to other experiments before the study. All efforts were made to minimize animal discomfort and limit the number of animals used. Study protocols were approved by the Institutional Animal Care and Use Committee (approval #12-12-7470-O) and were performed in accordance with the animal welfare bylaws of the Iowa State University which are in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) regulations. The studies were also approved by the Institutional Biosafety Committee at Iowa State University.

Compounds

ALX-0171 is a trimeric Nanobody consisting of three RSV-targeting subunits linked by two glycine-serine linkers, and was produced using a *Pichia pastoris* X-33 expression system [16]. ALX-0171 formulation buffer which consists of NaCl as osmolality agent and phosphate as buffer component was used as a placebo. The formulation components and their concentration were selected based on their compatibility with pulmonary administration.

Infection of Lambs

PARI LC Sprint™ nebulizers (PARI Respiratory Equipment, Inc., Lancaster, PA, USA) were used to administer virus or cell-conditioned media (media from HEp-2 cells lacking hRSV) to each lamb. They were attached to a conical mask fitted with a round rubber diaphragm with a pre-cut center hole through which the nose and mouth of the lamb was inserted (MidWest Veterinary Supply, Inc., Burnsville, MN). Three 2-mL aliquots of virus-containing media or control media were administered to each animal over the course of 23 minutes at 4L/min at 16 PSI (Philips Respironics Air Compressor, Andover, MA, USA) resulting in the total inhalation of

about 6 mL by each lamb. Identical viral inoculum doses were used for each lamb (hRSV M37 strain at 1.27×10^7 FFU/mL in media with 20% w/v sucrose).

Drug Administration

An Aeroneb® Solo System (Aerogen Ltd, Galway, Ireland), consisting of the Aeroneb® Solo mesh nebulizer and the Aeroneb® Pro-X controller were used in accordance with the instruction manual as provided by the manufacturer. The estimated particle sizes obtained with these meshes were in the range of $3.27 \pm 0.13 \mu\text{m}$ (MMAD). The assembly and operation of the Aeroneb® Solo System was done according to the nebulizer instruction manual. The nebulizer and the T-piece were inserted into the breathing circuit. Air was supplied to the system at an airflow speed of 2L/min using a compressed air canister that was attached directly to the nebulizer T-piece.

Prior to dosing, the nebulizer reservoir was filled with either 4 mL (3 mg/kg target inhaled dose), 1.3 mL (1 mg/kg target inhaled dose) or 0.4 mL (0.3 mg/kg target inhaled dose) of ALX-0171 or ALX-0171 formulation buffer (placebo). A cone mask (Cat # 05305, A.M. Bickford, Inc, US) was attached to the nebulizer T-piece and was placed over the lamb's nose, mandible and maxilla. The nebulizer was turned on at a constant nebulization mode and the cone mask was firmly held in place during the duration of the nebulization. Once the dose had been nebulized (i.e. when the nebulizer reservoir was empty) the face mask was removed and the nebulizer switched off. The lamb was then returned to its cage and general health (alertness, responsiveness, ability to stand and move) was monitored for 10 minutes.

Calculation of Inhaled Dose

The total dose emitted by the nebulizer was determined per administration by weighing the nebulizer before and after the administration and deducing the nebulized volume. The nebulized volume of ALX-0171 was then multiplied by the concentration of ALX-0171 to determine the delivered dose. The inhaled dose per lamb (i.e. the dose reaching the tip of the snout) was estimated to be 11% of the delivered dose based on experiments in which a similar nebulizer setup as the one that was used in these studies was connected to a breathing simulator programmed for neonatal breathing. The inhaled fraction was defined as the fraction that was found back on the inhalation filter in relation to the total nebulized dose. The bodyweight adjusted inhaled doses were expressed as mg/kg and were calculated as the inhaled dose on each specific day divided by the measured bodyweight on the corresponding dosing day.

Experimental Design

Three independent studies were performed in which a total of fifty five lambs were randomly assigned based on weight and sex to three or four groups depending on the study (Table 1). In study 1, thirteen lambs were assigned to four groups (groups 1, 2, 3 and 4). Three lambs were in group 1 (RSV-infected placebo treated), four lambs were in group 2 (RSV infected 0.3 mg/kg ALX-0171 treated), three lambs were in group 3 (RSV infected 1 mg/kg ALX-0171 treated) and three lambs were in group 4 (RSV infected 3 mg/kg ALX-0171 treated). In study 2, ten lambs were assigned to three groups (groups 1, 2 and 3). Three lambs were in group 1 (RSV infected placebo treated), four lambs were in group 2 (RSV infected 0.3 mg/kg ALX-0171 treated) and three lambs were in group 3 (RSV infected 3 mg/kg ALX-0171 treated). In study 3, twenty two lambs were assigned to four groups (groups 1, 2, 3 and 4). Five lambs were in group 1 (uninfected placebo treated), six lambs were in group 2 (uninfected 0.3 mg/kg

ALX-0171 treated), five lambs were in group 3 (RSV infected placebo treated) and six lambs were in group 4 (RSV infected 0.3 mg/kg ALX-0171 treated) (see Table 1).

Table 1. Summary of studies and groups

Study	Group number	Number of lambs		Infection	Dose level	Inhaled dose (mg/kg)	
		Total	For analysis			Target	Mean achieved
1	1	3	3	RSV	Vehicle	0	0
	2	4	4	RSV	ALX-0171 0.3 mg/kg	0.3	0.3
	3	3	3	RSV	ALX-0171 1 mg/kg	1	0.8
	4	3	2	RSV	ALX-0171 3 mg/kg	3	2.7
2	1	3	3	RSV	Vehicle	0	0
	2	4	2	RSV	ALX-0171 0.3 mg/kg	0.3	0.3
	3	3	3	RSV	ALX-0171 3 mg/kg	3	2.9
3	1	5	5	Mock	Vehicle	0	0
	2	6	5	Mock	ALX-0171 0.3 mg/kg	0.3	0.2
	3	5	5	RSV	Vehicle	0	0
	4	6	6	RSV	ALX-0171 0.3 mg/kg	0.3	0.3

On day 0 all lambs in study 1, study 2 and study 3 (except group 1 and 2) were infected with RSV Memphis 37 strain by the inhalation route whereas uninfected control lambs (study 3 - groups 1 and 2) received nebulized cell-conditioned media as described above. ALX-0171 treatment started either on Day 1 (study 1) or Day 3 (study 2 and 3) post-infection and was repeated daily until Day 5 post-infection. In total, four lambs were excluded from the studies on various days post-infection because of secondary bacterial infections. Two lambs were from study 2 group 2, one lamb was from study 1 group 4 and one lamb was from study 3 group 2. On day 6 post-infection, all other lambs were euthanized with an intravenous injection of sodium

pentobarbital (Beuthanasia®, Schering-Plough Animal Health Corporation) overdose (1 mL/5 kg). During necropsy, tissue samples were collected from each lung lobe of all animals in the same manner, with uniform sampling of each lobe.

Preparation of hRSV Memphis 37 Virus Stock

Memphis 37 (M37) RSV is a wild type RSV-A, first isolated from a 4 month old infant [20] and used in human clinical studies [21-24]. HEp-2 cells were infected in 300 cm² flasks at 0.5-1 multiplicity of infection (MOI) at a confluence of 80-90% by applying 4-5 mL of hRSV stock (Memphis 37 hRSV that was used originated from Meridian LifeScience, Memphis, TN, USA). Usually within 48-60 hours abundant syncytia appeared and close to 100% CPE was reached (as ascertained by periodic inverted microscope inspections). The monolayers of cells were then scraped off the bottom of each 300 cm² flask using a large rubber policeman (sterile plastic tool commonly used for cell scraping). The collected cell solutions were transferred to polypropylene 15 mL conical centrifuge tubes (~1 tube per 300 cm² flask), vortexed for 10 seconds at high speed and centrifuged at 2500 rpm (~1260 x g) for 10 minutes. Supernatants were then collected from each tube and kept in separate tubes while each resulting pellet was resuspended in 5 mL of PBS pH 7.4. Each cell pellet sample was then sonicated for 5 x 2-second pulses with 5 seconds in between (to avoid overheating samples) using a tip-style sonicator (Sonic Dismembrator Model 500, FisherScientific) with a pre-sterilized tip (tip was pre-soaked in 70% ethanol for 10 minutes followed by air-drying). Sonicated samples were then centrifuged again at 2500 rpm (~1260 x g) for 10 minutes and the resulting supernatants were added to each of the corresponding supernatants collected initially. The samples were then diluted with a solution of 60% sucrose, 10% FBS in PBS to obtain 20% w/v total sucrose concentration which was shown to be beneficial in preserving virulence post freeze-thaw and post-nebulization [25].

The titer of virus was subsequently determined, after one freeze-thaw cycle at -80°C , in a small portion of the resulting stocks that were aliquoted into cryovials for this purpose using the infectious focus forming unit (FFU) assay. The remainder of the stock was frozen/stored at -80°C until needed for use in the lambs. The Memphis 37 RSV strain used in this study was passaged 6 times on Vero cells, then twice on HEp-2 cells. Sucrose was added to 20% and the virus stock was frozen at -80°C and titered for infectivity on HEp-2 cells as has been characterized previously in this model [25].

Monitoring of Clinical Parameters

Lambs were monitored daily for body weight, rectal temperature, heart rate and percent blood oxygenation measurements (PalmSAT[®] 2500A VET pulse oximeter, Nonin Medical Inc., Plymouth, MN, USA), and manual heart and respiratory rates (by auscultation). Increased expiratory effort (forced expiration) was scored daily as were animal “wheeze” scores, using criteria previously reported [26].

Blood Collection

Approximately 1-1.5 mL of blood (at least 500 μL of plasma) was drawn from the external jugular vein with a syringe, and placed in spray coated K₂EDTA vacutainer tubes. The fur at the sampling site was shaved and extensively washed prior blood withdrawal to avoid any contamination of deposited ALX-0171. The blood samples were then centrifuged at $1,600 \times g$ for 10 min at 4°C in order to obtain plasma. Samples were stored at $\leq -60^{\circ}\text{C}$ prior to analysis.

Collection of Bronchoalveolar Lavage Fluid (BALF)

Following euthanasia the lungs of each lamb were removed and each left and right lung was separated and weighed. BALF was collected and processed from the right lung lobe as previously described [26].

Gross Lesions Evaluation and Scoring

Following euthanasia, the thorax was opened and the heart and esophagus were removed from the lungs. The percentage parenchymal involvement of gross hRSV lesions was scored for each individual lung lobe. The percentage of a specific lobe tissue that was affected by RSV in relation to the overall lobe tissue being scored was estimated. Mean percentage averages per lobe were calculated for each day of necropsy.

Histologic Lung Evaluation and Scoring

A histologic score was determined by evaluating percent involvement. This histologic score is an overall score based on the percentage of lung involvement in areas with hRSV lesions. Alveolar consolidation was defined by reduced expansion of alveolar lumen due to alveolar septal infiltration of neutrophils, lymphocytes, plasma cells, and type II cell hypertrophy along with intraluminal accumulation of neutrophils, macrophages, and small amounts of cell debris. The score was defined by converting the observed percentage ranges to a simple integer based consolidation scale: 0% consolidation = 0, 1-9% consolidation = 1, 10-39% consolidation = 2, 40-69% consolidation = 3, 70-100% consolidation = 4. Group averages were calculated for the alveolar consolidation score. In addition to the alveolar consolidation score, bronchitis, bronchiolitis, neutrophil infiltration, peribronchiolar and perivascular infiltration of lymphocytes,

syncytial cell formation, and epithelial alterations were also individually scored as previously published [26].

Immunohistochemistry for Viral Antigen Detection

Immunohistochemistry for the detection of RSV antigen was performed on 5 µm-thick formalin-fixed paraffin-embedded (FFPE) lamb lung tissue sections taken from the right and left cranial, left middle, and left caudal lung lobes of each animal in accordance with methods published previously [26]. 20 unique 10X fields on each slide (containing two lung sections each) were assessed for RSV antigen staining by counting positively-stained cells within bronchioles and alveoli. The mean number of stained bronchi/bronchioles and alveoli per field were counted.

Reverse Transcription Polymerase Chain Reaction (RT-qPCR) Assessment of RSV RNA Expression Levels in Lamb Lung Tissue.

For each animal, tissue samples from right and left cranial, left middle and left caudal lung lobes (0.3-0.4 g of each lobe) were homogenized for total RNA isolation in TRIzol (Invitrogen), assessed for quantity and purity by spectrometry, and then RT-qPCR was performed using a One-Step Fast RT-qPCR kit master mix (Quanta, BioScience, Gaithersburg, MD) in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Carlsbad, CA) with PREXCEL-Q for all set up calculations as previously described [27, 28]. Primer and probe sequences for all targets were designed with ABI Primer Express 2.0, and have been used previously [27, 28]. All samples were diluted to achieve a final RT-qPCR concentration of 0.784 ng/µL. Thermocycling conditions were 5 minutes at 50°C; 30 seconds at 95°C; and 45 cycles of 3 seconds at 95°C and 30 seconds at 60°C. Samples and standards were assessed in duplicate, and each target gene quantification cycle (C_q) value was converted to a relative quantity (Q_r)

based on each target's standard curve using: $Q_r = E_{AMP}^{(b-Cq)}$, wherein "b" and "E_{AMP}" are the y-intercept and exponential PCR amplification value, respectively. E_{AMP} values were obtained from the slope (m) of each target standard curve by: $E_{AMP} = 10^{(-1/m)}$, and all Q_r values interpolated from standard curves were normalized to total lung RNA per RT-qPCR. No-RT control (NRC) reactions gave either no signal or generated C_q values greater than 13 cycles later than those in the corresponding RT-qPCR target reactions.

Reverse Transcription Polymerase Chain Reaction (RT-qPCR) Assessment for RSV RNA in Bronchoalveolar Lavage Fluid

Viral RNA was quantified by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in BALF obtained from the right caudal lung lobe of each animal at necropsy as previously described [26]. RT-qPCR for RSV was then carried out as described above.

Focus Forming Unit Assay

Viral titers in bronchoalveolar lavage fluid (BALF) from the right lung caudal lobe were determined using an infectious focus assay (FFU) on HEp-2 cell monolayers as previously described [26].

Quantification of Urea in BALF and in Plasma

Quantification of urea in both plasma and BALF was done using the QuantiChrom® Urea Assay Kit (BioAssay Systems) according to the manufacturer's instructions. The method was validated specifically for lamb BALF and plasma. During urea sample analysis, the calibrator inter-curve precision did not exceed 4.1% and the intercurve accuracy was between -3.2% and 2.3%. The inter-assay precision of the QC samples did not exceed 2.4% and the inter-assay relative error was between -1.9% and 1.6% for plasma and BALF combined.

Quantification of ALX-0171 in BALF and Plasma

Plasma samples from hRSV-infected lambs were treated by UV-irradiation for 1 hour in a biosafety cabinet prior to analysis in the ELISA. During qualification of the assay it was demonstrated that this treatment did not affect the accuracy of ALX-0171 determination in plasma. For BALF samples ALX-0171 quantification was performed in a biosafety cabinet. Qualified ELISA methods were used for the quantification of ALX-0171 in lamb plasma and BALF, respectively. In brief, an anti-Nanobody Nanobody (Ablynx) for plasma or a Nanobody specific mouse monoclonal antibody generated against ALX-0171 (Ablynx) for BALF were coated overnight on a 96 well Maxisorp plate (Nunc). Samples were applied on the coated plate and ALX-0171, present in the sample, was detected with a biotinylated monoclonal antibody generated against ALX-0171. This mAb was then detected by horseradish peroxidase (HRP) - labeled streptavidin (Thermo Scientific). Bound streptavidin-HRP was revealed by adding 100 μ l/well of soluble high-sensitivity tetramethylbenzidine (s(HS)TMB; SDT Reagents) for 15min, followed by 1N HCl. The absorbance was read at 450 nm using a spectrophotometer, and the reference wavelength was 620 nm. The sensitivity of the ELISA methods was 21.5 ng/mL and 10.7 ng/mL in plasma and BALF, respectively. During ALX-0171 sample analysis, the calibrator inter-curve precision did not exceed 4.5% and the intercurve accuracy was between -4.8% and 1.7%. The inter-assay precision of the QC samples did not exceed 14.2% and the inter-assay relative error was between -6.9% and 6.6% for plasma and BALF combined

Calculation of ALX-0171 Concentration in Lung Epithelial Lining Fluid (ELF)

The ALX-0171 concentration in the epithelial lining fluid at necropsy was calculated based on the ALX-0171 concentration measured in BALF and following normalization by the Urea method [29]. The urea concentration in BALF was first corrected for possible

contamination with urea from blood using the method described previously [30, 31]. The concentration of ALX-0171 in epithelial lining fluid was calculated as described in different studies [30-33] using the following formula: $ELF_{[ALX-0171]} = BALF_{[ALX-0171]} \times ([Urea_{Plasma}]/[Urea_{BALF\text{ Corr}}])$. Where: $ELF_{[ALX-0171]}$ is the ALX-0171 concentration in epithelial lining fluid, $BALF_{[ALX-0171]}$ is the ALX-0171 concentration in recovered BALF, $[Urea_{Plasma}]$ is the concentration of urea in plasma and $[Urea_{BALF\text{ Corr}}]$ is the corrected urea concentration in BALF.

Statistical Analysis

All results were analyzed and interpreted using methods appropriate for the type of response analyzed. All statistical analyses were performed using SAS 9.4. Methods used and interpretation of outcome (e.g. statement on significance including significance level as a p value) are given in the text or in the caption of figures.

Results

Pharmacokinetics of ALX-0171 in Neonatal Lambs

In all three of the performed studies blood samples were taken at selected time points following the first dose and all the subsequent doses for pharmacokinetic purposes. On Day 6, bronchoalveolar lavage fluid (BALF) sampling was performed post-mortem for PK analysis in the lung compartment. Once daily administrations of ALX-0171 via inhalation for 5 (Figure 1) or 3 (Figure 2) consecutive days resulted in high concentrations of ALX-0171 in lung epithelial lining fluid (ELF). A dose-dependent increase in ELF concentrations was seen on day 6. All the assessed lambs had quantifiable ALX-0171 concentrations with the notable exception of lamb

number 10 from the 1 mg/kg ALX-0171-treated group. This lamb had ALX-0171 levels that were below the quantification limit. The reason for this is unknown but an inadequate BALF retrieval cannot be excluded. This particular lamb, however, had been adequately exposed to ALX-0171 as shown by the systemic profile. Plasma ALX-0171 concentrations were roughly 3 log lower than those observed in the lung compartment following pulmonary delivery to neonatal lambs (Figure 1 and Figure 2). The systemic pharmacokinetics was linear with dose and time and the clearance was dependent on the animal weight. Although no intravenous data are available from neonatal lambs for ALX-0171, it is likely that absorption is also the driving force of ALX-0171 pharmacokinetics in lamb.

Effect of Daily ALX-0171 Administration to Neonatal Lambs When Started on Day 1 Post-Infection

To assess the therapeutic efficacy of ALX-0171 when administered by inhalation, thirteen lambs were inoculated with RSV on day 0. The day after infection (day 1), the lambs were randomized in either the placebo group or in one of the three ALX-0171 dose groups (Table 1) and were treated daily by inhalation for 5 consecutive days. Lambs underwent daily physical examinations and body weights, heart rates, rectal temperatures, respiratory distress and RSV-infection related symptoms were recorded. On day 6, the animals were euthanized and lung lavage samples as well as lung tissues were obtained for analysis of viral load in lung, histopathology and immunohistochemical analysis.

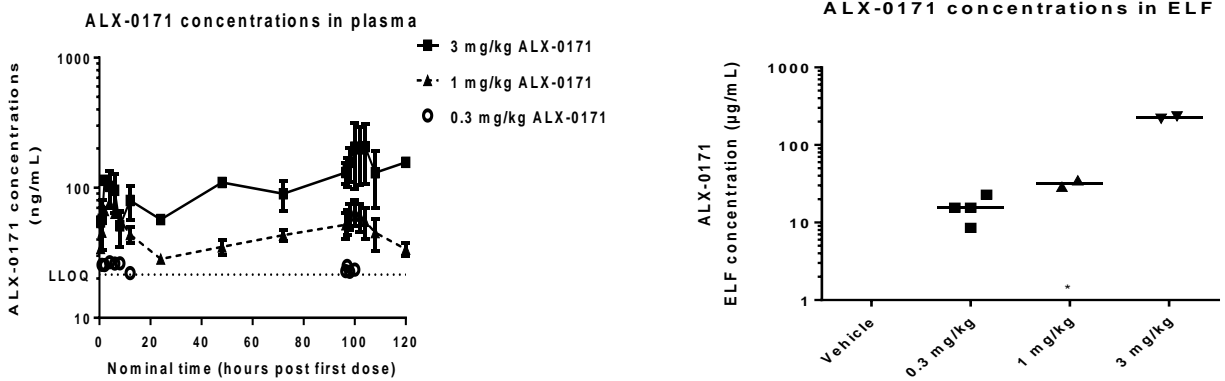


Figure 1. Pharmacokinetics study 1. Mean plasma concentration-time profiles of ALX-0171 (left graph) and ALX-0171 concentrations in epithelial lung lining fluid (ELF) (right graph) after five consecutive daily administrations by inhalation to neonatal lambs. ALX-0171 concentrations in ELF were derived from concentrations measured in BALF, which was sampled postmortem, after normalization for dilution based on the Urea correction method [29] (values were red blood cell corrected). Bronchoalveolar lavage fluid (BALF) was sampled 24 hours after the last dose. Results are expressed as mean \pm standard error for plasma curves and as individual lamb results with mean indicated as horizontal line for ELF. The hatched line represents the lower limit of quantification (LLOQ) of the assay. *ALX-0171 levels were below quantification limit for lamb N°10 from the mid-dose group.

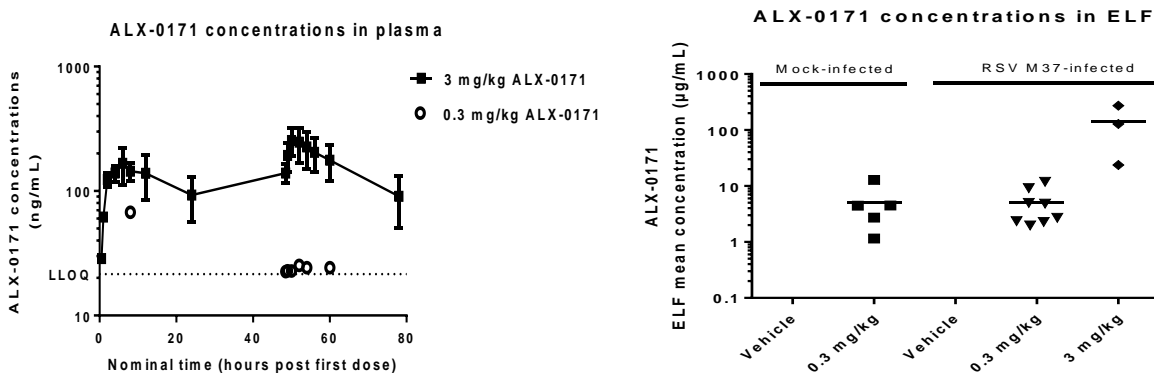


Figure 2. Pharmacokinetics study 2 and 3 combined. Mean plasma concentration-time profiles of ALX-0171 (left graph) and ALX-0171 concentrations in epithelial lung lining fluid (ELF) (right graph) after three consecutive daily administrations by inhalation to neonatal lambs for studies 2 and 3 combined. ALX-0171 concentrations in ELF were derived from concentrations measured in BALF, which was sampled postmortem, after normalization for dilution based on the Urea correction method [29] (values were red blood cell corrected). BALF was sampled 24 hours after the last dose. Plasma ALX-0171 concentrations that were below the lower limit of quantification were excluded from the analysis and the results are expressed as mean \pm standard error. The hatched line represents the lower limit of quantification (LLOQ) of the assay. ALX-0171 concentrations in ELF are shown for each individual lamb with the mean indicated as horizontal line.

hRSV inoculation by inhalation resulted in robust infection in all the analysed lambs as confirmed by RT-qPCR performed on BALF and lung tissue (Table 2).

Table 2. Lung viral loads in hRSV infected neonatal lambs for the three performed studies

Study	ALX-0171 target inhaled dose ^s	Viral load (BALF)				Viral load (lung tissue)	
		Viral culture (log ₁₀ FFU/mL ± SEM)	Reduction versus placebo (log ₁₀ FFU/mL)	RT-qPCR (Log ₁₀ viral RNA copies/mL ± SEM)	Reduction versus placebo (Log ₁₀ viral RNA copies/mL)	RT-qPCR (Log ₁₀ viral RNA copies/mg lung ± SEM)	Reduction versus placebo (Log ₁₀ viral RNA copies/mg lung)
1	Placebo	4.49 ± 0.43	NA	6.39 ± 0.10	-	6.02 ± 0.13	-
	0.3 mg/kg	UD	-	4.57 ± 0.16	1.82***	4.82 ± 0.17	1.21**
	1 mg/kg	UD	-	4.95 ± 0.09	1.44***	5.19 ± 0.36	0.83*
	3 mg/kg	UD	-	4.60 ± 0.06	1.79***	4.14 ± 0.16	1.88**
2	Placebo	4.83 ± 0.04	-	7.15 ± 0.20	-	7.63 ± 0.07	-
	0.3 mg/kg	UD (0.7)	4.13	5.90 ± 0.14	1.25*	5.79 ± 0.21	1.84***
	3 mg/kg	UD (0.7)	4.13	7.01 ± 0.18	0.14	7.09 ± 0.11	0.54*
3	Placebo	4.98 ± 0.41	-	7.26 ± 0.30		5.47 ± 0.21	
	0.3 mg/kg	0.87 ± 0.17	4.11	6.71 ± 0.25	0.55	5.00 ± 0.24	0.47

^sInhaled dose is defined as the total nebulised drug reaching the nose of the lamb (ie nebulised volume x concentration of ALX-0171 x 0.11 divided by body-weight).

UD: Undetected foci

Note: The mean number of focus forming units (FFU) and the RNA copies were calculated on the log₁₀ scale as these parameters are known to be lognormally distributed. The limit of quantification of the FFU assay was 5 FFU/mL or 0.7 log₁₀ FFU/mL. For descriptive statistics 0 foci (i.e. undetectable virus) were counted as 0.7 log₁₀ FFU/mL of BALF.

For the cultivatable viral titers in lung no statistical analysis was performed because all but 1 observations in the ALX-0171-treated groups were below the limit of quantification. For viral RNA data an ANOVA type model was used for statistical analysis where dose was a categorical variable. Correction for multiple testing was performed using the Hommel procedure. *p<0.05, **p<0.01, ***p<0.0001

In the placebo-treated lambs, RSV infection induced gross and microscopic lung lesions. Gross lung lesions were typical of experimental hRSV infection and consisted of locally extensive dark plum-red foci of pulmonary consolidation on each lung lobe which ranged from 8.3% to 13.3% (mean percent consolidation per lung of 11.7%). Microscopic examination of

lung tissue revealed the presence of locally extensive alveolar consolidation with mild epithelial necrosis in multifocal bronchioles, syncytial cell formation, infiltrates of neutrophils into the bronchiolar lumens, peribronchiolar infiltrates of lymphocytes and plasma cells and multifocal bronchioles had mild to moderate hyperplasia of epithelia. RSV antigen, assessed by immunohistochemistry, was present in areas with lesions and was localized to epithelial cells lining the bronchi, bronchioles and the alveoli as well as the cytoplasm of occasional macrophages. These gross and microscopic lesions and RSV antigen distribution were consistent with those observed previously in a study assessing hRSV kinetics in lambs [26].

Treatment of lambs with ALX-0171 at all three doses resulted in significant reductions in viral RNA titers that ranged from 1.44 to 1.82 Log₁₀ viral RNA copies/mL in BALF ($p < 0.0001$) and between 0.83 to 1.88 Log₁₀ viral RNA copies/mg in lung tissue ($p = 0.035$ and $p = 0.003$, respectively) depending on the dose (Table 2). All treated lambs had undetectable cultivatable virus however, during staining of the foci, instead of using the polyclonal antibody (Goat polyclonal Ab to hRSV [all antigens]) a monoclonal antibody was mistakenly used (Mouse anti Fusion protein Meridian MAb to hRSV Fusion Protein, Cat. No. C87610M 1 mg/mL, Clone: RSV 3216 [B016]). This tool was shown to compete with ALX-0171 binding and was not used in subsequent assays and studies.

Consistent with these reductions in viral loads, gross lung lesions were absent in all ALX-0171 treated lambs (Figure 3A) and microscopic alveolar consolidation scores were significantly reduced ($p < 0.0001$, Poisson regression) following treatment (Figure 3B). Only in lamb number 10, from the 1 mg/kg ALX-0171-treated group, was there observable alveolar consolidation (score of 0.5). The mean number of bronchi/bronchioles and alveoli per field in which viral antigen was detected was also significantly reduced ($p = 0.007$) (Figure 3C). Lamb 10

had the highest viral antigen expression although levels remained well below those seen in the placebo-treated lambs. Two lung lobes from this lamb showed some consolidated areas. Grossly and microscopically, this lamb also had lesions in these lobes that were not consistent with hRSV infection but, rather, bacterial infection. This lamb was not excluded from analysis because (i) the bacterial cause was not confirmed, (ii) systemic PK profiles indicated that this lamb had been adequately exposed, (iii) no other findings were seen and (iv) only regions (a minority of the surface area) of the lung had these lesions.

Clinically, each clinical sign was individually scored which may not be the best measure for the severity of disease as it was shown that single clinical signs do not correlate well with the degree of dyspnoea and airway narrowing in acute wheeze in human infants [34]. In infants with acute respiratory infections, clinical scoring usually relies on a combination of clinical symptoms and signs (feeding intolerance, medical intervention, respiratory difficulty, respiratory frequency, apnoea, general condition, fever) [34]. For this reason, an exploratory composite clinical score was calculated post-hoc for each lamb in the post-treatment initiation period. The clinical parameters included in the clinical score were changes in blood oxygenation levels, body temperature and respiratory rates; presence or absence of expiratory effort and wheeze. Each of these parameters were either scored 1 or 0 based on pre-defined criteria (see Figure 3) with a possible maximal clinical score of 6. The hRSV-infected, placebo-treated lambs displayed increased clinical scores throughout the post-treatment initiation period (Figure 3D) with 3 out of 3 lambs having a score of ≥ 1 . These increased clinical scores were mainly driven by labored breathing as expiratory effort was apparent on day 6 post-infection in 2 out of 3 lambs (maximum score of 2) but was absent on other days (except on day 0 where 1 lamb displayed expiratory effort). Wheeze was absent in all placebo-treated lambs throughout the study.

Respiratory rates in the placebo-treated lambs increased on day 3 post-infection and reached a peak on day 4 with a mean of 66 breaths/min (% increase of 18.3% versus day 0). In the ALX-0171-treated lambs 6 out of 9 (67%) lambs had a score of 0 and 3 lambs had a score of 1. Neither expiratory effort nor wheeze were detected at any time during the study in the ALX-0171-treated lambs whilst mean respiratory rates remained constant throughout the study where the mean respiratory rates on day 4 in the high-dose group was 56 breaths/min (% increase of 5.7% versus day 0). In the 3 lambs with a clinical score of 1, the relative body weight change between day 0 and day 6 ranged from -2% to 17%. When considering mean body weight evolution of all lambs throughout the study duration there were no significant differences in amongst the groups.

Delayed ALX-0171 Treatment Start to Day 3 Post-Infection Effectively Reduces Infection Related Changes

Following the first study, 2 additional studies were performed to evaluate the therapeutic activity of ALX-0171 when administered close to the viral peak and onset of symptoms. In study 2, 2 tested dose levels from the previous study were used. Based on the results of study 2, the lowest tested dose was selected for study 3, where appropriate mock-infected control groups were included to evaluate safety and efficacy of ALX-0171. Clinical parameters of lambs in study 2 were assessed as in study 1. However, for study 3, the general health-status of the lambs was additionally scored daily and is referred to as malaise score. This additional evaluation was included because of the observed variability in clinical parameters observed in study 1 and 2 which seemed to be associated with a more intense sampling regimen (multiple timepoint blood draws, holding the animals during respiratory rate auscultations, possible use of smaller rooms during housing etc). The procedure to observe the lambs was adapted in study 3 which involved observing the lambs for increased periods of time without picking them up to make the

behavioural clinical assessments and respiratory rate assessments and by reducing the blood sampling to only 3 over the 6 days.

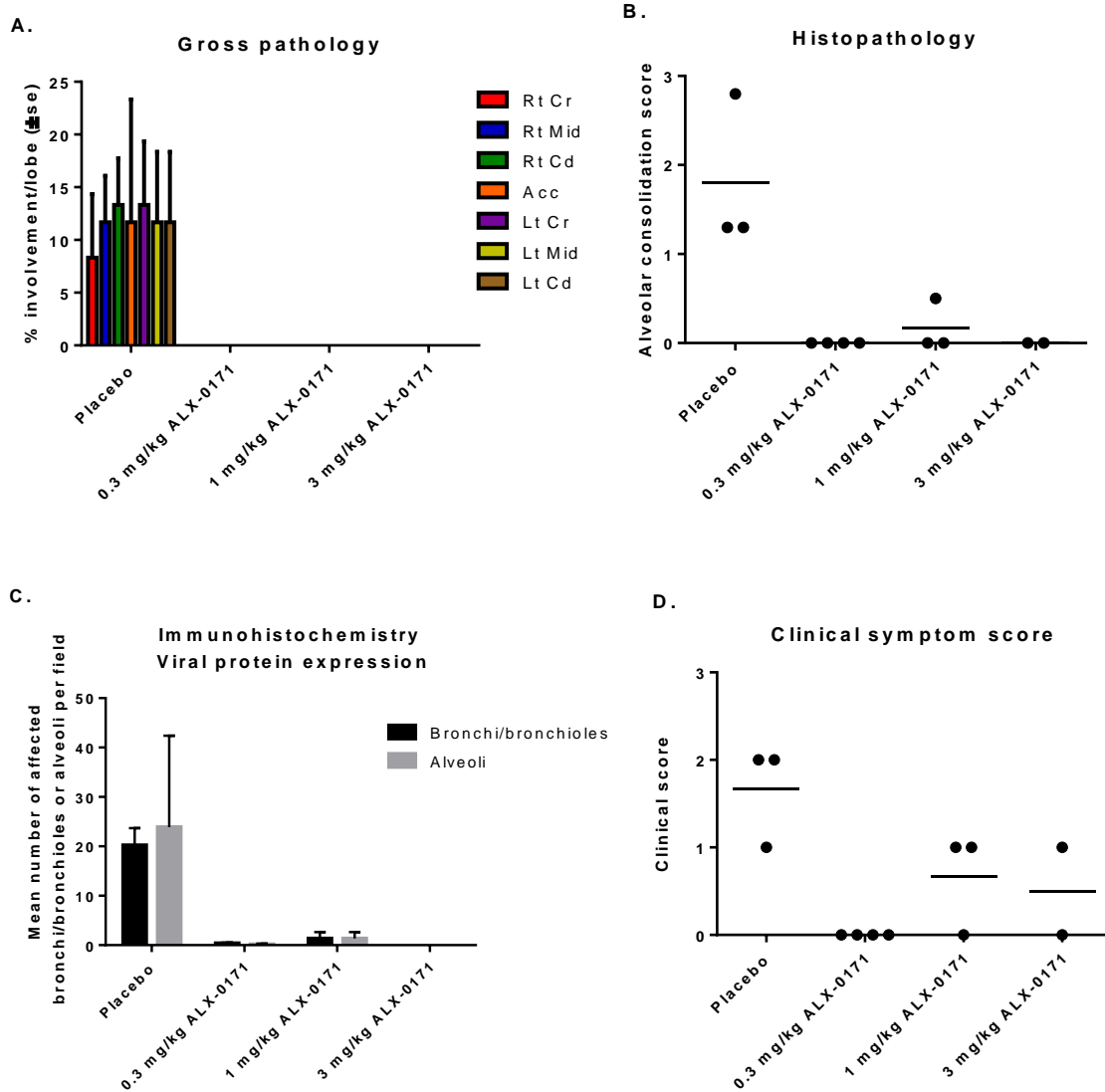


Figure 3. Gross lesions, histopathology lesions, immunohistochemical determination of viral antigen distribution and clinical scores summarizing clinical findings in lambs of Study 1. Gross and microscopic scores of lungs from hRSV-infected lambs treated with either placebo or ALX-0171 from study 1. (A) Viral-related lung gross lesions were scored and percentage parenchymal involvement was estimated for each lung lobe. Mean percentage averages per lobe were calculated. Legend: Rt Cr = Right cranial lobe; Rt Mid = Right middle lobe; Rt Cd = Right caudal lobe; Acc = Accessory lobe; Lt Cr = Left cranial lobe; Lt Mid = Left middle lobe; Lt Cd = Left caudal lobe.

Figure 3. (continued) (B) Microscopic (histopathologic) alveolar consolidation score was determined as percent area of RSV lesions followed by conversion to an integer-based consolidation scale as described in materials and methods section. (C) RSV antigen expression in lung tissue was determined by counting the number of affected bronchi/bronchioles or alveoli per field. (D) Clinical scores for lambs in study 1. For each lamb relative changes from baseline (day 0) in body-weights, respiratory rates, body temperature and blood oxygen saturation levels were calculated for each day. The presence or absence of expiratory effort or wheeze at any day after start of treatment was recorded. Each of the parameters were given a score of 0 or 1 based on pre-defined criteria as follows: If body weight increase between day 0 and day 6 $>20\%$ - score 0, if $\leq 20\%$ - score 1; if body temperature increase $>10\%$ at any day post treatment start - score 1, if $\leq 10\%$ - score 0; if respiratory rates increase $>10\%$ at any day post treatment start - score 1, if $\leq 10\%$ - score 0; if blood oxygenation levels increase $>10\%$ at any day after treatment start - score 1, if $\leq 10\%$ - score 0; if expiratory effort present at any day after treatment initiation - score 1, if absent - score 0; if wheeze present at any day after treatment initiation - score 1, if absent - score 0. The clinical score was the sum of these individual scores with a maximum of 6. Results are expressed as mean \pm standard error for panels (A) and (C); group averages with individual lamb scores are indicated by bullet points for panels (B) and (D).

RSV-infected placebo-treated lambs from both studies had comparable lung viral loads to placebo-treated lambs from study 1 whereas mock-infected placebo-treated lambs from study 3 had no detectable virus as expected. Consistent with findings from study 1, RSV-infection resulted in the presence of gross and microscopic lung lesions which was paralleled by lung epithelial cell viral protein expression (Figure 4 and Figure 5) ALX-0171 treatment initiated on day 3 post-infection, reduced infectious virus titers by more than 4 Log₁₀ FFU/mL at both tested doses. The effect on viral copy numbers was less pronounced as the reductions ranged from 0.14-1.25 Log₁₀ in BALF ($p=0.6$ and $p=0.019$, respectively) and 0.54-1.84 Log₁₀ in lung tissue ($p=0.017$ and $p<0.0001$) in study 2 and 0.55 Log₁₀ in BALF ($p=0.2$) and 0.47 Log₁₀ in lung tissue ($p=0.2$) in study 3. Despite these more modest reductions in viral copy numbers, ALX-0171 treatment significantly reduced microscopic alveolar consolidation score in both study 2 and 3 ($p<0.0001$ for combined ALX-0171 treated groups versus placebo) as well as for most of the individually scored microscopic lesions (Figure 4B, Figure 5B, Figure 6B and Figure 7). Reductions in the number of bronchi/bronchioles and alveoli expressing viral protein were also

noted in RSV-infected ALX-0171 treated groups from both studies, albeit non-significantly for study 2 ($p < 0.01$ for study 3 for RSV-infected ALX-0171 treated group versus RSV-infected placebo-treated group) (Figure 4C and Figure 5C). Gross lung examination in treated lambs confirmed the antiviral effect of ALX-0171-treatment as mean percent lung consolidation was 40.7% for the placebo-treated lambs compared to 0% and 7.6% for low and high ALX-0171 dose treated lambs in study 2 (Figure 4A). In study 3, the mean percent lung consolidation was 39% for the RSV-infected placebo-treated lambs compared to 4.9% in the RSV-infected ALX-0171 treated lambs (Figure 5A and Figure 6A).

Clinically, the hRSV-infected placebo-treated lambs in study 2 all had a clinical score of 3. This increased clinical score was driven by labored breathing as expiratory effort was evident in all 3 lambs (expiratory score score of 1) in at least one occasion during the post-treatment initiation period (i.e. day 3 to day 6) and in 1 lamb on day 0. In contrast to study 1, wheeze was also present on day 0 (1 lamb), day 3 (in 1 lamb), day 4 (in 1 lamb) day 5 (in 3 lambs) and day 6 (in 2 lambs) with a wheeze score of 3 on all occasions. In the ALX-0171-treated lambs, clinical scores were reduced compared to the placebo-treated lambs (Figure 4D) with 1 lamb scoring 0, 3 lambs scoring 1 and 1 lamb scoring 2. Expiratory effort and wheeze were only present in 1 lamb in the low dose ALX-0171 group on day 3 (expiratory effort and wheeze score of 1 and 2, respectively) and wheeze was present in 1 lamb in the high ALX-0171 dose group on day 6 (wheeze score of 1). Respiratory rates were relatively variable throughout the study duration with a mean relative change in respiratory rates from day 0 to day 6 for the placebo-treated lambs of $23.3\% \pm 3.2\%$ (mean \pm se) in contrast to $-10.6\% \pm 10.6\%$ and $-6.2\% \pm 9.4\%$ for low and high ALX-0171 treated lambs, although this difference was not significant.

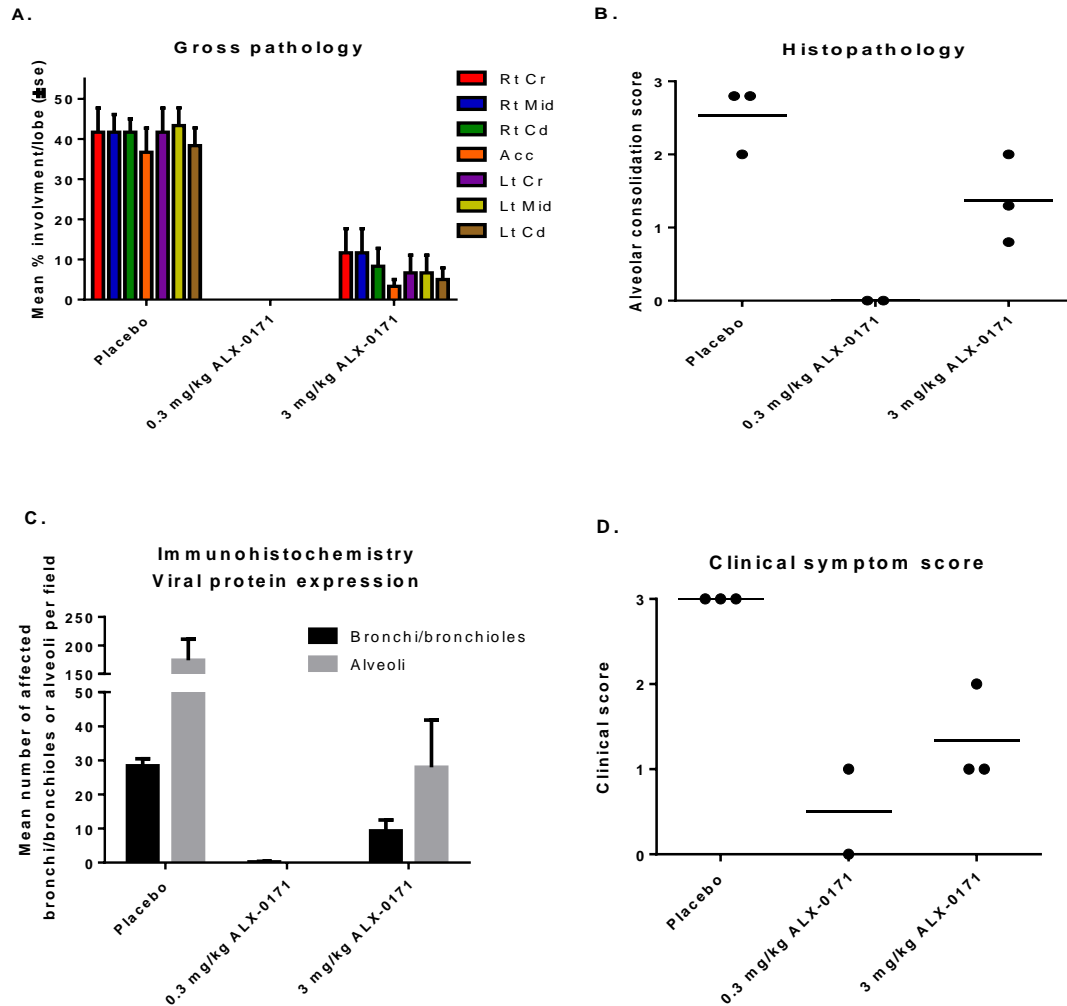


Figure 4. Gross lesions, histopathology lesions, immunohistochemical determination of viral antigen distribution and clinical scores summarizing clinical findings in lambs of Study 2. Gross and microscopic evaluation of lungs from hRSV-infected lambs treated with either placebo or ALX-0171 from study 2. (A) Viral-related lung gross lesions were scored and percentage parenchymal involvement was estimated for each lung lobe. Mean percentage averages per lobe were calculated. Legend: Rt Cr = Right cranial lobe; Rt Mid = Right middle lobe; Rt Cd = Right caudal lobe; Acc = Accessory lobe; Lt Cr = Left cranial lobe; Lt Mid = Left middle lobe; Lt Cd = Left caudal lobe. (B) Microscopic (histopathologic) alveolar consolidation score was determined as percent area of RSV lesions followed by conversion to an integer-based consolidation scale as described in materials and methods section. (C) RSV antigen expression in lung tissue was determined by counting the number of affected bronchi/bronchioles or alveoli per field. (D) Lamb clinical scores. Clinical scores were calculated as described in Figure 3. Results are expressed as mean \pm standard error for panels (A) and (C); group averages with individual lamb scores are indicated by bullet points for panels (B) and (D).

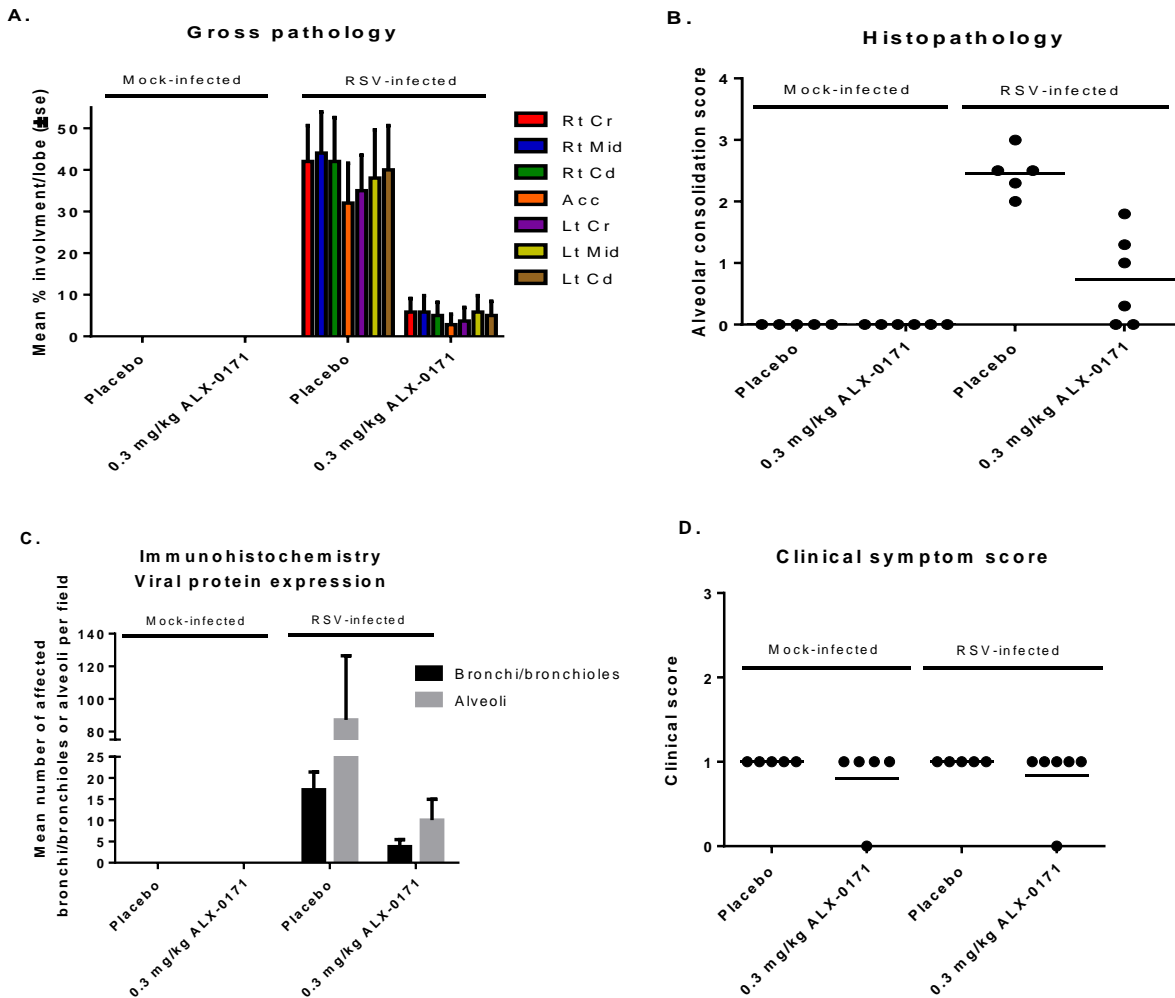


Figure 5. Gross lesions, histopathology lesions, immunohistochemical determination of viral antigen distribution and clinical scores summarizing clinical findings in lambs of Study 3. Gross and microscopic findings of lungs from mock-infected and hRSV-infected lambs treated with either placebo or ALX-0171 from study 3. (A) Viral-related lung gross lesions were scored and percentage parenchymal involvement was estimated for each lung lobe. Mean percentage averages per lobe were calculated. Legend: Rt Cr = Right cranial lobe; Rt Mid = Right middle lobe; Rt Cd = Right caudal lobe; Acc = Accessory lobe; Lt Cr = Left cranial lobe; Lt Mid = Left middle lobe; Lt Cd = Left caudal lobe. (B) Microscopic (histopathologic) alveolar consolidation score was determined as percent area of RSV lesions followed by conversion to an integer-based consolidation scale as described in materials and methods section. (C) hRSV antigen expression in lung tissue was determined by counting the number of affected bronchi/bronchioles or alveoli per field. (D) Lamb clinical scores. Clinical scores were calculated as described in Figure 3. Results are expressed as mean \pm standard error for panels (A) and (C); group averages with individual lamb scores are indicated by bullet points for panels (B) and (D).

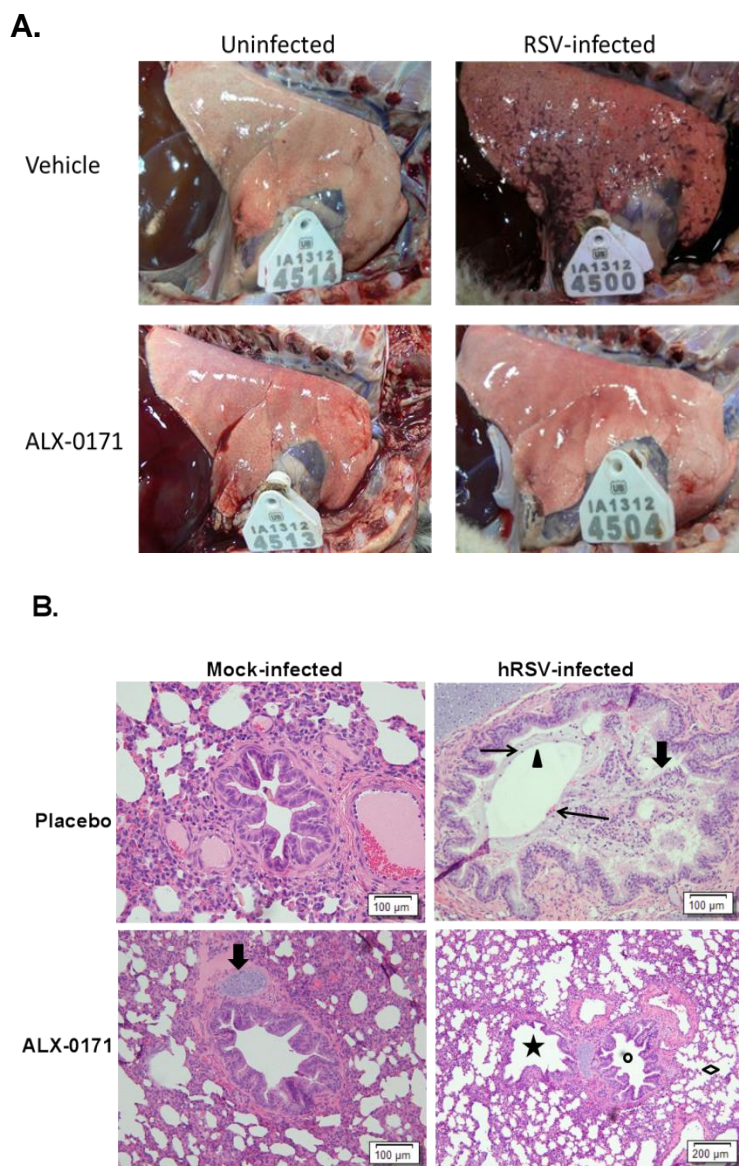


Figure 6. Images of gross lesions and histopathology in Study 3. Lung pathology in mock-infected or hRSV-infected lambs treated with either placebo or ALX-0171 from study 3. (A) Images of lungs from mock-infected lambs (left column) and hRSV-infected lambs (right column). Lambs were either treated with placebo (top row) or ALX-0171 (bottom row) for 3 days consecutively. Images were taken at necropsy on day 6 post-infection. Lungs from lambs 27 (tag ID 4514), 38 (tag ID 4500), 33 (tag ID 4513) and 44 (tag ID 4504) are shown. Viral lesions are visible as plum-red lesions in lamb 38. (B) Microscopic (histopathologic) lung lesions of hRSV infected lambs. Microscopic lung images are from the same lamb lungs shown in Figure 6A. There was no microscopic lung lesions seen in the mock-infected placebo and ALX-0171-treated groups.

Figure 6. (continued) The mock-infected placebo-treated group picture contains a normal bronchiole lacking lesions, while the mock-infected ALX-0171-treated group has a bronchus with surrounding cartilage (fat arrow) also lacks lesions. The hRSV-infected placebo-treated group had a wide spectrum of lung lesions, with bronchitis characterized by the lumen which contains neutrophils (thin short arrow), sloughed epithelial cells (fat arrow), mucin (arrowhead), and red blood cells (long thin arrow). The hRSV-infected ALX-0171-treated group had significantly reduced histopathological lesions when compared to the mock-infected placebo and ALX-0171-treated groups, which was evident by the clear bronchi (circle), bronchioles (star) and alveolar spaces (diamond) present in the picture.

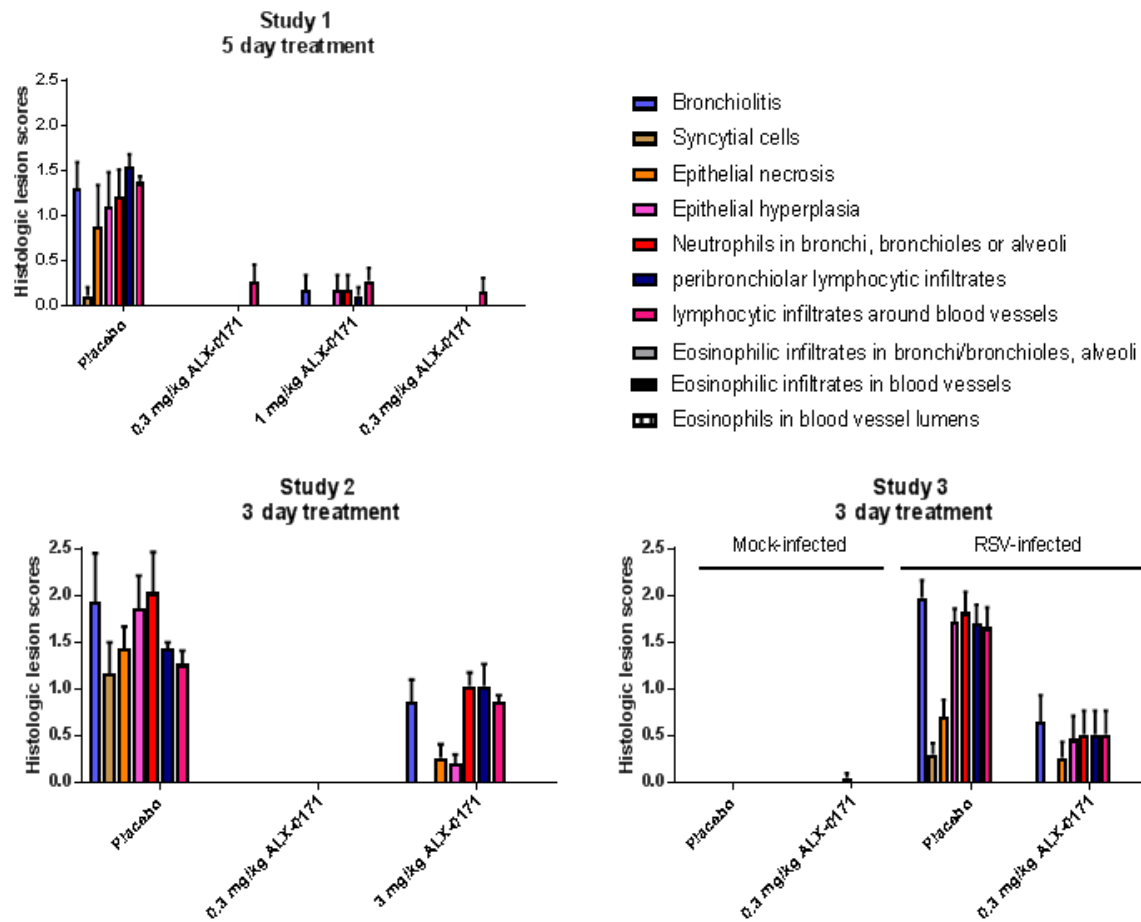


Figure 7. Individually scored histologic lung lesions in neonatal lambs. Bronchiolitis, neutrophil infiltration, peribronchiolar and perivascular infiltration of lymphocytes, syncytial cell formation and epithelial alterations were individually scored for each lamb in the three performed studies following the criteria reported previously [26]. Results are expressed as mean \pm standard error for each scored lesion.

In study 3, the hRSV-infected placebo-treated lambs and the mock-infected placebo-treated lambs all had a clinical score of 1 due to variable increases in respiratory rates. No noticeable expiratory effort and wheeze was apparent in these lambs. All hRSV-infected placebo-treated lambs on day 2 post-infection demonstrated unremarkable/normal behavior, energy level, perambulatory activity, and were alert and interactive. By the next day, 4 out of 5 hRSV-infected lambs clearly exhibited a decline in their general health status (Figure 8) On day 4 and 5 all the hRSV-infected placebo-treated lambs displayed some degree of reduced general health status as they were more lethargic in their movements, less prone to interact with each other and, when standing or laying down, drooped their heads and ears downward and appeared to be in a generally weakened and/or depressed state. In contrast, none of the mock-infected lambs treated with placebo displayed an altered general health status throughout the study duration. Clinical scores in hRSV-infected ALX-0171-treated lambs were comparable to the mock-infected ALX-0171-treated lambs where 5 out of 6 lambs and 4 out of 5 lambs, respectively had a score of 1. As in the placebo-treated lambs, the only noticeable finding was variably increased respiratory rates. None of the mock-infected ALX-0171-treated lambs displayed an altered general health status over the study duration. In the ALX-0171-treated lambs, a decline in the general health status of 3 out of 6 lambs was apparent on day 3 only which was the first treatment day. On the next and all the subsequent days, all the ALX-0171-treated lambs displayed normal behavior and general health status.

Discussion

In vivo efficacy of ALX-0171 has been previously tested in the cotton rat (*Sigmodon hispidus*) [16]. Although semi-permissive to hRSV infection, cotton rat studies have proven

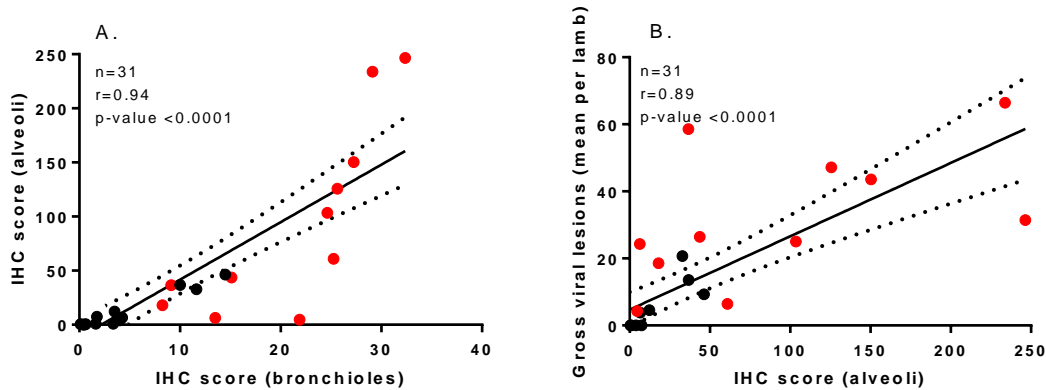
useful as both RSVIg (RespiGam®) and palivizumab (Synagis®) were advanced to clinical trials solely based on cotton rat data [9]. However, as the intended route of administration for ALX-0171 is by inhalation, adequate drug deposition in the lung is a prerequisite for efficacy. Because drug deposition is strongly affected by breathing patterns (respiratory frequency and breathing volume) which is dependent on body size and airway anatomy [18], the translational fidelity of rodent studies in terms of dose setting will be of limited value. In contrast, the respiratory system of lambs and human infants share many anatomical, physiological and developmental features that increase the translational value of studies performed in lambs for inhaled drugs [14, 18]. In addition, colostrum-deprived neonatal lambs are highly relevant for the study of RSV infection due to their natural susceptibility to ovine, bovine and human strains of RSV [27, 35, 36] and to the similarities in disease pathogenesis to that of human infants [12, 37]. This lamb RSV-infection model therefore constitutes a valuable tool for use in pre-clinical studies of vaccines or therapeutics.

Previous work in our laboratory has demonstrated that RSV-infection with Memphis 37 strain in neonatal lambs results in robust viral replication in the lungs that peaks around day 3 post-infection before subsiding by day 8. The viral replication was paralleled by an increase in RSV lung viral antigen expression, gross and histopathologic lesions and appearance of respiratory distress [12, 26, 27].

In the herein described studies, the administration of nebulized ALX-0171 resulted in systemic exposures that were indicative of absorption dependent pharmacokinetics. Overall, no unexpected retention of ALX-0171 in studied tissues or organs was observed. No difference in ALX-0171 deposition was noted between RSV-infected and uninfected lambs, although subtle differences may have been masked by the observed variability. Local lung ALX-0171

concentrations attained were adequate for reducing clinical or illness score (malaise), gross and microscopic lung lesions, viral titers, viral antigen, and viral RNA levels, even when administered around the peak of viral replication.

Previous studies have shown that RSV infects ciliated and bronchiolar airway epithelial cells in the respiratory tract [38-40] as well as type II pneumocytes [43, 44]. Consistent with these findings, viral antigen expression was present on these cell types in the hRSV-infected lambs with a significant correlation between hRSV expression in alveoli and bronchioles (Figure 9A). ALX-0171, greatly reduced the viral antigen expression both in the bronchioles and alveoli, which is indicative of a decreased number of infected epithelial cells. This decreased viral antigen expression was correlated with the decreased gross viral lesions (Figure 9B-C) and decreased viral titers (Figure 9D-E). There was also a significant correlation between infectious virus titers and gross viral lesions (Figure 9F).



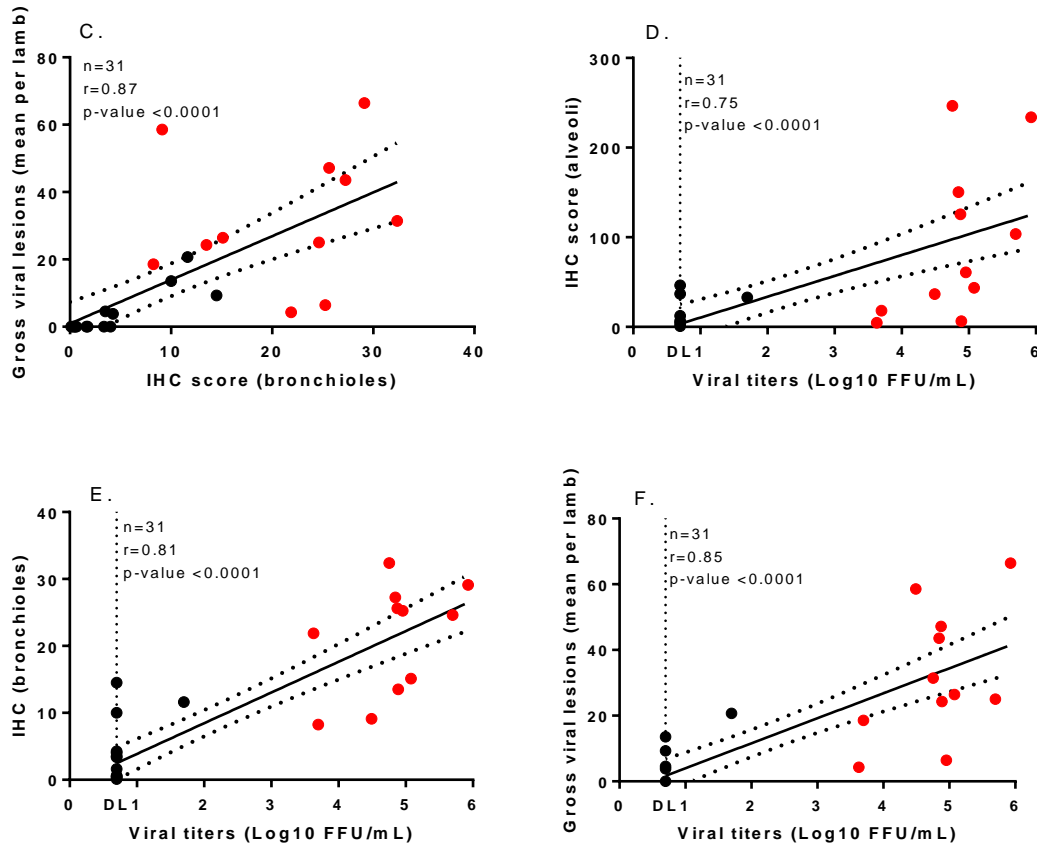


Figure 9. Correlation between independently scored readouts in hRSV – infected neonatal lambs. Correlation coefficient was calculated by Spearman's rank correlation test for the indicated parameters. Mock-infected lambs are excluded from the analysis.

Thus, measuring infectious virus titers in BALF could provide a reliable surrogate endpoint to monitor disease progression, at least in lambs. Similarly, viral loads were consistently correlated with increases in multiple different disease measurements (symptoms, physical examination, and amount of nasal mucus) in a human challenge study [21] and would seem to predict disease severity in previously healthy infants [41, 42]. ALX-0171 was less effective at reducing RSV RNA titers in lung tissue and BALF (as determined by RT-qPCR) than infectious virus titers as expected since ALX-0171 binds virus but does not directly inhibit transcription. Similarly, motavizumab, when given therapeutically to infants hospitalized with

RSV disease, resulted in a ≥ 2 Log₁₀ PFU/mL decrease in cultivatable virus between day 0 and day 1. This reduction ranged from 0.3 to 1 Log₁₀ PFU/mL for viral transcripts over the same period [43]. Likewise, in our studies cultivatable virus was reduced by >4 Log₁₀ FFU/mL on day 6 in the ALX-0171-treated lambs in comparison to the vehicle-treated lambs. On the same day viral RNA titers were reduced by 0.14 to 1.88 Log₁₀ viral RNA copies/mL or mg in both BALF and lung tissue. The likely explanation of the difference between the two measures is that, on the one hand, RT-qPCR not only quantifies fully replication-competent viruses but also complete viral particles unable to replicate, partially assembled virions, and whole and fragmented viral genome [44], on which ALX-0171 has no effect. Consequently, the natural rate of viral load decline is less steep when using a RT-qPCR method than when using quantitative culture, which is likely to confound antiviral efficacy determination of test compounds targeting RSV [44, 45]. The culture assay, on the other hand, quantifies fully infectious particles, but it is conceivable that viral particles that are neutralized by ALX 0171 and that are still present in the respiratory tract would not be quantified in the culture assay whereas they would be quantified by RT-qPCR. The culture assay would thus reflect the fact that either less virus is present in a sample or that any virus still present is effectively neutralized. The positive effects of ALX-0171 on the other viral related endpoints (viral antigen expression, gross lesions, histopathology) further substantiates the robust anti-viral effect of ALX-0171 in neonatal lambs.

Clinically, in the hRSV-infected neonatal lambs, a decline in respiratory function and general health status was observed in the herein described studies with observable expiratory effort and wheeze in some lambs and increased respiratory rates. However, these observations were not consistently present in all lambs thus making the analysis of ALX-0171 effects on altering clinical signs difficult. The natural individual susceptibility of hRSV infection in the

lambs may explain part of this observation. Similarly, most clinicians recognize bronchiolitis as a constellation of clinical symptoms and signs [46] with a high heterogeneity in disease severity likely due to a combination of host and viral factors [47]. Study procedures may also explain part of the variability seen in the clinical parameters as the procedures for clinical assessments were adapted for study 3 allowing for increased observation times and more robust scoring of lamb behavioural changes. One important challenge in the performance of these studies was the minimisation of any cross-contamination between not only hRSV-infected and uninfected lambs but also of ALX-0171 and placebo treated lambs making full blinding of the evaluation of individual clinical parameters difficult. The need to objectivate as much as possible the effects of ALX-0171 on these clinical parameters and their observed heterogeneity, led to the devise of a composite clinical score taking into account blood oxygen saturation, respiratory rates, body weight gain, body temperatures, expiratory efforts and wheeze using pre-defined scoring criteria which were considered meaningful. All these parameters have been shown to be important in hRSV-disease both in animal models and infants [27, 34, 46, 48, 49]. This analysis showed that ALX-0171 treatment reduced the clinical severity and had a positive effect on general health status.

In conclusion, when considering the totality of the results obtained from the studied effect measures (virology, observations of clinical signs, gross pathology and histopathology) which are known indicators of hRSV infection, a beneficial effect of ALX-0171 was noted on all established markers although the clinical and quantitative expression of hRSV infection differed somewhat in the different studies. Viral replication parameters were consistent with associated gross and microscopic lesions and viral antigen distribution. That is, in lambs not treated with ALX-0171, the high RSV titers and high levels of RSV RNA were associated with increased

severity of gross and microscopic lesions and viral antigen distribution. Following ALX-0171 treatment, reduced RSV parameters were associated with reduced lesions and antigen. There was no clear dose dependency seen which suggests that the lowest dose tested already provides full efficacy. ALX-0171 administered therapeutically may thus have the potential to be effective in the context of a developed hRSV infection in infants.

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CHAPTER 4. DETERMINING CLINICAL SIGNS AND SICKNESS BEHAVIOR IN A HUMAN RSV NEONATAL INFECTION LAMB MODEL

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Abstract

Rationale

The aim of this study was to determine the clinical symptomology and sickness behavior of RSV infected lambs through clinical data and video recording, without the heavy sampling schedule that previous lamb studies have included.

Methods

Colostrum-deprived newborn lambs were nebulized with M37 human RSV (infected) or cell-conditioned growth medium (control) at day 0, and were observed daily for general clinical signs of illness, and monitored for clinical data until day 8 p.i. Behavior data was collected by 10-minute instantaneous scan sampling from video recordings using Noldus Observer software with researcher blind to treatment group, lamb and trial day. Data was analysed using generalized mixed models (GLIMMEX) to compare changes in response across trial days and between control and inoculated lambs.

Results

General illness signs were sparse for the infected and non-infected groups. Mean respiratory rates (RR) and body temperatures (BT) differed on days 1 p.i. (RR and BT) and 5 p.i. (BT). Expiratory effort was only observed for 2 lambs in the infected group on days 4 (n=2) and 7 (n=1) p.i. Contrary to our hypothesis, infected lambs spent significantly less time lying and more time standing than control lambs. However, both treatment groups displayed tremoring and lameness, and bacterial pneumonias were observed at necropsy in the control group, confounding the clinical data and lamb behaviors due solely to RSV infection.

Conclusion

Clinical signs and sickness behavior in both groups were confounded by non-RSV clinical signs of tremors and lameness, as well as bacterial pneumonias. Bacterial infections are an inherent problem of the hRSV infection neonatal lamb model, and as long as they are present, interpretation of clinical data and lamb behaviors should be done with caution when drawing conclusions. Future lamb studies should make an effort to solely demonstrate RSV infection in lambs by identifying and preventing secondary or opportunistic pathogens.

Introduction

Human Respiratory Syncytial Virus (hRSV) is a major cause of respiratory disease in infants and young children in the United States and worldwide. Each year in the United States alone, more than 2 million children need medical intervention to treat the symptoms and complications of RSV infection [1, 2]. Clinical signs develop four to six days after infection with RSV, and usually subside after one to two weeks [3]. These signs vary with severity of disease

and range from mild flu-like symptoms (coughing, sneezing, fever, and loss of appetite) in 25% to 40% of first-time exposed infants to severe bronchiolitis with or without pneumonia (rapid breathing, difficulty breathing, and wheezing) necessitating hospitalization in 0.5% to 2% of infants [4]. In very young infants, irritability, decreased activity, and apnea may be the only symptoms of infection. These clinical symptoms together comprise the sickness behavior, and have been attributed to both the immune response to RSV, as well as the direct damage to RSV-infected bronchiolar epithelium [5, 6].

Sickness behavior is an adaptive organized immune response that leads to physiological and behavioral changes that are manifested in animals commonly as fever, depression, decreased lipido, adipsia, anorexia, and decreased grooming and social interaction due to potentially injurious stimuli, such as microbial agents (e.g. virus, bacteria, fungal, parasites) [7-9]. This evolved behavioral response helps to conserve energy through lethargy and fatigue and fight bacterial and viral infections by increasing body temperature through fever [7].

Important immune mediators that contribute to the sickness response include proinflammatory cytokines IL-1, IL-6, and tumor necrosis factor [7-10]. Together these cytokines are able to mediate local, systemic, and central nervous responses during acute and chronic infection that leads to sickness behavior [7]. However, fever can be metabolically expensive, and if prolonged animals may succumb to the disease, or become susceptible to predation [8, 9].

Sickness behavior can be easily induced and studied in animals with exposure to lipopolysaccharide (LPS) endotoxin. For example in rodents, fever, anorexia, and decreased activity and grooming are at their highest at increased lipopolysaccharide (LPS) endotoxin doses [11]. Therefore, sickness behavior varies with disease severity. This can also be seen in cattle

with mastitis and bovine respiratory disease, where the sickness behavior was greater for severe disease when compared to mild mastitis and bovine respiratory disease [12, 13]. Sickness behavior can be used to detect infected animals; however, identifying animals with mild disease and subclinical signs may be more challenging [14].

Using neonatal lambs to study RSV infection in human infants has proven to be a relevant animal model, with lambs having several biological features that closely mimic RSV infection in human infants. These similarities to human infants include lung development, lung structure and airway branching, cellular composition and immune responses, susceptibility to hRSV strains (Long, A2, and Memphis Strain 37), clinical symptomology, gross and histologic lesions, lung size to human infants, and the ability to obtain lambs lacking maternal antibodies [15-23]. These features are important for using RSV lambs to assess vaccines and antiviral drug safety and efficacy.

Determining the clinical symptoms associated with hRSV infection in lambs has been challenging. Previous studies done in our laboratory group using different strains of RSV (A2, Memphis 37) have identified several clinical signs including fever, coughing, expiratory effort, and wheezing [15, 16, 17, 18, 24]. Clinical parameters such as respiration rate, heart rate, pulse oxymetry, and weight loss have not been statistically significant between RSV infected and non-infected lambs. The only clinical sign that has been observed the most in lambs as a significant clinical marker to monitor for RSV disease is expiratory effort [16, 25-27]. Overall, clinical signs in lambs infected with RSV can be variable and non-predictable, and interpretation can be confounded by many variables that include the strain of RSV, experience of the interpreter observing clinical signs, handling of lambs, sampling schedule, experience in getting respiration and heart rate, unknown side effects of therapeutic drugs, administration of anesthetics, and

secondary bacterial infections. To add to this list, lambs are herd animals and don't express clear signs of sickness until severe disease is present or near death. The aim of this study is to better define the clinical symptomology and sickness behavior of RSV infected lambs through clinical data and video recording of lamb behavior without the heavy sampling schedule that previous lamb studies have included.

Material and Methods

Experimental Design

Fourteen colostrum-free neonatal lambs (1–2 days of age) numbered 1 through 14 received daily antibiotics (Ceftiofur, Pfizer, New York, NY; 1–2 mg/kg, intramuscular) to prevent secondary bacterial infections. They were randomly assigned based on age and sex into two groups of seven, groups labeled 1 through 6, with the RSV infected group containing groups 4, 5, and 6, and the non-infected group containing groups 1, 2, and 3. RSV infected and non-infected groups were then placed into separate rooms, with the RSV infected group containing lambs numbered 8-14 and the non-infected group containing lambs 1-7. Groups 1, 2, and 3 contained lambs numbered 1 and 2, 3 and 4, and 5, 6, and 7, respectively, with the infected groups having the same arrangement, 8 and 9 (group 4), 10-11 (group 5), and 12, 13, and 14 (group 6). Each room contained two pens, with one pen containing two groups, and the second adjacent pen containing only one group. The RSV infected group had the first pen with groups 4 and 5 and the last pen contained group 6, while the non-infected group had one pen with groups 1 and 2, and the other with group 3. The pens that contained two groups of lambs were separated by a center divider with a communal heating lamp that did not hinder socialization. Two video recording cameras (non-audio) were strategically placed at each end of the pens to fully capture

the observations of the lambs over a 24 hour continuous period, beginning at day -1 through day 8 post infection (p.i.), for a total of ten days (Figure 1). Each group contained one milk bucket with the nipples specifically positioned where the camera would be able to record the lambs feeding. Milk consumption was measured every day, starting at day 0 and ending at day 7 p.i. for each group. Each morning before feeding, clinical signs (general illness, cough, eye or nasal discharge, expiratory effort, wheezing, auscultated respiration, heart rate, temperature, and body weight) were collected after waiting 20-30 minutes for the lambs to settle down while being inside the room.



Figure 1: Pen and video recording camera set up for both infected and non-infected rooms. Each pen contained two cameras at each end with a center divider and a communal heating lamp, and one milk bucket per pen group.

At day 0, each nebulized group (RSV infected and non-infected) received either three successive 2-mL nebulizations of RSV M37 1.27×10^7 FFU/mL (in DMEM growth media +

20% sucrose w/v) or cell-conditioned growth media (DMEM + 20% sucrose) over a 23-minute period using a PARI LC Sprint™ nebulizer (PARI Respiratory Equipment, Inc., Lancaster, PA, USA) at 4L/min at 16 PSI (Philips Respironics Air Compressor, Andover, MA, USA) attached to a conical mask fitted with a round rubber diaphragm with a pre-cut center hole through which the nose and mouth of the lamb was inserted (MidWest Veterinary Supply, Inc., Burnsville, MN), respectively. Lambs were then euthanized by sodium pentobarbital overdose and necropsied at day 8 p.i. After euthanasia the thorax was opened, lungs were removed, and gross lesions were scored and photographed *ex vivo*. Tissue samples were collected from each lung lobe of all animals in the same manner, with uniform sampling of each lobe. Before lung dissection, bronchoalveolar lavage fluid (BALF) was collected from the right caudal lung lobe (in double-modified Iscove's media (DMIM) for infectious Focus-Forming Unit (FFU) assay and RT-qPCR for M37. Three samples from each lobe were snap frozen in liquid nitrogen for reverse transcription quantitative polymerase chain reaction (RT-qPCR), and two samples from each lobe were placed in tissue cassettes and put in 10% neutral-buffered formalin for histological and immunohistochemical analyses. Animal use and experimental procedures were approved by Iowa State University's Animal Care and Use Committee (IACUC).

Monitoring of Clinical Signs

Clinical signs were taken every morning before feeding and after waiting 20-30 minutes for the lambs to settle down while being inside the rooms of RSV infected and non-infected lambs. A clinical chart was developed to record clinical signs in order of less to more stressful, beginning with recording of general illness signs (Table 1), then followed by cough, eye or nasal discharge (Table 2), and expiratory effort (forced expiration) and wheezing (Table 3). Finally, respiration, heart rate, rectal body temperature, and body weights were taken, with all lambs

receiving a daily antibiotic (Ceftiofur, Pfizer, New York, NY; 1–2 mg/kg, intramuscular) at the end to prevent secondary bacterial infections. After observing lambs for general illness signs and collecting clinical data, lambs were fed.

Table 1. Scoring criteria for general illness

Score	General illness score
0	No clinical signs
1	Reluctant to move
2	Reluctant to move, head and ears down, not interested in eating
3	Down, unwilling to get up or difficulty standing, not eating, +/- isolated
4	Down, isolated, not eating, and should be euthanized

Table 2. Scoring criteria for cough and ocular/nasal discharge

Score	Cough	Eye or nasal discharge
0	No cough	No discharge
1	Occasional cough (once/5 minutes)	Earliest detectable watering
2	Repeated cough (more than once/5 minutes)	Consistent watering
3	Repeated, harsh cough, dyspnea	Copious watering

Table 3. Scoring criteria for lung function by auscultation

Score	Expiratory efforts	Wheezing (High-pitched whistling sound made while breathing)
0	No expiratory effort	No wheeze
1	Earliest detection of increased expiratory effort	Earliest detectable wheeze by auscultation
2	Moderate expiratory effort (>1 sec) observed with some abdominal effort	Audible wheeze in all lung fields by auscultation
3	Expiratory effort (>3 sec) with hard abdominal effort	Wheeze audible without stethoscope

Auscultation of Respiration and Heart Rate

The lungs were first auscultated for expiratory efforts and wheezing. Respiratory rates were then calculated by auscultating the lungs and counting breaths for 15 seconds and then multiplying the number of breaths by 4 to get breaths per minute (bpm). Heart rates were also taken and calculated in the same manner by auscultating the heart to get beats per minute (bpm). For both respiratory and heart rates, lambs were held gently in place standing up in their pen without picking them up.

Milk Consumption

Milk consumption was measured at the pen level every morning before feeding from day 0 through day 7 p.i. for both RSV infected and non-infected groups. The amount of milk given for each day (morning, mid-day, and evening) was based on the number of lambs in each group and their body weight of that morning. Milk consumption for each day was calculated by

subtracting the milk that was left in the bucket from the previous night that morning from the total amount of milk given for that day.

Behavioral Observations

Video recording of lambs occurred between 6:30 and 18:30 on days 0 through 7, relative to inoculation. Video was captured using two Noldus portable labs (Noldus Information Technology, The Netherlands), one in each of the treatment rooms. Cameras were placed in one corner of each pen (WV-CP484, Matsushita Co. Ltd, Kadoma, Japan), approximately 7 feet above the pen flooring (Figure 1). The cameras were connected to a multiplexer, which allowed the images to be recorded digitally onto a PC using HandiAvi (v4.3, Anderson's AZcendant Software, Tempe, AZ) at 30 frames/s.

Behavioral observations were collected using instantaneous scan sampling every 10 minutes beginning at 6:30 and ending at 18:30 [28]. All observations were collected by one trained technician blinded to animal ID, treatment and trial day (intra-observer reliability was 98%) using The Observer® software (Ver. 10 Noldus Information Technology, Wageningen, The Netherlands). Training consisted of familiarizing the technician with the ethogram and the software using representative video segments (Table 4). Once inter-observer reliability of 90% of achieved relative to experienced laboratory personnel, the technician commenced with formal data collection. A neutral technician performed the blinding procedures for the video recordings for all tests. The blinding procedures involved assigning a random number to each video and sorting for the purpose of providing a random sequence in which videos were to be scored. Five videos were selected at random and duplicated within the sequence for the purpose of determining intra-observer reliability. Behavior frequencies were summed for each trial day and subsequently analyzed.

Table 4. Ethogram used to evaluate lamb behavior following respiratory syncytial virus or control treatment

Behaviors	Behavior Description	Modifier
Feeding	Head within 2 inches of and oriented towards the milk bucket	
Locomotion/Active	Weight bearing on all four limbs and moving head and/or limbs	
Standing	Weight bearing on all four limbs and stationary	Social ¹ /Isolated ²
Lying	Belly and torso touching the ground	Social ¹ /Isolated ²

¹ Social: ≤1ft from pen mate and/or lambs in adjoining pen

² Isolated: >1ft from pen mate and/or lambs in adjoining pen

Virus

M37 hRSV is a wild type RSV-A first isolated from infected humans and used in human clinical studies [25]. The Memphis 37 RSV strain used in this study was passaged 6 times on Vero cells then twice on HEp-2 cells. Sucrose was added to 20% and the virus stock was frozen at −80°C and titered for infectivity on HEp-2 cells.

Gross Lesions Evaluation and Scoring

Following euthanasia, the thorax was opened and the heart and esophagus were removed from the lungs. The percentage parenchymal involvement of gross RSV lesions was scored for each individual lung lobe, and if present, the area and amount of lung lobes affected by bacterial pneumonia was also recorded. The percentage of a specific lobe tissue that was affected by RSV in relation to the overall lobe tissue being scored was estimated based on a score as done previously [16]. The percent total involvement of all lung lobe lesions was calculated by adding up estimated percentages of gross lesions given to each lung lobe and dividing by 7, which is the number of lung lobes present in lambs.

Histologic Evaluation and Scoring

A histologic score was given by determining percent involvement followed by conversion to an additional integer-based consolidation scale used by our laboratory previously [13] wherein: 0% consolidation = 0; 1%-9% consolidation = 1; 10%-39% consolidation = 2; 40%-69% consolidation = 3; 70%-100% consolidation = 4. Group averages were calculated for the alveolar consolidation score. In addition to the consolidation score, bronchitis/bronchiolitis, neutrophil infiltration, peribronchiolar and perivascular infiltration of lymphocytes, syncytial cell formation, and epithelial alterations were also scored according to criteria previously reported [16].

Immunohistochemistry

Immunohistochemistry for detecting RSV antigen was performed on 5 µm-thick formalin-fixed paraffin-embedded (FFPE) lamb lung tissue sections taken from the right and left cranial, left middle, and left caudal lung lobes of each animal in accordance with methods published previously [16]. Primary polyclonal goat anti-RSV (all antigens) antibody (EMD Millipore Corporation, Billerica, MA, USA) was applied for 90 minutes at room temperature diluted 1:500 in TBS-tween containing 10% NSS and 3% BSA. After rinsing with TBS-tween, biotinylated rabbit anti-goat secondary antibody (Kirkegaard-Perry Labs, Gaithersburg, MD, USA) diluted 1:300 in TBS-tween containing 10% NSS and 3% BSA was applied for 45 minutes, after which slides were rinsed with TBS-tween, treated with 3% H₂O₂ in TBS-tween for 25 minutes, rinsed and then incubated with streptavidin-conjugated HRP (Invitrogen) diluted 1:200 in TBS-tween for 30 minutes. Development of the color was performed in custom 12-slide plastic containers (Antibody Amplifier™ containers, ProHisto, LLC, Columbia, SC, USA) by

applying Nova Red (Vector Laboratories, Inc.) for about 90 seconds followed by copious rinses with ddH₂O, counterstaining with Harris' hematoxylin (for 2 minutes), bluing with alkaline Scott's water (for 1 minute), dehydration and coverslipping with Permount mounting medium (Sigma, St. Louis, MO, USA). 20 unique 10X fields on each slide (containing two lung sections each) were assessed for RSV antigen staining by counting positively-stained cells within bronchioles and alveoli.

Collection of Bronchoalveolar Lavage Fluid (BALF)

After the lungs of each lamb were removed, the right caudal lung lobe was excised and instilled with 5 mL of cold DMIM (42.5% Iscove's modified Dulbecco's medium, 7.5% glycerol, 1% heat-inactivated FBS, 49% DMEM, and 5 µg/ml kanamycin sulfate) after which 1 mL of the resulting BAL fluid was placed on ice and spun down for 5 minutes in a centrifuge at 3,000 x g to pellet large debris. Approximately 800–850 µL of each supernatant was collected and then spun through 850 µL-capacity 0.45 µm Costar SPIN-X filter (microcentrifuge 15,600 x g) for 5 minutes before being used in the standard infectious focus forming unit assay (FFU).

Focus-Forming Unit (FFU) Assay

Viral titers in bronchoalveolar lavage fluid (BALF) from the right lung caudal lobe were determined using an infectious focus assay (FFU). In brief, 200 µL of serially-diluted BALF samples were applied to HEp-2 cells grown to 70% confluence in 12-well culture plates (Fisher Scientific, Hanover Park, IL) in DMEM media (Mediatech, Inc., Manassas, VA) supplemented to 10% with heat-inactivated fetal bovine serum (FBS) (Atlanta Biologicals, Atlanta, GA) and 50 µg/mL kanamycin sulfate (Invitrogen/Life Technologies). Each sample was analyzed undiluted and at four additional serial-dilutions of 1:10, 1:100, 1:1,000 and 1:10,000 in duplicate.

Following a 48-hour incubation at 37°C, 5% CO₂, the cells were fixed with cold 60% acetone/40% methanol solution for 1 minute. Overnight primary polyclonal goat anti-RSV (all antigens) antibody (EMD Millipore Corporation, Billerica, MA, USA) incubation was followed by washing and secondary antibody (Alexa Fluor® 488 F(ab')₂ fragment of rabbit anti-goat IgG (H+L), Molecular Probes/Life Technologies) incubation for 30 minutes. Plates were rinsed and inspected for the presence of fluorescing foci of infection using the FITC/GFP filter on an inverted fluorescence microscope (Olympus CKX41, Center Valley, PA). Five or more fluorescing cells were counted as single focal events. An average of 40 counts in a 1:10-diluted (duplicate) sample indicated an original BALF sample “titer” of 2,000 [40 counts x dilution of 10 x 1,000 µL/mL]/200 µL assessed = 2,000 infectious focus-forming units/mL (FFU/mL).

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) for RSV in Bronchoalveolar Lavage Fluid

Viral RNA was quantified by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in BALF obtained from each animal at necropsy. BALF was obtained from the right caudal lung lobe of each animal (see section on BALF collection). Briefly, 100 µL of BALF was pipetted directly into 1 mL of TRIzol (Invitrogen/Life Technologies, Carlsbad, CA, USA) on ice, inverted to mix, and then transferred to -80°C for storage until RNA isolation and subsequent RT-qPCR. Upon thawing, each 1.1 mL sample was vortexed for 10 seconds and allowed to sit at room temperature for 10 minutes. RNA isolation from BALF samples continued as per manufacturer's instructions. The resulting (non-visible) RNA pellets, were each dissolved in 100 µL of nuclease-free water (Invitrogen/Life Technologies), vortexed thoroughly, microfuged briefly, warmed to 60°C for 3 minutes, vortexed for 5 seconds, microfuged briefly, then 80 µL of each was diluted 1:10 with a combination of 10 µL RNaseOUT™ (to 0.5 Units/ µL), and 710 µL

nuclease-free water, then stored at 4°C prior to RT-qPCR. RT-qPCR for RSV was then carried out as described above in the section: “Reverse transcription quantitative polymerase chain reaction (RT-qPCR) assessment of RSV and chemokine gene mRNA expression levels in lamb lung”.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) Assessment of RSV and Chemokine Gene mRNA Expression Levels in Lamb Lung

For each animal, tissue samples from right and left cranial, left middle and left caudal lung lobes (0.3–0.4 g of each lobe) were homogenized for total RNA isolation in TRIzol (Invitrogen) and previously described methods [26]. RNA was assessed for quantity and purity by spectrometry (Beckmann DU® 640B, Beckmann Coulter Inc., Brea, CA, USA) and all OD260nm/280nm values measured between 1.96 and 2.12. Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) analysis of RNA prior to DNase treatment consistently yielded RIN values ≥ 8.0 for all lamb lung RNA samples isolated this way [27]. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed using One-Step Fast qRT-PCR Kit master mix (Quanta, BioScience, Gaithersburg, MD, USA) in a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) and PREXCEL-Q for all set up calculations [29, 30]. Primer and probe sequences for all targets were designed with ABI Primer Express 2.0, and have been used previously [17, 26, 31]. Primers and hydrolysis probe for targeting M37 hRSV NP RNA were designed using ABI Primer Express version 2.0 based on RSV accession number M74568. Thermocycling conditions were 5 minutes at 50°C; 30 seconds at 95°C; and 45 cycles of 3 seconds at 95°C and 30 seconds at 60°C. Samples and standards were assessed in duplicate, and each target gene quantification cycle (Cq) value was converted to a relative quantity (Qr) based on each target’s standard curve using: $Qr = E^{AMP(b-$

C_q), wherein “b” and “EAMP” are the y-intercept and exponential PCR amplification value, respectively. EAMP values were obtained from the slope (m) of each target standard curve by: $EAMP = 10(-1/m)$, and all Q_r values interpolated from standard curves were normalized to total lung RNA per RT-qPCR (0.784 ng RNA/μL for all reactions). No-RT control (NRC) reactions gave either no signal or generated C_q values greater than 13 cycles later than those in the corresponding RT-qPCR target reactions.

Statistical Analysis

Data were analyzed using SAS® software (SAS® inst. Cary, NC) with generalized linear mixed models (GLIMMIX), to compare changes in behavior frequencies across trial days and between control and inoculated lambs. Behavior frequencies were divided by the total number of observations for each lamb and subsequently analyzed. The statistical model included the fixed effects of trial day, treatment and the interaction of trial and treatment. Treatment nested within Pen was included as a random effect. P-value ≤ 0.05 was considered to be significant. Longitudinal quantitative data were analyzed using repeated measures analyses of variance (ANOVA) with SAS software, and a P-value ≤ 0.05 was considered to be significant.

Results

General Illness

General illness signs were sparse and few for the RSV infected and non-infected groups. For the infected group, general illness signs were first observed on day 1 p.i. for lambs 8 and 9, which displayed general illness scores of 2 and 3, respectively (Table 1). Lamb 9 also had a fever of 104.8°F for day 1 p.i., before being euthanized on day 2 p.i. for seizing and having a fever of

107.0°F. This left lamb 8 by itself in group 4 with its neighboring group 5 to socialize with. There were no other general illness scores for the remaining days of the study for the infected group. For the non-infected group general illness signs were first observed on day 3 p.i. for lamb 3, which had general illness score of 3. On day 4 p.i., lamb 3 had a general illness score of 2. No other general illness signs were observed for the non-infected group.

Body Temperature

For the most part, the non-infected group had higher mean body temperatures than the RSV infected group, with the exception of days 1 and 2 p.i (Figure 2). However, mean body temperature for both groups were only statically significant at days 1 and 5 p.i. Mean body temperatures for the RSV infected group stayed within the normal limits of a newborn lamb's body temperature of 102.0°C-103.0°F, except for days 6 and 8 p.i where body temperatures were above 103.0°F. In comparison to the infected group, mean body temperatures for the non-infected group were above 103.0°F for days 1 through 8 p.i.

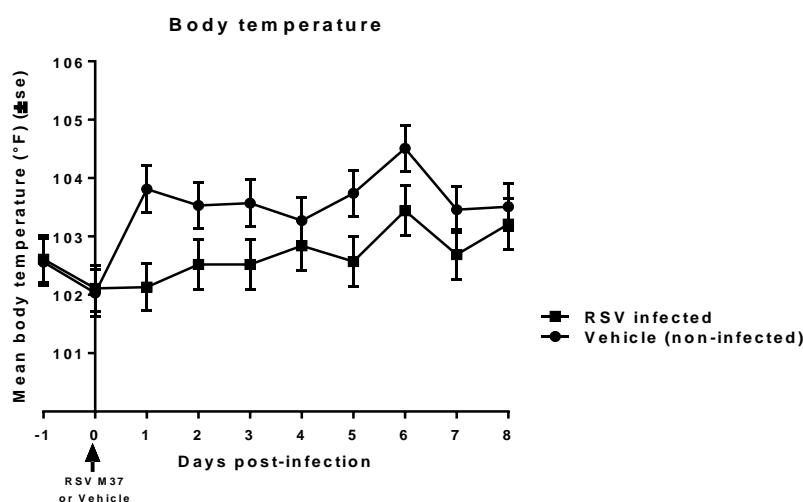


Figure 2. Mean body temperatures of RSV infected and non-infected (vehicle) groups. Mean body temperatures of RSV infected and non-infected (vehicle) lambs. Rectal temperatures were measured daily using a digital thermometer beginning at day -1 through day 8 post treatment. Results are shown as mean \pm standard error.

Looking at individual fevers in both groups, lambs 8, 10, and 12 in the infected group had 1 to 2 days of sporadic fevers. Lamb 14 had the most days of fever (4, 5, 6, 8) p.i., followed by lambs 13 (6, 7, 8 days p.i.) and 11 (-1, 3, 4 days p.i.). Lambs in the non-infected group had fevers that were more prevalent and consecutive than the infected group, with all lambs having fevers at day 1 p.i., except for lambs 7 and 6, which had fevers on day -1 and 5 p.i., respectively. Lambs 3 and 7 had fevers from days 1 through 8 p.i. Lamb 2 also had fevers for most days of the infection period (days 1 through 8 p.i.), except for day 5 p.i. Lambs 1 and 3 did have consecutive fevers from days 1 through 3 p.i, followed by two days of sporadic fever. Lambs 4 and 6 only had 3 days of fever, with lamb 6 having sporadic days of fever and lamb 4 having fever on days 5, 6, and 8 p.i.

Respiration Rates

Auscultated mean respiratory rates for the infected group peaked at day 3 (53 bpm) and day 7 (57 bpm) p.i., in comparison to the non-infected group which only had one peak at day 1 (55 bpm) p.i (Figure 3). At day 8 p.i., respiratory rates for both groups began to decrease. Between both groups, only day 1 p.i. was statistically significant.

High respiration rates were observed infrequently for both groups, with lambs 13 and 14 in the infected group having high respiration rates on days 7 (100 bpm) and 3 (80 bpm) p.i. Only lamb 2 in the non-infected group had high respiration rate on days 1 (120 bpm), 2 (80bpm), 4 (80 bpm), and 5 (80bpm) p.i. Expiratory efforts were only observed for the infected group, for lambs 13 and 14 on days 4 and 7 and 4 p.i., respectively.

There were also bilateral harsh lung sounds in the infected group that were sporadic for lambs 10, 12, 13, beginning on day 4 and ending on day 8 p.i., Lamb 11 had bilateral harsh lung sounds throughout this period of the study (days 4-8 p.i.). For the non-infected group, bilateral

harsh lung sounds were also sporadic and were only present on days 4 and 8 p.i. for lambs 2 and 1, respectively.

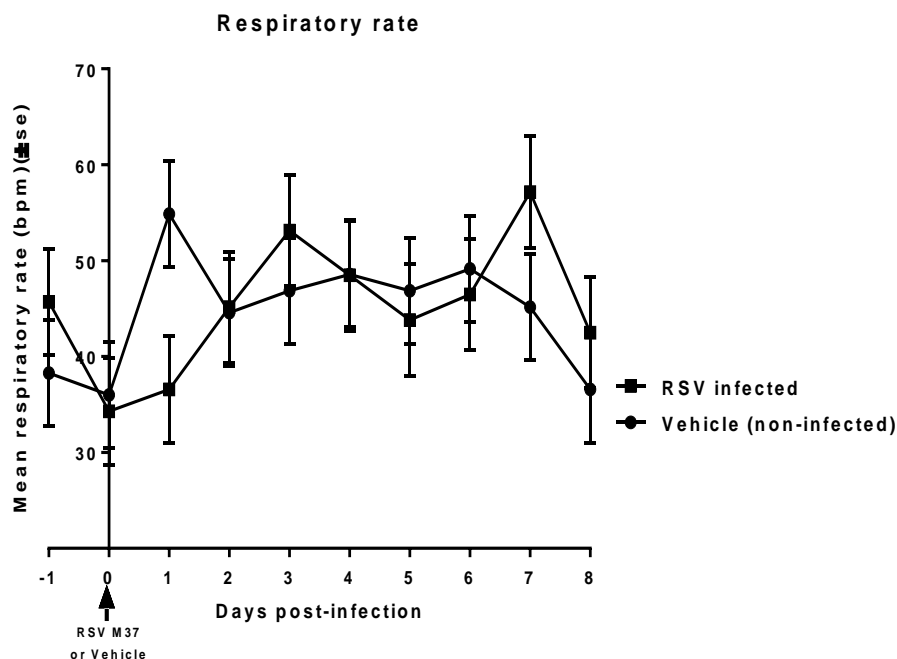


Figure 3. Respiratory rate assessment of RSV infected and non-infected groups. Respiratory rates were taken daily by auscultation using a stethoscope and breaths per minute (bpm) was calculated by counting the number of breaths in 15 seconds and multiplying it by 4. Results are shown as mean \pm standard error.

Behavioral Observations

There was no statistical difference in locomotion, socialization, and feeding between both infected and non-infected groups. Contrary to our hypothesis, infected lambs spent significantly less time lying and more time standing than the non-infected lambs (Figure 4A and 4B).

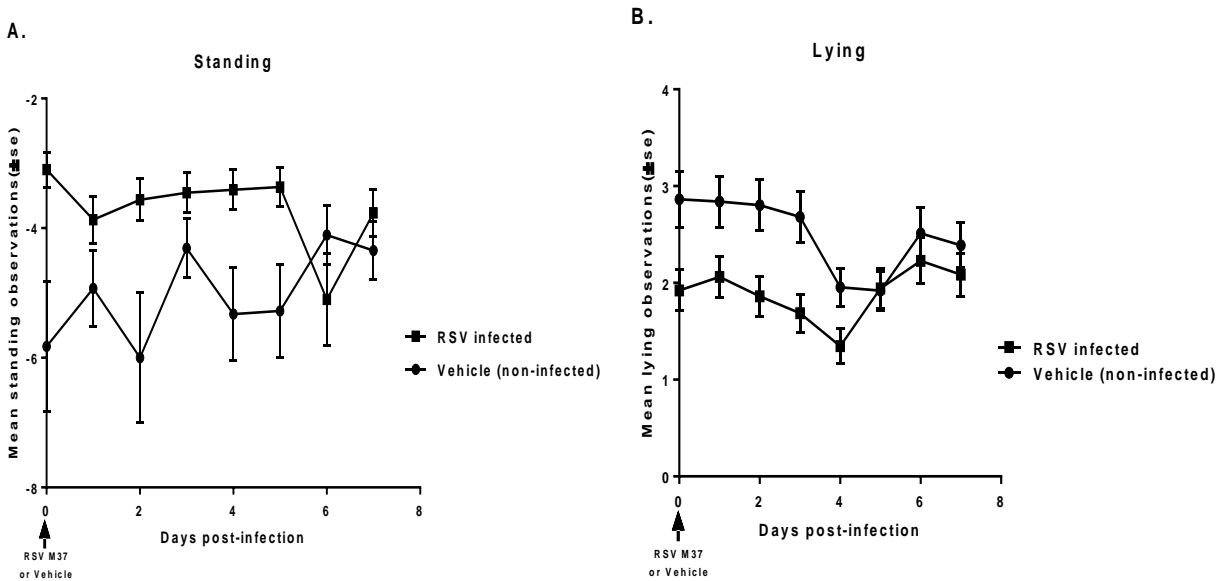


Figure 4 (A, B) sickness behavior. Behavioral observations (feeding, locomotion, standing, laying, socialization) for the infected and non-infected groups were collected using instantaneous scan sampling every 10 minutes from video recordings of lambs that occurred between 6:30 and 18:30 on days 0 through 7, relative to inoculation. All observations were collected by one trained researcher blinded to animal ID, treatment and trial day (intra-observer reliability was 98%). Behavior frequencies were summed for each trial day and subsequently analyzed.

Gross and Microscopic Lung Lesions

Gross lung lesions for the infected group were bilateral, evenly-distributed and characterized by multifocal to locally extensive dark plum-red, well demarcated foci of pulmonary consolidation which varied from moderate to severe; consistent with RSV infection in lambs as previously reported [16, 25, 26]. Lamb 11 had the highest percent total involvement of all lung lobe lesions, while lamb 8 had the lowest (Figure 5).

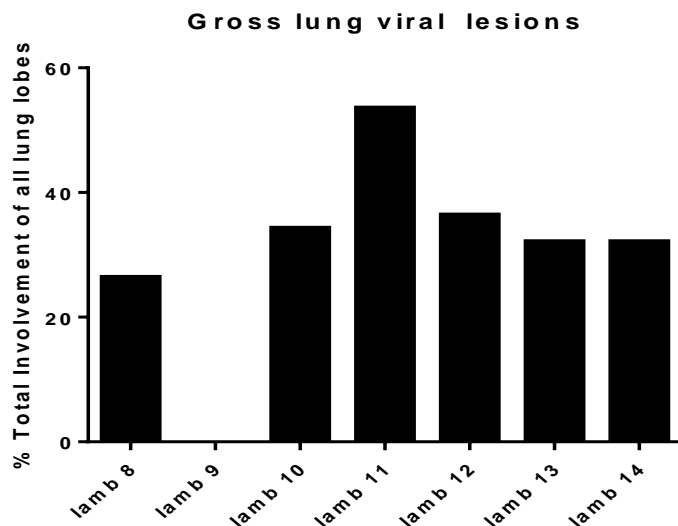


Figure 5. Scoring of gross lesions caused by M37 hRSV infection in neonatal lambs. The percent total involvement of all lung lobe lesions was calculated by adding up estimated percentages of gross lesions given to each lung lobe and dividing by 7, which is the number of lung lobes present in lambs. Lamb 9 was euthanized at day 2 p.i. for having a high fever and seizing.

Microscopic lesions for the infected group consisted mostly of consolidation, bronchitis/bronchiolitis, peribronchial and perivascular lymphocyte infiltration, and to lesser extent neutrophilic inflammation and epithelial hyperplasia as previously has been described for day 8 p.i.[16]. Gross lesions overlapped with the severity of microscopic lesions for each lamb, except for lambs 8 and 11. Lamb 11 had the highest total percent involvement of lung lobe gross lesions (53.57%), but did not have the highest score of microscopic lesions. Lamb 8 had the lowest total percent involvement of lung lobe lesions (26.43%), but had the highest score of microscopic lesions. Also, the microscopic lesions observed for lamb 14 are characteristic of an early RSV infection (Figure 6).

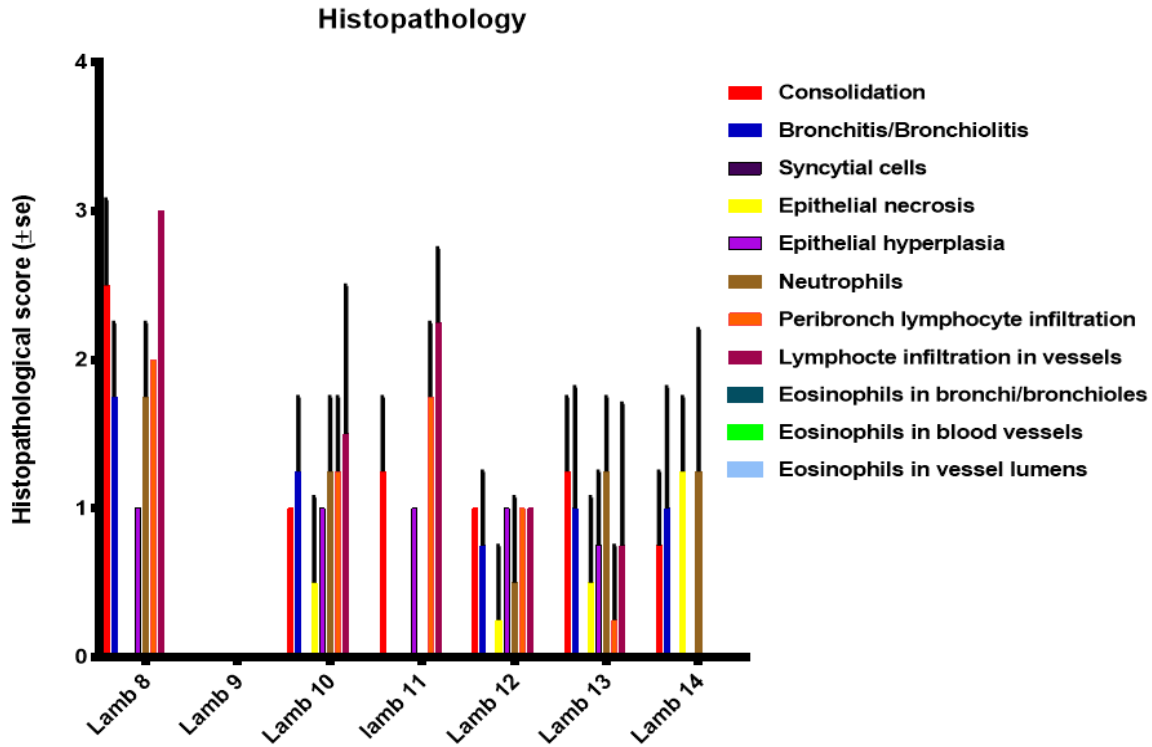


Figure 6. Microscopic lung lesions severity score in M37 hRSV infected neonatal lambs. A histologic score was given by determining percent consolidation followed by conversion to an integer-based consolidation scale used by our laboratory previously [1]: 0% consolidation = 0; 1%-9% consolidation = 1; 10%-39% consolidation = 2; 40%-69% consolidation = 3; 70%-100% consolidation = 4.

Figure 9 continue: Group averages were calculated for alveolar and bronchiolar consolidation scores. In addition to the consolidation score, bronchitis, bronchiolitis, neutrophil infiltration, peribronchiolar and perivascular infiltration of lymphocytes, syncytial cell formation, and epithelial alterations were also scored. Results are indicated as mean \pm standard error for each scored parameter. Lamb 9 was euthanized at day 2 p.i. for having a high fever and seizing.

Lambs 3, 6, and 7 in the non-infected group had gross lesions that were unilateral, and cranial ventral (right cranial and middle lung lobes being mostly affected), and were characterized by multifocal, locally extensive, firm, red-brown areas. These lesions are suggestive of a bacterial pneumonia and were not seen in the infected group. Other lesions in the non-infected group included, congestion and hemorrhage in all lobes of the left lung in lamb 5.

Both of these lesions were recorded and were not sampled for attaining experimental endpoints. Microscopically, only lamb 7 had areas of consolidation with neutrophilic inflammation.

Viral Titers, Viral RNA Replication Levels, and Viral Antigen Expression

The viral titers and viral RNA in BALF and lung tissue, as well as viral antigen expression coincided with each other, but did not overlap with the gross or microscopic lesions. For instance, lamb 14 had the highest viral titers (5.4 log₁₀ FFU/mL), viral RNA levels in BALF (2.1 log₁₀ RNA copies/mL) and lung tissue (2.9 log₁₀ copies/ mg lung tissue), and viral antigen expression in both bronchiolar and alveolar staining (Table 5 and Figure 7); however, lamb 14 did not have the highest gross viral lung lesions. As previously mentioned, lamb 14 also had microscopic lesions associated with early RSV infection. Another example is lamb 11, which had the highest gross viral lung lesions, with no detectable cultivable viral titers, and had the lowest viral RNA levels in BALF (6.63 log₁₀ RNA copies/mL) and lung tissue (4.38 log₁₀ copies/ mg lung tissue), and viral antigen expression in bronchioles.

Table 5. Quantification of RSV replication via infectious focus assay and RT-qPCR in lambs inoculated with M37 hRSV

Lamb #	BALF Viral Culture (log₁₀ FFU/mL)	BALF RT-qPCR (log₁₀ M37 RNA copies/mL)	Lung Tissue RT-qPCR (log₁₀ M37 RNA copies/mg lung)
8	2.68	7.33	5.93
9	0.00*	0.00*	0.00*
10	3.19	7.33	6.16
11	**BDL	6.63	4.38
12	1.60	7.15	5.71
13	2.37	7.17	5.54
14	5.36	9.31	7.46

* Lamb 9 was euthanized at day 2 p.i. for having a high fever and seizing.

** BDL= Below Detection Limit

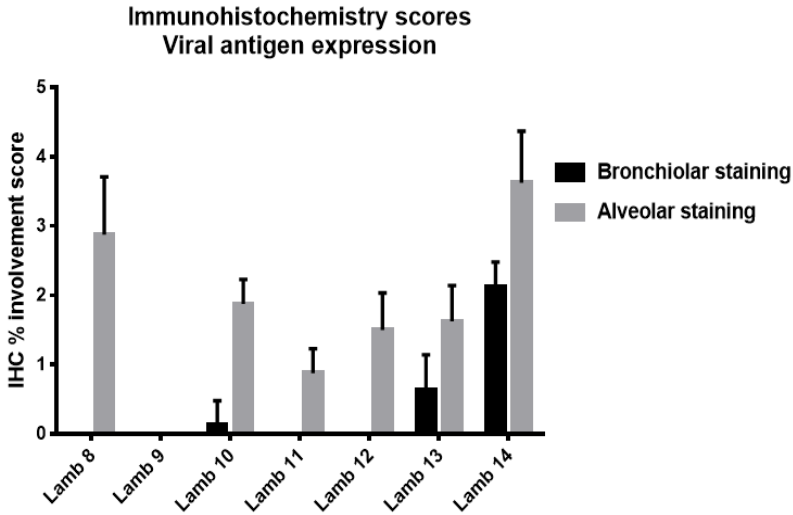


Figure 7. Scoring of RSV antigen expression in infected and non-infected groups. Immunohistochemistry was used to detect viral antigens using an all-antigens polyclonal antibody for RSV. Immunohistochemistry and scoring of RSV antigen expression in lambs inoculated with M37 hRSV. Immunohistochemistry was used to detect viral antigen using an all-antigens polyclonal antibody for RSV. The mean number of virally-infected bronchi/bronchioles and alveoli per field was counted for each day of necropsy.

Chemokine and Cytokine Expression of Lung Tissue

Lung cytokine and chemokine mRNA expression levels were quantified by RT-qPCR and demonstrated varying patterns for the infected and non-infected groups as previously reported for day 8 p.i [16] (Figure 8).

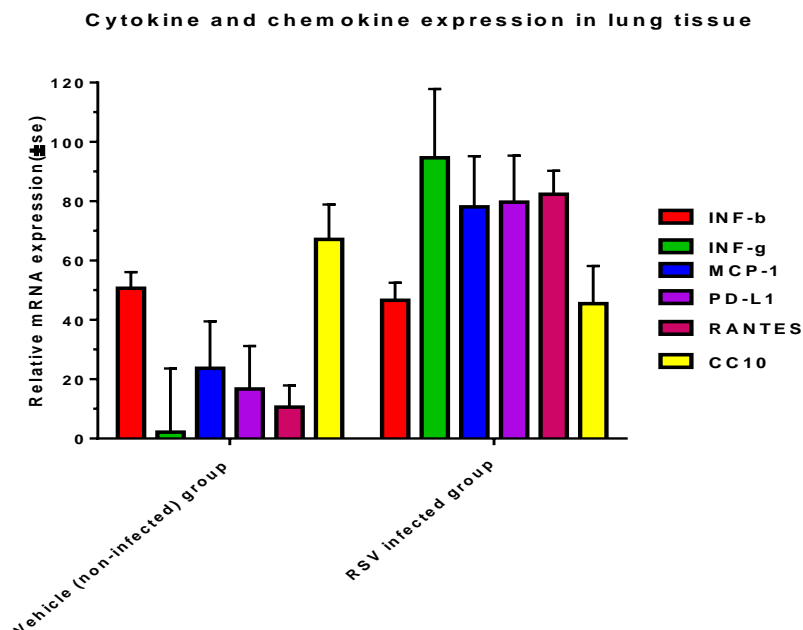


Figure 8. Lung chemokine mRNA expression assessed by RT-qPCR in lambs inoculated with M37 hRSV. Lung tissue obtained from each animal was evaluated for the following mRNA targets: monocyte chemotactic protein 1 alpha (MCP-1 α), interferon beta (IFN- β), interferon gamma (IFN- γ), programmed cell death 1 ligand 1 (PD-L1), and regulated on activation normal T-cell expressed and secreted (RANTES) and Clara cell secretory protein (CC10). Mean relative mRNA expression was calculated for each target with respect to each day of necropsy. Relative mRNA expression means: relative to the total amount of RNA loaded per reaction (which is kept constant) and relative to the values established by the standard curves for each target.

Discussion

The aim of this study was to determine the clinical symptomology and sickness behavior of RSV infected lambs.

Eliminating the heavy sampling schedule from this study still proved to be difficult when determining the clinical signs and sickness behavior of RSV infected lambs, as non-RSV clinical signs of tremors and lameness affecting both groups, as well as bacterial pneumonias in the non-infected group, and variability in RSV infection were present, which confounded the results. Bacterial infections are an inherent problem of the hRSV infection neonatal lamb model, and as

long as they are present, interpretation of clinical data and lamb behaviors should be done with caution when drawing conclusions.

Considering that both groups of lambs had fevers, high respiration rates, bilateral harsh lung sounds, and non-specific RSV clinical signs of lameness and tremors, it's evident that both groups were infected with a pathogen other than RSV. These non-specific RSV clinical signs are suggestive of a systemic bacterial infection. Eventhough, all lambs receive a daily intramuscular antibiotic, bacterial infections still remain a problem when dealing with colostrum free neonatal lambs. This brings up the importance of requiring bacterial culture and sensitivity for all lambs involved in the study. This would help with patterning common pathogens that the lambs succumb to, and hopefully be able to provide more effective antibiotics that could prevent bacterial infections, that in the long run would eliminate unwanted clinical signs and increase the survival rate of lambs to the end of the study.

Besides the non-RSV clinical signs present in both groups, necropsy findings of the non-infected group also had three lambs (3, 5, 7) with lung lesions that were suggestive of a bacterial pneumonia, as well as lamb 6 having congestion and hemorrhage in the lungs. Histopathology, only confirmed a bacterial pneumonia for lamb 7, which had lung consolidation with neutrophilic inflammation. In previous studies (not published), where lambs have died from non-RSV clinical signs and have been submitted to the veterinary diagnostic lab, bacteria culture has yielded for the most part *Escherichia coli*. This was also true for lamb 9, which was euthanized for having a fever and seizing on day 2 p.i. Gross and microscopic findings for lamb 9 were consistent with a meningoencephalitis and a mild interstitial pneumonia. Bacterial culture was done and lesions were attributed to a systemic bacterial infection with *Escherichia coli*. Furthermore, along with bacterial culture and sensitivity, all lambs should have full necropsies,

with histopathology to not only confirm secondary bacterial infections and RSV infection, but also determine if all lambs were infected with RSV at the same time.

Overall, clinical signs and sickness behavior were present in both groups, beginning with general illness signs that were sparse for both infected and non-infected lambs. Fevers were also present in both groups with the lambs in the non-infected group having more prevalent and consecutive fevers. The respiratory rates were variable and unpredictable. High respiration rates were present infrequently in both groups, with bilateral harsh lung sounds being more prominent, but sporadic in the infected group. There was no statistical difference in heart rates, body weights, and milk consumption between both groups.

Expiratory efforts were the only clinical sign observed in the infected group that was not seen in the non-infected group. There was no wheezing observed for either group. As previously mentioned, the only clinical sign that has been observed the most in lambs as a significant clinical marker to monitor for RSV disease is expiratory effort [16, 25-27]. Even though only two lambs in the infected group had expiratory efforts, our hypothesis was that expiratory efforts and wheezing would be more apparent by removing the heavy sampling schedule that previous lamb studies included. It's not certain if expiratory efforts and wheezing may have been exacerbated in previous lamb studies by the stress of handling lambs for blood withdrawals, drug delivery, vital parameters, etc., or by secondary bacterial pneumonias that are an inherent problem of the RSV neonatal lamb model, making these clinical signs more apparent [16].

Behavioral observations indicated that the infected group spent significantly less time lying and more time standing than the non-infected group. It's counterintuitive to the sickness response that sick animals would be standing more rather than lying and conserving energy. A

possible explanation for this behavior is that sick animals seek heat to facilitate fever during the immune response to an infection [7].

From the gross viral lung lesions, it appeared that all lambs had been infected with RSV. However, not all lambs were infected at the same time because the microscopic lesions, specifically for lamb 14, were characteristic of an early RSV infection. Interestingly enough, lamb 14 had the highest viral titers, viral RNA levels in BALF and lung tissue, and viral antigen expression in bronchioles and alveoli. From previous studies, viral titers and viral RNA levels in BALF and in lung tissue are decreasing by day 8 p.i. [16]. Due to the lambs not being infected with RSV at the same time, the gross and microscopic lesions did not overlap with each other or with the viral endpoints as previous studies have reported [16]. Moreover, gross lesions should not be looked at solely to determine lamb infectivity, but rather together with histopathology and viral endpoints to determine if lambs are infected with RSV at the same time.

Depending on the time course of RSV infection and diagnosis, infants can display a variety of clinical symptoms that make it difficult to assess the progression of clinical signs. For the most part, RSV clinical signs in infants and young children are variable, but include wheezing, crackles, rhonchi, tachypnea, nasal flaring, fever, coughing, and intercostal muscle retractions. These symptoms can be associated with bronchiolitis due to RSV and/or bacterial pneumonias [32, 33].

Future lamb studies should make an effort to identify and prevent secondary or opportunistic pathogens in RSV infected and non-infected lambs by doing full necropsies and bacterial culture and sensitivity, as well as viral and bacterial diagnostics to solely demonstrate RSV infection.

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CHAPTER 5. GENERAL CONCLUSION

Kinetics of RSV M37 Strain

In order to utilize the lamb model for therapeutic and vaccine studies, the viral kinetics of RSV M37 strain needed to be first characterized. This study demonstrated that RSV M37 replication in the lower airways of newborn lambs is robust with peak replication on day 3 and sustained until day 6. The viral kinetics coincided with that of lung gross viral and histopathological lesions, as well as viral antigen expression, and respiratory distress that included expiratory effort and wheezing. The chemokine and cytokine profile, specifically IL-8 and IFN- γ , also overlapped with the experimental endpoints discussed above. Peak expression of IL-8 was present at day 6 post-infection and coincided with peak neutrophil influx in the lungs of RSV-infected lambs which indicates that the increased chemokine expression contributed to neutrophil infiltration into the site of intense RSV infection. IFN- γ expression was maximal at day 8 post-infection at a time when peak lymphocyte lung infiltration was noted, which suggests that RSV infection in neonatal lambs results in an initial innate inflammatory response, the peak of which coincides to that of peak disease, and is characterised by IL-8 secretion and neutrophil influx. A suppressed lymphocyte function and increased plasma IL-8 levels were shown to be markers of severe disease in RSV bronchiolitis [1].

These findings, along with the similarities of lamb lung to those of infants in terms of alveolar development, airway branching and epithelium, susceptibility to human RSV strains, lesion characteristics (bronchiolitis), lung size, clinical parameters, and immunity, further establish the neonatal lamb as a model with key features that mimic RSV infection in infants.

Efficacy Evaluation of ALX-0171

The use of neonatal lambs to model RSV infection in human infants has provided a valuable tool to assess vaccines and antiviral drug safety and efficacy. A trivalent Nanobody, ALX-0171, targeting the RSV F-protein was developed by Ablynx and its therapeutic potential was evaluated in newborn lambs infected with RSV M37 strain, following daily ALX-0171 nebulization for 3 or 5 consecutive days. When compared to placebo-treated lambs ALX-0171 treated lambs had a >10,000-fold decline in cultivatable virus, markedly reduced lung viral antigen expression, reduced lung viral lesions and reduced histological changes. Clinical signs were reduced for ALX-0171 treated RSV infected lambs. Overall, ALX-0171 was well-tolerated in RSV infected newborn lambs and results are indicative of the therapeutic potential of ALX-0171 in infants.

RSV Clinical Signs and Sickness Behavior

To determine the clinical response of the therapeutic drugs or vaccines tested in the RSV infected lamb model, the clinical signs and sickness behavior also need to be fully characterized. This study solely looked at clinical signs and sickness behavior through clinical data and video recording of lamb behaviors, without the heavy sampling schedule of previous studies. The results of the clinical signs and sickness behavior were confounded by non-RSV clinical signs of tremors and lameness affecting both groups, as well as bacterial pneumonias in the non-infected group, and variability in RSV infection. Taking into account these non-RSV clinical signs, it's evident that both groups of lambs were infected with a pathogen other than RSV. It's not certain to what degree of impact these clinical signs had on the sickness behavior, but overall, RSV

infected lambs spent significantly less time lying and more time standing than the non-infected lambs. As far as the clinical signs, the only clinical sign that was not associated with the non-infected group was expiratory efforts, which were only seen in two lambs. Expiratory efforts in previous studies have been the most consistent clinical sign to monitor RSV disease [2, 3, 4]. However, the possibility of a bacterial pneumonia leading expiratory efforts should not be ruled out. In this study, only the non-infected lambs had bacterial pneumonias and no expiratory efforts were observed. Considering that not all infected lambs were infected with RSV at the same time, the clinical signs and sickness behavior were further confounded and did not overlap with the gross and histopathological lesions, and viral endpoints observed.

Bacterial infections are an inherent problem of the hRSV infection neonatal lamb model, and as long as they are present, interpretation of clinical data and lamb behaviors should be done with caution when drawing conclusions.

Future Lamb Studies

If the lamb model is to continue to be used in the evaluation of therapeutics and vaccines, future lamb studies should make an effort to identify and prevent secondary or opportunistic pathogens in RSV infected and non-infected lambs by doing full necropsies and bacterial culture and sensitivity, as well as viral and bacterial diagnostics to solely demonstrate RSV infection. If lambs are infected with other than hRSV, caution should be used when making interpretations or drawing conclusions.

Regarding the variability of infectivity in lambs, it might be worth while to characterize the viremia in serum to have a different experimental endpoint to follow the viral kinetics in lambs. This experimental endpoint can also be used to further corroborate the clinical signs and

sickness behavior for the RSV infected group. The cytokine and chemokine profile should also include IL-1, IL-6, and TNF- α for the RSV infected lambs, which are the primary cytokines for the sickness behavior. Along with measuring the virus in serum, the antibody profile of IgG, IgM, and IgA against RSV should be investigated not only in serum, but in also in BALF and lung tissue to further characterized the immune response in the lamb model. It has been reported that nasal IgA from experimentally infected adults with hRSV is the best predictor of RSV protection [5]. A lack of virus specific IgA memory cells in nasal samples has been shown to contribute to reinfection [5]. Virus specific IgA memory cells along with IgG and IgM memory cells should also be characterized in the lamb model.

Our lamb studies using RSV M37 have only gone up to day 8 p.i. It would be interesting to follow the histology, cytokine and chemokine profile, and viral endpoints passed day 8 p.i. to get an idea of the repair process. This may elude to the high risk factor to asthma that infants experience after long term severe RSV disease [6]. Extending the period of the lamb studies would also allow the investigation of reinfection as commonly occurs in humans [7, 8].

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