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Diagnostics in calves with Bovine Respiratory Disease

Usefulness of acute phase proteins and white blood cells in early diagnostics, and comparison of clinical signs and thoracic ultrasonography

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Front-page picture: "Brenda" by Anne Bang Jensen

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Preface

This Master Thesis is a part of the Master's degree in Veterinary Medicine at University of Copenhagen. It corresponds to 30 ECTS points.
The work performed in this project was a part of the project "Robust calves – well begun is half done".
Our project took place between Febuary 2019 and June 2019.

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Abstract

Background: Bovine Respiratory Disease (BRD) is a complex problem in Danish cattle herds. Bovine Respiratory Syncytial Virus (BRSV) is one of the pathogens contributing to BRD. BRSV causes an acute respiratory disease primarily in calves. It gives rise to an economic loss in Danish calf-fattening herds. The objective of this thesis was to determine the correlation between Serum amyloid A (SAA) and Haptoglobin (Hp) in serum to the concentrations in tracheal aspirate (TA) and pharyngeal swab in seemingly healthy calves between 3-34 days old. Furthermore, this thesis aimed to determine how SAA and Hp in serum correlated to lymphocytes and neutrophils in blood in seemingly healthy calves between 3-34 days old. In addition, another objective was to investigate the usefulness of clinical signs and thoracic ultrasonography (TUS) compared to macroscopic findings in calves between 2-23 days old experimentally inoculated with BRSV. Overall in order to identify whether acute phase proteins (APP) could be detectable in TA and pharyngeal swab, and whether this together with TUS could improve early detection of BRD in calves.

Results: Experiment 1: When comparing APPs in serum to APPs in TA or pharyngeal swab or comparing APPs to white blood cells (WBCs) no correlations were present. **Experiment 2:** Rectal temperature and respiration rate increased whereas other clinical signs were intermittent. The ultrasonography score (US) increased throughout the period. TUS compared to macroscopic findings showed some agreement (weighted kappa = 0,378).

Conclusion: The results showed no linear correlation between APPs (SAA and Hp) in serum, TA or pharyngeal swab. The results concerning WBC (lymphocytes and neutrophils) did not show a linear correlation to APPs (SAA and Hp). The results gave rise to reference values for healthy calves between 3-34 days old in Danish herds. The results also showed that mild clinical signs were present together with severe macroscopic findings. This could suggest that further diagnostics were indicated when mild clinical signs were present. Additionally, TUS correlates better with macroscopic findings when more severe pulmonary changes were present. Finally, the study suggests that APPs together with clinical signs and TUS could improve early diagnosis of calves with BRD.

Keywords: Acute phase proteins, Bovine Respiratory Disease, Bovine Respiratory Syncytial Virus, Serum amyloid A, Haptoglobin, White Blood Cells, Thoracic ultrasonography.

Resumé

Baggrund: Bovint Respiratorisk sygdom (BRD) er en kompleks sygdom som udgør en stor økonomisk udfordring specielt i danske slagtekalvebesætninger. Formålet med dette projekt var at undersøge akutfaseproteiner i kalve mellem 3-34 dage gamle i eksperiment 1. Herunder korrelationen mellem akutfaseproteiner, Serum Amyloid A (SAA) og Haptoglobin (Hp) i serum, tracheal aspirat (TA) og pharyngealsvab. Derudover blev korrelationen mellem henholdsvis SAA og Hp i serum og lymfocytter og neutrofile undersøgt. Et andet formål med projektet var at undersøge brugbarheden af kliniske tegn og thorax ultralyd sammenholdt med makroskopiske fund i kalve mellem 2-23 dage gamle eksperimentelt inokuleret med Bovint Respiratorisk Synsytial Virus (BRSV) i eksperiment 2.

Det overordnede formål med projektet var at afgøre om akutfaseresponset kunne detekteres i TA og pharyngealsvab, samt hvilken korrelation der var til serumkoncentrationer, og om dette sammen med kliniske tegn og thorax ultralyd kan forbedre tidlig diagnostik af BRD.

Resultater: Eksperiment 1: Ved sammenligning af akutfaseproteiner i serum med akutfaseproteiner i TA og pharyngealsvab og ved sammenligning af akutfaseproteiner og hvide blodlegemer, var der ingen lineær korrelation til stede. **Eksperiment 2**: Rektaltemperatur og respirationsfrekvens var stigende, mens andre kliniske tegn var intermitterende. Ultralydsscoren steg igennem forsøgsperioden. Der blev påvist en nogenlunde overensstemmelse (vægtet kappa = 0,38). mellem TUS og makroskopiske fund.

Konklusion: Ud fra resultaterne kan det konkluderes at der ingen lineær sammenhæng er mellem akutfaseproteiner (SAA og Hp) i serum, TA og pharyngealsvab. Ydermere blev det vist, at der ikke var nogen lineær sammenhæng mellem hvide blodlegemer (lymfocytter og neutrofile) og akutfaseproteiner (SAA og Hp). Resultaterne gav i stedet anledning til etablering af referenceværdier for raske kalve mellem 3-34 dage gamle i danske besætninger. Resultaterne viste også, at kliniske tegn varierede i kalve, som eksperimentelt var podet med BRSV, hvilket betyder, at milde kliniske tegn var til stede, når man fandt svære makroskopiske fund. Dette kunne tyde på, at yderligere diagnostik er indikeret, når kalven udviser milde kliniske tegn. Derudover korrelerer TUS (thorax ultralyd) bedre med makroskopiske fund, når der makroskopisk ses svære lungeforandringer. Slutteligt tyder resultatet på, at akutfaseproteiner sammen med kliniske tegn og TUS kunne forbedre tidlig diagnostik af kalve med BRD.

Abbreviations

APP = Acute Phase Protein

APR = Acute Phase Response

BAL = Bronchoalveolar lavage

BALF = Bronchoalveolar Lavage Fluid

BRD = Bovine Respiratory Disease

BRSV = Bovine Respiratory Syncytial Virus

CE = Clinical examination

DH = Danish Holstein

DTU = Technical University of Denmark

FBL = fetal bovine lung cells

Hp = Haptoglobin

ics = Intercorstal space

MS = Macroscopic score

PBS = Phosphate buffer saline

PID = Post inoculation day

SAA = Serum Amyloid A

TA = Tracheal aspiration

$TCID_{50}$ = 50% Tissue Culture Infective Dose

TUS = Thoracic Ultrasonography

US = Ultrasonoghraphy score

WBC = White Blood Cells

Introduction

Respiratory disease is a major problem among Danish calves. In Denmark, the incidence is around 30% in closed dairy herds, whereas a much higher incidence rate is seen in open calf-fattening herds [1]. In 2015, respiratory disease in calves accounted for the highest antibiotic use in both dairy herds (73%) and in beef cattle (86%) [2]. Bovine respiratory disease (BRD) is a complex, multifactorial disease process with several factors involved; bacteria, viruses and management [3]. According to one study Bovine Respiratory Syncytial Virus (BRSV) is one of the most common pathogens associated with BRD [1]. Other pathogens often associated with BRD are *Mycoplasma ssp.*, *Mannheimia Haemolytica* and *Pasteurella Multocida*, they can occure as primary or secondary infections, together or seperatly [4, 5]. The overall mortallity rate according to BRD is 5 – 10 %, dependent on the pathogen involved, but [4]. As earlier mentioned management including season of birth, navel dipping, other diseases before the calves turned two weeks, colostrum quality, amount of colostrum given are all part of the BRD complex [3, 6] .

BRD is a complex issue in Danish agriculture and it gives rise to economic losses [7]. In addition to the treatment, the disease also affects the long-term milk production outcome. It has a negative effect on reproduction, increases mortality, reduces growth, and possibly leads to a negative impact on milk production in the first lactation period [2, 5]. Pathologic pulmonary changes are seen in calves suffering from BRD. These changes include atelectasis, edema and interstitial emphysema [8, 9].

Technical University of Denmark (DTU) and SEGES found in 2018 that respiratory disease was the major cause of antibiotic use in feedlot calves <1 year (82%) in Denmark [2]. It is unlikely to avoid all respiratory disease, instead it is more realistic to optimize the strategy of diagnostics and treatment. One study has described that 22% of the calves treated for BRD, were treated more than once [6]. If BRD is detected ealier, it might bring down the risk of repeated treatments. This could result in a reduced antibiotic use, which is of great interest. Furthermore, antibiotics are only indicated when bacteria are prensent. However it is assumed that antibiotics are also used when atelectasis is present, even though atelectasis is not influenced by this treatment. It might be assumed that this use of antibiotics could be avoided if the calves were diagnosed before secondary bacterial infection were suspected. In order to make an early diagnosis, other diagnostic tools such as thoracic ultrasonography (TUS) and pharyngeal swab could be considered. Pharyngeal swab is of great interest, as it is non-invasive, easy to handle and the farmer would get a quick respond whether treatment was indicated. This would open the

possibility that the farmer could screen calves at risk and not only induviduals already showing severe clinical signs.

1. White blood cells

Through the years white blood cells (WBCs) have been used in bovine veterinary medicine. WBCs are often useful when the clinical examination is vague, or when a prognosis is needed [10]. Differential count is more significant than the total WBC count, as the increase and decrease in individual cell types at the same time, could result in an unchanged total count. Therefore, it is important to look at the individual changes [10]. A study imitating the cytokine release according to tissuedamage, showed that cattle injected with interleukin 1 increased in WBC within 48 hours [11]. Neutrophils are the most common WBC and is dominant in young calves [10]. The mature neutrophils are stored in the bone marrow and responds quickly in case of infection. Normally, neutrophils will not be found in healthy tissue but will be highly represented in infected tissue and therefore seems more reliable [12]. Besides neutrophils, lymphocytes are of great interest because they, as mentioned earlier, are initiated by cytokines which are known to react quickly, resulting in a rapid lymphocyte response [12]. The quick response and the presence in infected tissue, makes lymphocytes and neutrophils of great interest in this thesis.

2. Acute phase response

The Acute phase response (APR) is another mechanism worth investigating according to BRD. The APR is one of the major and immediate mechanisms induced and is a complex series of reactions to prevent further tissue damage [13]. When tissue damage occur cytokines among these Interleukin 1, Interleukin 6, and Interleukin 12 are released [12]. One study showed that calves injected with Interleukin 1 had an increase in acute phase proteins (APPs) at different times after injection, one APP after 18 hours and another after 48 hours [11]. Organs such as the brain and the liver initiate the APR, causing fever, anorexia, somnolence, and increased liver metabolism, affecting the APP concentration [13–15]. According to one study, the APR reflects the intensity of inflammation in humans [16]. Further, supported by two studies who proved that APP (Haptoglobin) concentration in serum correlated with the severity of BRD in calves [3, 17]. This correlation together with the rapid response are of great interest, as small changes in APP concentration could be due to infection.

It is well understood that some APPs decrease (negative APPs) and some APPs increase (positive APPs) during an APR, but it is uncertain whether the decrease is advantages to the host [16, 18]. Two positive APPs described in cattle are Serum Amyloid A (SAA) and Haptoglobin (Hp) [3, 18–20].

2.1 Haptoglobin

Hp is an important APP in cattle [3, 19]. It is favorable because it is present at insignificant levels in healthy cattle and is able to increase 100 fold in cattle suffering from acute infectious conditions [21, 22]. It is demonstrated that Hp is detectable even before clinical signs appear [17]. Another study showed that Hp concentration in serum increased significantly after 24 hours in calves injected with Interleukin 1, whereas inappetence and increase in rectal temperatur were present after four and six hours after injection, respectively [11]. Calves suffering from BRD had higher levels of Hp in serum than healthy controls [23], further a correlation was seen between Hp concentration in serum and severity of BRD [3, 17]. Therefore, Hp may be useful as a diagnostic tool with its large increase and relation to sick calves.

2.2 Serum Amyloid A

Another important APP in cattle is SAA [3, 19]. There is a significant difference in SAA concentrations between healthy cattle and those with infectious diseases [24]. One study showed that mild BRSV disease was associated with a low concentration of SAA in serum and severe BRSV disease was associated with a high concentration of SAA in serum. The study also showed that SAA increased five days after the calves were inoculated with BRSV [3]. This could propose that SAA is a useful APP in early diagnostics of BRD, because it does not recuire severe respiratory clinical signs to be detectable.

Usually, Hp and SAA are detected in serum [3, 18–20]. A study found that SAA in serum was more sensitive to BRSV-infection compared to Hp in serum, even though Hp had the relatively largest increase. Their study also showed that Hp concentrations in serum followed SAA concentrations in serum, although it was not possible to detect Hp in calves who had a low SAA concentration [3]. SAA and Hp are assumed to increase around the same time, SAA might even increase slightly before Hp, as SAA seem more sensetive to BRSV. Knowing that Hp starts increasing 18 hours after tissuedamage occur [11], it might be assumed that SAA increses slightly before.

Besides APPs in serum, studies have detected both SAA and Hp in bronchoalveolar lavage (BAL) [25, 26]. In addition, one of the studies concludes, that some APPs in bronchoalveolar lavage fluid (BALF) are useful markers in calves with BRD, because SAA and Hp did increase significantly in calves suffering from a bacterial (e.g. *mycoplasma*) bronchopneumonia [25]. As a local APP respons is detectable in BALF in BRD it is likely that APPs can be detected easier. Besides Hp detection in serum and BAL, some studies proved it possible to detect Hp in porcine saliva samples [27–29]. They used both serum and saliva to detect Hp changes in pigs suffering from Porcine Respiratory and Reproductive Syndrome virus and in healthy pigs. They found a statistically significant increase in the diseased group and not in the healthy group. This increases the likelihood that SAA also is detactable in saliva. Furthermore, that both SAA and Hp can be detected in bovine saliva, and therefore be assumed to be used in an early and non-invasive diagnosis of BRD.

Overall a better understanding of BRD would increase health and future production outcome in Danish herds of cattle. In order to reach a greater understading of BRD, it is nessesary to focus on minor parts of the disease. Earlier mentioned BRSV is one of the factors contributing to the complex BRD. The following will focus on BRSV in order to accomplish a better understanding of BRD [1].

3. Bovine Respiratory Syncytial Virus

BRSV is an enveloped RNA virus which belongs to the family Paramyxoviridae, subfamily Pneumovirinae and genus Pneumovirus [4].

3.1 Epidemiology

BRSV affects a wide age range, though mostly calves between 3-9 months [4, 30]. Less severely affected calves show tachypnoea, ocular serous discharge, dry muzzle, reduced activity, anorexia and fever up to 40°C, but sometimes more severe disease outbreaks with dyspnea and death are seen [7]. The incubation period of BRSV is between 2-5 days [4, 31]. The transmission occurs via aerosols and direct contact, therefore management procedures play a major role in limiting disease outbreaks. Many animals of different age groups housed together increase the risk of disease outbreaks. BRSV outbreaks occur more frequently from October to March when rain and wind is intermittent, and temperature varies [4, 30]. The mortality rate can reach up to 20% [4, 31]. The mortality rate depends on how many of the calves develop respiratory distress or acquire a secondary bacterial infection [32].

3.2 Pathogenesis

BRSV replicates mostly in the ciliated epithelium of the respiratory tract. There is destruction of the bronchiolar epithelium, which results in necrotizing bronchiolitis. BRSV causes an induction of pro-inflammatory chemokines and cytokines [4]. It is uncertain whether the host's immune response is responsible for the pathological changes seen in the pulmonary tissue [31]. Necrosis, cellular debris, and exudate in the pulmonary tissue facilitate a potential secondary bacterial infection [4]. Additionally, BRSV is most often isolated from the cranioventral part of the lungs [33].

3.3 Clinical observations

Infection with BRSV can cause a wide range of respiratory clinical signs often seen in a biphasic pattern, meaning mild respiratory disease followed by apparent recovery but then clinical signs develop again [4]. The clinical signs in calves include fever, nasal discharge, coughing [4, 34], lacrimation, respiratory distress [4], sensitivity of the larynx/and or trachea, abnormal and/or abdominal breathing, abnormal lung sounds [34], and anorexia [7].

3.4 Thoracic ultrasonography

In addition to clinical signs, TUS can be used as a non-invasive diagnostic tool. It has been proven accurate and successfully confirms lung consolidation in calves with a respiratory score = 0 [35], the respiratory score was based on normal rectal temperature, no nasal or ocular discharge, no coughing and normal ear position [36]. Furthermore, one study suggested that Se = 79,4% and Sp = 93,9 % [37], another study found Se = 94% (95% CI: 69 - 100%) and Sp = 100% (95% CI: 64 - 100%) [38]. Consolidation is seen in BRSV as atelectasis [9], which increases the likelihood that TUS also can detect consolidation in relation to BRD. Peripheral consolidation can be seen as comet tail artifacts. Comet tails may also occur because of edema, exudate, pneumonia, mucus, pleuritis, neoplastic infiltration or interstitial fibrosis [39–41].

3.5 Macroscopic pulmonary findings

Characteristically, naturally occurring BRSV often causes lobular bronchointerstitial pneumonia. The lesions in the cranioventral part and the caudodorsal part of the lungs often differ in appearance. The lesions in the cranioventral part appear as atelectasis, deep red color or dappled look and a rubbery texture [9]. Caudodorsal lesions appear as interstitial emphysema, edema, more heavy and firmer texture [9, 42]. During a necropsy of sixteen calves inoculated with BRSV, multifocal, firm, purple-red areas of consolidation which did not cross lobular separation

were found. Furthermore, the study showed lesions primarily in the cranioventral lobes which support the characteristic findings in calves infected with BRSV [43].

The clinical examination is the basis of veterinary diagnostics. WBC works as supplementary diagnostics if clinical signs are vague or if a prognosis is relevant [10]. To make the diagnostics according to BRD more precise TUS and APP concentrations are of interest. APPs was detectable in pig saliva but the litterature in bovine medicine is inadequate, which leads to the aims of the thesis.

4. Aim and objective of the study

The aim of this thesis was to improve early and non-invasive detection resulting in early diagnosis of BRD. This knowledge could be used in the detection of BRD in calves and thereby increase health and future production outcome.

Therefore, the objectives of the study presented in this thesis are:

- To determine the correlation between APPs (SAA, Hp) in serum, tracheal aspirate (TA) and pharyngeal swab in healthy calves between 3-34 days old.
- To determine the correlation between APPs (SAA, Hp) in serum and WBCs (neutrophils, lymphocytes) in blood in healthy calves between 3-34 days old.
- To determine if pulmonary ultrasonography findings and clinical signs in calves between 2-23 days old suffering from BRD, as a consequence of BRSV, reflect postmortem macroscopic findings.

The aim and objectives result in the following h_0:

1. There is no correlation between SAA in serum, TA and pharyngeal swab in 90 healthy calves between 3-34 days old.
2. There is no correlation between Hp in serum, TA and pharyngeal swab in 90 healthy calves between 3-34 days old.
3. There is no correlation between APPs (SAA, Hp) in serum and WBC (neutrophils, lymphocytes) in blood in 90 healthy calves between 3-34 days old.
4. Ultrasonography and clinical signs do not reflect postmortem lesions in calves between 2-23 days old experimentally inoculated with BRSV.

Materials and methods

Data for this thesis were collected from two different populations. Experiment 1 contained a reference group that consisted of 90 presumed healthy Danish Holstein (DH) calves 3-34 days old. Experiment 2 was comprised of eight calves 2-23 days old participating in a challenge study where they were experimentally inoculated with BRSV.

Experiment 1

1.1 Study design and herd selection

Data for this thesis were collected as a cross sectional study in nine Danish dairy herds from October 2018 to February 2019. The participating herds are a convenience sample based on: the farmers' willingness to participate, obtaining a herd size large enough to ensure enough calves at sampling time, and location. The study population was organized as a cluster of dairy farms that all delivered calves to the same feedlot.

1.2 Animals and sampling

All single housed calves were taken into consideration. The single huts were not isolated, so the calves were able to have contact with the calves on both sides. Both bulls and heifers were included. The calves underwent a clinical examination (CE) and TUS. Based on the CE, calves were only included if they did not show any of the following clinical signs: purulent nasal and/or ocular discharge, a rectal temperature above 39,0°C, abnormal breathing or abnormal sounds during auscultation and were given a ultrasonography score (US) of 0 or 1 [35]. TUS was performed on non-sedated calves, the hair coat was not clipped and ethanol was used as contact medium. The entire lung field was scanned from the 12th intercostal space (ics) cranial to the first ics on both sides. The scoring system and where the calves were scanned can be seen in appendix A.

The included calves were sedated with Xylazine (0,06 mg/kg). Blood was drawn from v. jugularis (three serum: 8,5mL, SST^{TM}II *Advance*, Ref.: 367953, one citrate: 2,7 mL, 9NC 0.109M Buffered Trisodium Citrate, Ref.: 363048 and two EDTA: 4mL, K2E (EDTA) 7,2mg, Ref.: 368861, BD vacutainer, UK) with a vacutainer system and a nonguided TA was performed with a calf flush catheter (proVETnordic, Ref.: 20096). The catheter was placed in the trachea until resistance. When feeling resistance, 50 mL isotonic NaCl was flushed into the lungs and as much as possible was aspirated and distributed in to a 50 mL plain tube (Ref.: 62.547.254,

Sarstedt AG & Co. KG, Germany) and two EDTA tubes. Finally, the calves were fixated, and four cotton swabs were placed as far in pharynx as possible and rotated until soaked in saliva. The cotton swabs were placed in cryotubes containing 1 ml phosphate buffer saline (PBS) each so the cotton was covered. After the samples were taken, they were marked for later identification

1.3 Preparation and sample management

The performed diagnostic tests and following processes carried out in the laboratory are described in the following as well as the focus on the different samples.

Pharyngeal swab

The sample was stored for 24 hours at room temperature, then the cotton swab was removed, and the fluid was frozen at -80°C until analysis. The purpose of the pharyngeal swab was to analyze APPs (SAA and Hp).

Blood samples

The blood was centrifuged (Hettich EBA 20, Andreas Hettich GmbH & Co., Tuttlingen, Settings: equilibrium, Speed = 1800G, RPM = 43, 10 min, room temperature), and serum was removed to four 2 mL cryotubes and stored in the freezer (-80°C) until analysis for APPs (SAA, Hp). From the EDTA stabilized blood, two hemacolor (Color steps: Methanol reinst, Chemsolute, Art.: 1462.2511, Hemacolor solution 2, 1.11956.2500, Merck KGaA, Hemacolor solution 3, 1.11957.2500, Merck KGaA) stained blood smears (Microscope slides, Labsolute, Cover glasses, Th. Geyer GmbH & Co. KG) were made, marked and fixated with glue (Pertex, Ref: 801, Histolab) for later reading. WBC count and differential count for neurophils and lymphocytes were performed on the blood smear.

Tracheal aspirate

The TA fluid was centrifuged (Hettich EBA 20, Andreas Hettich GmbH & Co., Tuttlingen, Settings: equilibrium, Speed = 300G, RPM = 18, 20 min), and the supernatant was distributed in four 2 mL cryo tubes, labeled and frozen until an ELISA analysis for APPs (SAA, Hp).

Data analysis

Data management and analysis were performed in the open-source statistical programe “R”. Linear regressions were made from the data collected. All regressions were tested by a residual and a quantile-quantile plot to confirm whether a linear regression was likely. Both plots are part of a model control. If the values distributes equally around zero in the residual plot a quantile-quantile plot is indicated. The quantile-quantile plot will establish if the data are normal distributed. If the

data are distributed equally around zero in the residual plot and are normal distributed, it might be assumed that the model/regression is linear.

Experiment 2

1.1 Study design and herd selection

The data of this thesis were collected in the period between 28/3-2019 and 8/4-2019 with calves purchased from one dairy herd on Zealand, Denmark. The calves had to be clinically healthy and born within the same month. The dams had a positive BRSV titer, which confirmed that the herd had BRSV.

1.2 Animals and housing

Eight conventional, colostrum-fed 2-23 days old DH calves were purchased from one herd. Five days (day -5) before inoculation with BRSV, the calves arrived and were stabled in an isolation unit. The isolation unit was a calf hut (13,5m^2) (CNAgro, Denmark). The following management procedures were made: fed unlimited milk replacer (Elitekalv1, Trouw Nutrition) three times a day using a calvex milk taxa (Calvex, Denmark), free access to water, feed concentrate, silage, and straw was given when needed. The calves were clinically healthy at arrival meaning no fever (<39.0°C), no nasal or ocular discharge, no coughing, a normal vesicular respiration based on auscultation, and no pathological pulmonary findings on TUS using a GE LogiqE portable scanner (Chicago, IL) equipped with a 14 × 48 mm GE i739L-RS linear transducer with bandwidth of 3.5 to 10.0 MHz..

1.3 Inoculation

The experimental model is described in detail elsewhere [44]. In summary, the calves were sedated with Xylaxin (0,05 mg/kg) IV and inoculated once by aerosol exposure (Waechtomat inhalator VM 82, Kruuse, with most droplets less than 3 μm, 5 ml (2.3×10^5 50% Tissue Culture Infective Dose ($TCID_{50}$)/ml) loaded into the chamber) in 12 minutes combined with administration of five ml (2.3×10^6 $TCID_{50}$/ml) of the inoculum trough a AMBU bronchoscope (Ambu® aScope™ 3 Large 5.8/2.8, Bending capabilities: 140° up, 110° down, Channel average inner diameter: 2.8 mm, Insertion cord length: 600 mm, Insertion cord diameter: 5.8 mm, Distal end diameter: 6.2 mm, Min. instrument channel width: 2.6 mm). The titer of the inocula was confirmed by titration before inoculation. The inoculum consisted of a low cell culture passage of BRSV in fetal bovine lung cells (FBL) of the BRSV isolate designated DK9402022 [45, 46].

To obtain sufficient amounts of isolate for inoculation and a higher virual titer, the BRSV cell culture isolate from FBL was further passaged on Vero cells (African green monkey kidney cells).

1.4 Sampling

Before inoculation all calves had a TUS and a CE performed (T_{-96}). After the inoculation (T_0), the calves were monitored every eight hours. This monitoring consisted of CE (shown in appendix B) performed by two different examiners, blood sampling and pharyngeal swab. Furthermore, TUS and TA were performed every 48 hours. The calves had a long-term high flow 16G catheter (HiFlow long-term IV catheter 16G x 3.00", Equivet, Denmark) in v. jugularis to ease the blood sampling. The calves were fixated, and blood was drawn (30 mL (34mL), single use, exentric 3-comp, syringe, Kruuse, Denmark and 19gx1½, 1,1x40mm, white, needle, Kruuse, Denmark) and put into two serum tubes (8,5 mL, SST^{TM}II *Advance*, Ref.: 367953), one citrate (2,7 mL, 9NC 0.109M Buffered Trisodium Citrate, Ref.: 363048) and one EDTA (4 mL, K2E (EDTA) 7,2 mg, Ref.: 368861) The catheter was flushed with NaCl with Heparin added afterwards. TUS and TA were always performed last, because of sedation. The entire lung field was scanned from the 12th ics cranial to the first ics on the right side, with the hair coat untouched and ethanol used as contact medium [35]. Scoring systems can be seen in appendix A. A guided bronchoscope (Ambu® aScope™ 3 Large 5.8/2.8, Bending capabilities: 140° up, 110° down, Channel average inner diameter: 2.8 mm, Insertion cord length: 600 mm, Insertion cord diameter: 5.8 mm, Distal end diameter: 6.2 mm, Min. instrument channel width: 2.6 mm) with a flushing mechanism was placed as far down in the trachea as possible. A maximum of 130 mL isotonic NaCl was flushed into the lungs to aspirate at least 15 ml. TA. TA was distributed into two 4 mL EDTA tubes to stabilize the cells and a plain tube. The tubes were marked with identification.

The calves had a CE (shown in appendix B) and each category was given a specific score. If the calves did not drink when they were fed milk replacer it was noted by the feeder. While the calves were eating, the rectal temperature was measured to avoid added stress. The amount and character of nasal- and/or ocular discharge were noted. If possible, the respiration rate was counted, and the cleanliness caudally checked before entering the calf hut. After entering the hut, the calves were captured one at a time with as minimal stress as possible. The pulse was counted, the larynx was palpated to provoke coughing if any and the lnn. mandibularis and lnn. cervicalis superficiales were palpated. The conjunctiva were inspected, degree of dehydration was determined, and auscultation of the lungs was performed.

The monitoring lasted until the calves developed consolidation on TUS or developed three or more of the following criteria: purulent nasal discharge, dyspnea, was depressed, stopped eating or had a rectal temperature >40,5°C. When the calves developed consolidation or three or more of these criteria, they were sedated with Xylazine (0,05 mg/kg) IV and humanely euthanized with Pentobarbital (125 mg/kg) IV. Ultrasound was always performed before euthanizing the calf. In this thesis, only CE, TUS and macroscopic findings were included.

Postmortem analyses

Euthanized calves were stored in a cold-storage room and had a necropsy within 24 hours. The "plucks" (tongue, cervical and thoracic vicera *en mass*) [47], were removed, the lungs were inspected and the lesions was scored in reference to appearance and distribution, cf. Table 3. The macroscopic score (MS) was determined by the authers of this Master Thesis. The macroscopic findings are described and scored in Table 4.

Results

Experiment 1

In total 92 DH calves from nine different herds met the inclusions criteria. Two calves at age 39 and 50 days were excluded to minimize the age range, resulting in 90 calves between 3-34 days old (14,14 ± 6,57). The experiment included 40 bulls and 50 heifers. There were included 13 calves, who had pus on the flushing catheter after it was flushed, serous nasal or ocular discharge, or a swollen umbillicus.

1. Serum Amyloid A and Haptoglobin compared to age

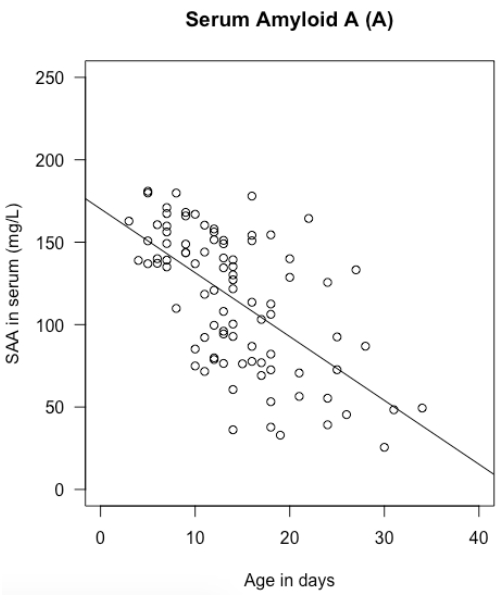
The SAA concentration in serum ranged from 25,57 mg/L up to 180,94 mg/L (115,41 mg/L ± 41,68 mg/L) (Fig. 1A). It was likely that there was a linear correlation between SAA in serum and age. The linear regression was testet with a residual plot and the residual plot and a quantile-quantile plot. The plots did not reject the linear regression model. Furthermore, there was a significant correlation between SAA in serum and age ($p = 1{,}67*10^{-10}$), resulting in a decreasing SAA concentration in serum as age increased.

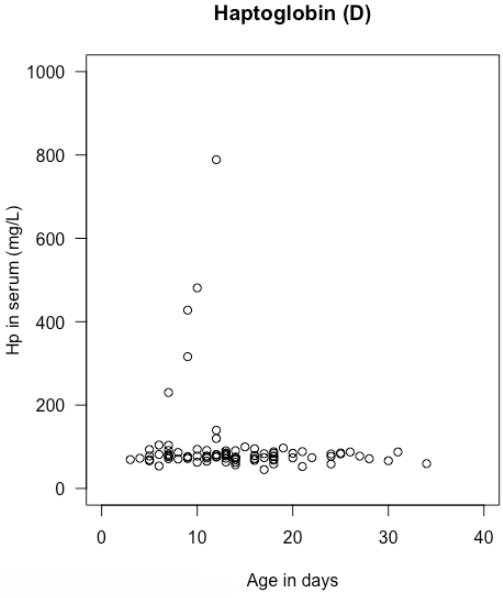
In TA the SAA concentration ranged from 0,0 mg/L up to 0,24 mg/L (0,054 mg/L ± 0,045 mg/L) (Fig. 1B). In the pharyngeal swab the SAA concentration ranged from 0,0 mg/L up to 0,73 mg/L (0,19 mg/L ± 0,13 mg/L) (Fig. 1C).

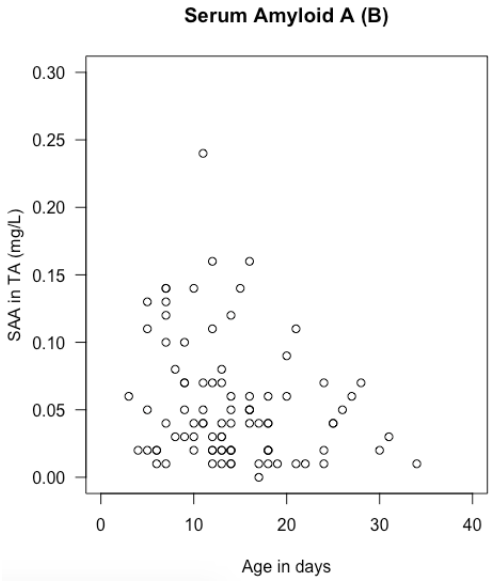
There was no linear correlation between age and SAA concentration in TA and SAA concentration in pharyngeal swab, respectively, because the residual plot rejected the linear regression model. The values were diffusly distributed within 0,0 mg/L – 0,16 (Fig. 1B) mg/L and 0,0 mg/L – 0,48 mg/L (Fig. 1C). There was respectively one calf with a higher SAA concentration in TA and two calves with a higher SAA concentration in the pharyngeal swab compared to the rest of the calves.

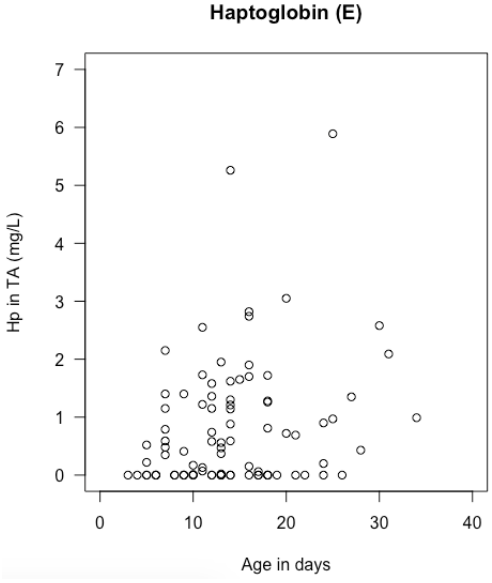
The Hp concentration in serum ranged from 45,14 mg/L up to 788,75 mg/L (98,67 mg/L ± 97,59 mg/L.) (Fig. 1D). In TA the Hp concentration ranged from 0,0 mg/L up to 5,89 mg/L (0,80 mg/L ± 1,08 mg/L) (Fig. 1E). In the pharyngeal swab it is shown that the Hp concentration ranged from 0,0 mg/L up to 5,53 mg/L (1,90 mg/L ± 1,32 mg/L) (Fig. 1F). There was no linear correlation between age and Hp concentration in both serum, TA and pharyngeal swab, because the residual plot rejected the linear regression model.

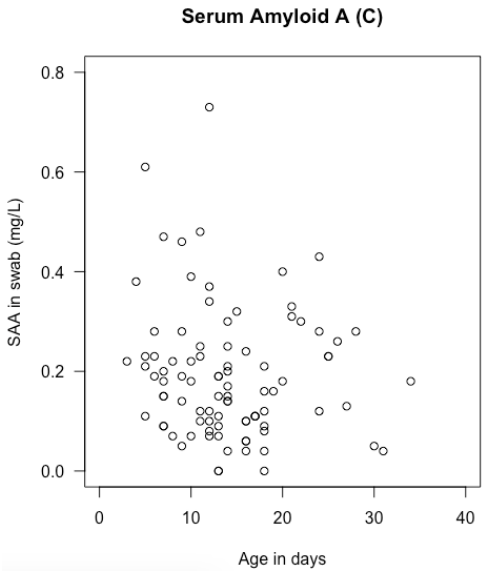
The Hp concentrations in serum in the majority of the calves were closely distributed within 45,14 mg/L – 139,71 mg/L, but five calves differed (Fig. 1D). Both Hp concentration in TA and pharyngeal swab values were diffusly distributed within 0,0 mg/L – 3,05 mg/L and 0,0 mg/L – 3,80 mg/L. However, there were respectively two calves with a higher Hp concentration in TA and six calves with a higher concentration in swab (Fig. 1E and 1F).


Serum Amyloid A (A)
SAA in serum (mg/L)
Age in days


Haptoglobin (D)
Hp in serum (mg/L)
Age in days


Serum Amyloid A (B)
SAA in TA (mg/L)
Age in days


Haptoglobin (E)
Hp in TA (mg/L)
Age in days


Serum Amyloid A (C)
SAA in swab (mg/L)
Age in days

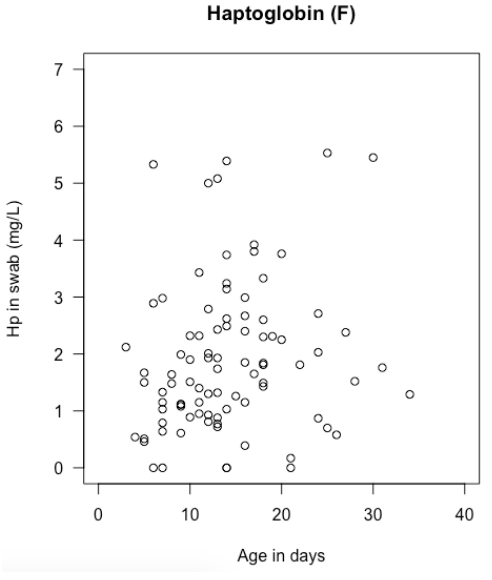

Haptoglobin (F)
Hp in swab (mg/L)
Age in days

Fig. 1 Plots of (**A**) Serum Amyloid A concentraion in serum (mg/L), (**B**) Serum Amyloid A concentration in tracheal aspirate (mg/L), (**C**) Serum Amyloid A concentration in pharyngeal swab (mg/L), (**D**) Haptoglobin concentration in serum (mg/L), (**E**) Haptoglobin concentration in tracheal aspirate (mg/L), and (**F**) Haptoglobin concentration in phanryngeal swab (mg/L) in 90 seemingly healthy Danish Holstein calves between 3-34 days old in experiment 1.

Concentrations of SAA and Hp in TA from this thesis and two other studies are listed in Table 1 [25, 26].

Table 1. Concentrations of Serum Amyloid A (SAA) and Haptoglobin (Hp) in tracheal aspirate (TA) and method from 90 seemingly healthy Danish Holsteins calves between 3-34 days old and reference values and methods according to Coskun *et al.*, (2012) and Prohl *et al.*, (2015).

	This study	Coskun *et al.* (2012)	Prohl *et al.* (2015)
SAA in TA	**0,0 – 0,24** mg/L (**0,053 ± 0,045** mg/L)	Healthy: **0,032 ± 0,01** mg/L Bronchopneumonia: **0,737 ± 0,1** mg/L	-
Hp in TA	**0,0 – 5,89** mg/L (**0,80 ± 1,08** mg/L)	**0,03 ± 0,007** mg/L	**0,003 – 0,347** mg/L
Method	Endoscopic. Inserted until resistance.	Feeding tube with inner diameter = 2,8mm, trans tracheal. Inserted until resistance	Endoscopic. Inserted until resistance.

2. Serum Amyloid A and Haptoblobin correlations

There was no linear correlation between SAA concentration in serum and TA with values diffusely distributed (Fig. 2A) or between Hp concentrations in serum and pharyngeal swab (Fig. 2B). There was neither a linear correlation between SAA in serum and pharyngeal swab or between Hp in serum and TA. The graphs are shown in Appendix C. There were no linear correlations, as the residual plot rejected the linear regression models.

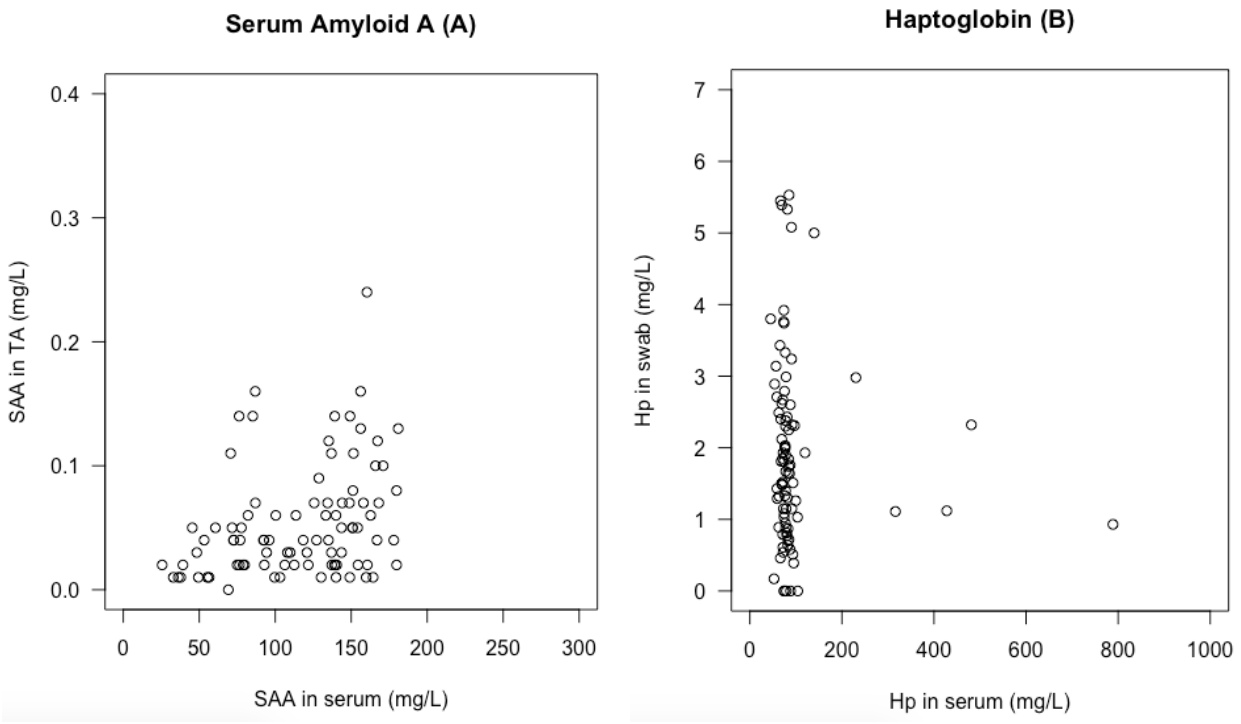


Fig. 2 Plots of (**A**) Serum Amyoloid A concentraion in serum (mg/L) compared to Serum Amyloid A in tracheal aspirate (mg/L) and (**B**) Haptoglobin concentration in serum (mg/L) compared to Haptoglobin in a pharyngeal swab (mg/L) in 90 seemingly healthy Danish Holstein calves between 3-34 days old.

3. Lymphocyte and neutrophils compared to age

The lymphocyte concentration in blood ranged from $1{,}7\times10^9$/L up to $7{,}7\times10^9$/L ($3{,}98\times10^9$/L ± 1,25 10^9/L) in calves between 3-34 days old (Fig. 3A). The neutrophil concentration in blood ranged from $0{,}46\times10^9$/L up to 8.57×10^9/L ($4{,}61\times10^9$/L ± $2{,}57\times10^9$/L) (Fig. 3B).

Values were diffusely distributed, lymphocytes within $1{,}7\times10^9$/L – $7{,}7\times10^9$/L and neutrophils within $0{,}46\times10^9$/L – $8{,}57\times10^9$/L (Fig. 3A and 3B). However, there were respectively four calves with higher neutrophil concentrations in blood. It is likely that there is a linear correlation between age and lymphocytes in blod. The linaer regression was testet with a resiudal plot in R and the residual plot did not reject the linear regreession model. Neither did the quantile-quantile plot. There was a significant linear correlation between lymphocytes in blood and age (p = 0,033), resulting in an increasing lymphocyte concentration in blood as age increased.

There was no linear correlation between age and neutrophil concentration in blood, because the residual plot rejected the linear regression model.

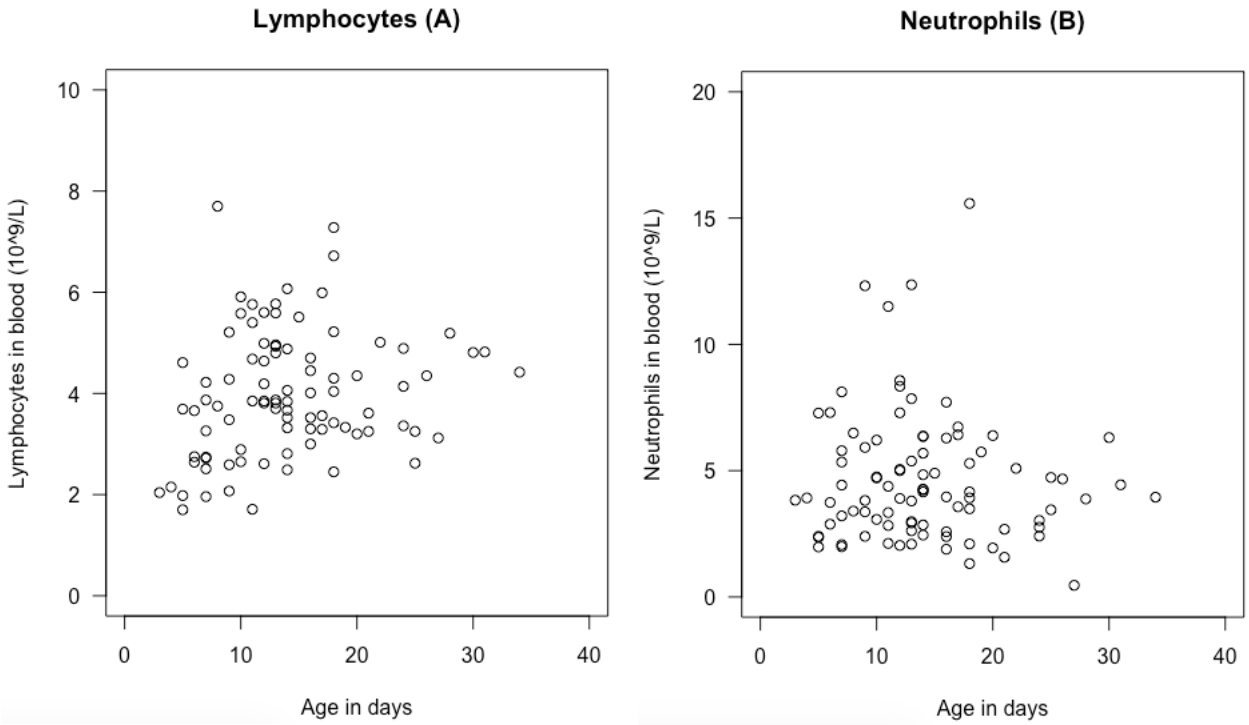


Fig. 3 Plots of (**A**) Lymphocyte concentration in blood (10^9/L) and (**B**) Neutrophil concentration in blood (10^9/L) in 90 seemingly healthy Danish Holstein calves between 3-34 days old.

4. Acute phase proteins and white blood cell correlations

There was no correlation between SAA in serum and lymphocytes in blood with values diffusely distributed (Fig. 4A) and no correlation between Hp in serum and neutrophils in blood as there was generally seen a constant Hp concentration in serum, but increasing neutrophil concentration in blood (Fig. 4B). The same was found when comparing Hp in serum to lymphocytes in blood or when comparing SAA in serum and neutrophils in blood, graphs are in Appendix C. When comparing APPs (SAA and Hp) to WBC (lymphocytes and neutrophils) no linear correlation was found, as the residual plot rejected the linear regression model.

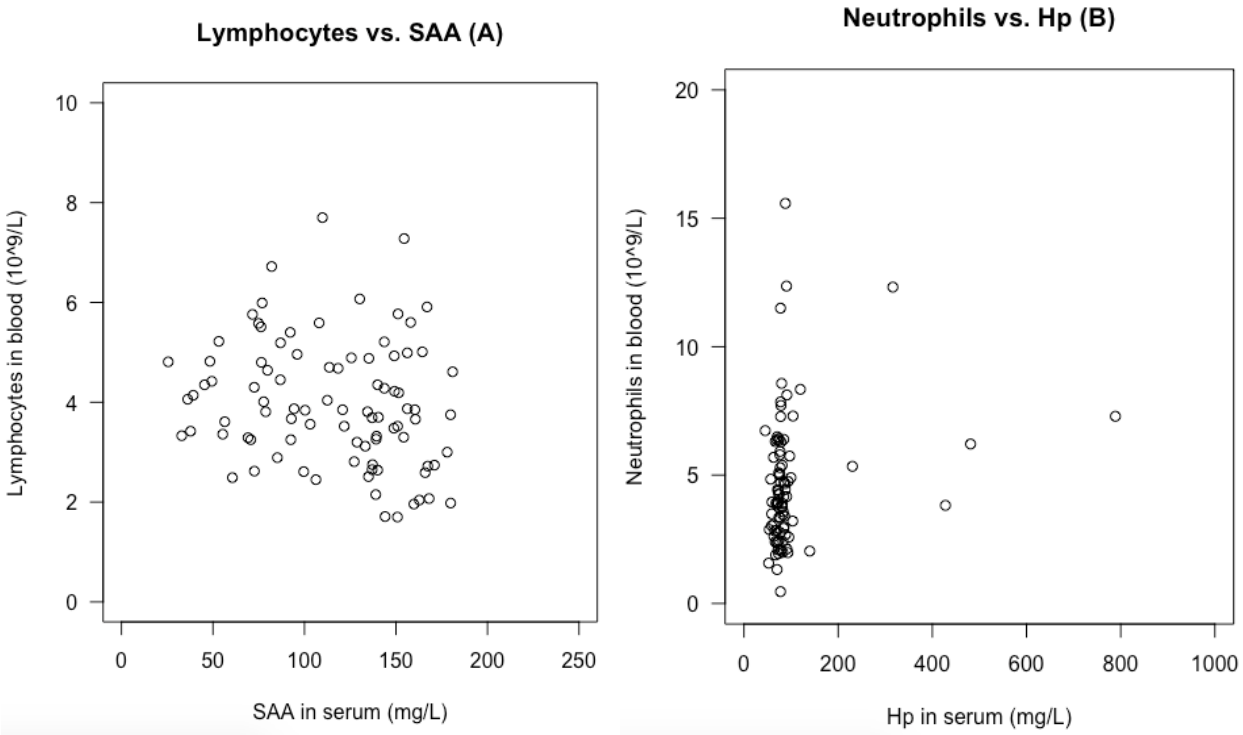


Fig. 4 Plots of (**A**) Serum Amyoloid A concentraion in serum (mg/L) compared to lymphocytes in blood (10^9/L) and (**B**) Haptoglobin concentration in serum (mg/L) compared to neutrophils in blood (10^9/L) in 90 seemingly healthy Danish Holstein calves between 3-34 days old.

Experiment 2

Experiment 2 was a challenge study, where the calves were inoculated with BRSV at day 0. The experiment consisted of eight healthy DH calves between 2-23 days old. The experiment included 4 bulls and 4 heifers. The calves were monitered as described under sampling.

1. Ultrasonography

All calves had a TUS before being euthanized. The ultrasonography findings in this thesis have been categorized in a scoring system as described in [35] and shown in Table 2.

Table 2. Classification (0-5) used to define pulmonary lung lesions on Thoracic ultrasonography in Danish Holstein calves between 2 - 23 days old experimentally inoculated with Bovine Respiratory Syncytial Virus.

Ultrasonography Score	Definition
0	Normal or none to a few comet tails
1	Diffuse comet tails
2	Lobular pneumonia: consolidation ≥ 1 cm^2
3	Lobar pneumonia, 1 entire lung lobe consolidated
4	Lobar pneumonia, 2 entire lung lobes consolidated
5	Lobar pneumonia, ≥3 entire lung lobes consolidated

Through the study the US has increased until euthanasia was indicated. All calves were categorized with a score ≤1 until day one after inoculation. Besides showing a moderate amount of comet tails, [6/8] calves developed consolidation (score ≥ 2). The majority [5/8] of the calves developed a score ≥ 3 before euthanizia. The highest US in this thesis (US = 4) was reported in calf 49 and calf 72. Two calves (no. 77 and 78) appeared normal or with some comet tails (score ≤ 1) on the ultrasound until euthanizia. The US are shown in Figure 5. .

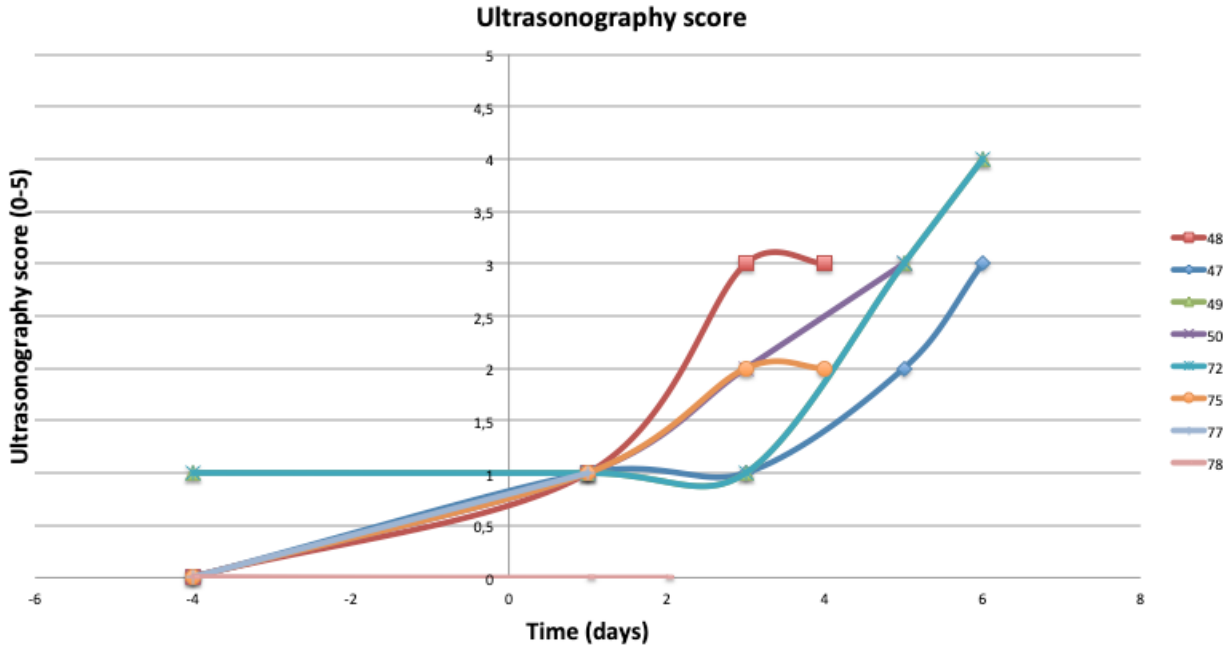


Fig. 5 Ultrasonography score over time in eight Danish Holstein calves between 2 – 23 days old experimentally inoculated with Bovine Respiratory Syncytial Virus.

2. Clinical signs

Every eight hours a CE was performed on all calves. The clinical findings in this thesis have been categorized in a scoring system as described in Appendix B. The following clinical signs were highlightet because they relate directly to the respiratory system. Some calves [5/8] had intermittent purulent ocular discharge after inoculation. All calves [8/8] showed intermittent purulent nasal discharge in various amounts. The earliest sign of purulent nasal discharge was recorded in calf no. 48, 50 and 77 before inoculation at t_{-48}. Pulmonary auscultation showed that all calves [8/8] at some point differed from normal vesicular respiration. The coughing reflex was noted but the scores varied with no distinct correlation. The respiration rate was increasing through the period $t_0 - t_{144}$ (Fig. 6). All calves showed an increase in respiratory rate at t_{-48}. Through the period $T_0 - T_{144}$ the temperature was varying but in generel increasing (Fig. 7). Half of the calves (no. 47, 48, 75 and 77) developed a temperature ≥ 40,7°C before euthanasia.

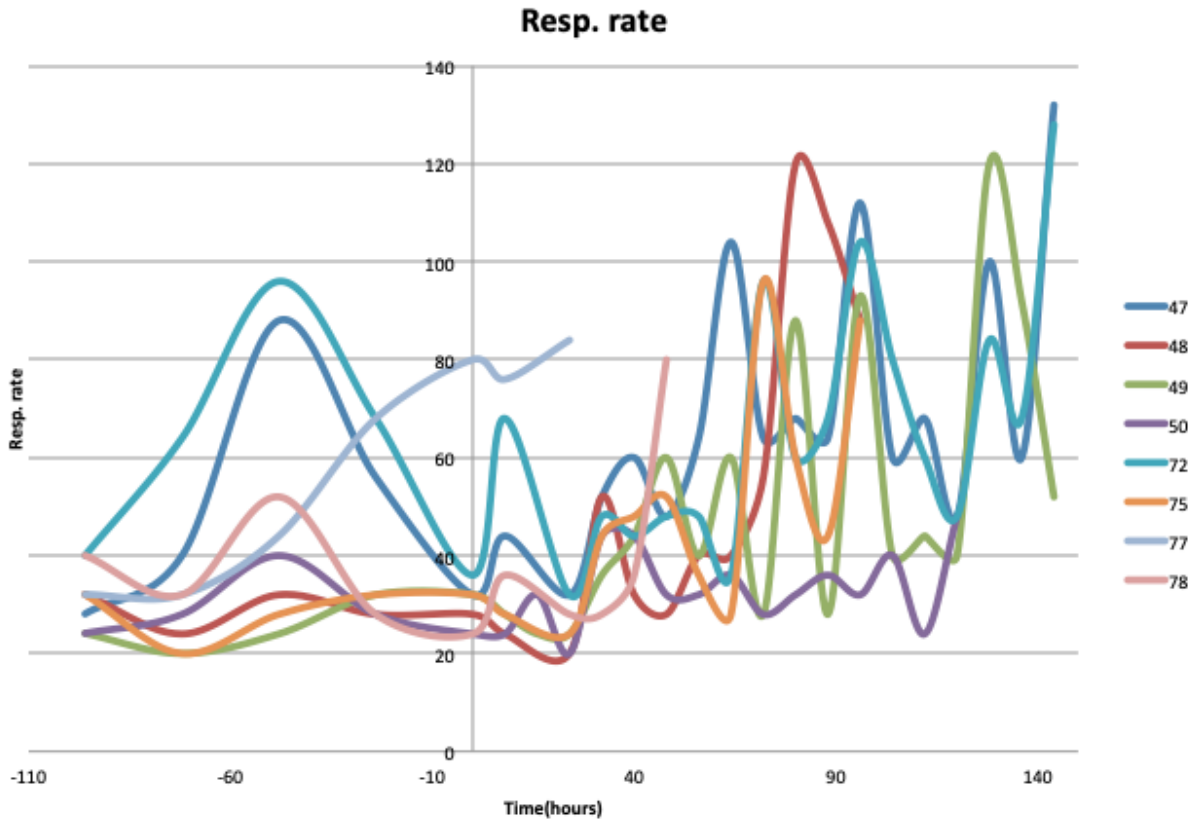


Fig. 6 Respiratory rate (Resp. rate) over time in eight Danish Holstein calves between 2-23 days old experimentally inoculated with Bovine Respiratory Syncytial Virus.

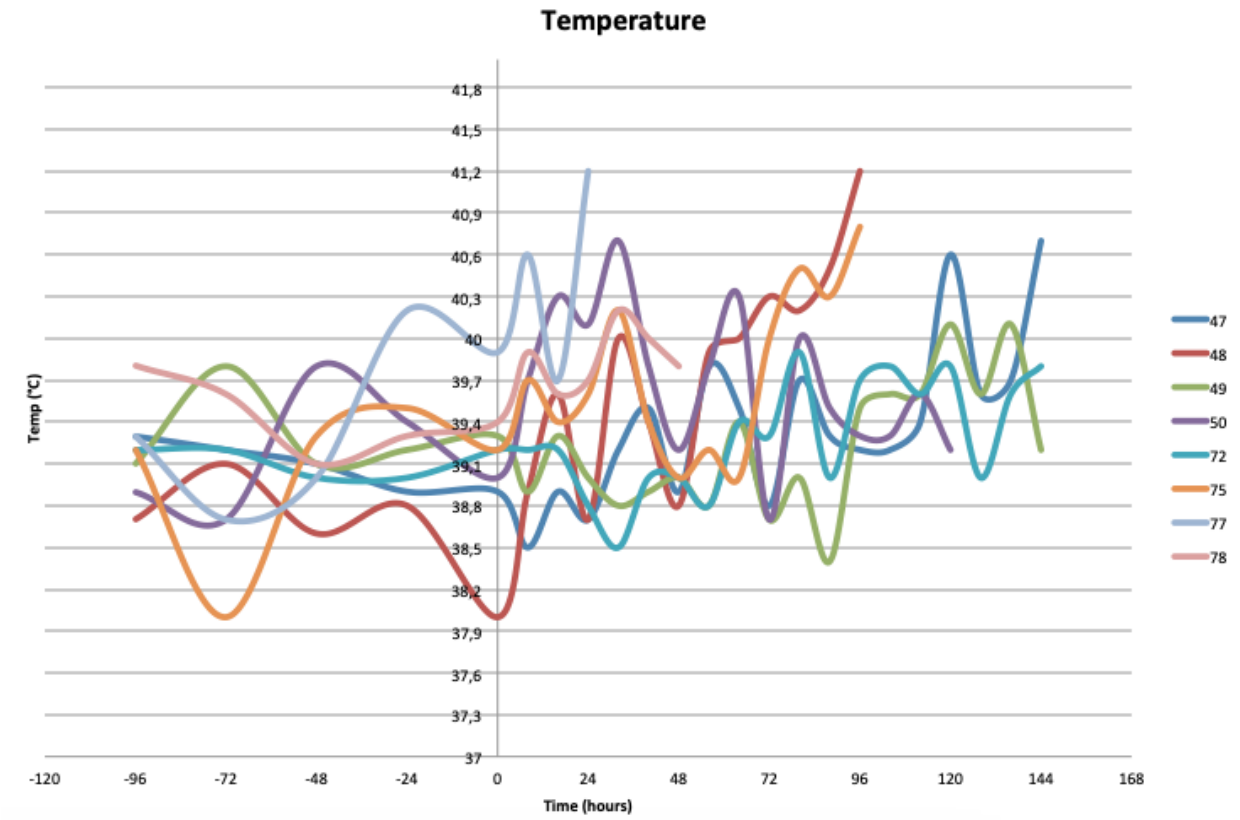


Fig. 7 Rectal temperature (Temp) over time in eight Danish Holstein calves between 2-23 days old experimentally inoculated with Bovine Respiratory Syncytial Virus.

3. Macroscopic findings post mortem

The calves were euthanized when they showed signs of consolidation on the TUS or if clinical signs were severe. Day 1+ and 2+ post inoculation day (PID) calves 77 and 78 were euthanized with four clinical signs (4+) noted related to respiration, MS = 3, MS = 1, US = 1 and US = 0, respectively. In calf no. 77 acute, lobular, hemorrhage, edema, consolodation(atelectasis, sublevel) were located in lung cranial 1, 2 and 3 (Fig. 8). Edema was located in lung cranial 4, 5 and 6 (Fig. 8), and lung caudal was reported unremarkably. In calf no. 78 acute, lobar, hemorrhage in level and edema was reported in lung cranial 1, 2 and 3 and the rest of the lung lobes (4 – 7) appeared unremarkable. The reported findings in calves 48, 75, 50, 47, 49 and 72 are all seen in Table 4.

The scores of MS and the US were compared in a Kappa calculation to determine the agreement of the two methods. As there was no MS = 5 or US = 5, the kappa is made with 5 levels, 0-4. The US and MS in this thesis gave a weighted Kappa = 0,378.

Post mortem the lungs were inspected. The lungs were divided into sections [33] and shown in Figure 8. The lesions found in this thesis appeared purple-red or dark red, well defined (lobular), consolidated including atelectasis. The results are listed in Table 4. Pictures of all macroscopic findings are shown in Appendix D. Some of the calves (no. 47 and no. 72) had mucopurulent exudate in the bronchies. The macroscopic findings were scored 0-5. The MS were categorized in Table 3.

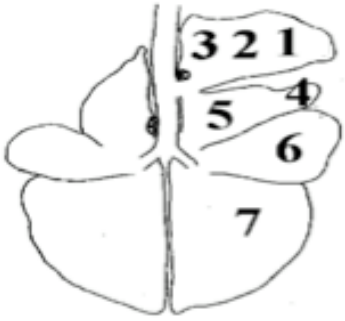


Fig. 8 Dorsal view of the bovine lung. Right side divided into seven different areas, to Ease the location of pulmonary findings [33].

Table 3. Classification (0-5) used to define post mortem pulmonary macroscopic lesions in Danish Holstein calves between 2 - 23 days old experimentally inoculated with Bovine Respiratory Syncytial Virus.

Macroscopic Score	Definition
0	Normal
1	Lobular changes without consolidation
2	Lobular pneumonia: consolidation ≥ 1 cm^2
3	Lobar pneumonia, 1 entire lung lobe consolidated
4	Lobar pneumonia, 2 entire lung lobes consolidated
5	Lobar pneumonia, ≥3 entire lung lobes consolidated

Table 4. Macroscopic lesions compared to clinical signs and ultrasonography score on the day of euthanasia in eight Danish Holstein calves between 2-23 days old experimentally inoculated with Bovine Respiratory Syncytial Virus.

* marked – = No signs; + = One of the following clinical signs was present: rectal temperature > 40,5°C or pulmonary auscultation variation or mucopurulent ocular or nasal discharge or respiration rate >40; ++ = Two of the clinical signs were present; xxx = Three of the clinical signs were present; ++++ = Four of the clinical signs were present; +++++ = All clinical signs were present.

** Un = Unremarkably

Experiment 2. Macroscopic and ultrasonography findings together with clinical signs on the day for euthanasia post inoculation.								
Post inoculation day (PID)	+1	+2	+4		+5	+6		
Calf No.	77	78	48	75	50	47	49	72
Clinical signs*	++++	++++	+++++	++++	+	+++	++++	++
Macroscopic score	3	1	2	3	2	3	4	4
Ultrasonography score	1	0	3	2	3	3	4	4
Macroscopic findings and location								
Lung cranial 1	Acute, lobular, hemorrhage, edema, consolidation (atelectasis, sublevel)	Acute, lobar, hemorrhage, in level, edema	Edema	Acute, lobular, hemorrhage, edema, consolidation (atelectasis, sublevel)	Edema	Un	Acute, lobular, hemorrhage, edema, consolidation (atelectasis, sublevel)	Acute, lobular, hemorrhage, edema, consolidation (atelectasis, sublevel)
Lung cranial 2			Acute, lobular (focal lesions), hemorrhage, in level, edema			Acute, lobular, hemorrhage, edema, consolidation (atelectasis, sublevel)		
Lung cranial 3			Un					
Lung cranial 4	Edema	Un**	Acute, lobular (focal lesions), hemorrhage, in level, edema	Un		Edema	Acute, Lobular(multi-focal), hemorrhage, edema, consolidation (atelectasis, sublevel)	
Lung cranial 5	Edema	Un	Un	Un				Un
Lung medial 6	Edema	Un	Acute, lobular (focal lesions), hemorrhage, in level, edema	Un	Acute, lobular, hemorrhage, edema, consolidation (atelectasis, sublevel)	Acute, lobular, hemorrhage, edema, consolidation (atelectasis, sublevel)	Acute, lobular, hemorrhage, edema, consolidation (atelectasis, sublevel)	Acute, lobular, hemorrhage, edema, consolidation (atelectasis, sublevel)
Lung caudal 7	Un	Un	Un	Un	Un		Un	Edema

Discussion

The objectives of this thesis were 1) To determine the correlation between APPs (SAA, Hp) in pharyngeal swab, TA and Serum in healthy calves between 3-34 days old, 2) To determine the correlation between APPs (SAA, Hp) in serum and WBCs (neutrophils, lymphocytes) in blood in healthy calves between 3-34 days old and 3) To determine if pulmonary ultrasound findings and clinical signs in calves between 2-23 days old suffering from BRD as a consequence of BRSV reflects postmortem macroscopic findings. The approach to analyzing the available data and the interpretation of the results will be discussed in this part.

1. Acute phase proteins and white blood cells

1.1 Acute Phase Proteins

APPs seem promising in the diagnostics of BRD, as the results in this thesis showed it was possible to detect SAA and Hp in both TA and pharyngeal swab which correlates with earlier studies [25, 27–29, 48]. It was not possible to establish a linear correlation between APPs (SAA and Hp) in serum and TA or between APPs (SAA and Hp) in serum and pharyngeal swab. Instead, a linear correlation was found between age and the SAA concentration in serum ($p = 1{,}67\times10^{-10}$). It seems like SAA concentration decreases, as age increases, which correlates with earlier studies [49].

A study established the following references for SAA in serum < 178 mg/L and Hp in serum < 196 mg/L [50]. The results presented in this thesis showed that [88/90] of the calves had a SAA concentration in serum and [85/90] of the calves had a Hp concentration in serum both consistent with the references established by [50]. The five Hp concentrations in serum above reference values could be due to stressful events earlier in life [49]. Four of the five calves were recorded with symptoms of diarrhea and one was seemingly healthy. Not all five calves showed signs of diarrhea as well as not all calves with diarrhea had Hp concentrations in serum above reference, making it uncertain whether diarrhea was the reason.

A higher Hp and SAA concentration in TA was found in this thesis (Table 1) compared to healthy calves in other studies [25, 26]. These two studies found reference values in BAL, but the results are still worth comparing to the results in TA in this thesis, as their method described, did not differ notable from the TA procedure described in this thesis. The results of Hp and SAA concentration in TA in this thesis were based on a sample size of 90 calves compared to eight

healthy calves in another study [25]. It was not well explained how the calves in the other study were housed and how the management procedures were [25], but we expect the number of calves housed together were less compared to the calves included in this thesis. This together with the sample size could indicate that the Hp and SAA concentration in TA in this thesis might be more suitable for calves held in Danish herds.

One calf had a SAA concentration above the rest of the calves (0,0 – 0,16 mg/L) (Fig 1B). In the pharyngeal swab, two calves had a higher SAA concentration than the rest of the calves (0,0 – 0,48 mg/L) (Fig 1C). These three calves were all reported healthy prior to sampling.
In this thesis two calves had a higher Hp concentration in TA than the rest of the calves (0,0 – 3,05 mg/L) (Fig 1E). There is no clear reason why these two calves had a higher Hp concentration in TA. One was reported healthy and the other one had pus on the flushing catheter. In pharyngeal swab six calves had a higher Hp concentration compared to the rest of the calves (Fig 1F). Three of these six calves were reported healthy, whereas one had purulent nasal discharge, one with serous ocular discharge and the last one had pus on flushing catheter. Some of the calves within reference values did also show these clinical signs, making it uncertain whether the high Hp concentration in the pharyngeal swab was due to the clinical signs. The results could either suggest that the calves were actually affected or that the real references are higher than the main group of calves suggests.
It seems like the calves with higher APP concentrations in serum, TA and swab, only do show higher levels in one of the three medias. Only two calves showed both higher Hp concentrations in TA together with higher Hp concentrations in pharyngeal swab. One of the two calves was reported healthy and the other one had pus on the flushing catheter. This does not clarify why both calves showed higher Hp concentrations in both TA and pharyngeal swab, making it uncertain what underlying reason the higher Hp concentration was due to. The higher values cannot be explained by age, as the age differ from 5 – 30 days (12,5 ± 6,95).

The TA was of interest because of the local APR in the lungs. TA demands more resources compared to blood sampling because the calves must be sedated prior to TA. Furthermore, the TA procedure is more invasive, because not all fluid down in the trachea can be aspirated, thereby leaving NaCl in the lungs. In our study TA had an unknown and varying dilution rate compared to the pharyngeal swab. The swab was always placed in 1 mL PBS that standardized the samples more compared to the TA. When TA was performed the amount of NaCl flushed into the lungs could vary between 50-130 mL. According to the results, it seemed that TA had a

lower SAA and Hp concentrations compared to pharyngeal swabs, but it was unknown whether this was due to the varying dilution rate or if it reflected the actual APP concentration in the lungs.

The APPs appear to be a good diagnostic tool if it is possible to detect and establish some cut off values early. The APPs make a rapid response [13], making them a possible diagnostic option for early detection of inflammation. An early detection is desirable right after tissue damage has occurred, and an increse in APPs is detectable, but before irreversible and cronic changes arises. Unfortunately, APPs appear during many cases of inflammation and other underlying reasons. APPs are therefore not specific for respiratory disease, which makes it nessesary to compare the values with clinical signs. This was supported by a study, who suggested that APPs were influenced by subclinical infection or other disease processes at the time of sampling [49].
The golden standard in diagnostics of BRD is not yet established [37]. The literature showed many examples of that APPs could be detected in serum [3, 19]. TA was in this thesis chosen to determine whether there was a local response to detect. The pharyngeal swab was chosen from an expectation based on two studies that there might be an easier and less invasive way to detect APPs and thereby an addition to diagnostics relative to BRD [28, 29]. The pharyngeal swab is a quick procedure, it is easy to handle, and it makes it possible for the farmer to do it himself, if he suspects infection in the respiratory tract. If the farmer can diagnose BRD with the pharyngeal swab, it could be assumed that he could not only diagnose individuals but screen calves at risk.

1.2 White Blood Cells

It was not possible to establish a linear correlation between APPs (SAA and Hp) in serum and WBC (lymphocytes and neutrophils) in EDTA-stabilized blood. A linear correlation was seen between age and lymphocyte concentration in blood ($p = 0{,}033$). Because the p-value associated with the lymphocyte concentration in blood compared to age was close to 0,05 the linear correlation is weaker compared to the correlation between SAA concentration in serum and age ($p = 1{,}67\times10^{-10}$). This could signify that more research is indicated.
The data in this thesis gave rise to reference values for WBC in blood which supported earlier studies [51]. A study found that calves ≤ 7 weeks had a neutrophil concentration in blood up to 10×10^9/L and a lymphocyte concentration in blood up to 9×10^9/L [51], all lymphocyte and [86/90] neutrophil values in this thesis were both consistent with these results. The four calves varying from the neutrophil reference values were all reported healthy without signs of disease.

The age of these four calves varied from 9 – 18 days (12,75 ± 3,86). Studies indicates that neutrophils are the dominant WBC in young calves [10], this could explain the higher values, but it is uncertain.

Through the years WBC has been used in bovine medicine as a diagnostic tool of respiratory infection. The WBCs have been preferred because of easy handling and low cost.

2. Clinical examination, thoracic ultrasonography and macroscopic findings

2.1 Clinical Examination

In this thesis nasal and ocular discharge were intermittent clinical signs to observe, as the calves could influence on wether it was present or not. The calves could have licked their muzzle, put their heads in the milk replacer when they were fed, or could have sucked on each other etc. before the CE was made. Respiratory rate and heart rate varied depending on whether the calves were sleeping or if they recently had been physically active. The coughing reflex was diffucult to score accurately. It was often experienced that the coughing reflex was negative, which resulted in a low score, but ten minutes later the same calf coughed spontaneously, resulting in a higher score. This raised the question: how accurate and significant was the test of the coughing reflex? It also led to speculation on how often the score was false negative. Environment played a role as well, if straw was given recently the environment became dusty which could increase the likelyhood of coughing.

The CE was fundamental in this thesis. However, it is important to keep in mind that the clinical examinations were subjective and performed by two different examiners, as earlier studies have described, observer variation was present when veterinarians performed clinical examinations [52]. The two examiners supervised each other while performing the CE and were calibrated by discussing the approch and the findings continuously. The examiners could also be biased because of the knowledge about inoculation time, which could have been avoided in a blinded experiment. The findings might not have been categorized under the same score despite attempts to be standardized beforehand. This could have resulted in a variation in the noted scores related to pulmonary auscultation and assesment of lymph nodes.

Overall, clinical signs are dynamic and should be a part of a whole and not on an individual basis.

2.2 Thoracic ultrasonography and macroscopic findings

Overall, the results showed that the accuracy of TUS compared to macroscopic findings, was higher in those euthanized last, and more inaccurate in those euthanized early in the study. This indicated that TUS was more accurate when pulmonary changes were more severe.

This thesis showed varying correlation when comparing MS and US to clinical signs. Three calves had higher MS compared to US and they all had clinical score = 4+. Two calves scored 3 on ultrasound compared to MS = 2 and with respectively 1+ and 5+ in clinical signs. Three calves had individually same MS and US but with varying clinical signs (2+ up to 4+). These three calves were the last ones to be euthanized (+6 PID). As earlier mentioned BRSV incubation time has been shown to be 2-5 days [4, 31], this could indicate that pulmonary pathology becomes more advanced over time, resulting in easier detection using TUS, and a stronger correlation between macroscopic findings and TUS findings. A weighted kappa was calculated = 0,378. This could indicate that the agreement might be worse than first assumed. It is important to notice that the weighted kappa was calculated based on subjective values and that the weighted kappa takes into consideration how much the two methods agree.

The TUS findings were difficult to interpret. Two experienced examiners did the scans and remarking, but the definition of mild, moderate and severe was subjective. In this thesis it was likely that TUS did not show the entire pathology because it was only performed on the right side. The clinical signs could therefore be severe without any severe TUS findings. An example of this was calf 77 who had severe clinical signs compared to a US = 1. The macroscopic findings showed lungs remarkably affected with MS = 3. The affected lung tissue could explain the clinical signs and the low US could be due to narrow ics and the calfs recumbency, making it hard to scan the cranioventral part of the lung properly. Calves no. 77 and 78 were euthanized at day +1 and +2 PID, making it unlikely that the calves showed severe clinical signs because of the BRSV inoculum. The macroscopic findings in calf no. 77 were consistent with specific pulmonary BRSV lesions. This, together with the knowledge of the presence of BRSV in the herd, could indicate that the calf was already infected before arrival. Unlike calf no. 77 nothing specific indicated that calf no. 78 developed severe clinical signs because of the BRSV inoculum.

TUS was chosen because the pulmonary changes were expected to occur fast because of the rapid realease of cytokines as described earlier in this thesis. In diagnostics comet tails are not BRSV specific, because the artifacts can occur because of variable circumstances including edema, exudate, pneumonia, mucus, pleuritis, neoplastic infiltration or interstitial fibrosis [39–

41]. Therefore, comet tails might be used as an indicator that pathology might be present in the respiratory tract, but it would not be conclusive.

Overall, the results could suggest that additional diagnostics were required along with TUS, and supports the need for early diagnostics, so treatment could be initiated before severe changes occur.

3. Inclusions criteria, herd selection and sample size

3.1 Experiment 1

It was comprised of a convenience sample, instead of a random sample. The calves included in this thesis were between 3-34 days old. To make the sample more representative for the Danish population of DH calves, it would be necessary to increase the sample size with calves randomly selected from more herds of all sizes from all over Denmark, instead of only parts of the country. The calves were included in this thesis even if they showed diarrhea, pus un the flushing catheter, and serous nasal or ocular discharge. Any previous diseases and prior treatments were unknown. Treatment and muzzle hygiene could have masked potential clinical signs, so a sick calf could have been included in the study. This could have biased the APP concentrations, resulting in unreliable high APP concentrations.

3.2 Experiment 2

If this experiment had included calves from a larger herd, it would have been possible to be more critical in selecting the calves. With a larger selection of calves, calves with diarrhea could have been avoided, the age difference could be minimized and only calves from dams with a negative BRSV titer would have been taken into consideration. This would have resulted in a more homogenous sample and fewer variables. In this thesis the BRSV titer in the dams was not taken into consideration, because otherwise the amout of calves born within 30 days would have been too few. The BRSV titer indicates that BRSV was present in the herd. This could suggest that the calves could have been infected even before arrival, this could explain why calf no. 77 developed macroscopic findings consistent with BRSV.

The calves included in experiment 2 were 2–23 days old. The age range was wide, and the youngest ones were expected to have a more naive immunity, whereas the older ones were expected to be more resistant. Added stress factors and environmental changes contributed to increased likelihood that the youngest calves in this experiment were more likely to contract

other diseases than the BRSV inoculum. Therefore, it would be preferable to avoid the youngest calves and thereby get a more homozygous group to minimize variation in the sample.

4. Study design

Experiment 1 was a cross sectional study with strengths and weaknesses. A larger sample size would have increased the power of the study. The study design imitated practice conditions, as this thesis only showed a snapshot of the calf health status at sampling time. The same applies to conditions seen in practice. This did not provide any information about where in a possible inflammation process the calf was, compared to a cohort study, which would reflect the dynamics of inflammation. However, a cross sectional study made it possible to suggest reference values for calves between 3-34 days old. This could provide a basis for future studies or be compared to diseased calves.

Conclusion

The results from experiment 1 of this thesis confirmed that SAA and Hp in healthy calves between 3-34 days old both could be detected in serum, TA and pharyngeal swab. The results did not show a correlation when comparing SAA in serum to SAA in TA or pharyngeal swab. A slight correlation was seen between SAA in serum and age, it seemed that the SAA concentration decreased as age increased. When comparing Hp in serum to Hp in TA and pharyngeal swab no correlation was detected. The results from experiment 1 mainly gave rise to SAA and Hp reference values for healthy calves in Danish herds, SAA in serum ≤ 180,94 mg/L, SAA in TA ≤ 0,16 mg/L, SAA in swab ≤ 0,48, Hp in serum ≤200 mg/L, Hp in TA ≤ 3,0 mg/L and Hp in swab ≤ 4,0 mg/L, respectively.

Another aim in this thesis was to determine the correlation between APPs (SAA and Hp) and WBC (lymphocytes and neutrophils) in healthy calves between 3-34 days old. The results did not show a clear correlation. Again, the results gave rise to reference values for calves in Danish herds for lymphocytes and neutrophils, lymphocytes in blood 1,7 – 7,7x10^9/L and neutrophils in blood 0,46 – 8,57x10^9/L, respectively.

The results from experiment 2 showed that the accuracy of TUS compared to macroscopic findings, in calves between 2-23 days old experimentally inoculated with BRSV, was higher in those euthanized last, and more inaccurate in those euthanized early in the study. This indicated that TUS was more accurate when pulmonary changes were more severe. Clinical signs varied and indicated that mild clinical signs could suggest further diagnostics, because those with severe macroscopic findings did show mild clinical signs.

When comparing the results from both experiments, the results overall indicated that APPs together with clinical signs and TUS could contribute to a more precise and early diagnosis of BRD infection. These results also indicate that more research is needed.

Perspectives

Multiple researchers have performed studies to establish APP concentrations in serum, especially SAA and Hp reference values in healthy calves, but further investigation is still indicated. Other APPs could also be investigated such as Fibrinogen, as it contributes to the inflammation. Future studies could investigate the correlation between APPs in serum and APPs in pharyngeal swabs or nasal swabs. Furthermore, it would be of interest to establish reference values for APPs in pharyngeal swab and in nasal swab. The APP changes during the subclinical period until disease is present, is of great interest. The correlation between APPs and immunoglobulin's are also of great interest, as one study established that APPs seem to decrease as BRSV antibody status was high, and the APPs seemed to increase when the antibody status was low [53], but still further investigation is needed. This could improve future diagnostics, by using more non-invasive methods, and lead to an on-farm diagnostic tool, which could help the farmer without attendance of a veterinarian to detect calves that need treatment. If treatment was initiated early in relation to BRD, NSAID might be enough to avoid secondary bacterial infections. This would reduce the antibiotic use in Danish feedlot herds, which is of great interest.

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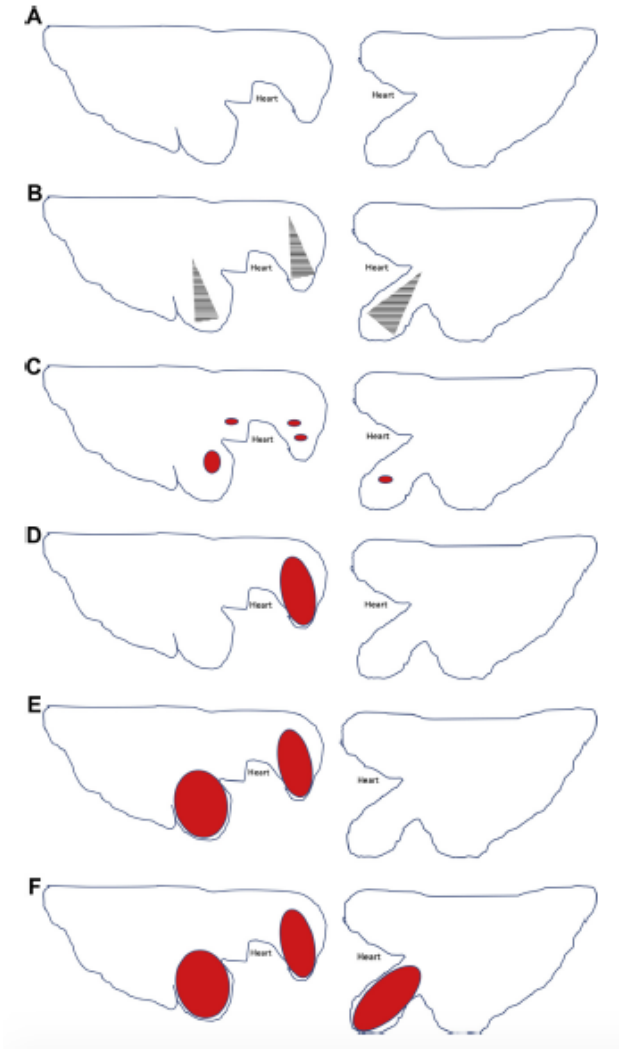
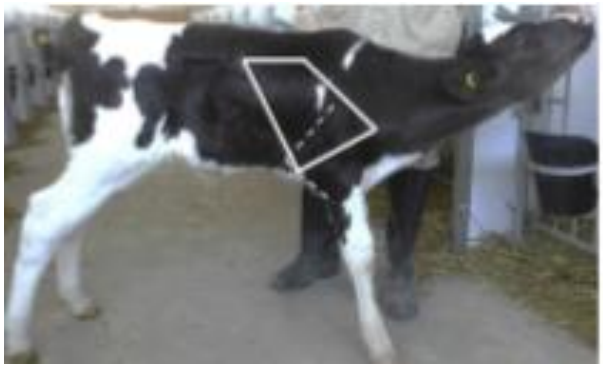
phase protein changes in calves during an outbreak of respiratory disease caused by bovine respiratory syncytial virus. Comp Immunol Microbiol Infect Dis. 2011;34:23–9. doi:10.1016/j.cimid.2009.10.005.

Appendix

Appendix A: Extract from ”On-Farm Use of Ultrasonography for Bovine Respiratory Disease”





The area where the calves were scanned.

Fig. 12. Ultrasonographic scoring system (0–5) used to categorize young cattle. (*A*) US score 0 indicates normal aerated lung with no consolidation and none to few comet-tail artifacts. Ultrasonographically, normal lung appears as a bright white, or hyperechoic, line. (*B*) US score 1 indicates diffuse comet-tail artifacts without consolidation. (*C*) US score 2 indicates lobular or patchy pneumonia. Small lobular lesions are most likely to be viral in nature and may not warrant treatment. (*D*) US score 3 indicates lobar pneumonia affecting only 1 lobe. (*E*) US score 4 indicates lobar pneumonia affecting 2 lobes. The cranial and caudal aspects of the cranial lobe are scored individually. (*F*) US score 5 indicates lobar pneumonia affecting 3 or more lobes.

Appendix B: Clinical examination in Experiment 1 based on Extract of ”Robuste kalve – klinisk protokol” and our own clinical scoring categories.

The following is an extract from ”Robuste kalve- klinisk protokol” together with our clinical scores used in this thesis.

General condition

Score	**Description**
0	Nothing to report (ntr)
1	Depressed

Appetite

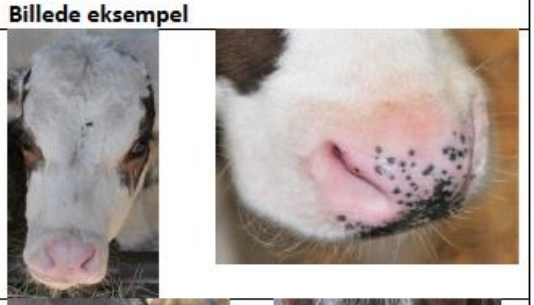
Score	**Description**
0	Nothing to report (ntr)
1	Barely or did not eat

10. Næseflåd – Nasal discharge

Kilde: Welfare Quality

Kig på kalven forfra. Vurder begge næsebor. Forandringer behøver ikke være bilaterale.

Look at the calf from in front. Examine both nostrils. Anomalies do not have to be bilateral.

Score	Beskrivelse	Billede eksempel
0	**Normal** Ingen flåd eller pus i næseborene *Normal, no signs of fluid, exudate or pus in the nostrils*	
1	**Serøst** Klart, vandigt (serøst) flåd i et eller begge næsebor. Ingen pus eller pus-tilblanding. *Clear, serous fluid/exudate in one or both nostrils. No sign of mucopurulent or purulent exudate*	
2	**Muko-purulent/purulent** Uklart/pus-tilblandet eller rent pus (mukopurulent). Frisk pus og/eller klatter af indtørret pus. *Cloudy (mukopurulent) or coupious discharge in one or both nostrils. Fresh pus and/or dry crusts.*	

The nasal discharge amount

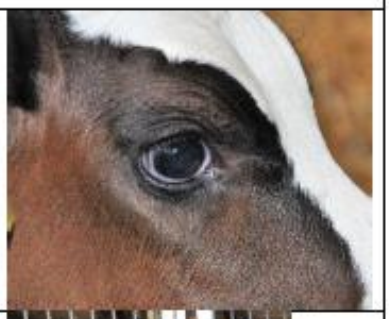
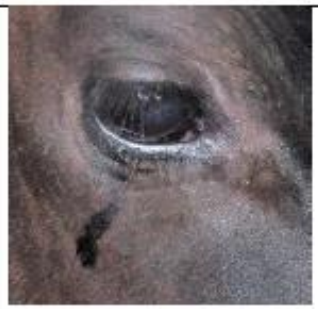
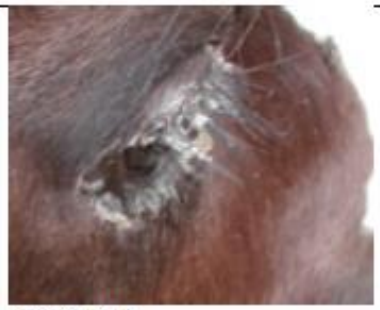
Score	Amount
0	Little (dried/fresh stain)
1	Large (running discharge)

12. Øjenflåd – Eye discharge

Kilde: Welfare Quality

Kig på kalven forfra. Vurder begge øjne og deres omgivelser. Forandringer behøver ikke være bilaterale.

Look at calf from the front. Examine both eyes and their surroundings. Anomalies do not have to be bilateral.

Score	Beskrivelse	Billedeksempel
0	**Normal** Ingen flåd, tørre øjenomgivelser *Normal, no discharge, dry eye surroundings*	 
1	**Serøst** Klart, tyndt/vandigt flåd *Serous discharge (transparent, thin)*	 
2	**Muko-purulent/Purulent** Uklart/pus tilblandet eller rent pus. Ofte sammenklistrede øjenvipper. Frisk pus og/eller klatter af indtørret pus. *Mucopurulent or purulent discharge. Often sticking in the eyelashes. Fresh pus and/or dry crusts.*	  Kilde: University of Wisconsin

The eye discharge amount

Score	Amount
0	Little (dried/fresh stain)
1	Large (running discharge)

Respiratory rate

Count	(min)

Respiratory caracteristics

Score	Description
0	Thoracoabdominal
1	Abdominal/ forced/ troubled

20. Tilsmudsning, hele kalven – Cleanliness

Kalven skal stå op og betragtes over hele kroppen ovenfor forknæ/has og uden hoved og distale del af benene. Med tilsmudsning menes friske eller indtørrede kager/stænk/områder af skidt og/eller fugt Det er det samlede areal af alle beskidte områder, der scores. Bærer kalven dækken tjekkes kalven under dette og er den mere beskidt under dækkenet (hvis den lige har fået den på fx) scores kalven. Ellers scores tilsmudsningen af kalven uden på dækkenet.

Calf must be standing. All of the body is examined except from the head and the legs from and below the the hocks/knees. Soiled means fresh and/or dried cakes/stenches and/or moisture on shoulders, belly, sides and/or hindquarter/tail. The total area of all soiled areas is scored. Calves with rugs are checked under the rug. If it is more soiled beneath the rug, it is scored without the rug.

Score	Beskrivelse	Billedeksempel
0	Ren Under 2 håndflader (minus fingre) tilsmudset *Less than the area of 2 palms soiled in total*	
1	Moderat tilsmudset Areal svarende til sammenlagt 2 håndflader op til 25 % *Area of in total 2 palms up to 25% soiled*	
2	Svært tilsmudset Mindst 25% af kalvens overflade er tilsmudset *At least 25% percent of the calfs' surface is soiled*	

Pulse

Beats	**(min)**

21. Hoste - *Coughing*

Vurdering foretages på stående kalv som led i den kliniske undersøgelse. Hvis der ikke inden eller under den kliniske undersøgelse er observeret spontan hoste forsøges den fremkaldt ved at klemme på strubehovedet.

During the clinical examination of the calf, it is observed whether the calf coughs spontaneously. If not, try to provoke coughing by manipulating the larynx from the outside with two fingers.

Score	Beskrivelse	Billedeksempel
0	Ingen hoste – hverken spontan eller induceret *No coughing observed*	
1	Et enkelt induceret host *A single cough when provoked*	
2	Gentagne host efter induction eller et enkelt spontan host *Repeated coughs when provoked OR a single spontaneous*	
3	Gentagne spontane host *Repeated unprovoked coughs*	

Lymph nodes (mandibularis, cervicales superficiales)

Score	**Description**
0	ntr
1	1 of the 4 was swollen or sore

Mucosal membranes (conjunctiva)

Score	**Description**
0	ntr
1	Dry, hyperemic, pale

Pulmonary auscultation (10th intercorstal space(ics) dorsal aspect, 5th ics ventral aspect, 2nd ics ventral aspect)

Score	**Description**
0	Vesicular resp. /increased vesicular resp.
1	Differing resp. – rattling, rough

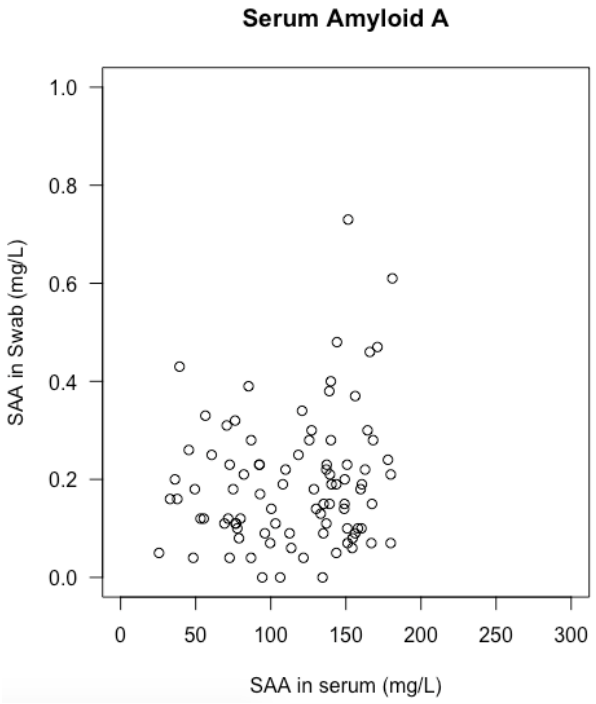
Dehydration (above eyelid)

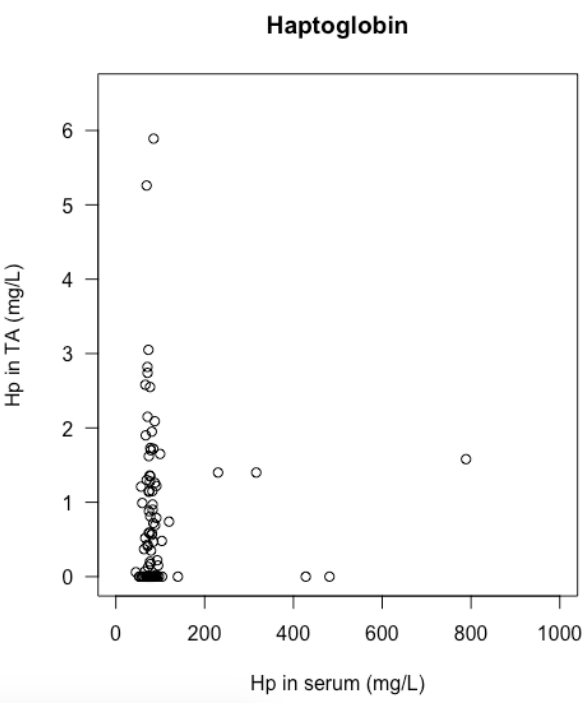
Score	**Description**
0	ntr
1	Dehydrated, skin elasticity > 3sec., dry mucosal membranes, sunken eyes

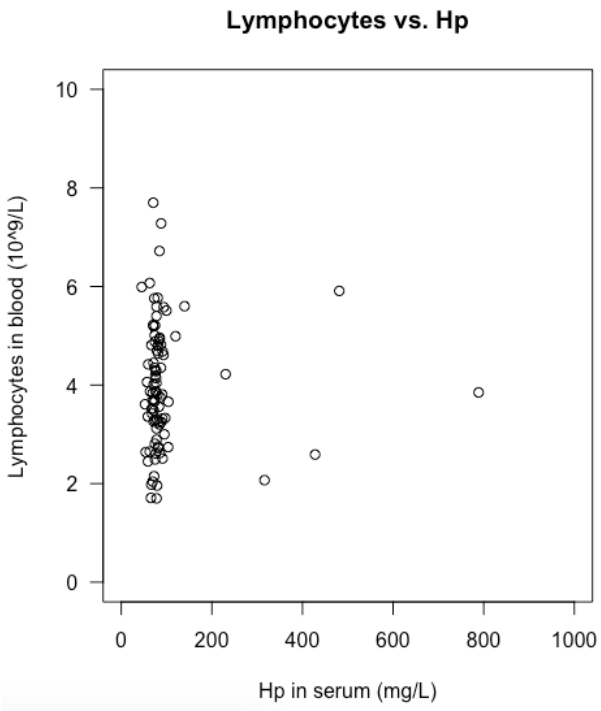
Dato: _____ Kl$_{start}$:_____ Kl$_{slut}$:_____

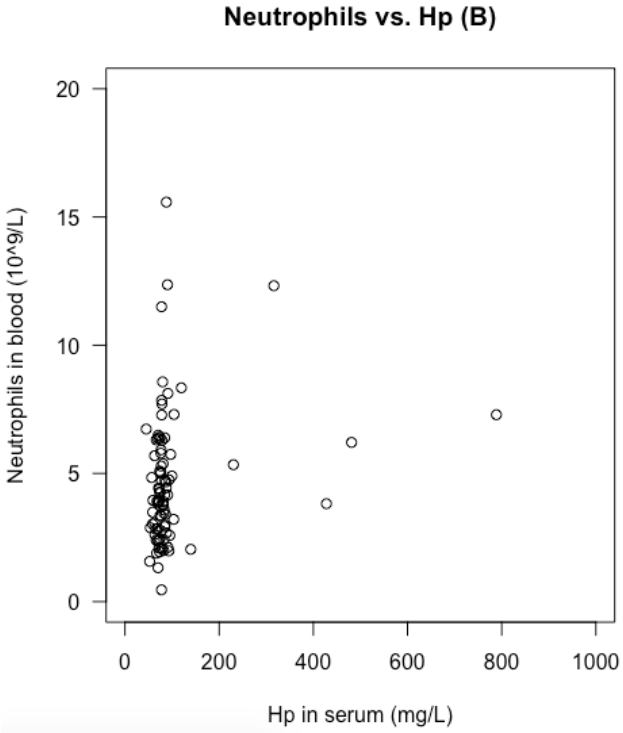
CHR – Nr	Kalv nr.	Alm. Befind (0-1)	Ædelyst (0-1)	Næseflåd (0-2)	Mængde (0-1)	Øjenflåd(0-2)	Mængde (0-1)	Resp. Rate (pr. min)	Tilsmudset bagtil(0-2)	Puls (slag/min)	Hosterefleks (0-3)	Lnn(madibularis cervicales superficiales) (0-1)	Slimhinder (konjuktiva) (0-1)	Ausk. Lunger (0-1)	Dehydrering (hudturgor over øjet)(0-1)	Temp rectal
	1															
	2															
	3															
	4															
	5															
	6															
	7															
	8															
	9															
	10															
	11															
	12															

Appendix C: Results “Experiment 1”



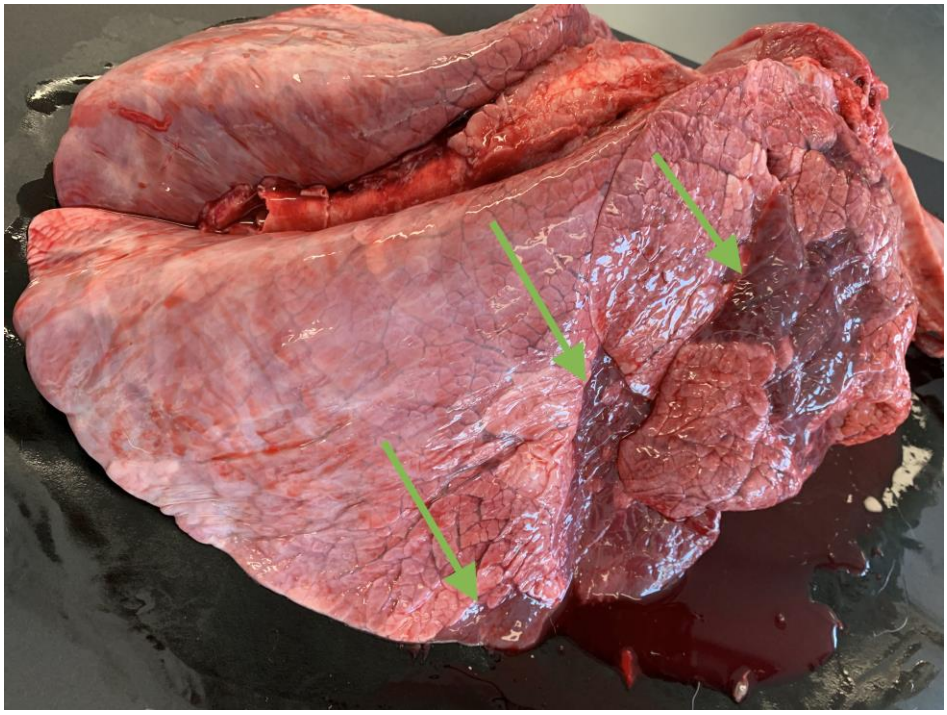


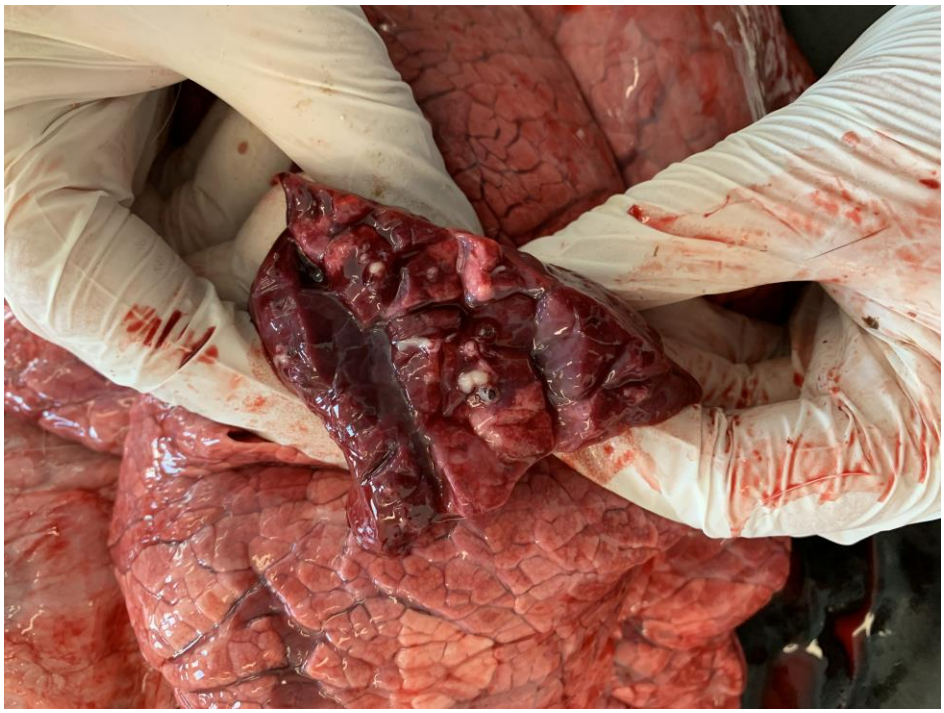




Appendix D: Macroscopic findings

Calf 47





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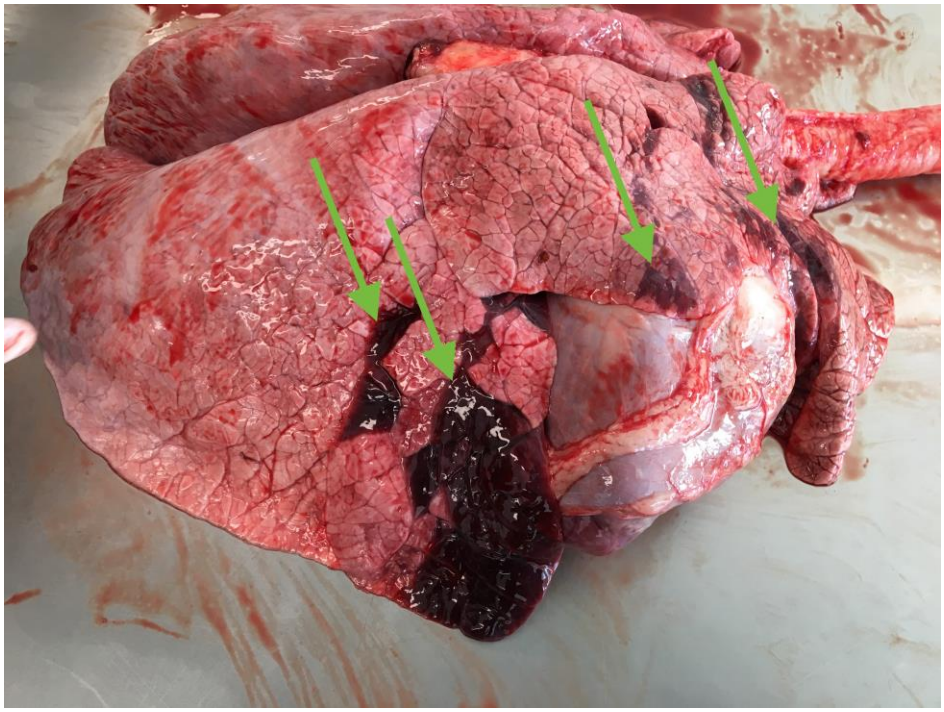

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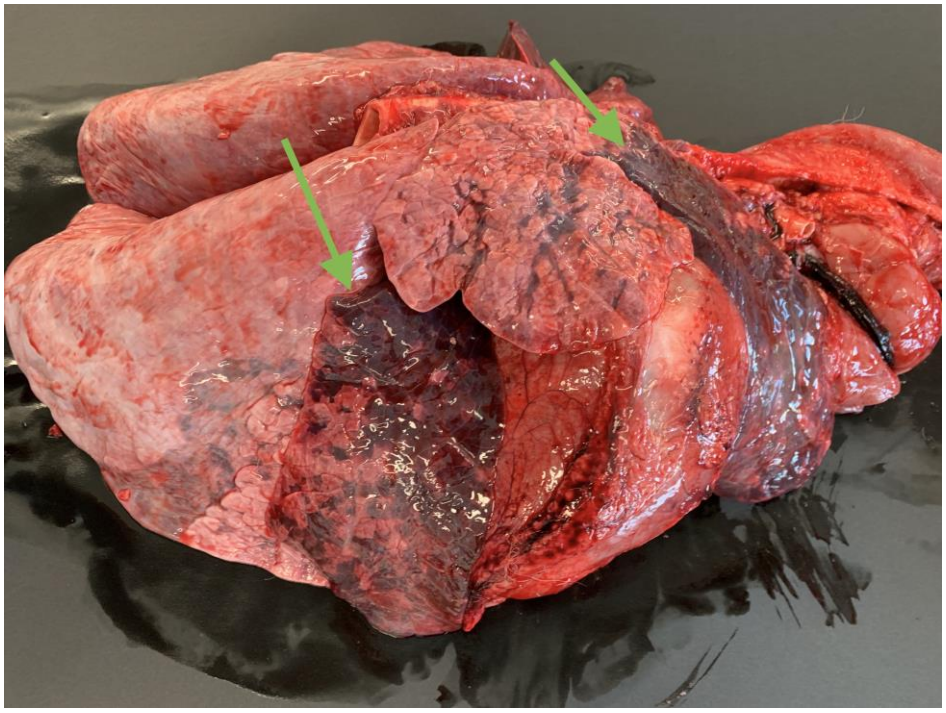
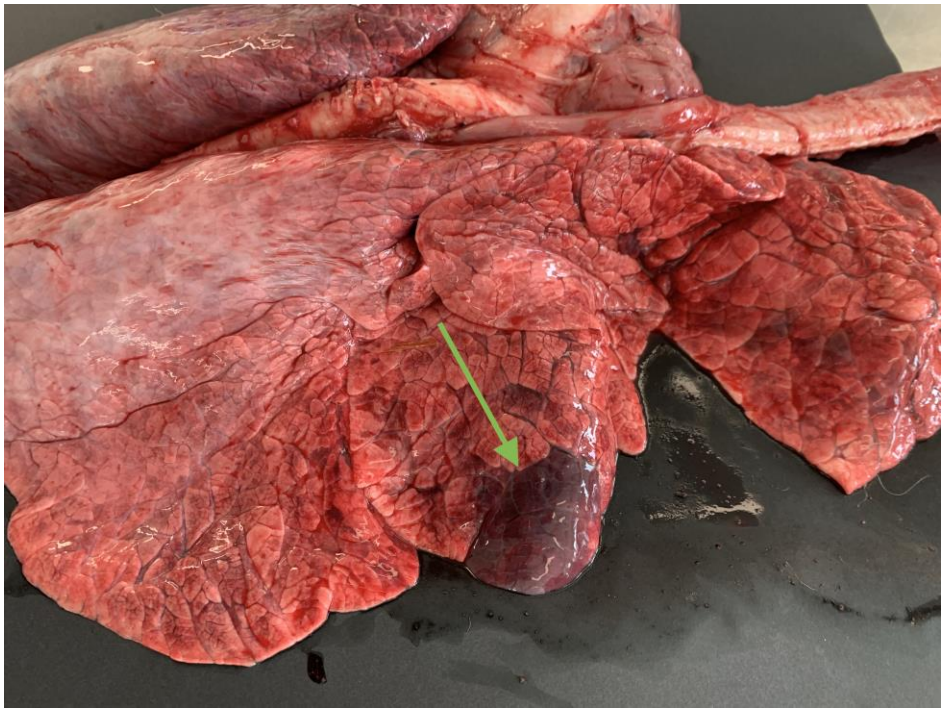
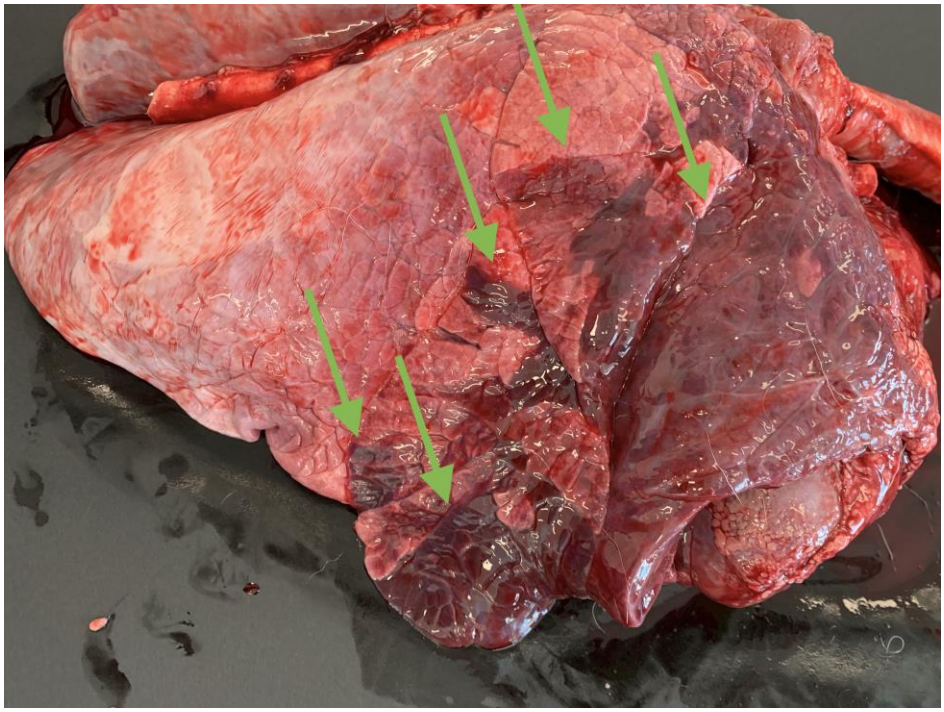
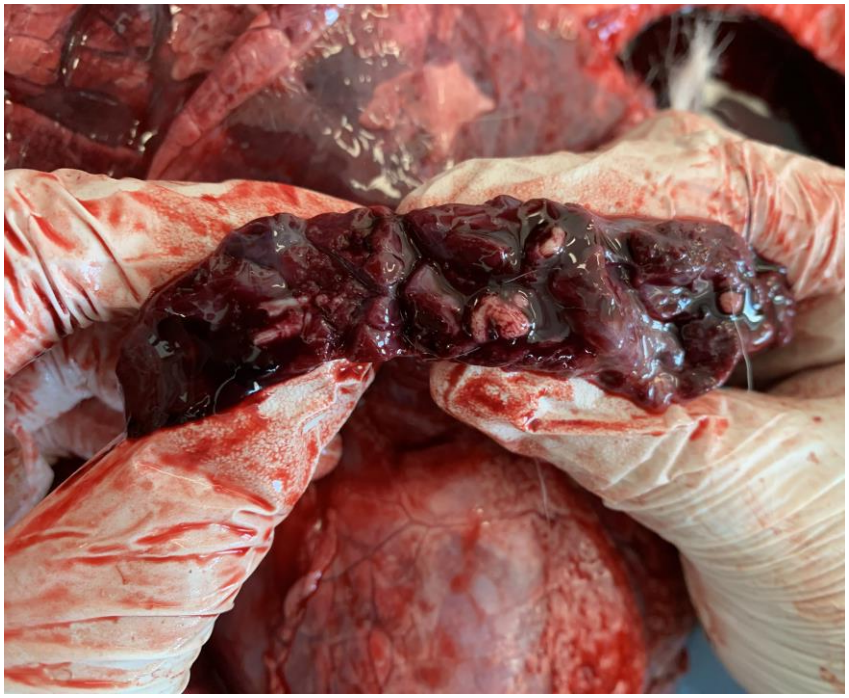


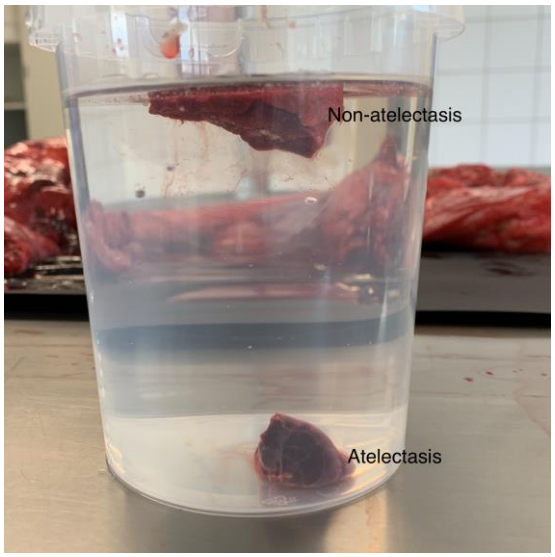
Calf 50



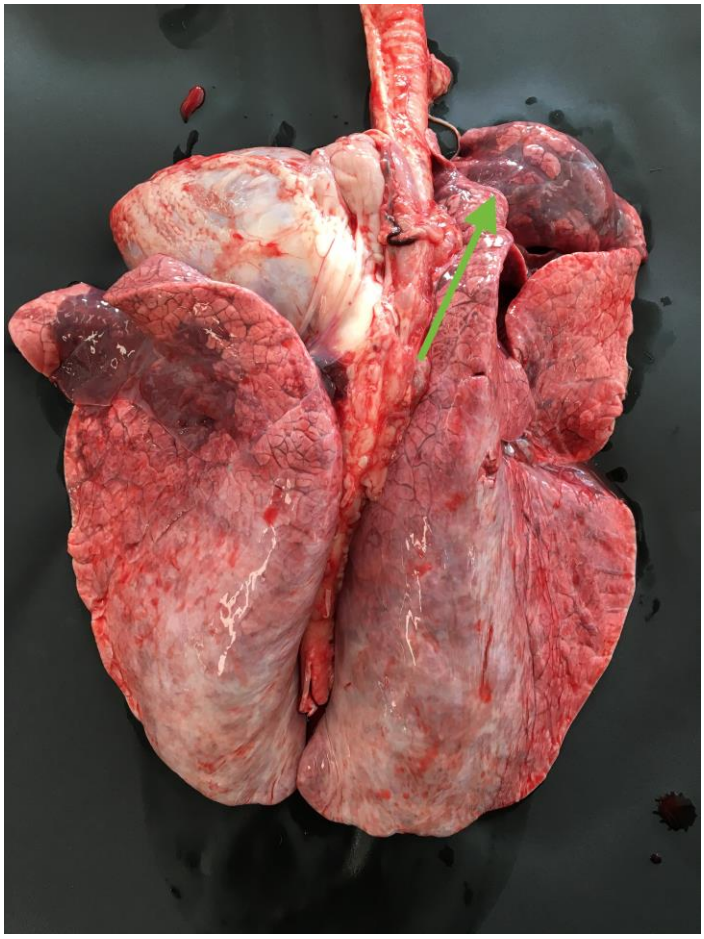
Calf 72



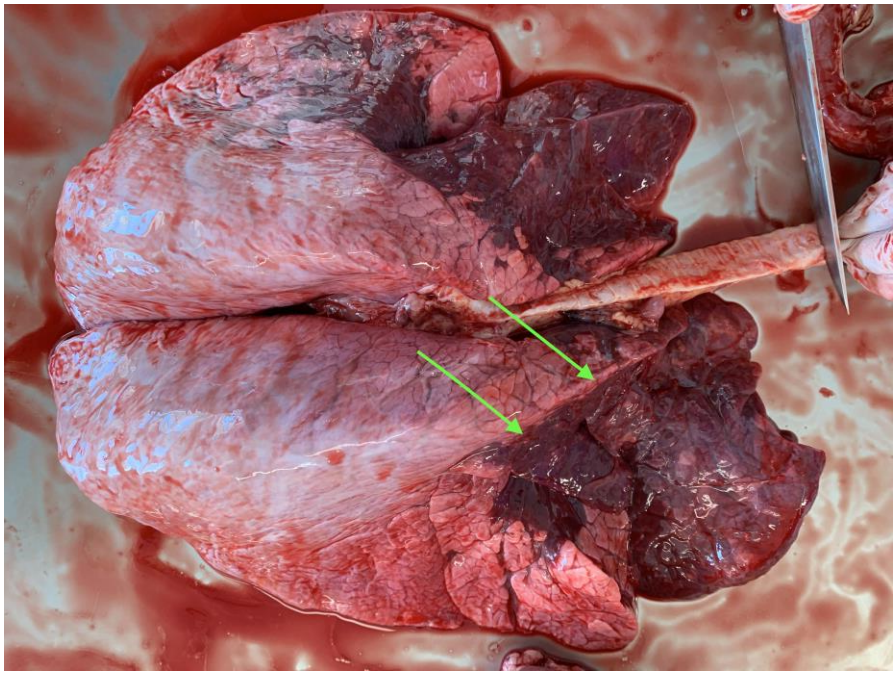




Calf 75



Calf 77



Calf 78