UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

Associations between respiratory disease and risk factors including pathogens detected in an observational cross–sectional study in two different age groups of Danish dairy calves

Master Thesis in Veterinary Medicine
30 ECTS

Authors
Franziska Helene Pedersen, pjq864 & Henrik Hjul Møller, cft936

Main Supervisor
Professor Liza Rosenbaum Nielsen, KU-SUND

Submission date
15th of February 2019

Faculty:	Faculty of Health and Medical Sciences
Authors:	BSc.med.vet Franziska Helene Pedersen, pjq864 BSc.med.vet Henrik Hjul Møller, cft936
Project type:	Master Thesis
ECTS:	30 ECTS
Title:	Associations between respiratory disease and risk factors including pathogens detected in an observational cross–sectional study in two different age groups of Danish dairy calves
Main Supervisor:	Professor Liza Rosenbaum Nielsen Department of Veterinary and Animal Sciences Section for Animal Welfare and Disease Control, KU-SUND
Co-supervisors:	Assistant Professor Nina Dam Otten Department of Veterinary and Animal Sciences Section for Animal Welfare and Disease Control, KU-SUND Professor Lars Erik Larsen, National Veterinary Institute, DTU
Submission date:	15th of February 2019

Copenhagen, 15th of February 2019

Franziska Helene Pedersen

Copenhagen, 15th of February 2019

Henrik Hjul Møller

Front-page picture: Nasal swab of a calf by Bodil Højlund Nielsen (AU Foulum).

Preface

This Master Thesis is a part of the Master´s Degree in Veterinary Medicine at University of Copenhagen and is conducted at the Department of Veterinary and Animal Sciences, Section for Animal Welfare and Disease Control. 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", a collaboration project between Aarhus University, SEGES, Technical University of Denmark (DTU) and University of Copenhagen (KU-SUND). The project was led by Jaap Boes from SEGES and funded by the Danish Cattle Levy Fund (Kvægafgiftsfonden) and the Danish Milk Levy Fund (Mælkeafgiftsfonden) from 2018 to 2021. The project has four different working packages (WP): WP1: Calf cluster sampling, WP2: Calf health status, WP3: Alternatives to antibiotics and WP4: Effective management systems. Our project took place between September 2018 and February 2019 and was part of WP1 and WP2.

A big thank you to our main supervisor, Professor Liza Rosenbaum Nielsen (KU-SUND) for her dedicated and committed supervision during the project period.

Further thanks to our co-supervisors Assistant Professor Nina Dam Otten (KU-SUND), for her great guidance and hospitality during the herd visits and to Professor Lars Erik Larsen (DTU) for good cooperation.

We would also like to thank Research Assistant Anne Marie Michelsen (KU-SUND), Special Consultant Henrik Læssøe Martin (SEGES), Postdoc Bodil Højlund Nielsen (AU Foulum), Technician Stine Lindgren (AU Foulum) and BSc.med.vet Jensine Wilm (KU-SUND), BSc.med.vet Jesper Kjærgaard Davidsen (KU-SUND) and BSc.med.vet Thomas Damm Poulsen (KU-SUND) for great cooperation in collecting the data and sharing thoughts and ideas during the project period.

Also, thank you to Martin Bjerring (AU Foulum) for creating the database "EasyOn" and providing data support.

And thanks to Postdoc Nicole Bakkegård Goecke (DTU) for an introduction to the high-throughput qPCR platform (Fluidigm) and laboratory handling of the samples.

We also want to thank Professor Søren Saxmose Nielsen (KU-SUND), Associate Professor Matt Denwood (KU-SUND) and Senior Researcher Carsten Thure Kirkeby (KU-SUND) for statistical advice and inspiration.

Thanks to Veterinarian Hans Andersen, Halifax Veterinary Centre, Nelson, New Zealand for helping us with proofreading.

Also thanks a lot to the farmers and the herd personnel for their great hospitality and willingness to participate in the study.

Finally, we would like to thank our families and especially Gitte and Mads for great support during this Master Thesis and the entire veterinary education.

Abstract

This Master Thesis is a contribution to a Danish research project: "Robust calves – well begun is half done" performed in cooperation between Aarhus University, SEGES, Technical University of Denmark (DTU) and University of Copenhagen between 2018 and 2021. The objective of the work presented in this thesis was to 1) to describe the distribution of selected respiratory pathogens in the calves in the study herds, and 2) to analyse associations between respiratory calf disease, presence of pathogens and other host and environmental risk factors.

This cross-sectional study included calves from two different age groups 0 – 14 and 14 – 28 days of age. These calves came from nine commercial dairy herds. The data were collected between September and December 2018 and consist of clinical and environmental observations as well as laboratory results from pathogen testing of collected nasal swabs.

The nasal swabs were analysed at DTU by the high-throughput qPCR platform (Fluidigm). It was the first time this diagnostic technique, which allows for testing for multiple pathogens simultaneously, was used for detection of pathogens from calves. The following pathogens were included in the PCR test: *Histophilus somni, Pasteurella multocida, Mannheimia haemolytica, Trueperella pyogenes, Mycoplasma bovis, Mycoplasma spp.,* BRSV (Bovine Respiratory Syncytial Virus), BCoV (Bovine Corona Virus) and Influenza D.

The pathogens detected from the nasal swabs in this study included *Pasteurella multocida*, *Mannheimia haemolytica, Trueperella pyogenes, Mycoplasma bovis* and *Mycoplasma spp*. for calves aged 0 – 14 days, whereas for calves aged 14 – 28 days *Pasteurella multocida*, *Mannheimia haemolytica, Trueperella pyogenes* and *Mycoplasma spp*. were found in nasal swabs.

The Cycle threshold values (Ct-values) were inspected visually using graphics and plausible cut-off values were selected to classify all test results as positive or negative for each pathogen. Then factors associated with observed clinical signs of respiratory disease was analysed as a dichotomous outcome in a generalized mixed effects model where age, sex, breed, body condition score (BCS), housing location and pathogens with positive test results were tested for significant associations.

The results showed a significant association between having respiratory disease, being a bull and being tested positive for *Pasteurella multocida* for calves 0 – 14 days old. For calves in the age group 14 – 28 days of age, lower BCS and testing positive for *Pasteurella multocida* had a significant increased probability of having respiratory disease.

More studies are needed to validate the PCR Fluidigm platform and to test whether new and more observations will confirm the associations with respiratory disease found in this study. The causality behind the significant associations with respiratory disease found in this study should be investigated in future longitudinal studies to better understand how to prevent disease outbreaks prospectively and to contribute to increased calf health overall.

It has been possible to use the collected data in this study to analyse for risk factors for having respiratory disease based on a relatively practical applicable method including nasal swabs analysed by the PCR Fluidigm platform.

Resumé

Dette specialeprojekt er et delprojekt under det danske forskningsprojekt: "Robuste kalve – godt begyndt er halvt fuldendt", som er udført i et samarbejde mellem Aarhus Universitet, SEGES, Danmarks Tekniske Universitet (DTU) og Københavns Universitet og foregår fra 2018 til 2021. Formålet med denne specialeopgave var 1) at beskrive fordelingen af udvalgte respiratoriske patogener hos kalvene i de inkluderede besætninger, og 2) at analysere sammenhænge mellem respiratorisk sygdom, tilstedeværelse af patogener og andre værts- og miljømæssige risikofaktorer.

Dette tværsnitsstudie inkluderede kalve fra to forskellige aldersgrupper 0 – 14 dage og 14 – 28 dage gamle. Disse kalve kom fra 9 forskellige malkekvægsbesætninger. Data blev indsamlet i perioden fra september til december 2018 og inkluderede kliniske og miljømæssige observationer, samt laboratorieresultater for fund af patogener fra indsamlede næsesvaberprøver.

Næsesvaberne blev analyseret på DTU ved hjælp af en high-throughput qPCR platform (Fluidigm). Det er første gang, at denne diagnostiske teknik, som tillader at teste for mange patogener på én gang, er blevet anvendt til påvisning af patogener fra kalve. Følgende patogener blev undersøgt ved PCR testen: *Histophilus somni, Pasteurella multocida, Mannheimia haemolytica, Trueperella pyogenes, Mycoplasma bovis, Mycoplasma spp.,* BRSV (Bovine Respiratory Syncytial Virus), BCoV (Bovin Coronavirus) og Influenza D.

Patogener fra næsesvaberprøverne i dette studie, som blev testet positiv var for kalve i alderen 0 – 14 dage *Pasteurella multocida*, *Mannheimia haemolytica, Trueperella pyogenes, Mycoplasma bovis* og *Mycoplasma spp*.. Hos kalvene i alderen 14 – 28 dage blev *Pasteurella multocida*, *Mannheimia haemolytica, Trueperella pyogenes* og *Mycoplasma spp*. fundet positive i næsesvaberprøverne.

Cycle threshold values (Ct-værdier) blev inspiceret visuelt ved hjælp af grafik og derved blev plausible cut-off værdier valgt for at kunne klassificere testresultaterne for hvert patogen som værende positive eller negative. En respiratorisk sygdomsdefinition blev sat ind som et dikotom outcome i en generaliseret mixed model baseret på en logistisk regressionsmodel. I modellen

blev alder, køn, race, huld, opstaldningssted og positivt påviste patogener testet for enhver signifikant sammenhæng mellem disse og respiratorisk sygdom.

Resultaterne viste en signifikant sammenhæng mellem at have respiratorisk sygdom og være en tyr samt teste positivt for *Pasteurella multocida* hos kalve i alderen 0 – 14 dage. For kalve i aldersgruppen 14 – 28 dage førte en lavere huld og positive testresultater for *Pasteurella multocida* til en signifikant øget sandsynlighed for at have respiratorisk sygdom.

Flere undersøgelser er indikeret for at kunne validere PCR Fluidigm platformen og for at kunne teste, om flere observationer ville kunne frembringe samme og andre signifikante sammenhænge med respiratorisk sygdom. Derudover bør kausaliteten imellem de signifikante sammenhænge i dette studie undersøges nærmere i fremtidige longitudinelle studier for at vide, hvor der skal sættes ind i forhold til forebyggelse af fremtidige sygdomsudbrud, og derved bidrage til øget kalvesundhed.

Det har i dette studie været muligt at anvende de indsamlede data til analyse af risikofaktorers association med respiratorisk sygdom baseret på en forholdsvis praktisk anvendelig metode, herunder næsesvaber, som blev analyseret ved hjælp af PCR Fluidigm platformen.

Contents

Abbreviations

AUC	Area under the curve
BAdV	Bovine Adenovirus
BCoV	Bovine Corona Virus
BCS	Body Condition Score
BHV	Bovine Herpes Virus
BPIV-3	Bovine Parainfluenza Virus
BRD	Bovine Respiratory Disease
BRDC	Bovine Respiratory Disease Complex
BRSV	Bovine Respiratory Syncytial Virus
BVDV	Bovine Virus Diarrhea Virus
CI	Confidence interval
Ct-value	Cycle threshold value
DNA	Deoxyribonucleic acid
DTU	Technical University of Denmark
H. somni	*Histophilus somni*
IgG	Immunoglobulin
M. bovis	*Mycoplasma bovis*
M. haemolytica	*Mannheimia haemolytica*
OR	Odds ratio
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
P. multocida	*Pasteurella multocida,*
p-value	Probability-value
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribo Nucleic Acid
ROC	Receiver operating characteristic
SD	Standard deviation
SE	Standard Error
T. pyogenes	*Trueperella pyogenes*
WP	Work package
˚C	Celsius degrees

1. Introduction

In Danish cattle farms mortality among calves has been relatively stable for the past 18 years even though the Danish dairy production has gone through large structural changes and a veterinary health advisory system has been implemented (Vaarst and Sørensen, 2009). In 1999, the mortality risk was 7% in calves from 0 to 180 days old compared to 2017, where it was 6.7% (Raundal and Nielsen, 2018).

Despite a small reduction in the calf mortality since 1999, it is still of societal and sectorial concern due to discussions about mortality associated disease prevalence, animal welfare and antibiotic usage. The Danish agriculture business organisation, "Danish Agriculture & Food Council", has set some strategic goals for reduction of calf mortality to be no more than 5.5% and the total antibiotic usage for calf production to be reduced to 1900 kg of active ingredients by 2020 (DAFC, 2018).

Nevertheless, the use of diagnostics for diseased calves is currently very limited in the Danish dairy herds and interest in making a feasible tool available for daily management decision-making is increasing. Due to political decisions strict regulation has been implemented on the Danish antibiotic usage and disease registration to keep control of antibiotic resistance (Retsinformation, 2018).

Improving the knowledge about the circulating pathogens in the herd would provide the farmer and the herd veterinarian with an improved possibility of making interventions for preventing diseases, which could lead to improved calf health and thereby reduced mortality.

Respiratory disease is the biggest disease problem in Danish calves and inventories have shown that 71% of all antibiotic use in Danish calves is prescribed for respiratory disease (Jensen et al., 2018).

Bovine Respiratory Disease (BRD) is an acute disease complex that includes uncertain diagnosis with a range of clinical signs related to the respiratory tract in a group of cattle (Radostits et al., 2007). The aetiology of BRD is mentioned as multifactorial and develops as an outcome of interactions between predisposing factors due to management, environment and pathogens (Van der Fels-Klerx et al., 2002).

The most common pathogens involved in development of respiratory disease in Denmark are *H. somni*, *P. multocida, M. haemolytica, T. pyogenes* and BRSV respectively (Tegtmeier et al., 1999; Larsen, 2000) . Beside these pathogens *M. bovis* is recorded as causing BRD in slaughter

calves (Wiggins et al., 2007). Beside these mentioned pathogens, Bovine Virus Diarrhea Virus (BVDV), Bovine Herpes 1 Virus (BHV-1), Bovine Parainfluenza 3 Virus (BPIV-3), Bovine Corona Virus (BCoV) and Bovine Adenovirus (BAdV) have been related to BRD in calves globally (Härtel et al., 2004; Ellis, 2009).

1.1. Known and potential respiratory pathogens in calves

H. somni is a commensal of the mucosal surface of the respiratory tract and is usually spread by direct contact (Pérez et al., 2010). *H. somni* can cause respiratory infection with mild to severe clinical signs consisting of fever, tachypnoea, coughing, nasal discharge, depression and can rarely cause fibrinous pleuritis. Often when *H. somni* causes respiratory disease there is disease in another organ system. *H. somni* is not divided into different serogroups, but there seem to be some isolates that are more virulent than others (Groom and Little, 1988; Woolums, 2015).

P. multocida infections may develop as pneumonia with clinical signs such as fever, depression, reduced appetite, tachypnoea, dyspnoea and with mucopurulent lacrimal and nasal discharge (Dowling et al., 2002; Woolums, 2015). *P. multocida* has been described as a commensal and a pathogen that has a horizontal transmission pattern. It has been suggested that the change between pathogenic and non-pathogenic behaviour of *P. multocida* occurs because of changes in environmental signals, stress, co-mingling, overcrowding and exposure to other bacterial or viral infections (Dabo et al., 2007).
Although *P. multocida* is dominant it is not necessarily the primary cause of disease. A study has shown that *P. multocida* has overcome an introduced pure culture with *M. haemolytica*. *P. multocida* includes 5 different serotypes and 16 different somatic serotypes. The primary serotype in cattle is serotype A and serotype A3 is predominant in calf pneumonia (Davies et al., 2004; Woolums, 2015).

M. haemolytica infections look similar to an infection caused by *P. multocida* but can present as a longer and more severe illness period. The symptoms including fever, tachycardia, tachypnoea, salivation, pale mucous membranes, extended capillary filling time and cold extremities are caused by the release of endotoxins (Ames et al., 1985). *M. haemolytica* includes 12 serotypes but not all are pathogenic. The most frequent isolated from sick calf lungs are serotype A1 and A6, while serotype A2 is most common in healthy calf lungs (Al-Ghamdi et al., 2000; Woolums, 2015).

T. pyogenes is a commensal of the nasopharyngeal mucosa and other mucosal surfaces in cattle and invades the lung as a pathogen after other primary infectious agents already have introduced pneumonia (Confer, 2009; Woolums, 2015).

M. bovis is a pathogenic organism which is characterized by the lack of a cell wall and a very small degree of a genome (Razin et al., 1998). It is difficult to estimate the precise role of *M. bovis* in the development of respiratory disease in cattle because of a complex pathogenesis, diffuse pattern of disease spread and clinical symptoms (Caswell and Archambault, 2007). The infection with *M. bovis* is described as a direct transfer by contact between cows and calves and as an indirect transfer by intake of contaminated milk or contaminated surroundings (Maunsell and Donovan, 2009). Stress and many movements, as in case with slaughter calves, are also described as predisposing factors for infection with *M. bovis* (Castillo-Alcala et al., 2012). Farm prevalence is estimated between 0 – 4% but the results also differ between PCR and culturing (Wiggins et al., 2007). The most frequent clinical manifestations of infection by *M. bovis* in calves are respiratory disease, arthritis and otitis media (Maunsell and Donovan, 2009). Other non-specific symptoms are nasal discharge, dyspnoea, decreased appetite, fever, head shaking and head tilt (Maunsell et al., 2011).

Bovine Respiratory Syncytial Virus (BRSV) is an enveloped RNA virus and belongs to the Paramyxoviridae family, subfamily Pneumovirinae, genus Pneumovirus. The transmission of BRSV occurs by contact between infected calves and aerosols. BRSV can lead to subclinical, mild or severe clinical signs including fever, depression, decreased appetite, elevated respiratory rates, hypersalivation, coughing and nasal and lacrimal discharges (Ellis et al., 1996; Woolums, 2015).

1.2. Other potential risk factors of respiratory disease in calves

Other factors such as age, sex, breed, BCS and housing type might have an influence on the development of respiratory diseases in calves and are described in the following.

The risk of illness and death in calves changes with the age of the calves as shown in several studies. The highest morbidity and mortality risks have been found to be during the first two weeks of life (Sivula et al., 1996; Wells et al., 1997).

It is an open question whether sex is a risk factor for respiratory disease among young calves. A literature review by Taylor et al. (2010) reported on different studies with conflicting results. Some studies found significant higher risk for bulls to have BRD while other studies found significantly higher risks among heifers.

Snowder et al. (2006) made a study of the influence of breed on prevalence of BRD. No significant results were found. However, this study only included beef-cattle breeds, so an association between breed and prevalence of BRD in dairy calves cannot be rejected.

Body condition score (BCS) is primarily used as a risk factor in mature heifers and cows to monitor of their health status (Markusfeld et al., 1997). It is a challenge to estimate the health status of young calves by using BCS. Weight measurement is more often used in young calves. A significant relationship between weight and BRD has probably not been described. However, a study found a significant relationship between the arrival weight of slaughter calves into a slaughter herd and the presence of Bovine Herpes 4 Virus (BHV-4). At arrival the calves with higher weight had a lower risk of morbidity compared to the calves with lower weight at arrival (Murray et al., 2018).

Calves housed in groups have a higher risk of getting respiratory diseases (Svensson et al., 2003; Marcé et al., 2010). Furthermore, a study by Gulliksen et al. (2009) found that calves housed in groups at an early age had a higher mortality risk during the first month of life compared to calves housed in individual pens. In a Danish study by Pedersen et al. (2009), it was found that the prevalence of respiratory disease was more than twice as high among calves housed in groups where the introduction and removal of new calves occurred continuously compared to groups of calves where the introduction and removal happened at one point in time (i.e. all-in-all-out system). Calves housed outside generally have a lower risk of

respiratory disease compared to calves housed inside according to one Danish study (Dalgaard, 2005). In that study the number of treatments of respiratory and gastrointestinal diseases was 50% lower in calves housed outside compared to calves housed indoors.

1.3. Aim and objective of the study

The aim in this thesis was to identify pathogens and other risk factors that are involved in respiratory disease in young calves. This knowledge could be used to prevent disease outbreaks prospectively and to contribute to increase calf health overall.

Therefore, the objective of the study presented in this thesis was 1) to describe the distribution of selected respiratory pathogens in the calves in the study herds, and 2) to analyse associations between respiratory calf disease, presence of pathogens and other host and environmental risk factors.

2. Materials and methods

2.1. Study design and herd selection

The data of this study were collected for a cross-sectional study in the period between September 2018 and December 2018 from nine Danish dairy herds. Six of the herds were located in the southern Jutland and three on Zealand. Every herd was visited at least two times and the visits included clinical observations and diagnostic testing in two age groups: 0 – 14 and 14 – 28 days of age. Some calves could be observed in both age groups but every observation was evaluated individually.

For the first age group 10 heifers and 10 bulls were observed and if there were not enough calves represented, the age span was extended or extra visits were done the week after. The same number of heifers and bulls was sought. For the second age group 10 heifers were observed and if some bulls were still at the dairy herd and not sent to the slaughter herd they were included as well. The calves in each age group were randomly selected for observation.

The herd selection criteria were based on the farmers' willingness to participate, enough calves in the included age groups and also on a convenient distance to the living address of the main responsible project observers for the practical implementation. Thus, the herds were not randomly chosen but selected by "convenience sampling".

2.2. Clinical scoring protocol

The clinical scoring was done using a pre-prepared protocol to which the participating team of technicians, veterinarians and veterinary students had been calibrated during workshops (one just before start of data collection and one in November 2018).

At every herd visit clinical scores were performed and diagnostic samples were collected. A clinical protocol was developed for the project with guidance from Welfare Quality (Blokhuis, 2008) and Love et al. (2014) and data were directly entered into a digital online registration platform called "EasyOn". The protocol contains descriptions of each scoring pattern and how the diagnostic samples were supposed to be collected (see Appendix A).

Every single calf was registered for its age, sex, breed and the housing location on the registration date. The clinical observation involved scoring of nasal and lacrimal discharge and ear and head position. An attempt to provoke coughing by laryngeal manipulation was made if

the calf had not coughed spontaneously during the examination. The BCS was evaluated and scored as normal, lean or fat.

2.3. Materials and sample collection

Every sample was manually labelled with a unique number and was synchronised with "EasyOn". The nasal swabs were collected with sterile polyester-tipped swabs with a wooden shaft taken from the dorsal conchae. The swab was guided in until resistance was obtained and rotated few times against the walls of the animal´s nares. The tip of the swab was placed in an Eppendorf tube containing PBS. All samples were cooled immediately and sent for laboratory PCR analysis at DTU.

2.4. Preparation of samples

At arrival at the laboratory, all samples were transferred to NUNC tubes and placed at –80 ˚C until analysis. RNA and DNA were extracted and preamplified according to the procedures listed in Appendix B. In order to test the samples for both DNA (bacteria and parasites) and RNA targets (viruses) in the same test, the samples were both preamplified and reverse transcribed/preamplified after purification. After the samples were preamplified and reverse transcribed/preamplified they were stored at –20 ˚C.

2.5. High-throughput qPCR Fluidigm platform

PCR is a popular diagnostic tool due to higher sensitivity and speed compared to viral and bacteriological cultivation (Koskinen et al., 2009). Real-time PCR is a very sensitive and specific method for detection and quantification of DNA and RNA because it is possible to detect few or even as low as one copy per reaction cycle. Many different PCR platforms have been developed of which the most commonly used are low-throughput platforms. The numbers of samples that a low-throughput platform can test at once are limited (Spurgeon et al., 2008; Ishii et al., 2013).

The high-throughput qPCR platform has been used for detection of multiple food and waterborne pathogens (Ishii et al., 2013) as well as detection of tick-borne pathogens in Western Europe (Michelet et al., 2014). Furthermore, a high-throughput real-time PCR array (Fluidigm, South San Francisco, CA) for detection of multiple swine pathogens has previously been designed and validated on samples from pigs in Denmark as a part of an objective health monitoring study performed in nursery and finisher pigs (Goecke, 2018). The sensitivity of the

Biomark chip for swine pathogens was found to be comparable to the traditional real-time assays routinely used (Goecke, 2018).

This system greatly reduces the time, labour and reagent requirements compared with conventional PCR platforms (Spurgeon et al., 2008; Ishii et al., 2013; Michelet et al., 2014). The fact that the high-throughput PCR assay panel is easy to modify because of the possibility to add or remove primers or probes according to the relevant pathogens makes it a very beneficial tool in a large-scale pathogen-monitoring programme (Ishii et al., 2013; Michelet et al., 2014).

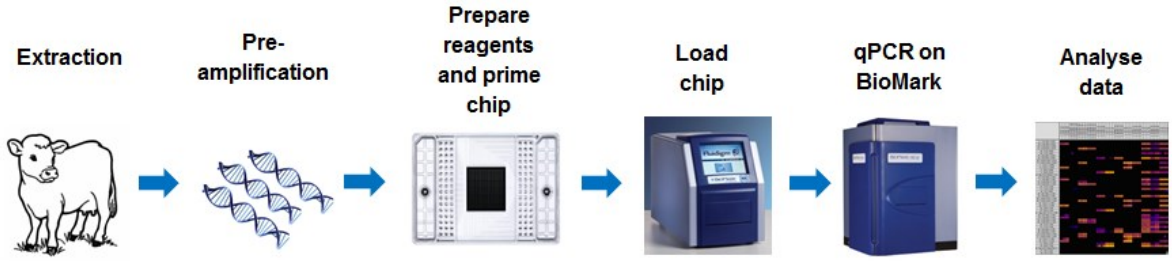


Figure 1: Flowchart of the high-throughput qPCR Fluidigm System. The system has never been used for detection of cattle pathogens before (Source: Nicole Bakkegård Goecke).

For this project a Fluidigm chip to detect the bovine pathogens *H. somni, P. multocida, M. haemolytica, T. pyogenes, M. bovis*, *Mycoplasma spp.,* BRSV, BCoV and Influenza D virus has been developed. The chip used can dispense 24, 48 or 96 PCR assays and 48, 96 or 196 samples into individual chambers prior to thermal cycling resulting in 2304 and 9216 individual reactions (Michelet et al., 2014). In this study we used a chip containing 24 individual assays and 192 samples including control samples that resulted in 4608 individual PCR reactions.

2.6. Input for interpreting PCR test results

A PCR result needs a specific cut-off value to tell if the result is positive or not. To know when a positive result is true positive a "gold standard" for the laboratory test is preferred. A "gold standard" is a method that tests for the same agent and preferably rarely becomes positive if the animal is uninfected, or negative if the animal is infected (Houe et al., 2004). This could be tested by other diagnostic tests, such as bacteriological culturing. In reality, good "gold standard" tests are often hard to find, and for this study none were used.

Because the high-throughput qPCR platform (Fluidigm) has not been used in bovine research before and such a validation has not been performed in a field setting, we received other raw

PCR results from the "Robust calves – well begun is half done" project and had to make our own interpretation of the PCR results. For this we used an independent source of data, namely PCR results from 120 samples originating from 83 slaughter calves sampled in two slaughter herds and tested on the same PCR platform used in this study. These PCR results were then used for decision-making and interpretation when defining Ct-value cut-offs for each pathogen by visual inspection of the distributions of Ct-values (see Appendix C).

2.7. Data management and statistical analysis

The clinical and environmental observations were converted from "EasyOn" into an Excel document together with laboratory results and then the statistical programmes; R® (version 3.5.2) and R Studio (version 1.1.463) were used for performing statistical analysis. All observations and laboratory results were distributed on single animal level.

2.7.1. Descriptive statistic

The dichotomous outcome variable was described as respiratory disease (yes/no) and defined as clinical signs including coughing either spontaneously or provoked, mucopurulent nasal or lacrimal discharge, ear tilt either unilateral or bilateral, or head tilt.

The age of the calves at the time for the clinical and environmental observations was expressed as a continuous variable and analysed by the mean, median, minimum and maximum values, 95% confidence intervals and the standard deviation.

For the categorical variables a prevalence for each variable in each outcome group; Respiratory disease positive and Respiratory disease negative, has been analysed. The categorical variables included sex (heifers or bulls), breed (Holstein, Danish red, Cross-breed), housing location (calf housed either outside with no roof (e.g. huts in the open), under a roof with minimum one side open or in a barn/stable with all sides closed), BCS (normal, lean or fat) and the positive tested pathogens from the nasal swabs with individual Ct-values. All the categorical variables were included as factor variables.

This study included nine different Danish dairy herds where different environment, management and biosecurity may have had an influence on the risk of getting respiratory disease for the calves in this study. In Appendix D a table is shown with the comparison between selected conditions for the nine herds. To meet this diversity we added *herd effect* as a *random effect.*
Every herd has a responsible main observer, and an eventual disagreement between them has to some extent been taken into account indirectly by using the *herd effect*.

2.7.2. Multivariable analysis

To clarify whether the risk factor variables have a significant impact on the probability of having respiratory disease or not a generalized mixed model, based on a logistic regression model was made using the "glmer" function in R®.

Because this model includes both qualitative and quantitative variables, the generalized mixed effects model is given by (Houe et al., 2004):

$$logit(p_{ij}) = \alpha + B_{ij} + \gamma x_{ij} + v_j$$

α	Intercept
B_{ij}	Effect of the qualitative explanatory variable for calf *j* in herd *i*
γ	Slope for the continuous variable
x_{ij}	Continuous variable for calf *j* in herd *i*
v_j	Random variation for herd *i*

The model was specified as a generalized mixed effects model with a transformed y-axis using logit-transformation and involving both fixed effects as explanatory variables and a *random effect* of herd, because calves within herds were expected to be more similar than calves between herds. The final model included the significant variables (p-value ≤ 0.05) and a *random effect* all compared by the fact of having respiratory disease.

To find the final model with the best fit to the data in each age group every significant variable was analysed individually and in combination in "receiver operating characteristic" (ROC) curves with the "roc.test" function in R® (see Appendix E).
The ROC curves work by plotting sensitivity values against 1–specificity. To interpret a ROC curve consider "the area under the curve" (AUC). The AUC of the ROC curve of a worthless

test is 0.5 (including 50% sensitivity and 50% specificity) while the AUC of the ROC curve for a perfect test is 1 (100% sensitivity and 100% specificity) (Houe et al., 2004).
The highest AUC values between the analysed significant variables either individually or in combination were chosen for the best fit model.

The definition of having respiratory disease is a subjective definition and included coughing either spontaneously or provoked, mucopurulent nasal or lacrimal discharge or ear tilt either unilateral or bilateral, or head tilt (Definition 1). Therefore the robustness of the model results to a changed outcome definition (Definition 2) was tested. The definition was rephrased with milder thresholds for the including variables to see if it would give other results in the final model by comparing the different AUC out of ROC curves for the two definitions (see Appendix F). The milder definition included coughing, either spontaneous or provoked, serous or mucopurulent nasal or lacrimal discharge or ear tilt either unilateral or bilateral, or head tilt.

Age was also included in the final model as the only continuous variable that gave a distribution of the results according to age. This variable was included as two effects; age and age * age.

Parameter estimate, standard error (SE), p-value, odds ratio (OR) and 95% confidence interval (CI) for the OR were indicated in the final model. The p-value is significant when the p-value is < 0.05. Additionally, the variance and standard deviation (SD) for the *random effect* was added too.

3. Results

3.1. Descriptive statistics

In total, 202 observations were performed out of 121 calves. Out of these, 81 calves had a second observation in the oldest age group.

3.1.1. Continuous variable

The age of the calves at the time for the clinical and environmental observations was expressed as a continuous variable.

Table 1: The distribution of age (in days) for all the observations (N) in respiratory disease positive or negative calves described by mean, median, minimum and maximum values, 95% confidence intervals and the standard deviation (SD).

	Age in days					
	Mean (median)	**Min**	**Max**	**95% CI**	**SD**	**N**
Respiratory diseases +	11.6 (9)	0	33	[1 ; 24.1]	7.8	75
–	9.9 (7)	0	26	[1 ; 24.8]	6.9	127

From Table 1 it can be seen that the age of the included calves ranged from 0 – 33 days. The sick calves were on average around 2 days older than the healthy calves.

3.1.2. Categorical variables

Descriptive analyses were prepared for calves with respiratory disease (respiratory disease +) and without respiratory disease (respiratory disease –) and divided into selected variables.

Calves aged 0 – 14 days

Table 2: Number of observations (n) and prevalence distributions (%) for respiratory disease for each of the categorical variables including the pathogens based on PCR results divided into positive results (+) and negative results (–) with their pathogen specific Ct-value cut-offs in calves from 0 – 14 days of age.

		Respiratory disease +	Respiratory disease –
Variable	**Category**	**n (%)**	**n (%)**
Observations		41 (33.9)	80 (66.1)
Sex	**Heifer**	17 (24.6)	52 (75.4)
	Bull	24 (46.2)	28 (53.8)
Breed	**Holstein**	32 (33.3)	64 (66.7)
	Danish Red	2 (50.0)	2 (50.0)
	Cross-breed	7(33.3)	14 (66.7)
BCS	**Normal**	35 (33.3)	70 (66.7)
	Lean	6 (37.5)	10 (62.5)
	Fat	0 (0.0)	0 (0.0)
Housing location	**Outside**	15 (34.1)	29 (65.9)
	Under a roof	11 (44.0)	14 (56.0)
	In a barn/stable	15 (28.8)	37 (71.2)
Herd	**A**	3 (23.1)	10 (76.9)
	B	2 (11.1)	16 (88.9)
	C	1 (25.0)	3 (75.0)
	D	5 (31.2)	11 (68.8)
	E	9 (69.3)	4 (30.8)
	F	7 (38.9)	11 (61.1)
	G	5 (50.0)	5 (50.0)
	H	6 (30.0)	14 (70.0)
	I	3 (33.3)	6 (66.7)
H. somni	**+ (Ct ≤ 25)**	0 (0.0)	0 (0.0)
	– (Ct > 25)	41 (33.9)	80 (66.1)
P. multocida	**+ (Ct ≤ 25)**	5 (71.4)	2 (28.6)
	– (Ct > 25)	36 (31.6)	78 (68.4)
M. haemolytica	**+ (Ct ≤ 30)**	3 (75.0)	1 (25.0)
	– (Ct > 30)	38 (32.5)	79 (67.5)
T. pyogenes	**+ (Ct ≤ 27)**	6 (37.5)	10 (62.5)
	– (Ct > 27)	35 (33.3)	70 (66.7)
M. bovis	**+ (Ct ≤ 35)**	1 (100.0)	0 (0.0)
	– (Ct > 35)	40 (33.3)	80 (66.7)
Mycoplasma spp.	**+ (Ct ≤ 35)**	3 (100.0)	0 (0.0)
	– (Ct > 35)	38 (32.2)	80 (67.8)
BRSV	**+ (Ct ≤ 25)**	0 (0.0)	0 (0.0)
	– (Ct > 25)	41 (33.9)	80 (66.1)
BCoV	**+ (Ct ≤ 40)**	0 (0.0)	0 (0.0)
	– (Ct > 40)	41 (33.9)	80 (66.1)
Influenza D	**+ (Ct ≤ 40)**	0 (0.0)	0 (0.0)
	– (Ct > 40)	41 (33.9)	80 (66.1)

Table 2 shows that 33.9% of all the observations were defined as having respiratory disease and there was an uneven distribution between heifers and bulls on 24.6% and 46.2%, respectively. Most of the included calves are Holsteins. Very few of the calves were scored as lean and none was scored as fat. About 1/3 of the calves with a normal BCS has respiratory disease and about the same proportion in calves with lean BCS. The calves were registered in three different housing locations of which about 1/3 of the calves that were housed outside, about 1/2 of the calves housed under a roof and 1/4 of the calves housed in a barn or stable with four solid walls had respiratory disease. Table 2 also shows the distribution of having respiratory disease or not in the nine dairy herds involved. The prevalence ranged between 11.1% and 69.3% of having respiratory disease in the participating herds. Out of the tested pathogens from the nasal swabs *P. multocida, M. haemolytica, T. pyogenes, M. bovis* and *Mycoplasma spp.* were detected in the analysis.

Calves aged 14 – 28 days

Table 3: Number of observations (n) and prevalence distributions (%) for respiratory disease for each of the categorical variables including the pathogens based on PCR results divided into positive results (+) and negative results (–) with their pathogen specific Ct-value cut-offs in calves from 14 – 28 days of age.

		Respiratory disease +	Respiratory disease –
Variable	**Category**	**n (%)**	**n (%)**
Observations		34 (42.0)	47 (58.0)
Sex	**Heifer**	28 (48.3)	30 (51.7)
	Bull	6 (26.1)	17 (73.9)
Breed	**Holstein**	25 (40.3)	37 (59.7)
	Danish Red	2 (28.6)	5 (71.4)
	Cross-breed	7 (58.3)	5 (41.7)
BCS	**Normal**	25 (36.8)	43 (63.2)
	Lean	9 (69.2)	4 (30.8)
	Fat	0 (0.0)	0 (0.0)
Housing location	**Outside**	23 (48.9)	24 (51.1)
	Under a roof	1 (11.1)	8 (88.9)
	In a barn/stable	10 (40.0)	15 (60.0)
Herd*	**A**	6 (54.5)	5 (45.5)
	B	2 (15.4)	11 (84.6)
	C	7 (50.0)	7 (50.0)
	D	6 (50.0)	6 (50.0)
	G	2 (50.0)	2 (50.0)
	H	3 (23.1)	10 (76.9)
	I	8 (57.1)	6 (42.9)
H. somni	**+ (Ct ≤ 25)**	0 (0.0)	0 (0.0)
	– (Ct > 25)	34 (42.0)	47 (58.0)
P. multocida	**+ (Ct ≤ 25)**	12 (85.7)	2 (14.3)
	– (Ct > 25)	22 (32.8)	45 (67.2)
M. haemolytica	**+ (Ct ≤ 30)**	1 (20)	4 (80)
	– (Ct > 30)	33 (41.2)	47 (58.8)
T. pyogenes	**+ (Ct ≤ 27)**	6 (42.9)	8 (57.1)
	– (Ct > 27)	28 (41.8)	39 (58.2)
M. bovis	**+ (Ct ≤ 35)**	0 (0.0)	0 (0.0)
	– (Ct > 35)	34 (42.0)	47 (58.0)
Mycoplasma spp.	**+ (Ct ≤ 35)**	7 (63.6)	4 (34.4)
	– (Ct > 35)	27 (38.0)	44 (62.0)
BRSV	**+ (Ct ≤ 25)**	0 (0.0)	0 (0.0)
	– (Ct > 25)	34 (42.0)	47 (58.0)
BCoV	**+ (Ct ≤ 40)**	0 (0.0)	0 (0.0)
	– (Ct > 40)	34 (42.0)	47 (58.0)
Influenza D	**+ (Ct ≤ 40)**	0 (0.0)	0 (0.0)
	– (Ct > 40)	34 (42.0)	47 (58.0)

* Calves in 14 – 28 days of age from herd E and F were excluded because of absence of PCR results.

Table 3 shows that 42.0% of all the observations were defined as having respiratory disease and there was an uneven distribution between heifers and bulls on 48.3% and 26.1%, respectively. Most of the included calves were Holsteins and of these 40.3% had respiratory disease. The proportion of lean calves with respiratory disease was relatively high (69.2%) and 36.8% of the calves with normal BCS had respiratory disease. Most of the calves were housed outside and the distribution of respiratory disease is almost equal in this group. Compared to the calves in 0 – 14 days of age two of the participating herds (E and F) had missing laboratory results and were excluded in this age group. The prevalence of having respiratory disease ranged between 15.4% and 57.1% in the involving herds. Out of the tested pathogens from the nasal swabs *P. multocida, M. haemolytica, T. pyogenes* and *Mycoplasma spp.* were detected in the analysis.

3.2. Multivariable analysis

A final model for each age group was made. To specify which variables should be included in the final models every variable was tested individually against the fact of having respiratory disease and from that significant results were included in the models. For calves in 0 – 14 days of age sex ($p = 0.02$) and *P. multocida* ($p = 0.05$) had a significant result. For calves in 14 – 28 days of age BCS ($p = 0.03$) and *P. multocida* ($p = 0.04$) had a significant result.

To find the strongest final model for each age group every significant variable was analysed by ROC curves and AUC values were compared. The combinations with the highest AUC values were chosen for the final models.

Table 4: AUC and the related p-value for the including significant variables in the final model for calves in 0 – 14 days of age:

Variable	AUC	p-value
Sex	0.71	0.02*
P. multocida	0.71	0.049*
Sex + *P. multocida*	0.73	0.005*

* The p-value is significant (the p-value is significant when it is ≤ 0.05).

Table 4 showed that sex in combination with *P. multocida* worked out strongest in the final model for showing an association between respiratory disease and the risk factor variables.

Table 5: AUC and the related p-value for the including significant variables in the final model for calves in 14 – 28 days of age:

Variable	AUC	p-value
BCS	0.75	0.03*
P. multocida	0.76	0.002*
BCS + *P. multocida*	0.82	0.00005*

* The p-value is significant (the p-value is significant when it is ≤ 0.05).

Table 5 showed that BCS in combination with *P. multocida* worked out strongest in the final model for showing an association between respiratory disease and the risk factor variables.

Rephrasing the definition of having respiratory disease (Definition 1) into a milder definition with lower thresholds for the including variables (Definition 2) was tested to see if it would give other results in the final model by comparing the different AUC out of ROC curves for the two definitions (see Appendix F).

Table 6: Comparison of the two definitions for the outcome tested in the final model for calves 0 – 14 days of age:

Model	AUC	p-value
Definition 1	0.73	0.02*
Definition 2	0.60	0.57

* The p-value is significant (the p-value is significant when it is ≤ 0.05).

Definition 1 had the highest AUC and was therefore chosen as the final model.

Table 7: Comparison of the two definitions for the outcome tested in the final model for calves 14 – 28 days of age:

Model	AUC	p-value
Definition 1	0.81	0.0005*
Definition 2	0.71	0.03*

* The p-value is significant (the p-value is significant when it is ≤ 0.05).

Both Definition 1 and Definition 2 were significant but because Definition 1 had the highest AUC and the lowest p-value it was therefore chosen as the final model.

3.2.1. Final models

Calves aged 0 – 14 days

Table 8: Results from the final logistic regressions model of the probability of having respiratory diseases by different explanatory variables in Danish dairy calves in 0 – 14 days of age in September – December 2018. The table includes variance and standard deviation (SD) of the *random effect* and the parameter estimate, standard error (SE), odds ratio (OR) with 95% confidence interval (CI) and the p-value of the fixed effects.

Variable			**Variance**		**SD**	
Random effects		**Herd**	0.31		0.56	
		Parameter estimate	**SE**	**p**	**OR**	**95% CI of OR**
Intercept		–1.3	0.4	0.0004	–	–
Sex	**Heifer**	0	–	–	1	–
	Bull	1.0	0.4	0.02*	2.8	[1.2 ; 6.6]
Age		-1.3	2.3	0.6	0.3	[0.0 ; 25.2]
Age * age		1.7	2.3	0.5	5.6	[0.1 ; 599]
P. multocida	**+ (Ct ≤ 25)**	2.1	1.0	0.04*	8.3	[1.3 ; 81.5]
	– (Ct > 25)	0	–	–	1	–

* The p-value is significant (the p-value is significant when it is ≤ 0.05).

In Table 8, sex and *P. multocida* are in combination with age significant for the probability of having respiratory disease in calves in 0 – 14 days of age. If the calf has respiratory disease there is 2.8 times higher probability for it to be a bull than a heifer. And if a calf has respiratory disease it has 8.3 times higher probability of testing positive for *P. multocida* compared to testing negative for *P. multocida*.

Calves aged 14 – 28 days

Table 9: Results from the final logistic regressions model of the probability of having respiratory disease by different explanatory variables in Danish dairy calves at 14 – 28 days of age in September – December 2018. The table includes variance and standard deviation (SD) of the *random effect* and the parameter estimate, standard error (SE), odds ratio (OR) with 95% confidence interval (CI) and the p-value of the fixed effects.

Variable			**Variance**		**SD**	
Random effects		**Herd**	0.23		0.48	
		Parameter estimate	**SE**	**p**	**OR**	**95% CI of OR**
Intercept		–1.0	0.4	0.005*	–	–
BCS	**Normal**	0	–	–	1	–
	Lean	1.9	0.9	0.03*	6.5	[1.4 ; 50.1]
Age		0.8	2.5	0.8	2.2	[0.0 ; 436]
Age * age		0.2	2.8	0.9	1.2	[0.0 ; 1614]
P. multocida	**+ (Ct ≤ 25)**	2.6	0.8	0.002*	14.0	[3.1 ; 101]
	– (Ct > 25)	0	–	–	1	–

* The p-value is significant (the p-value is significant when it is ≤ 0.05).

In Table 9, BCS and *P. multocida* are significant for the probability of having respiratory disease in calves in 14 – 28 days of age when accounting for age. If the calf had respiratory disease there was 6.5 times higher probability for it to be lean than to have normal BCS. Also, if a calf had respiratory disease, it had 14 times higher probability of testing positive for *P. multocida* compared to testing negative for *P. multocida*.

4. Discussion

The objective of this thesis was 1) to describe the distribution of selected respiratory pathogens in the calves in the study herds, and 2) to analyse associations between respiratory calf disease, presence of pathogens and other host and environmental risk factors. The interpretation of the results and the approach to analysing the available data will be discussed in this section.

4.1. Inclusion criteria, herd selection and sample size

In this study the only inclusion criteria for the included calves were that they should fit into the mentioned age groups. Their disease and treatment history were unknown and possible unknown treatments could have affected which clinical observations could be observed. For example in two of the herds the heifer calves were vaccinated against pathogens that are causing respiratory disease. In one "Bovalto Respi Intranasal" was used. This is a vaccine that gives active immunization to calves from 10 days of age against BRSV and BPIV-3. A side effect of this vaccine is that it can cause nasal discharge within 3 days after introduction. Unfortunately, the clinical observations and the PCR results from this specific herd (F) were missing (see Table 3) and we can therefore not tell whether this circumstance would have had any impact on the results.

The included herds were selected by "convenience sampling" and not by random selection. To be able to comment on the results at national level more herds should have been included, randomly selected and in addition the sample size should have been increased. But in the real world such studies are difficult to achieve due to the willingness of farmers to participate and funding availability for large field studies. Furthermore, it is difficult to find herds that are big enough so not too many herd visits are needed. When many visits are needed, other risk factors will be added like the season of the year.

In a study like this the optimal sample size could have been calculated beforehand by knowing the prevalence of having respiratory disease as a calf in a given age group. As our study was part of the "Robust calves – well begun is half done" project, where the sample size already was decided, it was not possible to calculate the optimal sample size.

4.2. High-throughput qPCR Fluidigm platform as a diagnostic tool

Using the PCR Fluidigm as a diagnostic tool is a great opportunity to test many samples for several pathogens on a large scale in short time. The PCR Fluidigm diagnostic test of the nasal swab samples has an unknown sensitivity and specificity, because no field study for diagnostic performance evaluation has been performed on bovine pathogens for this test before. Nevertheless, this diagnostic tool was chosen for this project because of its large capacity. The PCR Fluidigm is the main diagnostic tool in work package (WP)1 and WP2 in the "Robust calves – well begun is half done" project and is also used for developing a diagnostic platform. A large capacity/high throughput system is desirable because many BRD outbreaks are undiagnosed due to the limited capacity on conventional PCR platforms (Thonur et al., 2012). Therefore, this way of doing large-scale diagnostic testing can make it more cost-effective and encourage more diagnostic testing of BRD outbreaks in the future. In discussion about using PCR Fluidigm both as a diagnostic and a monitoring tool in modern veterinary practice, Horwood and Mahony, (2011) claimed that the high sensitivity of real-time qPCR in general can make this tool very beneficial in testing clinically ill and healthy individuals and thus determine at which concentration the pathogens will be pathogenic or not. In such a setup with regular sample collection during a specified time period, it could indeed be a helpful tool for herd veterinarians and farmers to follow the disease status in a herd over time.

Generally, PCR has a high sensitivity compared to bacteriological culture but does not distinguish between functioning pathogens and inactive DNA residues (Koskinen et al., 2009). It could have been of interest to compare the PCR Fluidigm results with culturing by the finding of bacterial pathogens. Likewise, a comparison between PCR Fluidigm results and serology could have been of interest with finding viruses.

4.3. Ct-value cut-off

The use of data from slaughter herds to set a realistic Ct-value cut-off for the laboratory results is a questionable approach. Slaughter herds and dairy herds might not be sufficiently comparable, e.g. the housing of a slaughter herd can be different, the calves have been mingled with a high number of calves from other herds and the slaughter calves in this study were older (22 – 96 days of age) than the dairy calves at the time of sample collection.

In this and most other studies, a Ct-value cut-off is often chosen but it has to be done with care. Burns and Valdivia (2008) made a modelling study of finding the limit of detection of positive samples in a real-time qPCR reaction. They concluded that choosing a very low cut-off value, there was a risk of incorrectly rejecting a true positive test result. On the other hand, a high Ct-

value can incorporate false positive test results. Angen et al. (2001) described how important it is to make a thorough evaluation of the criteria for defining a positive test in PCR results. It is important to have in mind what the primary intention with the laboratory testing is. If the intention was to find all possible present agents (both pathogens and commensals), a higher Ct-value cut-off was indicated. If the purpose was to be sure that the positive test results were true positive results, a lower Ct-value cut-off was indicated.
The PCR results from the slaughter herds were used for decision-making and interpretation of the Ct-value cut-offs for each pathogen by visual inspection of the distributions of Ct-values. Another method of choosing the Ct-value cut-offs could have been by making ROC curves analysis and calculating the AUC. This has been tried but was not possible with this data because of the lack of a suitable "gold standard".

4.4. Interpretation of test results

The descriptive analysis showed the prevalences of the pathogens detected in the nasal swab samples. Only *P. multocida* had a significant association with calves having respiratory disease in both age groups. In addition, *T. pyogenes, M. haemolytica and Mycoplasma spp.* were also found in both age groups and *M. bovis* was found in calves in 0 – 14 days of age. None of them were found to be associated with having respiratory disease in this study. It is not possible for us to claim that *P. multocida* has a causal association with calves that have respiratory disease in general, but what we can say is that it was the only statistically significant pathogen association we found in this study.

The other risk factors that had a significant association with calves that had respiratory disease were that bulls had a higher risk than heifers for having respiratory disease in calves aged 0 – 14 days. For calves in 14 – 28 days of age lean calves had a higher risk of having respiratory disease compared to calves with a normal BCS.

According to the farms' management procedures, all calves whether they were heifers or bulls were treated equally (see Appendix D). It was only in calves aged 0 – 14 days that sex had a significant association with respiratory disease. In the group with calves in 14 – 28 days of age, a predominance of heifers was seen because most bulls were moved to the slaughter herd around two weeks of age. Considering this fact, it did make sense that sex did not have a significant association in this age group. Hence, we cannot rule out that bulls might also be at higher risk in the oldest age group.

BCS had a significant association with respiratory disease in calves 14 – 28 days of age. First, it is questionable whether it is possible to determine BCS of a calf at such a young age. The body condition scoring system is well established and well defined as a clinical parameter among adult cattle. In adult cattle the differences in the BCS does not change very much along with age. But for young calves age changes the animal to such a large degree that it is debatable if it makes sense to use the BCS as a clinical parameter. A more valid variable could have been the measured weight, but this was not possible to use, because there were different breeds with too different starting weights included in this study. It was chosen to keep BCS as a risk factor because we wanted to see if the body condition of the calf had an association to its probability of having respiratory disease.
A study had found a significant association with lower feeding time and diagnosed respiratory disease (Toaff-Rosenstein and Tuckter, 2018). The study concluded that the feeding time was not affected on the last 2 days before clinical signs of respiratory disease was shown. That means that being lean might be caused by respiratory disease.

The overall causality of all the discussed risk factors about respiratory disease is very interesting and a very important issue to consider. The question is also of interest when being positive for *P. multocida* that is the case in both age groups of calves. It is an open question whether the calves having respiratory disease because they are tested positive for *P. multocida* or if they test positive for *P. multocida* because they have respiratory disease.
Dabo et al., 2007 has described many risk factors that have been present before *P. multocida* was detected. It suggests that predisposing factors as stress, co-mingling, overcrowding and bacterial and viral infections led to introduction of *P. multocida*.
A possible causality in this study could be that respiratory disease is predisposing for *P. multocida* to be pathogenic in the calves. Further, multiple infections such as gastrointestinal disease could possibly cause immunosuppression and in that way make *P. multocida* pathogenic. In several studies a significant association between clinical respiratory disease and history of having diarrhoea has been found (Curtis et al., 1988; Perez et al., 1990; Svensson et al., 2005). This substantiates a possible causative explanation of *P. multocida* as a secondary infection after predisposing risk factors.

Analysing the data used in this study as a cohort design could have been of great interest. In that way calves in the different age groups could be followed over time and thereby the impact of multiple infections or previously diseases e.g. gastrointestinal disease of getting respiratory disease, could have been evaluated.

The question about the causative role of a bacterial agent is interesting when the agent also can be detected in clinically healthy individuals as a commensal in the respiratory tract.
P. multocida is a bacterial commensal that can be detected in healthy calves. There might be a risk that our results were a coincidence and do not have any true causal association with calves having respiratory disease but because we used a relatively low Ct-value as a cut-off (positive = Ct ≤ 25), it is less likely.

5. Conclusion

In this thesis the distribution of selected respiratory pathogens in the calves in nine Danish dairy herds has been identified. In the group of calves that were 0 – 14 days of age at sampling, *P. multocida*, *M. haemolytica, T. pyogenes, M. bovis* and *Mycoplasma spp.* were detected in nasal swabs by PCR Fluidigm. *P. multocida*, *T. pyogenes, M. haemolytica* and *Mycoplasma spp.* were present in some of the calves 14 – 28 days of age. *H. somni,* BRSV, BCoV and Influenza D were not identified in any of the age groups in these herds.

A generalized mixed effects model was performed to investigate associations between signs of respiratory disease and the identified respiratory pathogens, age, sex, breed, BCS and housing location as fixed effects as well as herd as random effect. The results showed that bulls were significantly more likely (OR = 2.8) to have signs of respiratory disease than heifer calves ($p = 0.02$). In addition, presence of *P. multocida* in nasal swabs was significantly ($p = 0.04$) associated with respiratory disease in calves aged 0 – 14 days of age (OR = 8.3 in test-positive compared to test-negative calves). In the calves aged 14 – 28 days, lean BCS was significantly more likely (OR = 6.5) than calves with a normal BCS ($p = 0.03$). Additionally, *P. multocida* in nasal swabs was significantly ($p = 0.002$) associated with the fact of having a respiratory disease (OR = 14 in test-positive compared to test-negative calves).

The causality of the risk factors *P. multocida*, sex and BCS in association with the presence of respiratory disease needs to be investigated in future studies to analyse which risk factor has a significant influence on the other factors and the presence of respiratory illness as a calf. By knowing this, respiratory disease can be treated more correctly and a rational prophylaxis plan can be established for minimising future respiratory disease in calves.

Analysing the nasal swabs with the PCR Fluidigm platform was demonstrated to be useful in this study, even though the PCR Fluidigm platform has not been validated under field conditions for detection of bovine pathogens before. Results must therefore be interpreted with this knowledge in mind.

Overall, it has been possible to use the collected data in this study to analyse for risk factors for having respiratory disease based on a relatively practical applicable method.

6. Perspectives

Many studies have been performed to determine prevalence and description of BRD. However, further investigations of the BRD complex are indicated. In the future the cattle industry might be regulated to decrease antibiotic usage or at least need more documentation before initiating antibiotic treatments. To do so, more knowledge about the pathogenic bacterial, mycoplasma and viral agents is needed. For instance, a comparison between nasal swabs and tracheal and bronchoalveolar lavage would need to be evaluated to understand what nasal swab results actually mean in terms of infection of the calf. Moreover, an on-farm diagnostic approach would be of great interest so the sampling can be done without the attendance of a veterinarian. The nasal swab could be a good tool because of the easy and reasonably non-invasive approach.

An even less invasive approach to investigate pathogens could include air sampling and in that way look at pathogen concentrations in the environment by PCR testing. Another less invasive form of testing could be testing of milk and water troughs that the calves put their nose into. That could function as a screening tool beside the clinical screening and other diagnostic approaches on an animal level.

It would be interesting to see how the overall picture of the pathogen concentration is changing during a whole year. It is well known that respiratory disease is more present at autumn and wintertime but if a monitoring programme is going to be established as a result of this project more knowledge about the changing pathogen concentrations is needed.

Validating the PCR Fluidigm platform on bovine pathogens will be necessary if this technique is going to be a valuable diagnostic platform to use in the future. Not only for respiratory pathogens but also for the detection of gastrointestinal pathogens which cause significant disease among calves in dairy production.

Causality between respiratory disease, simultaneously multiple infections and other risk factor interactions as stocking density, environment scores, feeding analysis, calving difficulties and the parity number of the calf´s mother could be of interest to determine in future studies.

References

Al-Ghamdi, G.M., Ames, T.R., Baker, J.C., Walker, R., Chase, C.C.L., Frank, G.H., Maheswaran, S.K., 2000. Serotyping of Mannheimia (Pasteurella) Haemolytica Isolates from the Upper Midwest United States. Journal of Veterinary Diagnostic Investigation 12, 576–578.

Ames, T.R., Markham, R.J., Opuda-Asibo, J., Leininger, J.R., Maheswaran, S.K., 1985. Pulmonary response to intratracheal challenge with Pasteurella haemolytica and Pasteurella multocida. Canadian Journal of Comparative Medicine 49, 395–400.

Angen, O., Jensen, J., Lavritsen, D.T., 2001. Evaluation of 5' Nuclease Assay for Detection of Actinobacillus pleuropneumoniae. Journal of Clinical Microbiology 39, 260–265.

Blokhuis, H.J., 2008. International cooperation in animal welfare: The Welfare Quality® project. Acta Veterinaria Scandinavica 50, (Suppl I):S10.

Burns, M., Valdivia, H., 2008. Modelling the limit of detection in real-time quantitative PCR. European Food Research and Technology 226, 1513–1524.

Castillo-Alcala, F., Bateman, K.G., Cai, H.Y., Schott, C.R., Parker, L., Clark, M.E., McRaild, P., McDowall, R.M., Foster, R.A., Archambault, M., Caswell, J.L., 2012. Prevalence and genotype of Mycoplasma bovis in beef cattle after arrival at a feedlot. American Journal of Veterinary Research 73, 1932–1943.

Caswell, J.L., Archambault, M., 2007. Mycoplasma bovis pneumonia in cattle. Animal Health Research Reviews 8, 161–186.

Confer, A.W., 2009. Update on bacterial pathogenesis in BRD. Animal Health Research Reviews 10, 145–148.

Curtis, C. R., Erb, H. N., White, M. E., 1988. Descriptive epidemiology of calfhood morbidity and mortality in New York holstein herds. Preventive Veterinary Medicine 5, 293–307.

Dabo, S.M., Taylor, J.D., Confer, A.W., 2007. Pasteurella multocida and bovine respiratory disease. Animal Health Research Reviews 8, 129–150.

DAFC, 2018. Strategi, Landbrug og Fødevarer, Kvæg 2018-2022, Danish Agriculture & Food Council. https://www.seges.dk/fagomraader/kvaeg/~/media/6751842091cd48d6954444594e93c636.ashx (accessed 21.01.19).

Dalgaard, I., 2005. FarmTest: Kalvestalde kontra kalvehytter (No. 1.). Kvæg, nr. 18, 2005, Dansk Landbrugsrådgivning, Landscentret. https://www.landbrugsinfo.dk/.../FarmTest/Sider/FT_KVA_018_kalvestald_hytte.pdf (accessed 13.02.19).

Davies, R.L., MacCorquodale, R., Reilly, S., 2004. Characterisation of bovine strains of Pasteurella multocida and comparison with isolates of avian, ovine and porcine origin. Veterinary Microbiology 99, 145–158.

Dowling, A., Hodgson, J.C., Schock, A., Donachie, W., Eckersall, P.D., Mckendrick, I.J., 2002. Experimental induction of pneumonic pasteurellosis in calves by intratracheal infection with Pasteurella multocida biotype A: 3. Research in Veterinary Science 73, 37–44.

Ellis, J.A., 2009. Update on viral pathogenesis in BRD. Animal Health Research Reviews 10, 149–153.

Ellis, J.A., Philibert, H., West, K., Clark, E., Martin, K., Haines, D., 1996. Fatal pneumonia in adult dairy cattle associated with active infection with bovine respiratory syncytial virus. Canadian Veterinary Journal 37, 103–105.

Goecke, N. B. 2018. PhD thesis: Development and validation of a high-throughput real-time PCR systems for diagnosis and typing of pathogens in pigs, 50–77.

Groom, S.C., Little, P.B., 1988. Effects of vaccination of calves against induced Haemophilus somnus pneumonia. American Journal of Veterinary Research 49, 793–800.

Gulliksen, S.M., Lie, K.I., Løken, T., Østerås, O., 2009. Calf mortality in Norwegian dairy herds. Journal of Dairy Science 92, 2782–2795.

Härtel, H., Nikunen, S., Neuvonen, E., Tanskanen, R., Kivelä, S.-L., Aho, R., Soveri, T., Saloniemi, H., 2004. Viral and bacterial pathogens in bovine respiratory disease in Finland. Acta Veterinaria Scandinavica 45, 193–200.

Horwood, P.F., Mahony, T.J., 2011. Multiplex real-time RT-PCR detection of three viruses associated with the bovine respiratory disease complex. Journal of Virological Methods 171, 360–363.

Houe, H., Ersbøll, A.K., Toft, N. (Eds.), 2004. Introduction to veterinary epidemiology. 1. Edition, Biofolia, 3rd impression 2011, Frederiksberg.

Ishii, S., Segawa, T., Okabe, S., 2013. Simultaneous Quantification of Multiple Food- and Waterborne Pathogens by Use of Microfluidic Quantitative PCR. Applied and Environmental Microbiology 79, 2891–2898.

Jensen, V.F., Svensmark, B., Larsen, G., Pedersen, K., Toft, N., Jorsal, S.E., 2018. Diagnostiske undersøgelser af luftvejsinfektioner og antibiotikabehandling af kalve. Dansk Veterinærtidsskrift 6, 28–35.

Koskinen, M.T., Holopainen, J., Pyörälä, S., Bredbacka, P., Pitkälä, A., Barkema, H.W., Bexiga, R., Roberson, J., Sølverød, L., Piccinini, R., Kelton, D., Lehmusto, H., Niskala, S., Salmikivi, L., 2009. Analytical specificity and sensitivity of a real-time polymerase chain reaction assay for identification of bovine mastitis pathogens. Journal of Dairy Science 92, 952–959.

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

Love, W.J., Lehenbauer, T.W., Kass, P.H., Van Eenennaam, A.L., Aly, S.S., 2014. Development of a novel clinical scoring system for on-farm diagnosis of bovine respiratory disease in pre-weaned dairy calves. PeerJ 2, e238.

Marcé, C., Guatteo, R., Bareille, N., Fourichon, C., 2010. Dairy calf housing systems across Europe and risk for calf infectious diseases. Animal 4, 1588–1596.

Markusfeld, O., Galon, N., Ezra, E., 1997. Body condition score, health, yield and fertility in dairy cows. Veterinary Record 1997, 67–72.

Maunsell, F.P., Donovan, G.A., 2009. Mycoplasma bovis Infections in Young Calves. Veterinary Clinics of North America: Food Animal Practice 25, 139–177.

Maunsell, F.P., Woolums, A.R., Francoz, D., Rosenbusch, R.F., Step, D.L., Wilson, D.J., Janzen, E.D., 2011. Mycoplasma bovis Infections in Cattle. Journal of Veterinary Internal Medicine 25, 772–783.

Michelet, L., Delannoy, S., Devillers, E., Umhang, G., Aspan, A., Juremalm, M., Chirico, J., van der Wal, F.J., Sprong, H., Boye Pihl, T.P., Klitgaard, K., BÃ¸dker, R., Fach, P., Moutailler, S., 2014. High-throughput screening of tick-borne pathogens in Europe. Frontiers in Cellular and Infection Microbiology 4.

Murray, G.M., More, S.J., Clegg, T.A., Earley, B., O'Neill, R.G., Johnston, D., Gilmore, J., Nosov, M., McElroy, M.C., Inzana, T.J., Cassidy, J.P., 2018. Risk factors associated with exposure to bovine respiratory disease pathogens during the peri-weaning period in dairy bull calves. BMC Veterinary Research 14.

Pedersen, R.E., Sørensen, J.T., Skjøth, F., Hindhede, J., Nielsen, T.R., 2009. How milk-fed dairy calves perform in stable versus dynamic groups. Livestock Science 121, 215–218.

Pérez, D.S., Pérez, F.A., Bretschneider, G., 2010. Histophilus somni: Pathogenicity in Cattle. An Update. Actualización de la patogenicidad en vacuno | Anales de Veterinaria de Murcia 26, 5–21.

Perez, E., Noordhuizen, J.P.T.M., Van Wuijkhuise, L.A., Stassen, E.N., 1990. Management factors related to calf morbidity and mortality rates. Livestock Production Science 25, 79–93.

Radostits, O.M., Gay, C.C., Hinchcliff, K.W., Constable, P.D., dawsonera, 2007. Veterinary Medicine E-book: A textbook of the diseases of cattle, horses, sheep, pigs and goats. Elservier Health Science 2006.

Raundal, P., Nielsen, J., 2018. Kalvedødelighed i Danmark. Landbrug & Fødevarer, SEGES. https://www.landbrugsinfo.dk/Kvaeg/Tal-om-kvaeg/Sider/KododMain.aspx (accessed 12.10.18).

Razin, S., Yogev, D., Naot, Y., 1998. Molecular Biology and Pathogenicity of Mycoplasmas. Microbiology and Molecular Biology Reviews 62, 63.

Retsinformation, 2018. Bekendtgørelse om grænseværdier for antibiotikaforbrug og dødelighed i kvæg- og svinebesætninger - retsinformation.dk. https://www.retsinformation.dk/eli/lta/2018/1313 (accessed 12.10.18).

Sivula, N.J., Ames, T.R., Marsh, W.E., Werdin, R.E., 1996. Descriptive epidemiology of morbidity and mortality in Minnesota dairy heifer calves. Preventive Veterinary Medicine 27, 155–171.

Snowder, G.D., Van Vleck, L.D., Cundiff, L.V., Bennett, G.L., 2006. Bovine respiratory disease in feedlot cattle: Environmental, genetic, and economic factors. Journal of Animal Science 84, 1999–2008.

Spurgeon, S.L., Jones, R.C., Ramakrishnan, R., 2008. High Throughput Gene Expression Measurement with Real Time PCR in a Microfluidic Dynamic Array. PLoS ONE 3, e1662.

Svensson, C., Hultgren, J., Oltenacu, P. A., 2005. Morbidity in 3–7-month-old dairy calves in south-western Sweden, and risk factors for diarrhoea and respiratory disease. Preventive Veterinary Medicine 74, 162–179.

Svensson, C., Lundborg, K., Emanuelson, U., Olsson, S.-O., 2003. Morbidity in Swedish dairy calves from birth to 90 days of age and individual calf-level risk factors for infectious diseases. Preventive Veterinary Medicine 58, 179–197.

Taylor, J.D., Fulton, R.W., Lehenbauer, T.W., Step, D.L., Confer, A.W., 2010. The epidemiology of bovine respiratory disease: What is the evidence for predisposing factors? Canandian Veterinary Journal 51, 1095–1102.

Tegtmeier, C., Uttenthal, A., Friis, N.F., Jensen, N.E., Jensen, H.E., 1999. Pathological and Microbiological Studies on Pneumonic Lungs from Danish Calves. Journal of Veterinary Medicine, Series B 46, 693–700.

Thonur, L., Maley, M., Gilray, J., Crook, T., Laming, E., Turnbull, D., Nath, M., Willoughby, K., 2012. One-step multiplex real time RT-PCR for the detection of bovine respiratory syncytial virus, bovine herpesvirus 1 and bovine parainfluenza virus 3. BMC Veterinary Research 8, 37.

Toaff-Rosenstein, R. L., Tuckter, C. B., 2018. The sickness response at and before clinical diagnosis of spontaneous bovine respiratory disease. Applied Animal Behaviour Science 201, 85–92.

Van der Fels-Klerx, H.J., Martin, S.W., Nielen, M., Huirne, R.B.M., 2002. Effects on productivity and risk factors of Bovine Respiratory Disease in dairy heifers; a review for the Netherlands. NJAS - Wageningen Journal of Life Sciences 50, 27–45.

Vaarst, M., Sørensen, J. T., 2009. Danish dairy farmers' perceptions and attitudes related to calfmanagement in situations of high versus no calf mortality. Preventive Veterinary Medicine 89, 128–133.

Wells, S.J., Garber, L.P., Hill, G.W., 1997. Health status of preweaned dairy heifers in the United States. Preventive Veterinary Medicine 29, 185–199.

Wiggins, M.C., Woolums, A.R., Sanchez, S., Hurley, D.J., Cole, D.J., Ensley, D.T., Pence, M.E., 2007. Prevalence of Mycoplasma bovis in backgrounding and stocker cattle operations. Journal of the American Veterinary Medical Association 230, 1514–1518.

Woolums, A.R., 2015. Ruminant Respiratory System, in: Large Animal Internal Medicine. 5th Edition, Elsevier, St. Louis, Mo, 575–637.

Appendix

Appendix A: Extract of “Robuste kalve – Klinisk protokol”

The following, extract of the "Robuste kalve – Klinisk protokol" is shown including the clinical procedures used for this study.

Robuste kalve – Klinisk protokol

I protokollen gennemgås de enkelte scores – både hvordan de skal udføres og hvordan de skal scores.

Hvor ikke andet er angivet, er billederne i protokollen taget af Mari Reiten (AU), Kristoffer Eriksen, (SEGES) eller projektdeltagere (Mette Bisgård Petersen (KU), Per Spleth (SEGES), Henrik Læssøe Martin (SEGES) eller Bodil Højlund Nielsen (AU)).

Hvor ikke andet angivet er scorerne udarbejdet til projektet i samarbejde mellem projektdeltagere.

Scores og beskrivelser passer til registreringssystemet EasyOn, som vil blive benyttet til indtastning.

AU, Foulum 31. august 2018

1. Boks ID - *Pen ID*

Numerisk værdi, der henviser til angivelse på **'Oversigt, bedrift'.** Bokse nummereres fortløbende og angives tillige med nummer på oversigten.

Boksnummer der henviser til oversigtstegning. OMRÅDE, BOKSNR - tal med op til 3 decimaler ex: 1,001. Kan også være landmandes egen angivelse af boksnr.

Numeric value referring to drawing on 'Herd overview'

2. Antal kalve i boks – *Number of calves in pen*

Antal kalve i den boks aktuelle individ opholder sig i

Number of calves in the pen

3. Opstaldning, type – *Housing, type*

Type af opstaldning

Score	Beskrivelse	Billedeksempel
0	Enkeltboks *Single pen*	
1	Parvis opstaldning *Pairwise*	
2	Gruppeopstaldning *Group wise*	

4. Opstaldning, sted – *Housing, location*

Hvilke omgivelser befinder boksen sig i.

In which environment is the pen located

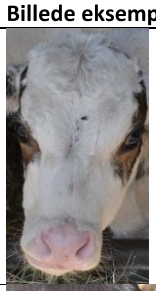
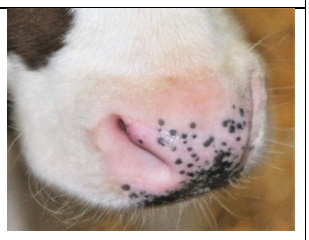
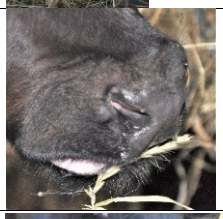
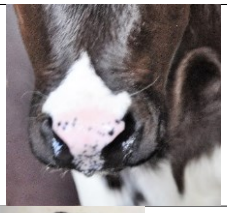
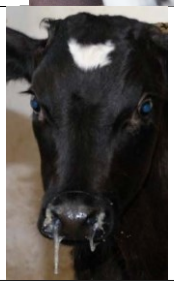
Score	Beskrivelse	Billedeksempel
0	Udenfor – ikke tag over (fx kalvehytter i det fri) *Outside – no roof (e.g. huts in the open)*	
1	Under tag – minimum 1 side åben *Under a roof – minimum 1 side open*	
2	I staldbygning med 4 lukkede vægge/sider *In a barn/stable – all sides closed*	

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.

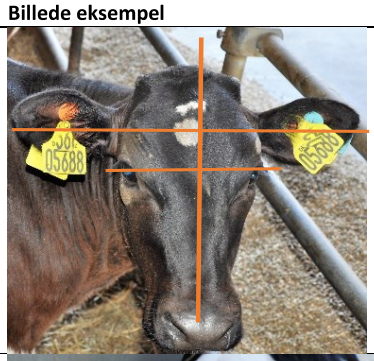
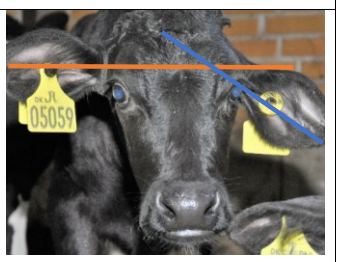
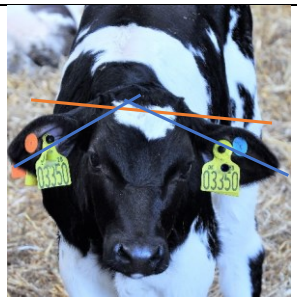
Score	Beskrivelse	Billede eksempel
0	**Normal** Ingen flåd eller pus i næseborene *Normal, no signs of fluid, exudate or pus in the nostrils*	 
1	**Serøst** Klart, vandigt (serøst) flåd i et eller begge næsebor. Ingen pus eller pus-tilblanding. *Clear, serous fluid/exudate in one or both nostrils. No 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.*	 

11. Øre- og hovedholdning – Ear drop and/or head tilt

Kilde: Welfare Quality

Kig på kalven forfra. Holdes begge ører i samme højde ved eller over en vandret linje gennem panden? Holdes hovedet lodret med øjnene på en vandret linje?

Look at the calf from the front. Examine the head and the ears. Are the ears held equally high (at or above a horizontal line between them)? Is the head held tilted (are the eyes at a horizontal line)?

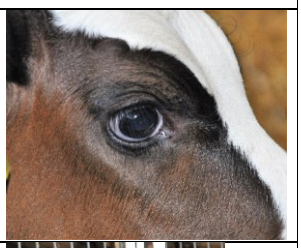
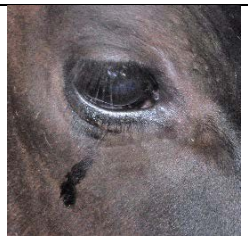
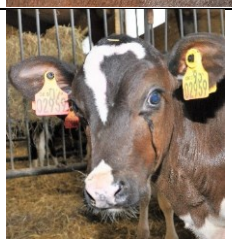
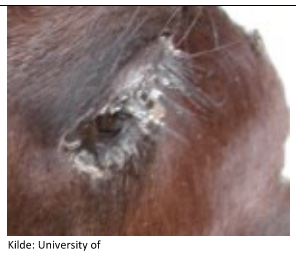
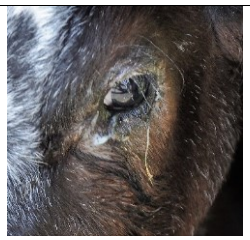
Score	Beskrivelse	Billede eksempel
0	**Normal øre- og hovedholdning** Der kan tegnes en vandret linje imellem øjne og imellem ører *Normal positioned ears and head: Horizontal line between ears and between eyes*	
1	**Unilateral øredrop** Det ene øre 'hænger', det andet på eller over vandret linje gennem panden. *Unilateral eardrop: One ear positioned lower than the other*	 
2	**Bilateralt øredrop eller skæv hovedholdning** Begge ører hænger under vandret linje gennem panden eller hovedet holdes på skrå. *Bilateral eardrop or head tilted*	 

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.

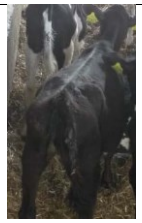
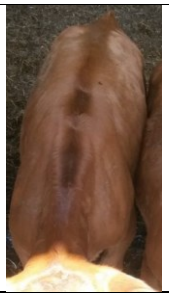
Score	Beskrivelse	Billedeksempel
0	**Normal** Ingen flåd, tørre øjenomgivelser *Normal, no discharge, dry eye surroundings*	 
1	**Serøst** Klart, tyndt/vandigt flåd *Serous discharge (transparent, thin)*	 
2	**Muko-purulent/Purulent** Uklart/pus tilblandet eller rent pus. Ofte sammenklistrede øjenvipper. 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 

19. Huld – Body condition

Kilde: Welfare Quality

Kig på kalven – helst bagfra. Vurder hoftehjørner, tværtappe og ryghvirvler efter beskrivelsen i skemaet nedenfor. Visuel vurdering alene – ingen palpering.

Look at the calf – preferably from behind. Examine hips (Tuber coxae), transverse processes and the spines from the criteria in the scheme below. Only visual examination – no palpation.

Score	Beskrivelse	Billedeksempel
0	**Normalt huld** Hoftehjørner synlige, men rundede. Ryghvirvler og tværtappe anes, men er ikke markerede. *Normal body condition – Tuber coxae visible and rounded. Spines and transverses processes are distinguishable but rounded.*	 
1	**Undervægtig/tynd** Fremstående hoftehjørner, markeret ryghvirvler og tværtappe på lændehvirvler. *Lean/very lean: Depression btw. backbone and tuber coxae. Spines visible. Ends of transverse processes distinguishable.*	  
2	**Overvægtig/fed** Hoftehjørner og tværtappe på lændehvirvler er næsten usynlige. *Fat: Tuber coxae and ends of transverse processes not/almost not distinguishable*	

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*	

26. Næsesvaber – *Nasal swab*

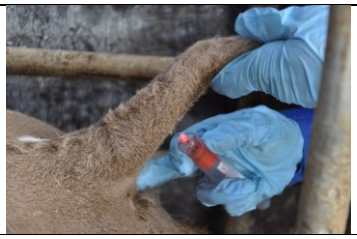
Kalven fikseres og steril svaber indføres i næseboret (dorsale conchae) indtil der mødes modstand (3-7 cm inde afhængig af kalvens størrelse). Her føres svaberen lidt rundt og tages ud og deponeres i røret, der lægges på køl hurtigst muligt.

Using sterile, polyester-tipped swabs with a wodden shaft, rub the swab tip gently but thoroughly against the walls of the animal's nares, about 3-7 cm from the opening (depending on the size of the calf), saturating the swab with mucus. The swab is placed in the vial and cooled immediately.

27. Blodprøve – *Blood sample*

Blodprøve kan udtages med vacutainer i V. jugularis eller ved halevenepunktur. Glasset fyldes minimum 2/3

Blood samples can be drawn from the jugular vein (Vena jugularis) or from the tail vein (Vena cocc.med.). Sample needs to be filled at least two thirds



Appendix B: Protocol for PCR Fluidigm

Dia.-61-nicbg 23-10-18

Protokol for Dia. fluidigm # 61

Lot nr.

1. Tænd for MX IFC controller
2. Fremstil Primer Stock (2 rør) i PCR-inderum i epp. Rør

Nr. 4 Assay mix	
100 µM Forward primer	10 µL
100 µM Reverse Primer	10 µL
30 µM Probe	10 µL
	30 µL

3. I en mikrotitterplade dispenseres følgende ud i venstre side af pladen:
   - 3 µL af 2x Assay loading reagent –
   - 3 µL af primer stock
4. Film sættes på pladen, som derefter sættes i køleskabet.
5. Fremstil sample-mix (til 48 prøver) efter nedenstående skema:

Sample-mix	48x48 chip (µL)
2x taqMan Gene Expression master mix	170
20x Sample loading reagent	17
Total	187 (3,89 pr. prøve)

6. Dispensere 3,3 µL af sample-mix pr. brønd i en mikrotitterplade.
7. Tilsæt 2,7 µL DNA pr. brønd
8. Film sættes på pladen, som derefter sættes i køleskabet.
9. Tilsæt ”control line fluid” til chippen, tjek at ventilerne er bevægelige med sprøjten. Væsken sprøjtes ind samtidig med, at man trykker let på ventilen, væsken skal løbe relativt let igennem.

10. Fjern beskyttelsesfilm fra chippen (skal gemmes) og præparer denne i MX IFC controller (tager 11 min). Vælg: chip Prime 113x, User, run script. Chippen skal bruges inden for en time efter endt priming. Sæt beskyttelsesfilm på igen.
11. Chippen placeres på mørkt underlag med "afskåret" hjørne øverst til venstre. Primer/assay mix vortexes og spindes ned (1000 RPM, 30 sek, 21°C) og der tilsættes 4,9 µL af dette mix i venstre side af chippen. Sample/prøve mix vortexes og spindes ned (1000 RPM, 30 sek, 21°C) og der tilsættes 4,9 µL af dette mix i højre side af chippen.
12. Tjek for luftbobler!
13. Chippen sættes tilbage i MX IFC controller og programmet Load Mix (113x) køres. Dette tager omkring en time.
14. Tænd BioMark, tænd computer og start "data collection" (det tager tid at starte programmet op), dobbelt klik på "turn on lamp" nederst.
15. Efter afsluttet loading og mixing fjernes støv fra oversiden af chippen med Stotch tape (3M grøn) og sættes ind i BioMarken Start New Run og vælg programmet Dia. Chip (nicbg). Opsamler på en kanal.

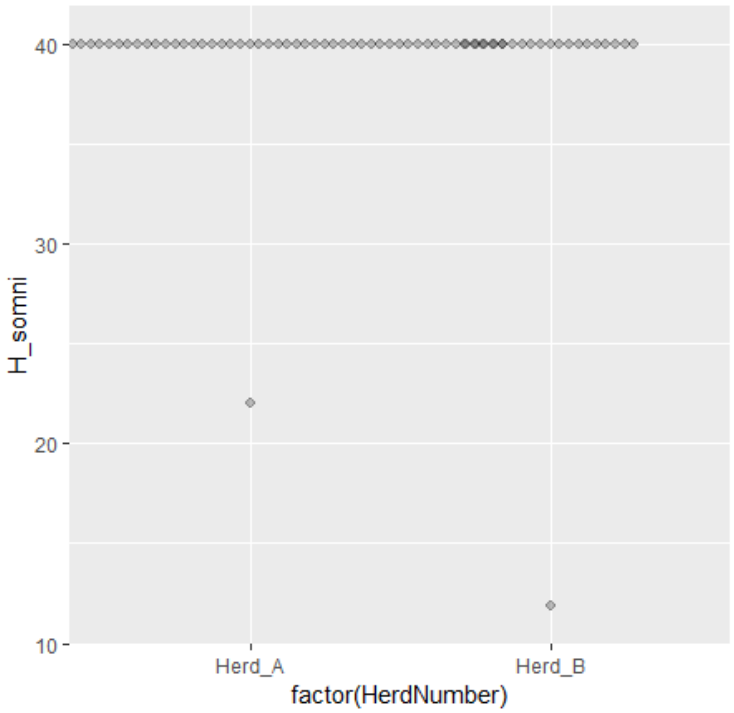
PCR Programmet:

Activation	94 °C	2 min
	94 °C	15 sec
40 cycles of:	60° C	60 sec

Appendix C: Ct-value cut-offs for the included pathogens

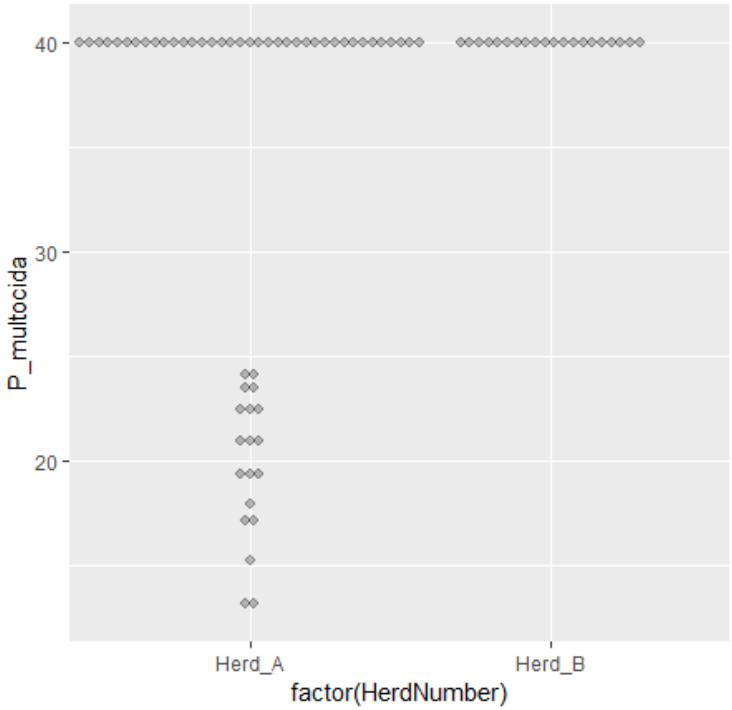
The graphs below shows raw PCR results from the "Robust calves – well begun is half done" project from 120 samples originating from 83 slaughter calves sampled in two slaughter herds and tested on the same PCR platform used in this study. These PCR results were used for decision-making and interpretation when defining Ct-value cut-offs for each pathogen by visual inspection of the distributions of Ct-values.

H. somni



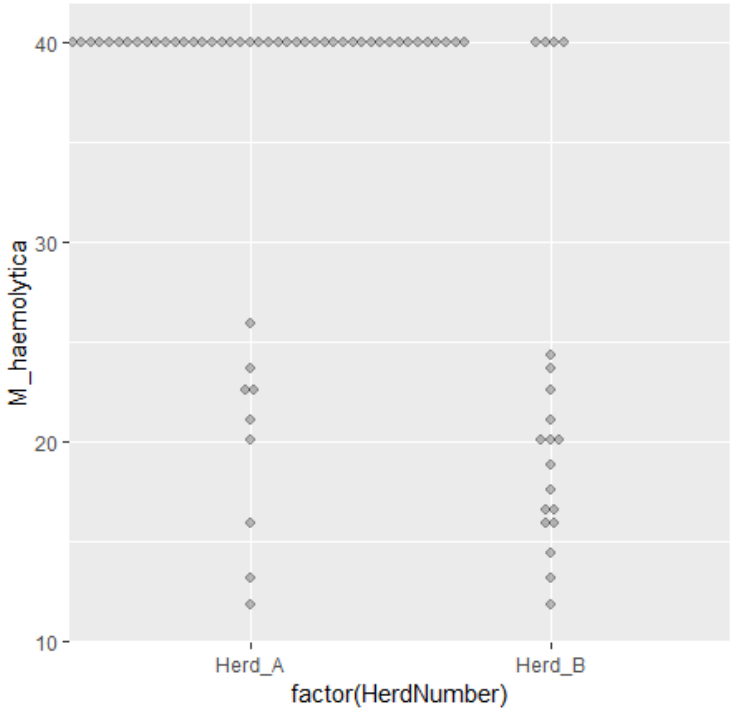
A Ct-value cut-off 25 is suggested for the threshold between positive ($Ct \leq 25$) and negative ($Ct > 25$) results with *H. somni*.

P. multocida



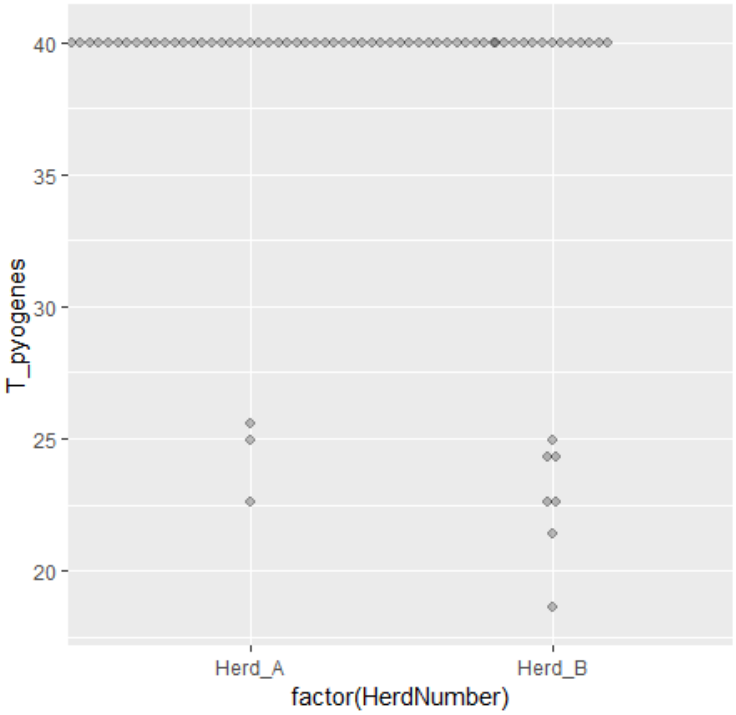
A Ct-value cut-off 25 is suggested for the threshold between positive (Ct ≤ 25) and negative (Ct > 25) results with *P. multocida*.

M. haemolytica



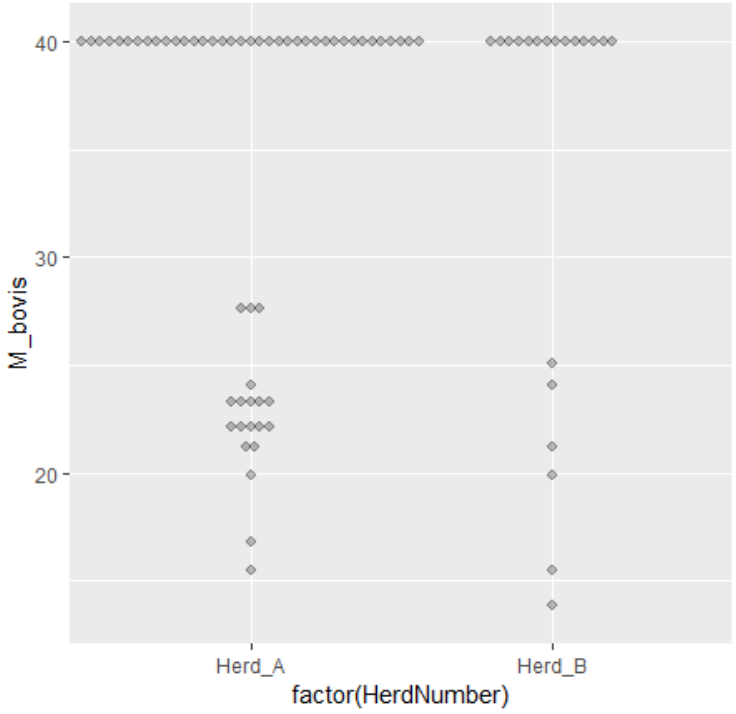
A Ct-value cut-off 30 is suggested for the threshold between positive (Ct ≤ 30) and negative (Ct > 30) results with *M. haemolytica*.

T. pyogenes



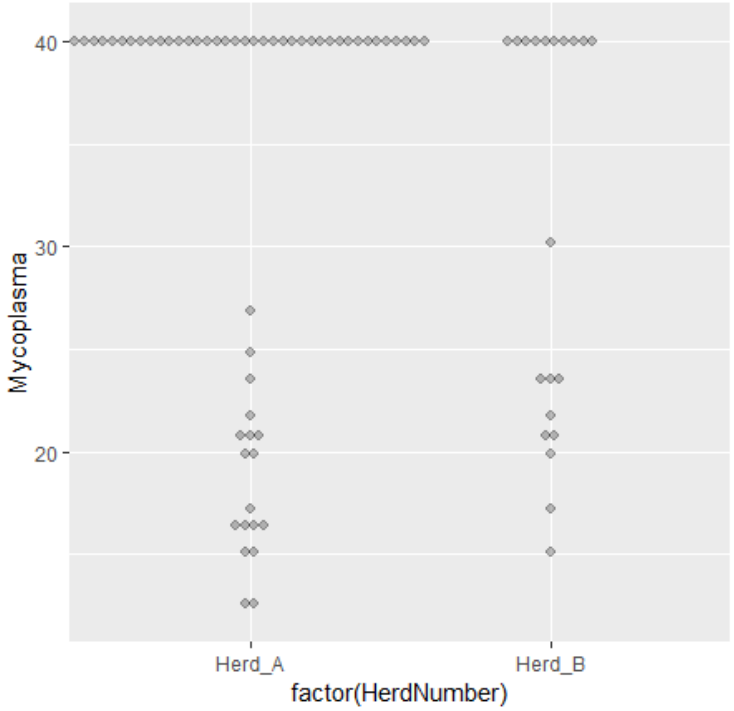
A Ct-value cut-off 27 is suggested for the threshold between positive (Ct ≤ 27) and negative (Ct > 27) results with *T. pyogenes.*

M. bovis



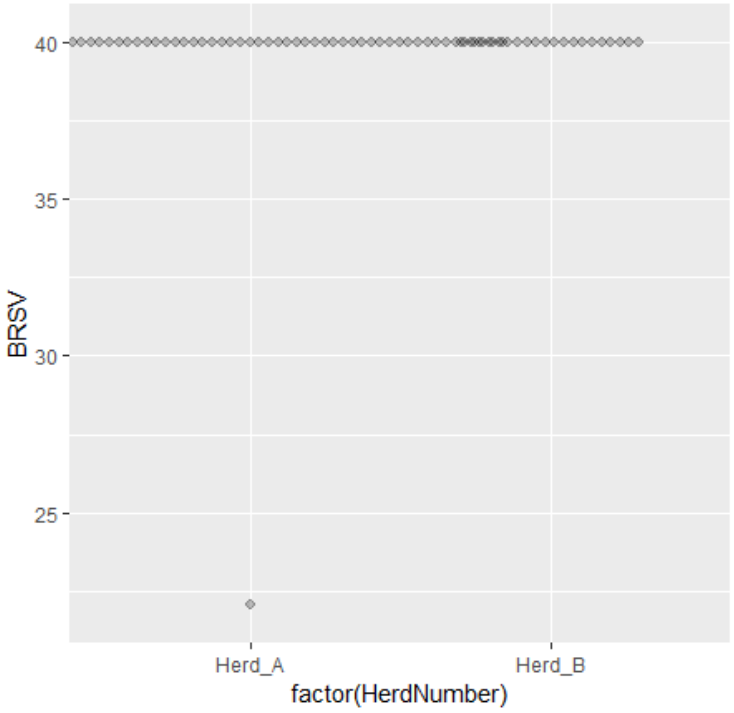
A Ct-value cut-off 35 is suggested for the threshold between positive (Ct ≤ 35) and negative (Ct > 35) results with *M. bovis*.

Mycoplasma spp.



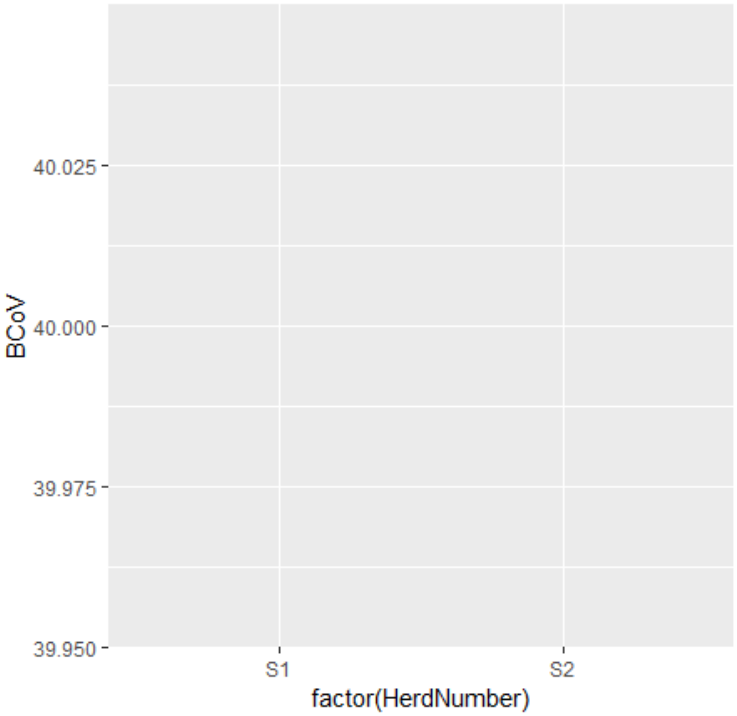
A Ct-value cut-off 35 is suggested for the threshold between positive (Ct ≤ 35) and negative (Ct > 35) results with *Mycoplasma spp*.

BRSV



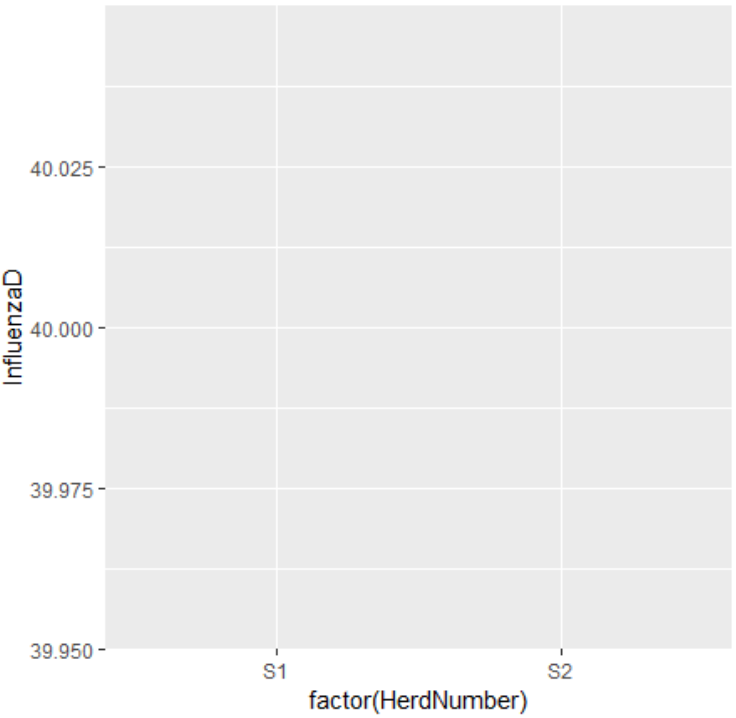
A Ct-value cut-off 25 is suggested for the threshold between positive (Ct ≤ 25) and negative (Ct > 25) results with BRSV.

BCoV



A Ct-value cut-off 40 is suggested for the threshold between positive ($Ct \leq 40$) and negative ($Ct > 40$) results with BCoV.

Influenza D



A Ct-value cut-off 40 is suggested for the threshold between positive ($Ct \leq 40$) and negative ($Ct > 40$) results with Influenza D.

Appendix D: Description of the participating dairy herds

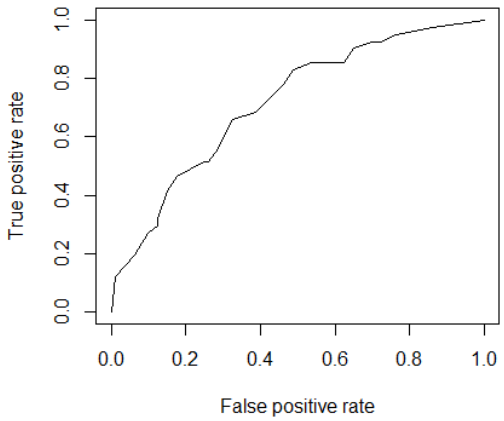
This table is based on qualitative questionnaires from "BioSecure®". This platform has been developed in a previous project about biosecurity in Danish dairy herds prepared in cooperation between University of Copenhagen, Technological Institute, SEGES and SAGRO (Nielsen et al., 2017). Further questions were answered by phone call to the involved farmers.

Description of the nine (A – I) Danish dairy herds that participated in the study:

	A	B	C	D	E	F	G	H	I
Representing calves (Holstein/Danish Red /Cross-breed)	24 (19/0/5)	31 (25/0/6)	18 (16/0/2)	28 (25/0/3)	13 (10/0/3)	18 (18/0/0)	14 (12/0/2)	31 (26/0/5)	23 (5/11/7)
Heifers / bulls	16/8	21/10	16/2	16/12	7/6	11/7	7/7	15/16	16/7
Housing location	Outside in hutches	Most: in a stable with 4 solid walls Few: in a big bulding	Outside in hutches	Outside in hutches	Outside in hutches	In a stable with 4 solid walls	Outside in hutches	Heifers: Inside in a big / open building Bulls: Inside in a big / open building and with several age groups in the same barn	Outside in hutches
Systematically calf care with regular daily procedures	Yes, no matter who takes care of the calves	Yes, no matter who takes care of the calves	Yes, no matter who takes care of the calves	Yes, no matter who takes care of the calves	Yes, no matter who takes care of the calves	Yes, no matter who takes care of the calves	Yes, no matter who takes care of the calves	Yes, no matter who takes care of the calves	Yes, no matter who takes care of the calves
High pressure washer in stable with calves	Rarely	No	Regularly	Never	Never	Never	Regularly	Never	Regularly
Can aerosols reach the calves during high pressure wash?	No	No	Yes	No	No	No	No	No	Yes
Vaccines of calves: **Heifers** **Bulls** **Age at vaccination**	No No –	No No –	No No –	No No –	No No –	Yes, Bovalto Respi Intranasal No 10 days	No No –	Yes, Hiprabovis Somni/Lkt No 3 weeks and again at 6 weeks	No No –
Litres milk/day under normal weather conditions at: **1 week of life** **2 – 4 week of life**	6 6	7 8	8 8	6 6	7 8	4 6	7 8	5 6	6 7
Average amount of IgG (g/L) in serum in cohort calves 0 – 11 days of age	N = 13 Mean: 22.7 SD: 12.8	N = 18 Mean: 18.4 SD: 10.3	N = 4 Mean: 9.69 SD: 2.76	N = 16 Mean: 22.2 SD: 11.7	N = 13 Mean: 23.1 SD: 5.71	N = 18 Mean: 12.5 SD: 6.59	N = 10 Mean: 12.1 SD: 10.6	N = 20 Mean: 18.4 SD: 10.6	N = 9 Mean: 18.7 SD: 10.2

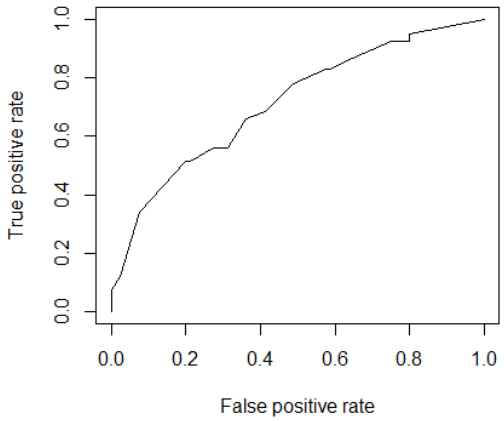
Appendix E: ROC curves for the variables in the final models

ROC curve for sex for calves at 0 – 14 days of age with respiratory disease:



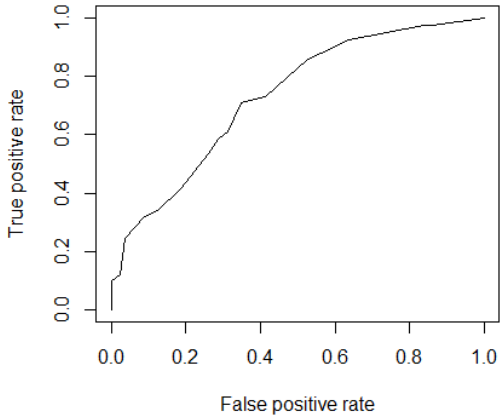
AUC = 0.71

ROC curve for *P. multocida* in calves at 0 – 14 days of age with respiratory disease:



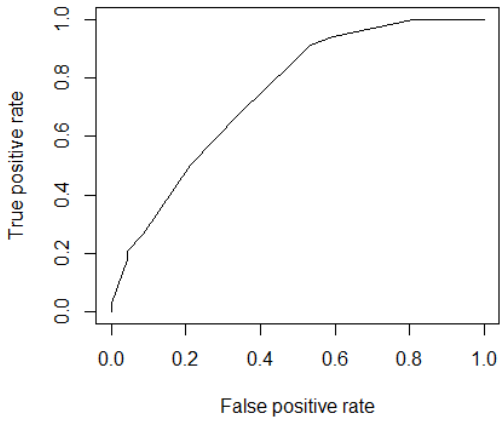
AUC = 0.71

ROC curve for sex and *P. multocida* in calves at 0 – 14 days of age with respiratory disease:



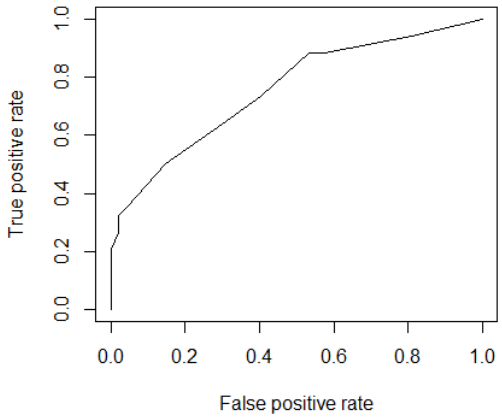
AUC = 0.73

ROC curve for BCS in calves at 14 – 28 days of age with respiratory disease:



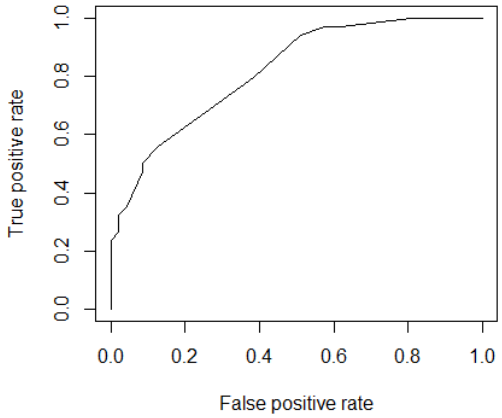
AUC = 0.75

ROC curve for *P. multocida* in calves at 14 – 28 days of age with respiratory disease:



AUC = 0.76

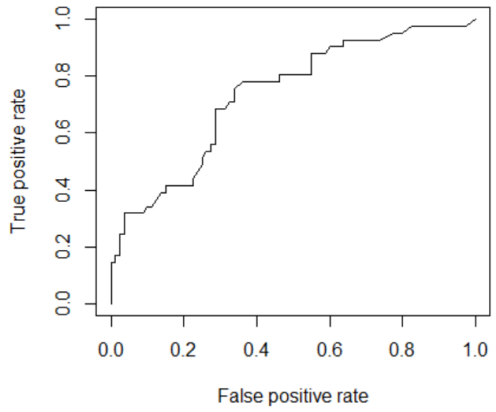
ROC curve for BCS and *P. multocida* in calves at 14 – 28 days of age with respiratory disease:



AUC = 0.82

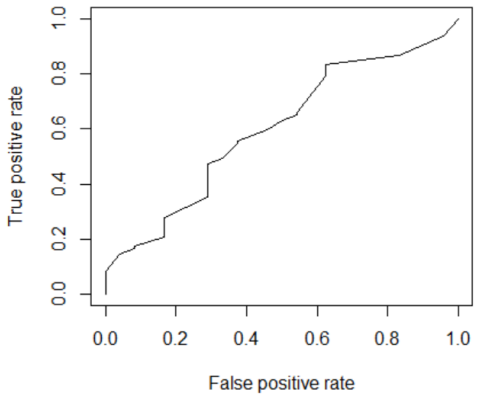
Appendix F: ROC curves for the two different respiratory disease definitions

ROC curve for 0 – 14 days of age with respiratory disease Definition 1:



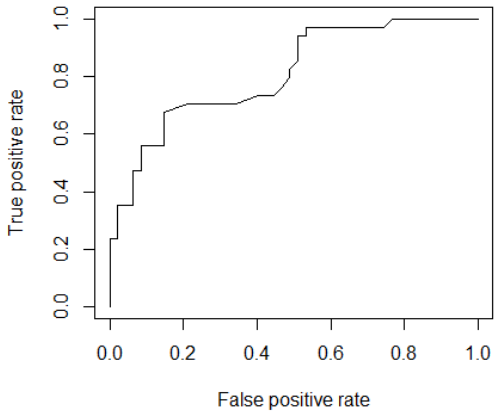
AUC = 0.73

ROC curve for 0 – 14 days of age with respiratory disease Definition 2:



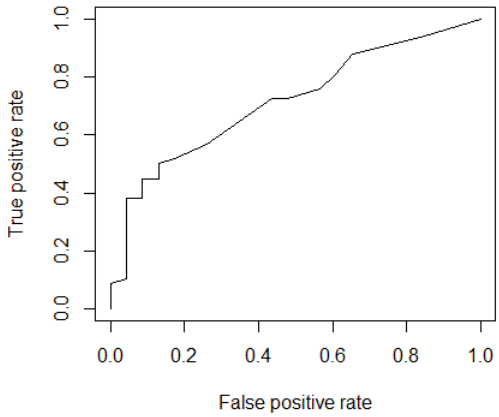
AUC = 0.60

ROC curve for 14 – 28 days of age with respiratory disease Definition 1:



AUC = 0.81

ROC curve for 14 – 28 days of age with respiratory disease Definition 2:



AUC = 0.71