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Diagnosing bovine respiratory disease in Danish calves less than four months old

Estimation of high-throughput real-time PCR cut-offs for detection of selected pathogens in nasal swabs, and comparison of two sampling methods

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Front page picture: From herd visit with acceptance from the respective owner.

Preface

This master thesis was made as a part of the master´s degree in Veterinary Medicine at the University of Copenhagen, Department of Veterinary and Animal Sciences, Section for Animal Welfare and Disease Control. The work took place between September 2019 and February 2020 and corresponds to 30 ECTS points.

The study conducted in this thesis is part of the four-year long project "Robust calves – well begun is half done" (2018-2021), which is financially supported by the Danish Cattle Levy Fund and the Danish Milk Levy Fund. The project is a coordinated research collaboration between the University of Copenhagen, the Technical University of Denmark, Aarhus University, and SEGES. The aim of the project is to improve the health management of calves. The project approaches this by focusing on four work packages: collecting knowledge from calf clusters of veal and dairy farms, development of diagnostic tools, exploring alternatives to antibiotics, and development of IT management tools.

First, a big thank you to our main supervisor Liza Rosenbaum Nielsen for guidance, support, and dedication. We would also like to thank our co-supervisors Anne Marie Michelsen, for useful advice both for this thesis and in the field, and Lars Erik Larsen, for providing help, perspective and consistent optimism. A special thank you to Nicole Bakkegård Goecke for answering our many questions regarding the qPCR system and for showing us the qPCR Fluidigm procedure. A big thank you to Matt Denwood for creating the statistical model that has been critical for our investigations and to Carsten Thure Kirkeby for answering many R related questions. Furthermore, we would like to thank everyone involved with the Robust Calves project who have been generous with their time and knowledge. We would especially like to thank Bodil Højlund Nielsen for sharing her expertise, as well as Henrik Læssøe Martin for his help and kind hospitality. Additionally, a thank you to Nina Dam Ottesen for making the field visits in Jutland joyful and instructive and to Masja Reipurth for her help and company, as well as Mette Bisgaard Petersen for enlightening advice. A big thank you to all the farmers for agreeing to participate in the comprehensive project. Thank you to our friends and beloved parents and siblings for their unwavering encouragement. Finally, a big thank you to Malte and Sebastian for their patience and never-failing support.

Abstract

Bovine respiratory disease (BRD) is a multifactorial disease involving interactions between a wide range of pathogens and stressors. It continues to be an issue in Danish calf production and impacts welfare and performance of the affected calves. Obtaining an accurate diagnosis of BRD is challenging, but the use of high-throughput real-time PCR (qPCR) can potentially advance the efficiency of preventive and therapeutic means. The aim of this study was to improve the interpretation of diagnostic results combined with clinical observations. To achieve this, the objectives of this thesis were to 1) determine relevant cut-offs associated with respiratory disease for the qPCR Fluidigm based on nasal swab (NS) samples, and 2) compare the qPCR results of two sampling methods for nine respiratory pathogens. This master thesis used data collected in a Danish field study which is a part of the Robust Calves project which focuses on improving the health of Danish calves. Data from 36 herds was used, where randomly selected calves were subjected to clinical assessment and sampling by NS. In 12 of these herds, tracheal wash (TW) was also performed. The samples were analysed with the novel qPCR system, Fluidigm. For the first objective, a clinical scoring system was created to classify 864 observation units as either sick with respiratory disease or not. Several statistical methods were then applied including using a model with an optimisation algorithm and a mixed effects logistic regression to determine the best qPCR cut-off associated with respiratory disease. Thereby, it was possible to determine the clinically relevant qPCR cut-off at Cq $\leq$ 21 for the three pathogens *Mycoplasma bovis, Histophilus somni,* and *Pasteurella multocida*. Furthermore, it was possible to estimate a cut-off for *Mannheimia haemolytica* at Cq $\leq$ 23, based on observation units with more severe disease.

For the second objective, 237 observation units with paired NS and TW samples were used. A comparison of the sampling results was carried out by calculation of prevalence and Cohen's Kappa for nine respiratory pathogens. The agreement between the sampling methods for the pathogens *Mycoplasma bovis, Mycoplasma* spp., *H. somni, Mannheimia haemolytica, Pasteurella multocida, Trueperella pyogenes,* and Influenza D virus was between 0.16 and 0.70. The results showed that NSs could not predict what could found by TW. The pathogens bovine respiratory syncytial virus and *Mycoplasma bovis* were found most frequently in TW samples, while the remaining pathogens including bovine coronavirus were found most frequently in NS samples.

In conclusion, this thesis has contributed new knowledge to increase the utility of qPCR for pathogens associated with BRD. However, further research is necessary in order to establish this system as a diagnostic mean in the Danish cattle sector.

Resumé

Luftvejslidelser hos kalve er en multifaktoriel sygdom, hvor interaktioner mellem en bred vifte af patogener og stressfaktorer er involveret. Det er et problem i den danske kalveproduktion og har betydning for de berørte kalves velfærd og præstation i besætningerne. Det er forbundet med udfordringer at opnå en præcis diagnose for BRD, men anvendelsen af high-throughput real-time PCR (qPCR) kan potentielt fremme effektiviteten af præventive og terapeutiske tiltag. Formålet med dette studie var at fremme tolkningen af diagnostiske resultater kombineret med kliniske tegn. Delmålene i dette speciale var derfor 1) at bestemme relevante cut-offs associeret med luftvejslidelse for qPCR Fluidigm baseret på næsesvaberprøver, og 2) at sammenligne qPCR resultater fra to prøvetagningsmetoder for ni luftvejspatogener. Der blev anvendt data indsamlet i et dansk feltstudie, som er en del af Robuste Kalve projektet, der har fokus på at forbedre danske kalves sundhed. Data fra 36 besætninger blev anvendt, hvorfra tilfældigt udvalgte kalve fik foretaget kliniske vurderinger samt prøvetagning med næsesvaber. I 12 af besætningerne blev trakealskyl også foretaget. Prøverne blev analyseret med det nye qPCR system, Fluidigm. I forbindelse med det første delmål blev et kliniske scoringssystem udviklet til at klassificere 864 observationsenheder som enten syge med luftvejslidelse eller ej. Flere statistiske metoder blev derefter anvendt, inklusiv en model med en optimeringsalgoritme og en mixed effects logistisk regression for at kunne bestemme det bedste qPCR cut-off associeret med luftvejssygdom. Det var derved muligt at fastsætte et klinisk relevant qPCR cut-off på Cq ≤ 21 for de tre patogener *Mycoplasma bovis, Histophilus somni* og *Pasteurella multocida*. Derudover var det muligt at fastsætte et cut-off for *Mannheimia haemolytica* på Cq ≤ 23, baseret på observationsenheder med sværere sygdom. I forbindelse med det andet delmål blev der anvendt 237 observationsenheder med parrede næsesvabre og trakealskyl. Til at sammenligne prøveresultaterne blev der beregnet prævalens og Cohen's Kappa for ni patogener. Enigheden mellem de to prøvemetoder for patogenerne *Mycoplasma bovis, Mycoplasma* spp., *H. somni, Mannheimia haemolytica, Pasteurella multocida, Trueperella pyogenes* og Influenza D virus var mellem 0.16 og 0.70. Resultaterne viste at næsesvabre ikke kunne forudsige, hvad der blev fundet ved trakealskyl. Patogenerne bovine respiratory syncytial virus og *Mycoplasma bovis* blev fundet hyppigst med trakealskyl, mens de resterende patogener inklusiv bovine coronavirus oftest blev fundet i næsesvaberprøver. Resultaterne i denne opgave har bidraget med ny viden i relation til brugbarheden af qPCR for patogener associeret med luftvejslidelser hos kalve. Dog er der brug for yderligere forskning for at kunne etablere systemet i den danske kvægsektor.

Table of contents

1. Introduction

Bovine respiratory disease (BRD) is a multifactorial disease, where multiple stressors, environmental factors and various infectious agents are involved either alone or in combinations. The disease is one of the most common health problems as well as a cause of mortality and welfare issues in calves between one and six months old (Brscic et al., 2012; Svensson et al., 2006). Bovine respiratory disease is associated with economic losses due to direct costs of lost calves and treatment, but also because of long-term impacts on animal performance e.g. reduced weight gain which in turn can impact age at first calving (Virtala et al., 1996). A Dutch study estimated that annual losses associated with clinical BRD on average was €31.2 (approx. 233 DKK) per heifer present on a farm (Van der Fels-Klerx et al., 2001).

Stressors such as transportation, weaning, dietary changes, and commingling of calves from different farms play an important part in predisposing to BRD (Taylor et al., 2010). Environmental conditions, for instance poor ventilation, overcrowding, and weather conditions are also recognised as risk factors for respiratory disease (Snowder, 2009). When predisposing conditions act together, they impair the exposed calves' immune systems and pulmonary defence mechanisms, making them susceptible to viral as well as bacterial infections (Taylor et al., 2010).

Although BRD is often caused by two or more infectious agents acting together, some agents can also cause disease alone. It has been suggested that bovine respiratory syncytial virus (BRSV) can cause an acute respiratory infection alone and without any predisposing conditions (Tjørnehøj et al., 2003). Viruses also act as a stressor and increase the susceptibility of the lungs to secondary infection with opportunistic bacteria such as *Pasteurella multocida* (*P. multocida*), *Histophilus somni* (*H. somni*), and *Mannheimia haemolytica* (*M. haemolytica*), thereby further worsening the condition (Griffin et al., 2010; Taylor et al., 2010).

The viral agents predominantly associated with BRD in Denmark are BRSV, bovine coronavirus (BCV), and occasionally bovine parainfluenza virus type 3 (BPIV-3) (DTU Veterinærinstitut, 2019; Larsen et al., 1999), all of which are enveloped RNA viruses. BRSV can infect all age groups, but severe clinical disease is primarily seen in calves. The virus replicates in ciliated epithelium as well as type II pneumocytes (Valarcher and Taylor, 2007). BPIV-3 is associated with a mild course of respiratory disease (Griffin et al., 2010). When inhaled, the virus primarily infects the epithelial cells of the tracheo-bronchial tree (Kin Son Tsai and Thomson, 1975). Bovine coronavirus also replicates in the epithelium, and antigen has been detected in both tracheal-, lung- and nasal epithelium. The virus has been detected in both healthy and sick calves (Boileau and Kapil, 2010). Recently, a newly

identified RNA virus called Influenza D virus (IDV) has been suggested to play a role in BRD by facilitating co-infection with other pathogens, and has been shown to cause mild clinical disease in experimentally infected animals (Ferguson et al., 2016).

Bacterial pathogens often isolated in Danish BRD cases are *P. multocida*, *H. somni*, *M. haemolytica*, *Trueperella pyogenes* (*T. pyogenes*), and bacteria from the *Mycoplasma* genus (DTU Veterinærinstitut, 2019; Larsen et al., 1999; Tegtmeier et al., 1999). *P. multocida* and *M. haemolytica* belong to the family Pasteurellaceae and are gram-negative, opportunistic commensal bacteria found in the nasopharynx in ruminants (Dabo et al., 2008; Rice et al., 2008). *H. somni* also belongs to the family Pasteurellaceae, but there is evidence that *H. somni* preferentially colonises the lower respiratory tract (Griffin et al., 2010). Several *M. haemolytica* serotypes exist, with some being routinely isolated from healthy cattle (Rice et al., 2008). In stressed animals, the pathogenic *M. haemolytica* serotypes colonise and replicate in the nasal mucosa, resulting in the inhalation of bacteria-containing aerosols into the lungs (Griffin et al., 2010). *T. pyogenes* is a gram-positive bacterium, which exists as a commensal in the upper respiratory tract, but is also identified as an opportunistic pathogen (Rzewuska et al., 2019). The bacterium within the Mycoplasma genus which is most often discussed in relation to BRD in Denmark is *Mycoplasma bovis (M. bovis). M. bovis* inhabits the upper respiratory tract and it has been suggested that the tonsils are the primary site of colonistion and may also act as a reservoir for the bacterium (Maunsell and Donovan, 2009). It can then adhere to the epithelium of the trachea and bronchi, from which it can colonise the lungs (Caswell et al., 2010). *M. bovis* has been isolated from the lower respiratory tract of clinically healthy calves (Maunsell and Donovan, 2009).

High antimicrobial usage in relation to respiratory disease has an economic impact but can also result in development of antimicrobial resistance. In 2015, antibiotics registered for respiratory disease covered 71% of the total amount of antibiotics registered for Danish calves less than one year old (Jensen et al., 2018). This indicates that there is a need for attention towards managing respiratory disease. However, a study on the laboratory diagnostics conducted at the Technical University of Denmark and SEGES suggested that of the herds that had antibiotics prescribed for respiratory disease in Denmark in 2015, only 4% of the veal herds and 2.7% of the dairy herds had conducted a diagnostic laboratory test for the causative pathogens (Jensen et al., 2018). The study does not include all laboratory diagnostics performed in Denmark but may give an indication of the use of diagnostics for respiratory disease. The prevention and treatment decisions for the individual herd and disease

case could be more effectively targeted if the veterinarian and farmer knew the aetiology of the respiratory disease.

Obtaining a timely and accurate diagnosis of BRD is challenging (Fulton and Confer, 2012). A timely diagnosis is reliant on recognition of BRD by the farmer or staff. This recognition was shown in a study to be 100% specific, but only 56% sensitive (Sivula et al., 1996). An accurate diagnosis is difficult to obtain due to the uncertainty about whether pathogens recovered from samples are in fact the cause of the respiratory disease or simply part of the microbiota. The gold standard for diagnosing BRD is necropsy and testing for relevant pathogens post-mortem (Love et al., 2014). Under field conditions, ante-mortem diagnosis of respiratory disease is typically based on clinical examinations (Buczinski et al., 2014), sometimes combined with laboratory investigations. Some of the most common clinical signs of BRD include fever, coughing, nasal- and ocular discharge, reduced appetite, depression, increased respiratory rate and laboured breathing. The course of disease ranges from subclinical to severe, and may lead to death (Griffin et al., 2010). Clinical respiratory scoring systems to detect calves with respiratory disease have been developed and scientifically validated with moderate sensitivity and relatively high specificity (Love et al., 2016). However, the clinically sick animal will often not display signs which are specific for a single aetiology (Fulton and Confer, 2012), and diagnostic laboratory testing is therefore necessary for identification of the pathogens associated with BRD (Love et al., 2014). Polymerase chain reaction (PCR) and culture-based microbiological investigations are examples of diagnostic laboratory methods for pathogen identification. Ante-mortem culture and antimicrobial sensitivity or PCR can be performed on nasal swab (NS) samples and samples from the lower respiratory system (Doyle et al., 2017). However, a swab from the nasal cavity is the cheapest, fastest and least invasive sampling technique and therefore more appropriate for sampling multiple calves (Van Driessche et al., 2017). Multiple studies have compared upper respiratory samples such as NS and/or nasopharyngeal swabs with lower respiratory samples such as bronchoalveolar lavage, lung samples or transtracheal aspirate for common bacterial respiratory pathogens (Allen et al., 1991; Doyle et al., 2017; Godinho et al., 2007; Thomas et al., 2002b; Van Driessche et al., 2017). These studies showed conflicting results regarding the extent of agreement for *H. somni* and *M. bovis,* but generally moderate to good agreement for *P. multocida* and *M. haemolytica* between upper and lower respiratory tract samples. However, these studies were primarily based on bacterial culture, where there is a risk of polymicrobial overgrowth which can give false negative results (Van Driessche et al., 2017). This could be avoided with the use of PCR. Infectious agents can be localized to different areas in the respiratory tract, which is important to

consider when choosing a sampling procedure or evaluating diagnostic output. It is therefore necessary to investigate the extent of agreement between sampling methods preferably using PCR for analysis. A newly established high-throughput real-time PCR (qPCR) detection system using the Biomark platform (Fluidigm, South San Francisco, USA) at the Technical University of Denmark can detect and quantify genetic material from multiple bovine viruses, bacteria and intestinal parasites in the same setup while running numerous samples at once. The Fluidigm qPCR system can identify relevant infectious respiratory agents, including BRSV, BCV, *M. bovis*, *Mycoplasma* spp., *H. somni*, *M. haemolytica*, *P. multocida*, *T. pyogenes* and IDV. Several studies in the veterinary and human medical field have shown that clinical presentation and/or disease severity can be related to qPCR quantitative results (Best et al., 2018; Dormond et al., 2015; Trang et al., 2015), and thereby pathogenic load. As described, some of the pathogens associated with BRD can also be found in clinically healthy animals, and to the authors' knowledge, clinically relevant qPCR cut-offs have not yet been defined for respiratory pathogens. Therefore, there is a need to determine cut-offs associated with respiratory disease, thereby improving the interpretation of molecular diagnostics. Improving the interpretation of diagnostic results for respiratory pathogens in calves combined with clinical observations could potentially assist veterinarians and farmers in making more objective and accurate interventions.

The purpose of this study was therefore to increase the utility of diagnostics regarding respiratory disease in calves, in order to improve treatment and prevention of BRD, and thereby increase growth, health and production. The specific objectives of this study were as follows:

1) To investigate whether it is possible to define relevant qPCR cut-offs associated with respiratory disease for the bovine respiratory pathogens tested by the qPCR system, Fluidigm, based on data available from a Danish field study providing paired clinical recordings with qPCR results from NS samples.
2) To compare the occurrence of nine respiratory pathogens between the two sampling methods, NS and TW.

2. Materials and Methods

2.1 Study design

The data used in this work originates from a field study which is a part of the Robust Calves project – a project aiming to improve the health management of calves. The project consists of four work packages and the current thesis falls under work package two which is concerned with diagnostic tools for surveillance of health status. For this thesis, two subsets of the collected data by the Robust Calves field study were used. The first subset was from calves that were sampled and examined repeatedly over time at different age groups. All available observation units from this subset were used. In this study, an observation unit was defined as a calf in a particular age group. The second subset of data consisted of calves where paired NS and TW samples were collected. The data used in the present study was collected between September 2018 and December 2019 from a total of 36 participating herds – nine veal herds and 27 dairy herds.

2.2 Herd and calf selection

The herd selection in the Robust Calves field study was planned as "calf clusters" - which are groups of herds, each consisting of a veal herd and five of its supplying dairy herds. These clusters allowed the calves to be followed from birth to slaughter or first lactation. A veal herd is defined as a Danish herd which produces rosé veal calves, where calves purchased from dairy herds are slaughtered as veal (8-12 months) or young bulls (>12 months) (Fertner et al., 2016). The veal producing herds were chosen based on convenience. The inclusion criteria were that they had regular dairy calf suppliers and a sufficient herd size in order to provide enough calves in all included age groups. The dairy farms were chosen based on how many calves they delivered to the veal herd. Veal herds included in the Robust Calves project needed to register production data in the Danish Cattle Database to participate. The mean size of the nine veal herds was 1092 (512-1629) veal calves, while the mean size of the 27 supplying dairy herds was 351 (114-705) milking cows. In Appendix A the herd sizes and geographical distribution of the 36 participating herds are shown.

The calves included in the first subset of data from the Robust Calves field study were randomly selected from each herd based on sex (12 heifer and 12 bull calves) and age (0 to 10 days) by the Robust Calves digital platform and database, "EasyOn". However, on most farms it was not possible to obtain the required number of calves at the first visit. Consequently, follow-up visits were made to sample and assess additional calves in order to gain the desired sample size for the age groups. However, on some farms the desired sample size of 24 calves was not achieved. In the Robust Calves

project, four age groups of calves were included. They were assigned the value 1 for calves 0-10 days old, 3 for calves three weeks old, 5 for calves two weeks past introduction to the veal herds, and 7 for calves three months old.

The calves included in the second subset (with paired NS and TW samples) were randomly selected based on sex and age by "EasyOn".

2.3 On-farm scoring

For the Robust Calves project, a standard protocol was developed with input from participating veterinarians. It describes how to evaluate pens and clinical signs as well as how to perform blood sampling, NSs, and faecal collection. In addition, the calf's sex, breed, and age on the registration date were recorded. An excerpt of the protocol relevant for this thesis is shown in Appendix B. At every visit, data collection was performed starting with the youngest age group. Data was registered on the "EasyOn" platform. The data collection was primarily carried out by two veterinarians involved in the Robust Calves project.

2.4 Sample collection

The TW and NS samples were marked with specific identification numbers synchronized with the "EasyOn" platform. All samples were stored in a polystyrene box containing cooling elements before delivery to the Technical University of Denmark. After delivery, all samples were kept under cooled conditions at approximately 5°C until processing.

2.4.1 Nasal swab

The NS samples were collected with a 15 cm unguarded sterile polyester-tipped swab. According to the Robust Calves protocol given in Appendix B, the swab was guided three to seven cm into a naris, and then rotated against the mucosal wall before withdrawal. The tip of the swab was stored in an Eppendorf tube containing PBS.

2.4.2 Tracheal wash

The TW samples were obtained by a technique adapted from a description shown in Appendix C by veterinarian Niels Peter Jensen. For the procedure a 120 cm long flush catheter (proVET®,*"Tracheal skyllekateter til kalv"*) was used. Prior to the procedure, each calf was sedated with 0.25 ml xylazine for approx. 80 kg calf. The sample was collected using a blind technique where the tongue was moved rostrally in order to guide the catheter down the trachea until resistance and an estimated position by the bifurcation was reached. With a 50 ml disposable syringe, 50 ml of isotonic sterile saline was

instilled and immediately aspirated. If no fluid was recovered, the catheter was repositioned until fluid was aspirated. The fluid was then transferred to a Corning 50 ml centrifuge tube.

2.5 Pathogen detection

For the diagnostic analyses of the TW and NS samples, the high throughput real-time PCR (qPCR) BioMark HD (Fluidigm, South San Francisco, USA) was used. In this study, qPCR was performed to detect and quantify the following respiratory pathogens: BRSV, BCV, *M. bovis, Mycoplasma* spp., *H. somni, M. haemolytica*, IDV, *P. multocida* and *T. pyogenes*. The steps for the diagnostic analyses are shown in Figure 1 and explained further in the following.

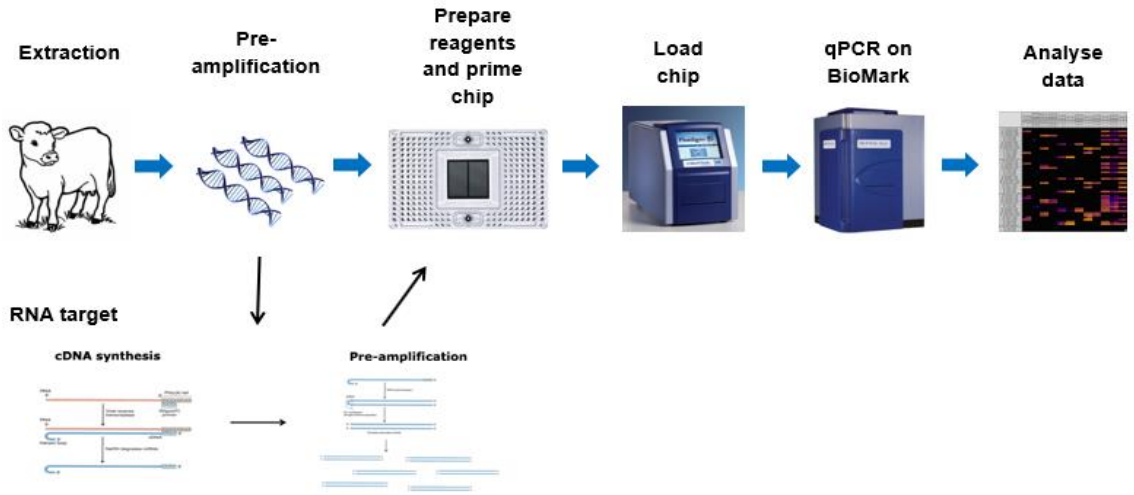

Figure 1. Flowchart illustrating each step for the diagnostic laboratory analyses associated with qPCR conducted by the Fluidigm BioMark HD system, at the Technical University of Denmark. By courtesy of Nicole Bakkegård Goecke.

2.5.1 Sample preparation, extraction and pre-amplification

The NS samples were prepared by vortexing the Eppendorf tube containing the swab-tip in order to transfer cell material from the swab into the PBS. No further preparation was carried out for the TW samples. Viral and bacterial DNA and viral RNA were extracted from the samples as described in the study by Goecke et al. (2020)(Goecke et al., 2020). Prior to high-throughput qPCR the extracted NS and TW samples were both reverse transcribed and pre-amplified or simply pre-amplified on a T3 Thermocycler (Biometra, Fredensborg, Denmark). The primer and probe sequences used for the pre-amplification steps and the high-throughput qPCR were either from previously published assays or designed for the Robust Calves project, not yet published (N.B. Goecke, personal communication, 20-01-2020).

2.5.2 High-throughput qPCR

For the high-throughput qPCR amplification, the BioMark 192.24 Dynamic Array (DA) Integrated Fluidic Circuit (IFC) chip (Fluidigm, South San Francisco, USA) was used. This platform contains fluidic networks that automatically combine 192 of the pre-amplified samples with 24 assays (primer and probe pairs), enabling 4,608 individual qPCR reactions simultaneously. The samples, assays and controls were dispensed into the respective inlets on the 192.24 DA. The 192.24 DA IFC chip (Fluidigm) was then placed in the RX IFC controller (Fluidigm) for loading and mixing. After approximately 30 minutes, the 192.24 DA was transferred to and run by the high-throughput qPCR BioMark platform (Fluidigm).

The output data, including the quantification cycle (Cq) values and amplification curves of the target, were acquired on the BioMark system as a heat map and analysed using the Fluidigm Real-Time PCR Analysis software 4.1.3 (Fluidigm). Before qPCR was performed, a threshold line was decided upon by the laboratory. The threshold line defines when a reaction reaches fluorescent intensity above background levels. The Cq value is the number of qPCR cycles needed to intersect the threshold line, meaning that it is the number of cycles needed for the sample to exceed background levels. Cq values are inverse to the amount of target-DNA in a sample. This means that a low Cq value indicates a high target-DNA concentration, while a high Cq value indicates a low target-DNA concentration. These Cq values were used for the statistical analyses.

2.6 Data management

Data management and statistical analyses were performed using the statistical software R (version 3.6.1) with RStudio (version 1.2.1335).

2.6.1 Collecting data into dataset

The data for this thesis was downloaded to Microsoft Excel from “EasyOn”. For NS and TW samples in which none of the investigated pathogens were detected (no target-DNA was detected), a value of 32 was assigned. The value 32 was chosen because no samples, in which the investigated pathogens were detected, reached this Cq value within 40 cycles.

Two datasets were produced. The first dataset consisted of the first subset of data collected by the Robust Calves field study with paired clinical scores and NS qPCR results. Subsequently, observation units were removed if any of the following variables were missing: Cq values, temperature, scores for coughing, nasal or ocular discharge. In total, 73 observation units were

deleted. The second dataset was produced by pairing all TW qPCR results with the corresponding NS results based on Animal ID and registration date.

2.6.2 Clinical scoring

For the first objective, a clinical scoring system was created in order to classify whether a sampled calf suffered from respiratory disease or not. It was developed based on two existing scoring systems for BRD developed by veterinarians at the University of Wisconsin-Madison (McGuirk, 2008) and University of California-Davis (Love et al., 2014) and communication with veterinarians participating in the Robust Calves project. It was aimed at utilizing the registered clinical observations while acknowledging the challenges and limitations of on-farm scoring. The clinical signs included in the scoring system were nasal and ocular discharge, coughing and temperature. For each clinical sign, different scores were assigned based on severity as shown in Table 1. Based on these scores, each observation unit was given a total clinical score for respiratory disease. In addition, one point was added to the total score if an observation unit had both nasal and ocular discharge. If an observation unit had a total clinical score equal to or exceeding the case definition of 5, it was classified as sick with respiratory disease. If the total clinical score was below 5, it was classified as not sick with respiratory disease, and referred to as healthy in this thesis.

Table 1. The clinical scoring system used to assign respiratory clinical status to the calves in this study.

Score	0	1	2	3	4	+1
Nasal discharge	None	Serous			Mucopurulent	If oculonasal discharge
Ocular discharge	None	Serous	Mucopurulent			
Temperature	< 39°C	39 - 39.3°C	≥ 39.4°C			
Coughing	None			≥ 1 spontaneous		

2.6.3 Explanatory and outcome variables

The explanatory variable “AgeGr” was a four-leveled ordinal variable describing the age group that the calf was in at the time of registration and sampling. It could take on the levels 1 (0-10 days old), 3 (three weeks old), 5 (two weeks after introduction to veal herd) or 7 (three months old). “HerdNumber” was a categorical explanatory variable and represented the herd identification number for the herd that the calf was located in at the time of registration and sampling. “GroupID” was a categorical explanatory variable describing which “HerdNumber” and “AgeGr” a calf belonged to at the time of sampling. “GroupID” thereby accounted for dependencies between calves from the same age group within a herd. The qPCR Cq values for each respiratory pathogen used in this study were included as continuous variables. The explanatory variable ”dich” was a dichotomous variable

(positive/negative) defining whether a sample's Cq value for a pathogen was ≤ or > a defined Cq cut-off.

A case definition of ≥ 5 total clinical score points classified the observed calves as sick with respiratory disease, and <5 as not being sick with respiratory disease. "Sick" was therefore a binary outcome variable (yes/no).

2.7 Statistical analysis

2.7.1 qPCR cut-off

Descriptive statistics were carried out by calculation of the prevalence for each pathogen on herd-level. The pathogens for which attempts were made to find a clinically relevant Cq cut-off were *M. bovis, H. somni, M. haemolytica, P. multocida* and *T. pyogenes.* The pathogens IDV, BCV and BRSV were not included in the analyses because there were too few samples in which target-DNA for these pathogens were detected. In addition, *Mycoplasma* spp. was also excluded because determining a cut-off on genus level was outside the scope of this thesis.

The mean age, median clinical score and the ratio of sick and healthy observation units for each of the 36 herds was calculated. Furthermore, for each herd, the number of samples in which each of the five pathogens were detected by qPCR was given. In addition, a summary for each of the five pathogens was given. The percentage of the observation units, in which the respective pathogen was detected, which were classified as sick with respiratory disease, was calculated. Furthermore, the pathogens' minimum and maximum Cq value were found for both the observation units classified as sick and as healthy. The median clinical score and mean age for the sick and healthy observation units were calculated for each pathogen. In addition, the number of sick and healthy observation units where the respective pathogen was detected as the only pathogen in the NS sample was calculated. Finally, for each pathogen the most frequent combinations with other pathogens were calculated.

Various statistical tests were used to analyse *M. bovis, H. somni, M. haemolytica, P. multocida* and *T. pyogenes* to find relevant clinical Cq cut-offs. They are explained in the following sections.

2.7.1.1 Scatter plots

Scatter plots depicting the total clinical score for each observation unit plotted against Cq values from the NS samples were created for each of the five pathogens. A data point was displayed in red if the observation unit was classified as sick with respiratory disease, or blue if classified as healthy. The values of 32, describing when target-DNA was not detected for the investigated pathogen, were included in the plots to visualize the distribution and ratio of qPCR results with and without target-

DNA detected. Based on the scatter plots, it was attempted to visually estimate a Cq cut-off for each pathogen.

2.7.1.2 Chi-squared test of independence

A chi-squared test of independence was carried out using the chisq.test function in R, on 2x2 contingency tables. In the cases where a cell in the table contained an expected frequency below five, the Fisher's exact test was applied using the fisher.test function. For the contingency tables, the variables used were sick with respiratory disease (yes/no) as defined by the case definition, and whether observation units were qPCR positive or negative for the specific pathogen at a given Cq value. Significance was set at $p < .05$.

2.7.1.3 Multivariable analysis

The analysis of the data was performed stepwise using a statistical model, referred to here as *the model*, which includes an optimisation algorithm to run through all relevant qPCR Cq values and test these as potential cut-offs in relation to the calves' respiratory health status. *The model* contained a mixed-effects logistic regression with binomial distribution and logit link function using the glmer() function in the lme4 package in R. Mixed-effects regression is an extension of fixed-effects linear regression which adjusts for data that contain nested grouping structures. The effects were described by log odds ratios. *The model* was used to test one pathogen (*M. bovis, H. somni, M. haemolytica, P. multocida*, or *T. pyogenes*) at a time. It was not possible to test combinations of pathogens due to data limitations.

Data hierarchies (e.g. data with a nested or clustered structure) were adjusted for in *the model* at herd- and age-group level by including GroupID as a random effects factor.

The dataset contained repeated measurements as the same individual calves were sampled up to four times during the study period with intervals from two to seven weeks. However, these were considered as single observation units.

The model is described stepwise in the following:

1) Data pertaining to the pathogen to be tested was extracted.
2) An interval was defined, which was then applied in step 5. The intervals of Cq values explored by the algorithms for each pathogen for a relevant cut-off had to be limited to intervals with sufficient data points. These intervals, as shown in Table 2, were determined by graphically considering the distributions of Cq values for each pathogen and choosing lower and upper

boundaries that excluded obvious outliers but left sufficient data. An example is given in Figure 2 where the interval is given between the dotted lines.

Table 2. Intervals for determination of clinically relevant Cq cut-offs for the five pathogens.

Pathogen	Interval (Cq)
M. bovis	(15-26)
H. somni	(15-25)
M. haemolytica	(14-26)
P. multocida	(15-25)
T. pyogenes	(20-26)

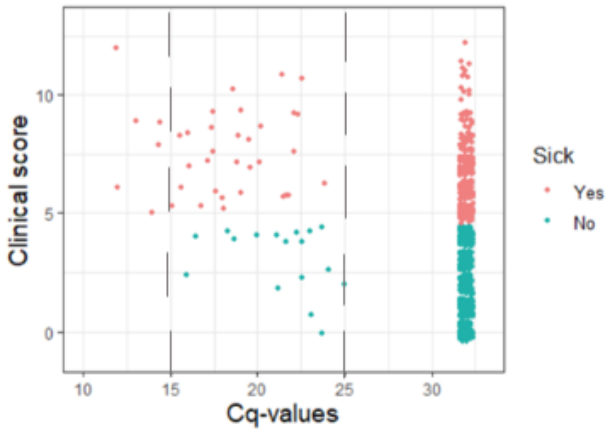

Figure 2. Distribution of clinical score against Cq values for *H. somni*. Grouped after case definition where sick with respiratory disease versus healthy is highlighted in the colours red and blue, respectively. The dotted lines illustrate the interval for Cq cut-off determined for *H. somni*.

3) A function was created in R for reuse in the optimisation step, with a single argument representing the test cut-off value ("dich"). It determined whether the qPCR Cq values were ≤ the specified cut off (positive/negative) for the pathogen specified in step 1. The function then ran a generalised mixed-effects logistic regression using the glmer() command in R based on this dichotomisation ("dich"). The model can be written as follows:

$$logit(p_{ij}) = \alpha + A_{ij} + \gamma x_{ij} + G_{kij}$$

where

p_{ij} is the probability for a calf *j* in an age group *i*, being scored as sick with respiratory disease given the explanatory variables

α is the intercept

A_{ij} is the fixed effect, age group, *i* = 1, 3, 5, 7 for calf *j*

x_{ij} is the fixed effect, Cq values being ≤ cutoff, *i* = true, false for calf *j*

γ is the slope

G_{kij} is the random effect, herd number, *k* =1…36, in combination with age group, *i* = 1, 3, 5, 7 for calf *j*

The function returned the maximised log-likelihood associated with the logistic regression.

4) "bestcutoff" was defined to maximize the object function from step 4 using the command optimise(), which searched the step 3 chosen interval from lower to upper boundaries for a

minimum qPCR Cq cut-off. Thereby, the best Cq cut-off associated with respiratory disease in calves was found.

5) The dichotomised test cut-off variable "dich", was redefined to describe whether the qPCR Cq values were ≤ bestcutoff (positive/negative) for the pathogen chosen in step 1.
6) The glmer() function was used on a similar model as the one presented in step 4 with the new definition of "dich" as a confounding variable replacing the original definition, in order to estimate the association between the explanatory variables and the outcome.

Based on *the model*, a plot for the predicted probability of being scored as sick with respiratory disease for the determined clinically relevant cut-off was created for each pathogen. Furthermore, a log-likelihood graph was produced for each tested pathogen where *the model* optimised for the highest point. This means the highest point in the graph was considered optimal clinically relevant Cq cut-off.

2.7.2 Agreement between sampling methods

The number of pathogens detected in each of the included 12 herds was calculated and scatterplots were created to illustrate the Cq values of the paired NS and TW samples for each of the nine pathogens. The occurrence and agreement beyond chance between NS and TW for the isolation of *M. bovis*, *Mycoplasma* spp., *M. haemolytica, H. somni, P. multocida, T. pyogenes,* BRSV, BCV and IDV was evaluated by calculation of the prevalence and Cohen's Kappa statistic in R, using the 'psych' package. The strength of agreement for the Kappa coefficient was interpreted as given in Table 3 (Landis and Koch, 1977). Positive results were defined as Cq values < 32. An additional calculation was made where a cut-off for positive Cq values was placed at ≤ 21. Significance was set at $p < .05$.

Table 3. Interpretation of the Kappa test statistic as described by Landis and Koch, 1977.

Cohen's Kappa coefficient	Interpretation
<0	Poor
0.01-0.20	Slight
0.21-0.40	Fair
0.41-0.60	Moderate
0.61-0.80	Substantial
0.81-1.0	Almost perfect

3. Results

3.1 Descriptive statistics for qPCR cut-off data

A total of 864 observation units were available from 308 individual calves from nine veal herds and 27 dairy herds. The pathogen that was detected in the most herds was *T. pyogenes*, which was present in 32 of 36 herds (88.9%). However, *P. multocida* was the pathogen detected in most observation units (25.9%), even though it was only present in 26 herds (72.2%). It was present in all nine veal herds. *M. haemolytica* was equally present in all nine veal herds, but only in ten of the 27 dairy herds (37%) with an overall herd prevalence of 52.8%. Both *M. bovis* and *H. somni* were found in 12 of the 36 herds (33.3%). *M. bovis* was found in four dairy herds (14.8%) and eight veal herds (88.9%) while *H. somni* was found in five dairy herds (18.5%) and seven veal herds (77.8%).

In Table 4 and 5, summaries of the data used in this part of the thesis are shown for veal and dairy herds separately. In total, 320 observation units were scored as being sick with respiratory disease. The median clinical score and mean age of the sick observation units were 7 points and 49.9 days, respectively. Of the 320 observation units which were scored as sick, 229 (71.6%) of the observation units were positive for at least one of the five investigated respiratory pathogens (as defined by Cq < 32). Of the 320 sick observation units, 91 (28.4%) did not test positive for any of the five pathogens. Of the 864 observation units, 544 (63%) were scored as without respiratory disease. Of these, in 337 (61.9%) observation units no pathogen was detected, while in 207 (38.1%) observations units at least one pathogen was detected.

Table 4. Summary showing the number of observation units (*n*) sampled from each veal herd. The number of observation units in each age group, the median clinical score and the observation units classified as healthy and sick with respiratory disease are shown. The total number of samples where each pathogen was isolated (Cq < 32) is shown for *M. bovis* (MB), *H. somni* (HS), *M. haemolytica* (MH), *P. multocida* (PM) and *T. pyogenes* (TP) for each veal herd.

		Age Groups			Clinical status # and (%)		# Pathogen (%)				
Herd No.	*n*	5	7	Median score	Healthy	Sick	MB	HS	MH	PM	TP
1	56	28	28	4	29 (52)	27 (48)	19 (34)	1 (2)	9 (16)	19 (34)	3 (5)
2	41	21	20	6	13 (32)	28 (68)	8 (20)	3 (7)	28 (68)	13 (32)	9 (22)
3	59	34	25	6	13 (22)	46 (78)	15 (25)	16 (27)	11 (19)	22 (37)	6 (10)
4	33	23	10	6	12 (36)	21 (64)	17 (52)	6 (18)	11 (33)	18 (55)	0 (0)
5	27	19	8	6	8 (30)	19 (70)	21 (78)	0 (0)	14 (52)	18 (67)	3 (11)
6	19	16	3	6	7 (37)	12 (63)	7 (37)	9 (47)	11 (58)	14 (74)	8 (42)
7	16	16	0	4.5	8 (50)	8 (50)	9 (56)	3 (19)	3 (19)	12 (75)	1 (6)
8	10	10	0	4	6 (60)	4 (40)	3 (30)	1 (10)	2 (20)	7 (70)	2 (20)
9	8	8	0	5.5	2 (25)	6 (75)	0 (0)	0 (0)	1 (13)	4 (50)	2 (25)
	269	175	94	6	98 (36)	171 (64)	99 (37)	39 (14)	90 (33)	127 (47)	34 (13)

Table 5. Summary showing the number of observation units (*n*) sampled from each dairy herd. The number of observation units in each age group, the median clinical score and observation units classified as healthy and sick with respiratory disease are shown. The total number of samples where each pathogen was isolated (Cq < 32) is shown for *M. bovis* (MB), *H. somni* (HS), *M. haemolytica* (MH), *P. multocida* (PM) and *T. pyogenes* (TP) for each dairy herd.

		Age groups			Median	Clinical status in # and (%)		# Pathogen (%)				
Herd No.	*n*	1	3	7	score	Healthy	Sick	MB	HS	MH	PM	TP
10	51	20	20	11	3	37 (73)	14 (27)	2 (4)	0 (0)	1 (2)	5 (10)	4 (8)
11	42	20	12	10	3.5	31 (74)	11 (26)	1 (2)	0 (0)	0 (0)	9 (21)	10 (24)
12	38	18	12	8	3	28 (74)	10 (26)	0 (0)	0 (0)	6 (16)	8 (21)	7 (18)
13	51	24	14	13	3	37 (73)	14 (27)	0 (0)	0 (0)	3 (6)	13 (25)	13 (25)
14	39	18	13	8	2	31 (79)	8 (21)	0 (0)	2 (5)	2 (5)	7 (18)	1 (3)
15	35	13	13	9	2	28 (80)	7 (20)	0 (0)	1 (3)	4 (11)	9 (26)	2 (6)
16	43	23	17	3	2	33 (77)	10 (23)	1 (2)	1 (2)	1 (2)	5 (12)	9 (21)
17	40	18	14	8	3	26 (65)	14 (35)	0 (0)	0 (0)	2 (5)	10 (25)	7 (18)
18	19	11	4	4	4	11 (58)	8 (42)	0 (0)	0 (0)	2 (11)	10 (53)	2 (11)
19	16	8	8	0	3	12 (75)	4 (25)	0 (0)	0 (0)	0 (0)	0 (0)	2 (13)
20	13	10	3	0	1	13 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
21	10	7	3	0	4	5 (50)	5 (50)	0 (0)	0 (0)	0 (0)	0 (0)	4 (40)
22	14	8	6	0	2	14 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (14)
23	13	10	3	0	3	8 (62)	5 (38)	3 (23)	0 (0)	0 (0)	1 (8)	2 (15)
24	8	5	3	0	3	4 (50)	4 (50)	0 (0)	0 (0)	0 (0)	2 (25)	1 (13)
25	17	9	8	0	4	10 (59)	7 (41)	0 (0)	10 (59)	2 (12)	7 (41)	3 (18)
26	10	5	5	0	6	4 (40)	6 (60)	0 (0)	0 (0)	0 (0)	5 (50)	1 (10)
27	3	3	0	0	0	3 (100)	0 (0)	0 (0)	0 (0)	0 (0)	2 (67)	1 (33)
28	15	7	8	0	3	12 (80)	3 (20)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
29	14	7	7	0	1	12 (86)	2 (14)	0 (0)	0 (0)	1 (7)	1 (7)	2 (14)
30	10	5	5	0	1	10 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (30)
31	24	12	12	0	3	19 (79)	5 (21)	0 (0)	0 (0)	0 (0)	0 (0)	5 (21)
32	19	10	9	0	3	15 (79)	4 (21)	0 (0)	0 (0)	0 (0)	1 (5)	4 (21)
33	15	8	7	0	3	15 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (7)
34	13	9	4	0	1	12 (92)	1 (8)	0 (0)	0 (0)	0 (0)	0 (0)	5 (38)
35	11	6	5	0	4	10 (91)	1 (9)	0 (0)	0 (0)	0 (0)	0 (0)	4 (36)
36	12	6	6	0	4.5	6 (50)	6 (50)	0 (0)	1 (8)	0 (0)	1 (8)	0 (0)
	595	300	221	74	3	446 (75)	149 (25)	7 (1)	15 (3)	24 (4)	96 (16)	95 (16)

In Table 6, a summary of data for the five investigated pathogens is given. Generally, the minimum and maximum Cq values for these pathogens did not differ much. For observation units which were positive for *M. bovis, H. somni, M. haemolytica* and *P. multocida,* approximately 60 to 70% were classified as sick with respiratory disease. However, only 32% of the observation units which were positive for *T. pyogenes* were classified as sick. The mean age for observation units positive (Cq value < 32) for *T. pyogenes* was lower for both healthy and sick calves, compared to the other four pathogens. *H. somni* was the pathogen which was detected the least times as the only pathogen in a sample. The five pathogens were generally most often seen in combination with *Mycoplasma* spp. and *P. multocida*.

Table 6. Summary of the five pathogens (*M. bovis* (MB), *H. somni* (HS), *M. haemolytica* (MH), *P. multocida* (PM) and *T. pyogenes* (TP)) with the percentage of the observation units, in which the respective pathogen was detected, which were classified as sick and the minimum and maximum Cq value for both healthy (H) and sick (S) observation units. The median clinical score and mean age for the healthy (H) and sick (S) observation units and the number of healthy (H) and sick (S) observation units where the pathogen was detected as the only pathogen. Finally, the pathogens which the respective pathogen is most often seen in combination with are shown (*Mycoplasma* spp. (M spp.))

		Cq value										
		Min		Max		Median score		Mean age		# detected as the only pathogen		
Pathogen	Scored as sick (%)	H	S	H	S	H	S	H	S	H	S	In combination with (%)
MB	63.2	13.1	12.8	27.5	27.8	4	7	48.9	52.9	16	16	PM (54) MH (33)
HS	68.5	15.6	11.9	24.8	24.1	4	8	50.8	73.9	1	1	PM (69) M spp. (67) MH (41)
MH	62.3	10.5	11.9	26.7	24.4	3	7	51.7	65.9	16	7	PM (53) M spp. (51) MB (31)
PM	62.3	12.5	12.9	25.2	26.6	2.5	7	49.7	64.4	32	37	M spp. (45) MH (27) MB (26)
TP	31.8	13.8	17.9	26.3	25.1	2	7	19.2	36.1	63	16	PM (24) M spp. (20)

3.2 qPCR cut-off results

Scatter plots, results of *the model* including predictive probability boxplots and log-likelihood graphs, as well as the results of tests for unadjusted associations (chi-squared test or Fishers exact test) are given in the following sections for each analysed pathogen (*M. bovis*, *H. somni*, *M. haemolytica*, *P. multocida* and *T. pyogenes*).

3.2.1 *M. bovis*

By visual inspection of Figure 3, the cut-off for *M. bovis* was placed at Cq value 21. As shown in Table 7 using the chi-squared test of independence and Fisher's exact test to test for unadjusted associations, significant associations between being sick with respiratory disease and cut-offs from Cq 14 to 26 were found, except at Cq value 17.

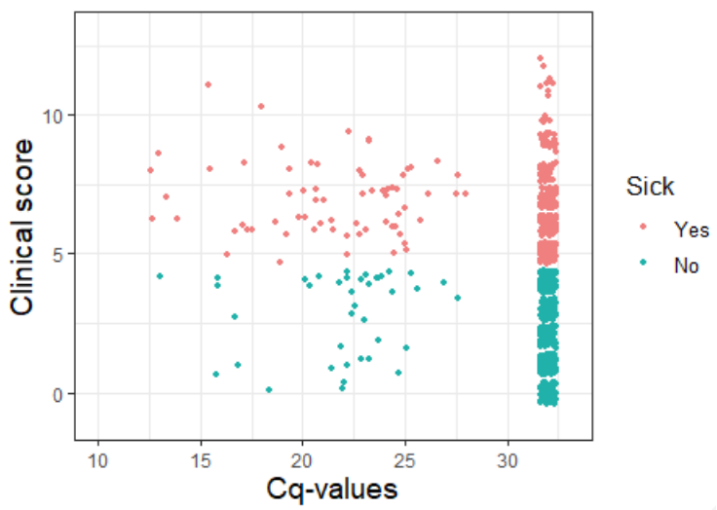

Figure 3. Distribution of respiratory clinical score against Cq values for *M. bovis* and additionally grouped by respiratory disease classification with red indicating a sick observation unit and blue indicating a healthy observation unit.

Table 7. Results for tests of unadjusted associations between *M. bovis* Cq values 14 to 26 and respiratory disease, n=864. The number of positive samples and the number of these which were classified sick is given. * = *p*-value < .05, ** = *p*-value < .01, *** = *p*-value < .001

Cq cut-off	#positive (#sick)	*P*
14	6 (5)	*
15	6 (5)	*
16	10 (7)	*
17	16 (10)	-
18	20 (14)	**
19	23 (16)	**
20	29 (21)	***
21	37 (28)	***
22	48 (33)	***
23	60 (36)	***
24	75 (44)	***
25	90 (56)	***
26	100 (63)	***

The model for *M. bovis* was run within the interval of Cq 15 to 26 and the results are shown in Table 8. A significant cut-off was found at Cq value 21, with a log odds ratio of 1.22. This corresponds to a probability of 0.76 for being classified as sick with respiratory disease if a given sample where *M. bovis* is detected has a Cq value ≤ 21.

Table 8. Results of *the model* for analysis of the optimal qPCR Cq cut-off for *M. bovis*. The estimates describe log-transformed fixed effects: Cq cut-off and age group. Standard error (SE) and *p*-value (*P*) were calculated for the estimates. Variance (σ^2) and standard deviation (SD) were calculated for the random effect, GroupID. * = *p*-value < .05, ** = *p*-value < .01, *** = *p*-value < .001

Variables	Estimate	SE	*P*	σ^2	SD
Fixed effects					
Intercept	-1.3636	0.1782	***		
Best Cq cut-off ≤ 21	1.220	0.4386	*		
Age Gr 3	-0.2332	0.2738	-		
Age Gr 5	1.5756	0.3008	***		
Age Gr 7	2.1627	0.2883	***		
Random effect					
Group ID				0.239	0.4889

In Figure 4, the predicted probability boxplot illustrates that *M. bovis* at Cq ≤ 21 was not detected in samples from observation units in age group 1. Figure 5 shows the log-likelihood graph, illustrating that the maximum likelihood was found at Cq 21. In summary, it was possible to determine a cut-off at Cq ≤ 21 associated with being classified with respiratory disease for *M. bovis*. Furthermore, the predicted probability graph illustrates that the probability of being scored as sick with respiratory disease for samples where *M. bovis* was detected was higher in older calves compared to younger.

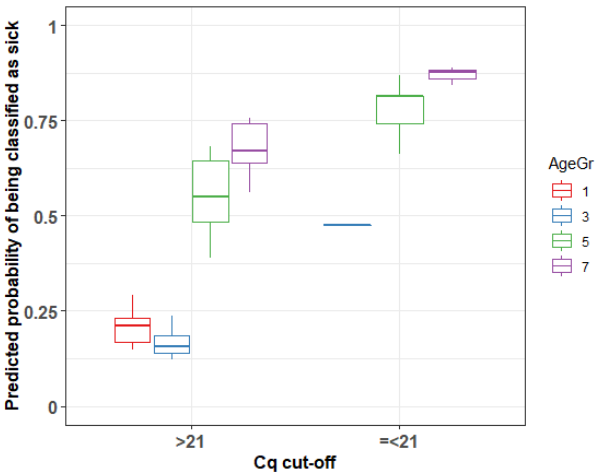

Figure 4. Predicted probability graph for *M. bovis.* The probability of being classified as sick with respiratory disease as predicted by Cq cut-off ≤ 21 in each age group.

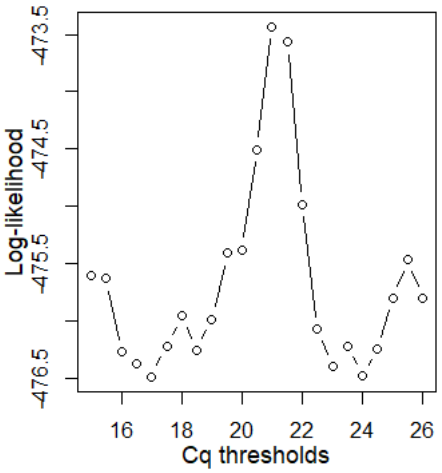

Figure 5. Log-likelihood graph for *M. bovis.*

3.2.2 *H. somni*

By visual inspection of Figure 6 the Cq cut-off for *H. somni* was placed at Cq 20. When testing unadjusted associations between being scored as sick with respiratory disease and various Cq cut-offs, significant associations were found for all Cq values from 14 to 26, as shown in Table 9.

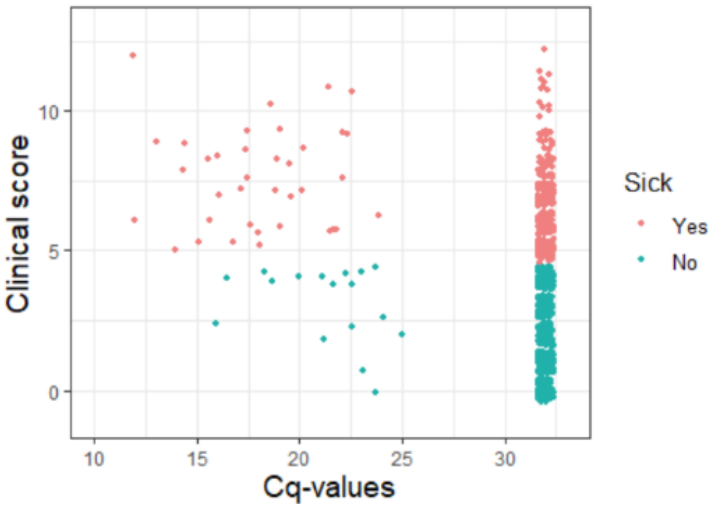

Figure 6. Distribution of respiratory clinical score against Cq values for *H. somni* and additionally grouped by respiratory disease classification with red indicating a sick observation unit and blue indicating a healthy observation unit.

Table 9. Results for tests of unadjusted associations between *H. somni* Cq values 14 to 26 and respiratory disease. The number of positive samples and the number of these which were classified sick is given. $n = 864$, * = p-value < .05, ** = p-value < .01, *** = p-value < .001

Cq cut-off	#positive (#sick)	*P*
14	4 (4)	*
15	6 (6)	**
16	11 (10)	***
17	15 (13)	***
18	21 (19)	***
19	27 (23)	***
20	31 (26)	***
21	34 (28)	***
22	40 (32)	***
23	48 (36)	***
24	52 (36)	***
25	54 (37)	***
26	54 (37)	***

The model was run within the interval 15 to 25 for *H. somni*. As seen in Table 10, a significant cut-off at Cq value ≤ 18.8 was found, with a log odds ratio of 2.79. This corresponds to a probability of 0.95 for being classified as sick with respiratory disease if a given sample where *H. somni* is detected has a Cq value ≤ 18.8.

Table 10. Results of *the model* for analysis of the optimal qPCR Cq cut-off for *H. somni*. The estimates describe the log-transformed fixed effects: Cq cut-off and age group. Standard error (SE) and p-value (*P*) were calculated for the estimates. Variance (σ^2) and standard deviation (SD) were calculated for the random effect, GroupID. * = p-value < .05, ** = p-value < .01, *** = p-value < .001

Variables	Estimate	SE	*P*	σ^2	SD
Fixed effects					
Intercept	-1.3861	0.1762	***		
Best Cq cut-off ≤ 18.8	2.7905	0.7361	***		
Age Gr 3	-0.3182	0.2750	-		
Age Gr 5	1.7357	0.2874	***		
Age Gr 7	2.1063	0.2853	***		
Random effect					
Group ID				0.214	0.4628

In Figure 7, the predicted probability boxplot illustrates that *H. somni* at Cq ≤ 18.8 was detected in few samples. As shown in Figure 8, the log-likelihood graph illustrates that maximum likelihood was found at Cq value 18. In summary, it was possible to determine a *H. somni* cut-off at Cq ≤ 19 significantly associated with being sick with respiratory disease. Furthermore, the boxplot showed

that the probability of being classified as sick for samples where *H. somni* was detected was generally higher in calves in the age groups 5 and 7 compared to age group 1 and 3.

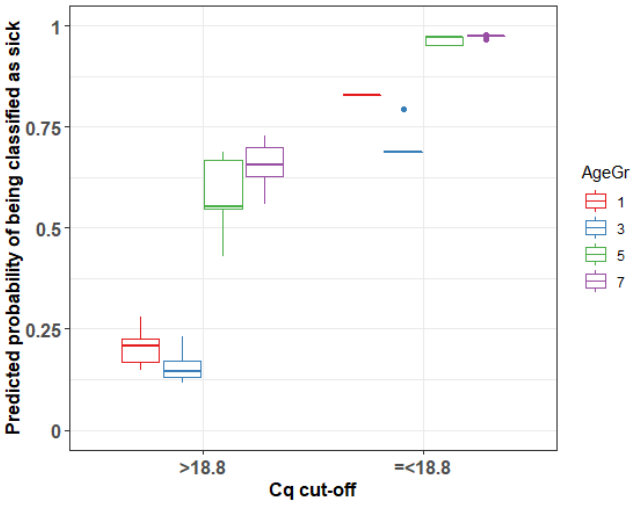

Figure 7. Predicted probability graph for *H. somni.* The probability of being classified as sick with respiratory disease as predicted by Cq cut-off ≤ 18.8 in each age group.

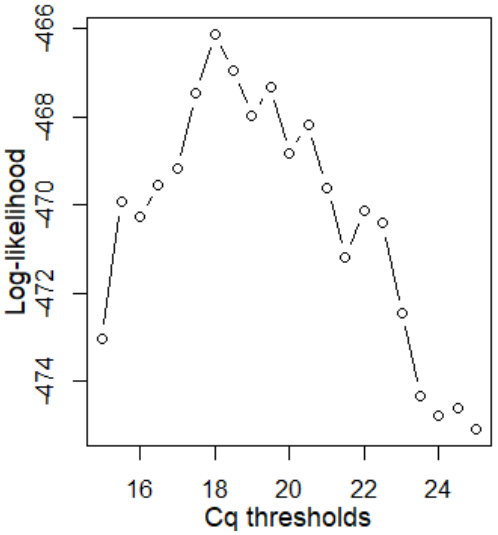

Figure 8. Log-likelihood graph for *H. somni*.

3.2.3 *M. haemolytica*

Based on Figure 9, it was not possible to place a cut-off for *M. haemolytica*. In Table 11, the results of the chi-squared test of independence and Fisher's exact test showed significant associations between being sick with respiratory disease and cut-offs from Cq 15 to 26.

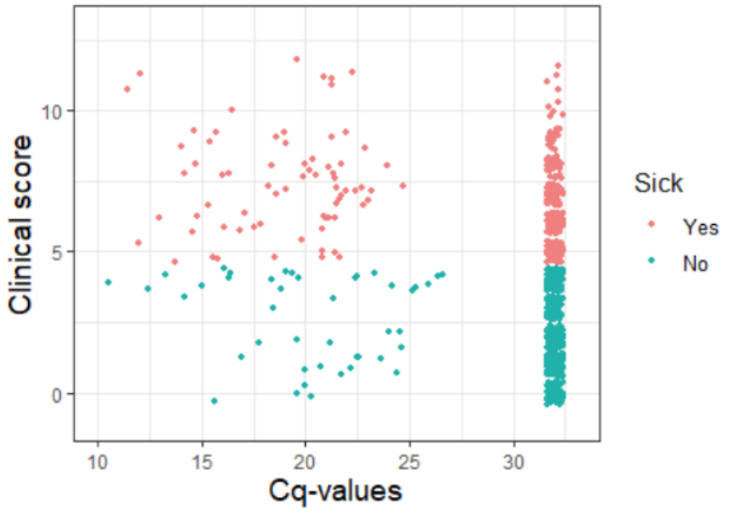

Figure 9. Distribution of respiratory clinical score against Cq values for *M. haemolytica* and additionally grouped by respiratory disease classification with red indicating a sick observation unit and blue indicating a healthy observation unit.

Table 11. Results for tests of unadjusted associations between *M. haemolytica* Cq values 14 to 26 and respiratory disease. The number of positive samples and the number of these which were classified sick is given. n = 864, * = *p*-value < .05, ** = *p*-value < .01, *** = *p*-value < .001

Cq cut-off	#positive (#sick)	*P*
14	9 (6)	-
15	15 (11)	**
16	25 (18)	***
17	30 (21)	***
18	36 (25)	***
19	43 (29)	***
20	53 (35)	***
21	68 (45)	***
22	89 (63)	***
23	100 (69)	***
24	102 (69)	***
25	110 (71)	***
26	112 (71)	***

The interval chosen for *the model* for *M. haemolytica* was from 14 to 26 and the results are given in Table 12. No significant Cq cut-off was found for *M. haemolytica*.

Table 12. Results of the *model* for analysis of the optimal qPCR Cq cut-off for *M. haemolytica*. The estimates describe the log-transformed fixed effects: Cq cut-off and age group. Standard error (SE) and *p*-value (*P*) were calculated for the estimates. Variance (σ^2) and standard deviation (SD) were calculated for the random effect, GroupID. * = *p*-value < .05, ** = *p*-value < .01, *** = *p*-value < .001

Variables	Estimate	SE	*P*	σ^2	SD
Fixed effects					
Intercept	-1.3665	0.1771	***		
Best Cq cut-off ≤ 22.5	0.5319	0.2829	-		
Age Gr 3	-0.2148	0.2719	-		
Age Gr 5	1.6329	0.2980	***		
Age Gr 7	2.0540	0.2943	***		
Random effect					
Group ID				0.230	0.4798

In Figure 10, the predicted probability boxplots show that for samples where *M. haemolytica* was detected at Cq ≤ 22.5 the corresponding observation units had a higher probability of being classified as sick compared to those with Cq values > 22.5, although this cut-off was not significant. Figure 11 shows the log-likelihood graph for *M. haemolytica*. It illustrates that maximum likelihood was found at Cq value 22. In summary, a Cq cut-off for *M. haemolytica* could not be determined when using the case definition of ≥ 5 points. However, a significant cut-off was found at Cq ≤ 22.9 using a clinical score of ≥ 9 to classify an observation unit as sick, see Appendix D.

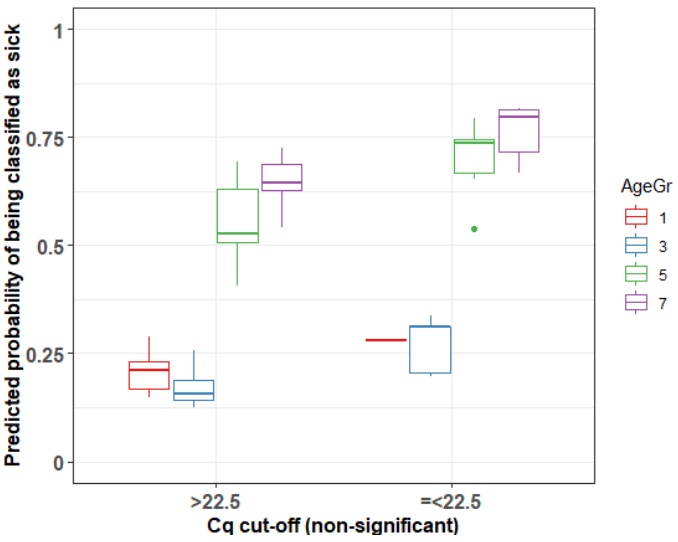

Figure 10. Predicted probability graph for *M. haemolytica*. The probability of being classified as sick with respiratory disease as predicted by Cq cut-off ≤ 22.5 in each age group. However, non-significant.

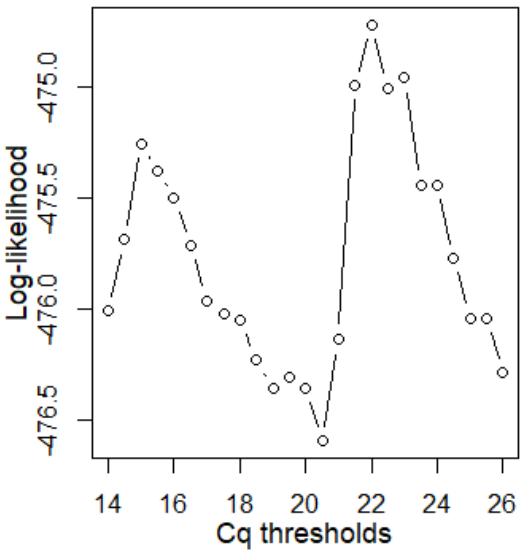

Figure 11. Log-likelihood graph for *M. haemolytica*.

3.2.4 *P. multocida*

Based on Figure 12, it was not possible to place a visual Cq cut-off for *P. multocida*. The chi-square test of independence and Fisher's exact test showed significant unadjusted associations between being sick with respiratory disease and Cq values from 15 to 26, as shown in Table 13.

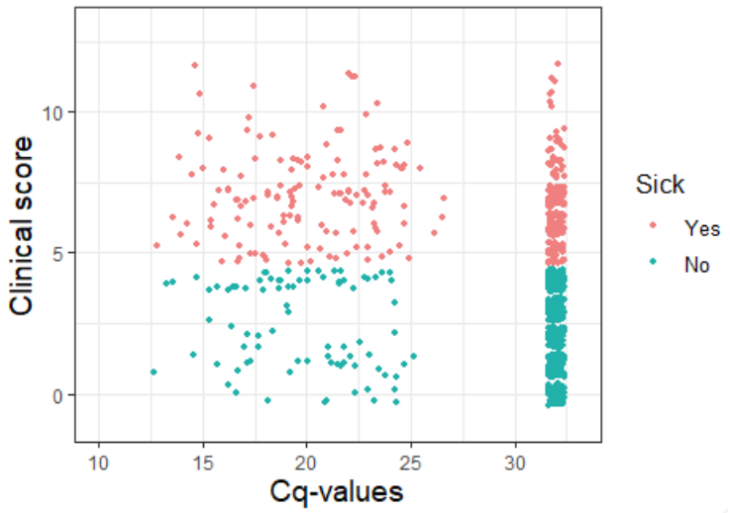

Figure 12. Distribution of respiratory clinical score against Cq values for *P. multocida* and additionally grouped by respiratory disease classification with red indicating a sick observation unit and blue indicating a healthy observation unit.

Table 13. Results for tests of unadjusted associations between *P. multocida* Cq values 14 to 26 and respiratory disease. The number of positive samples and the number of these which were classified sick is given. n = 864, * = p-value < .05, ** = p-value < .01, *** = p-value < .001

Cq cut-off	#positive (#sick)	*P*
14	7 (4)	-
15	14 (10)	*
16	25 (17)	**
17	46 (28)	**
18	67 (40)	***
19	89 (53)	***
20	115 (71)	***
21	130 (80)	***
22	156 (94)	***
23	176 (107)	***
24	199 (123)	***
25	218 (135)	***
26	221 (137)	***

The interval chosen for *the model* for *P. multocida* was 15 to 25 and results are given in Table 14. A significant cut-off was found at Cq ≤ 21.2 with a log odds ratio of 0.701. This corresponds to a probability of 0.67 for being classified as sick with respiratory disease if a given sample where *P. multocida* is detected has a Cq value ≤ 21.2.

Table 14. Results of *the model* for analysis of the optimal qPCR Cq cut-off for *P. multocida*. The estimates describe log-transformed fixed effects: Cq cut-off and age group. Standard error (SE) and p-value (*P*) were calculated for the estimates. Variance (σ^2) and standard deviation (SD) were calculated for the random effect, GroupID. * = p-value < .05, ** = p-value < .01, *** = p-value < .001

Variables	Estimate	SE	*P*	σ^2	SD
Fixed effects					
Intercept	-1.4053	0.1843	***		
Best Cq cut-off ≤ 21.2	0.7010	0.2336	**		
Age Gr 3	-0.2590	0.2820	-		
Age Gr 5	1.6238	0.3079	***		
Age Gr 7	1.9951	0.3031	***		
Random effect					
Group ID				0.281	0.5297

Figure 14 shows the log-likelihood graph for *P. multocida.* It did not illustrate a definite maximum likelihood, however Cq 20 the graph has a peak. In summary, it was possible to determine a cut-off at Cq ≤ 21 significantly associated with being classified with respiratory disease. Furthermore, as shown in Figure 13, the predicted probability graph illustrates that the probability of being classified as sick with respiratory disease for samples where *P. multocida* at Cq ≤ 21.2 was detected was slightly higher than when *P. multocida* was above >21.2.

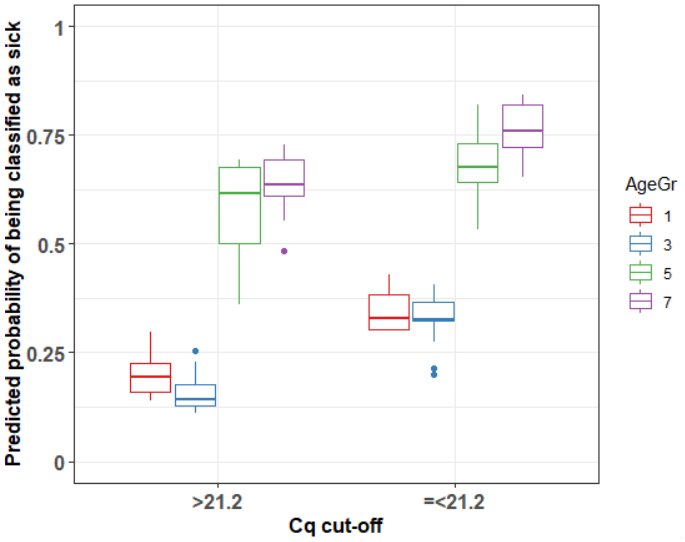

Figure 13. Predicted probability graph for *P. multocida.* The probability of being classified as sick with respiratory disease as predicted by Cq cut-off ≤ 21.2 in each age group.

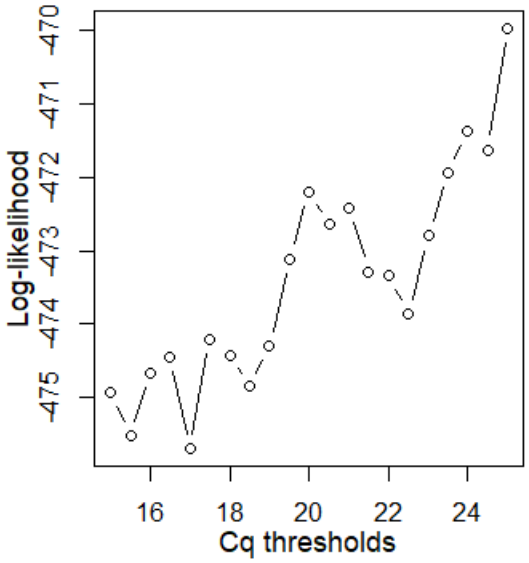

Figure 14. Log-likelihood graph for *P.* multocida.

3.2.5 *T. pyogenes*

By visual inspection of Figure 15, it was not possible to estimate a Cq cut-off for *T. pyogenes*. No significant unadjusted associations were found for *T. pyogenes* Cq values from 17 to 26 and being classified as sick with respiratory disease, see Table 15.

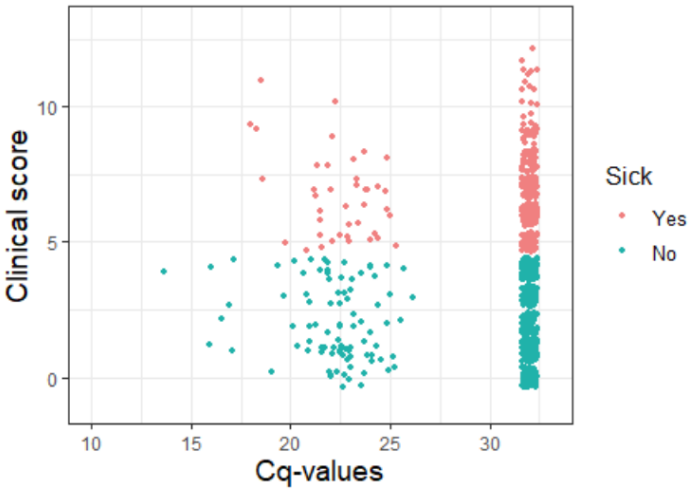

Figure 15. Distribution of respiratory clinical score against Cq values for *T. pyogenes* and additionally grouped by respiratory disease classification with red indicating a sick observation unit and blue indicating a healthy observation unit.

Table 15. Results for tests of unadjusted associations between *T. pyogenes* Cq values 17 to 26 and respiratory disease. The number of positive samples and the number of these which were classified sick is given. n = 864, * = *p*-value < .05, ** = *p*-value < .01, *** = *p*-value < .001

Cq cut-off	#positive (#sick)	*P*
17	5 (0)	-
18	8 (1)	-
19	12 (4)	-
20	14 (5)	-
21	21 (5)	-
22	45 (15)	-
23	79 (21)	-
24	104 (33)	-
25	121 (39)	-
26	128 (41)	-

The interval chosen for *the model* for *T. pyogenes* was 20 to 26. As seen in Table 16, a cut-off of ≤ 23.7 was found, but it was not significant. Furthermore, as shown in Figure 16, the predicted probability boxplots show that the probability for being classified as sick with respiratory disease is higher for the Cq values > 23.7. Figure 17 shows the log-likelihood graph for *T. pyogenes.* It illustrates that maximum likelihood could be found at Cq value 23, although as mentioned non-significant. In summary, it was not possible to find any significant Cq cut-off associated with respiratory disease for *T. pyogenes*.

Table 16. Results of *the model* for analysis of the optimal qPCR Cq cut-off for *T. pyogenes*. The estimates describe log-transformed fixed effects: Cq cut-off and age group. Standard error (SE) and p-value (P) were calculated for the estimates. Variance (σ^2) and standard deviation (SD) were calculated for the random effect, GroupID. * = *p*-value < .05, ** = *p*-value < .01, *** = *p*-value < .001

Variables	Estimate	SE	*P*	σ^2	SD
Fixed effects					
Intercept	-1.3452	0.1838	***		
Best Cq cut-off ≤ 23.7	-0.2053	0.2748	-		
Age Gr 3	-1.1983	0.2780	-		
Age Gr 5	1.7682	0.3009	***		
Age Gr 7	2.1777	0.2948	***		
Random effect					
Group ID				0.265	0.5152

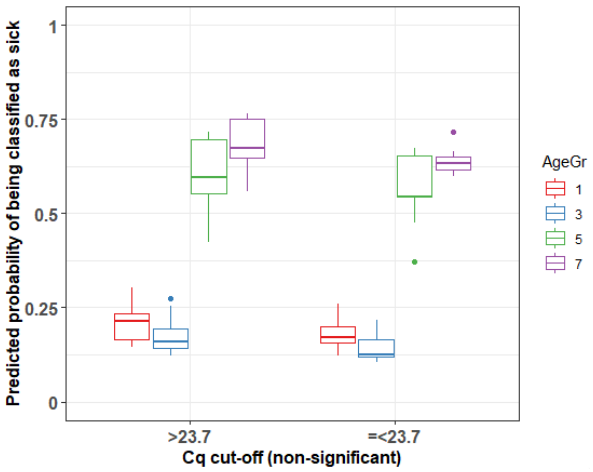

Figure 16. Predicted probability graph for *T. pyogenes.* The probability of being classified as sick with respiratory disease as predicted by Cq cut-off ≤ 23.7 in each age group. However, non-significant.

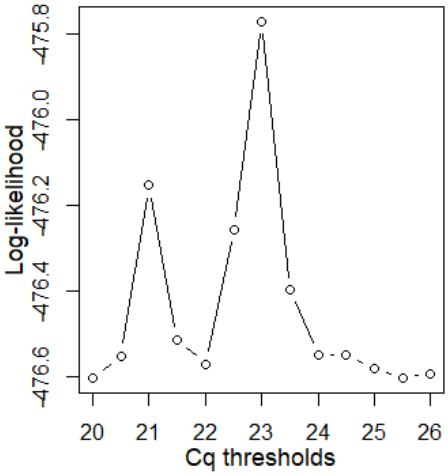

Figure 17. Log-likelihood graph for *T. pyogenes*.

3.3 Agreement of sampling methods

A total of 237 paired NS and TW samples were included in this study. The samples were taken from 157 individual calves from three veal herds (herd numbers one, two and three) and nine dairy herds (herd numbers 10 to 18). Scatter plots showing the Cq values of the paired NS and TW samples for each of the nine pathogens are illustrated in Appendix E, as well as summaries of the number of pathogens detected in each herd. The prevalence of the different pathogens found by the two different sampling methods are illustrated in Figure 18.

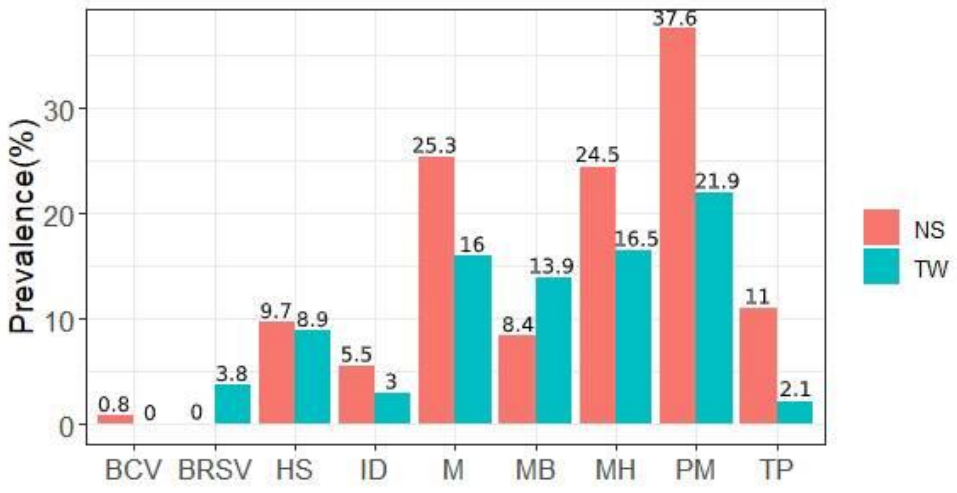

Figure 18. Prevalence (%) for the pathogens (HS: *H. somni*, ID: *Influenza D*, M: *Mycoplasma* spp., MB: *M. bovis*, MH: *M. haemolytica*, PM: *P. multocida* and TP: *T. pyogenes*) given for each sampling method (NS and TW) using Cq cut-off value <32, n = 237.

The prevalence for most of the pathogens was higher in NS than in TW samples, except for *M. bovis* and BRSV. Of the 237 paired samples, *M. bovis* was found in 20 NS samples (8.4%) and 33 TW samples (13.9%), while BRSV was found in zero NS and nine TW samples (3.8%). Notably, BCV was found in only 2 NS (0.8%) and no TW samples.

The results for the Cohen's Kappa statistic for the nine pathogens are shown in Table 19. It was not possible to estimate the agreement for the pathogens BRSV and BCV. For *M. bovis, M. haemolytica, P. multocida, Mycoplasma* spp. and *T. pyogenes* the agreement between the two sampling methods ranged between slight and moderate. However, for IDV there was moderate agreement between the two tests, with a kappa coefficient of 0.58. For *H. somni* the Kappa coefficient 0.70 indicated substantial agreement between NS and TW samples. In Appendix F, an additional calculation where positive results were defined as ≤ Cq 21 is shown. Here, the Kappa statistics were less than those presented in Table 17, except for IDV which was 0.62, making the agreement for this pathogen substantial.

Table 17. Agreement between the TW and NS samples for the identification of the nine respiratory pathogens from 237 paired samples, using Cq cut off value < 32, calculated using Cohen's Kappa.

	# Calves with each combination (TW/NS)					
Pathogen	+/+	+/-	-/+	-/-	Kappa (95% CI)	Kappa interpretation
BRSV	0	9	0	228	0 (0-0)	-
BCV	0	0	2	235	0 (-6.7^{-11} – 67^{-11})	-
M. bovis	11	22	9	195	0.35 (0.17-0.52)	Fair
Mycoplasma spp.	16	22	44	155	0.16 (0.03-0.3)	Slight
M. haemolytica	27	12	31	167	0.45 (0.31-0.58)	Moderate
H. somni	16	5	7	209	0.7 (0.54-0.86)	Substantial
IDV	6	1	7	223	0.58 (0.32-0.84)	Moderate
P. multocida	34	18	55	130	0.28 (0.16-0.41)	Fair
T. pyogenes	3	2	23	209	0.16 (-0.02-0.34)	Slight

4. Discussion

4.1 qPCR cut-offs

The current study has provided significant clinically relevant Cq cut-offs for three (*M. bovis, H. somni* and *P. multocida*) of the five investigated respiratory agents for the high-throughput qPCR system, Fluidigm. In addition, a significant Cq cut-off was found for *M. haemolytica* using another case definition for being classified as sick with respiratory disease. As these pathogens can be detected among the respiratory microbiota in healthy calves, the objective was to determine Cq cut-offs associated with respiratory disease. This knowledge can potentially assist veterinarians when interpreting diagnostic output from the qPCR Fluidigm system.

The lowest cut-off at Cq ≤ 19 was found for *H. somni*, accompanied by the highest probability of being scored as sick. However, because the Cq cut-off ≤ 21 was shown to be significant for both *M. bovis* and *P. multocida*, this cut-off was also investigated for *H. somni*. The calculations showed that a cut-off at Cq ≤ 21 for *H. somni* was also significantly associated with the probability of being scored as sick with respiratory disease. However, the probability was lower compared to a cut-off at Cq 19. This is most likely because a low Cq value equals a higher bacterial load.

The optimal Cq cut-off for *M. haemolytica* was difficult to determine although the unadjusted associations showed that there was a significant association between being scored as sick with respiratory disease and the detection of *M. haemolytica*. *The model* did not find a Cq cut-off significantly associated with being classified with respiratory disease by using the case definition applied in this study (≥ 5 in clinical score). However, by using a case definition of ≥ 9 points to classify respiratory disease, a significant association between detection of *M. haemolytica* at Cq ≤ 22.9 and the probability of being classified as sick, was found. By using this higher clinical score as case definition, the observation units classified with respiratory disease would on average be sicker than those with the case definition of ≥ 5. Furthermore, by using ≥ 9 as case definition, *M. haemolytica* was only detected in 18 samples from sick observation units. In ten of these observation units, *M. haemolytica* was detected in combination with *H. somni*, and in nine of the sick observation units the bacterium was detected in combination with IDV among others. Together, this suggests that *M. haemolytica* is most relevant if detected in combination with other pathogens and/or if the calf presents with more severe disease. Many different serotypes of *M. haemolytica* exist, for instance serotype A1, which is associated with clinical disease, and serotype A2, which occurs as a commensal (Cozens et al., 2019). Unfortunately, the assay used for the qPCR in this study did not distinguish between these. Basing the statistic calculations on the specifically commensal serotypes complicates

the matter of determining clinically relevant Cq cut-offs. For further investigation it would be relevant to only test samples for the pathogenic serotypes of *M. haemolytica*.

It was not possible to determine a significant Cq cut-off for *T. pyogenes* using *the model*, nor were any Cq cut-offs found to be associated with being scored as sick using the tests for unadjusted associations. In relation to this, only about 30% of the observation units where *T. pyogenes* was detected were scored as sick, whereas it was above 60% for observation units where any of the other pathogens were detected. Furthermore, respiratory disease is typically a problem for calves between one and six months of age (Svensson et al., 2006), however for the sick observation units which had samples where *T. pyogenes* was detected, the mean age only just fell within this interval, being 36 days old. The findings suggest that the majority of the *T. pyogenes* detected in this study were a part of the commensal nasal microbiota and it was therefore not possible to determine a clinically relevant Cq cut-off.

A limitation to take into account when interpreting the results of this thesis is that pathogens other than those tested for can be associated with BRD. BPIV-3 is an example of such, but no results were available due to the qPCR assay not running correctly (N.B.Goeke, Personal communication, 07-02-2020). *Salmonella Dublin* (*S. Dublin*) can be found in the respiratory tract in Danish calves in the age groups included in this study (Larsen et al., 1999), but it is not usually described as an aetiology for BRD. In Denmark, there is a national surveillance program for *S. Dublin*, which means that it is not included in routine diagnostics (DTU Veterinærinstitut, 2019), nor in the Robust Calves project.

It is important to note that the Cq values used to determine cut-offs associated with respiratory disease for the respiratory pathogens were derived from samples obtained by NS. However, as described in this thesis, some of these pathogens are also isolated from the nasopharynx and lower respiratory tract. Samples from the nasal cavity may not be fully representative of the true composition of microbials in the sampled calves. This could complicate the investigation of clinically relevant cut-offs for other pathogens, if based only on NS.

4.2 Agreement between sampling methods

Scientific literature shows conflicting results on whether an upper respiratory sample can predict the presence of pathogens in lower respiratory samples and vice versa. The results presented in this thesis add to the existing knowledge regarding the comparative value of upper and lower respiratory antemortem sampling methods. The agreement beyond chance between concurrently collected NS and TW samples for six (*M. bovis*, *Mycoplasma* spp., *M. haemolytica*, *P. multocida*, *T. pyogenes* and

IDV) of the nine investigated respiratory pathogens varied between slight and moderate. *H. somni* presented with the highest agreement of 0.70, corresponding to substantial agreement. There is no official guideline on how to interpret the Kappa test statistic, but most studies use the categorisation described by Landis and Koch (1977), as is done in this thesis. However, McHugh (2012) questions this interpretation of the Kappa test statistic and deems it problematic, especially in the healthcare field, as relatively low agreement could be considered adequate. For instance, a Kappa value of 0.50 would by Landis and Koch (1977) be considered moderate, but for 50% of the evaluations there would be disagreement. Instead, they suggest that any agreement below 0.60 should be deemed inadequate while agreement ≥ 0.80 should be considered strong (McHugh, 2012). The objective for this study was to simply compare the occurrence of pathogens for the two sampling methods. However, a practical aim could be to discuss if a NS could predict what was to be found by TW, as NSs are both cheaper and easier to carry out and might be preferable to use. For this, a threshold value for adequate agreement is needed, and following the suggestion of McHugh (2012), only *H. somni* (κ=0.70) met the threshold of 0.60, however as it did not reach an agreement of 0.80, it was not considered strong and therefore not considered adequate for the authors. Additionally, the confidence interval of *H. somni* was rather wide from 0.54 to 0.86, making the Kappa statistic less convincing.

To the authors' knowledge, no published literature exists which compares NS and TW samples nor any studies using qPCR as the only diagnostic detection method. However, other studies have compared upper and lower respiratory tract isolates using similar methods and the findings in these studies are discussed in relation to the results in this thesis. It is important to bear in mind that these methods may not be completely comparable to the ones used in this study.

Agreement between samples from the upper and lower respiratory tract regarding *H. somni* was higher in this work compared to a study by Van Driessche et al. (2017), where *H. somni* was found more frequently in bronchoalveolar lavage compared to deep nasopharyngeal swabs. This could be because the mentioned study used culture and *H. somni* can easily be overgrown by other resident bacteria (Angen, 2016). A study by Tegtmeier et al. (2000) in fact proved that detection by PCR is the most sensitive technique to detect *H. somni* (Tegtmeier et al., 2000). The agreement between NS and TW samples for *M. haemolytica* was moderate (κ = 0.45), which supports the work of Van Driessche et al. (2017) who compared bronchoalveolar lavage with deep nasopharyngeal swabs and found a kappa coefficient of 0.52 for *M. haemolytica*. Other studies have found a higher agreement for the isolation of *M. haemolytica* and *P. multocida* compared to this work, which could be due to their enrolment of clinically sick calves (Doyle et al., 2017; Van Driessche et al., 2017). In this thesis

calves were enrolled regardless of health status. This could have had an influence on the results as studies with sick BRD cases and healthy controls show that the detection of pathogens can be higher in cases compared to controls (Allen et al., 1991; Thomas et al., 2002a; Van Driessche et al., 2017). A second calculation of agreement was carried out using the cut-off Cq ≤ 21, based on the results of the first objective. However, this did not increase the agreement for *P. multocida* and *M. haemolytica* but instead decreased it to κ= 0.2 and 0.4, respectively. However, this may be due to a smaller number of positive samples when using this cut-off. The only agreement to increase using the Cq ≤ 21 cut-off was for IDV, possibly due to there being only few samples in which IDV was detected with only one sample exceeding a Cq value of 21.

In a study by Thomas et al. (2002), they found that NS culture results from sick calves were not predictive of the presence of *M. bovis* in the lower respiratory tract, as *M. bovis* was isolated more frequently in lower respiratory tract samples compared to samples from the nasal cavity. This concurs with the results of this study, in which the prevalence of *M. bovis* was higher in TW than in NS samples, and the agreement between NS and TW was only fair. *M. bovis* inhabit mucosal surfaces, but the tonsils have been described as the primary site of colonisation, from which *M. bovis* can subsequently colonise the lower respiratory tract (Maunsell and Donovan, 2009). This can explain the higher prevalence of *M. bovis* in TW samples.

T. pyogenes and the genus *Mycoplasma* spp. shared the lowest agreement beyond chance (κ = 0.16). The reason why *Mycoplasma* spp. did not show the same distribution as *M. bovis* could be explained by the fact that some of the other species in this genus (e.g. *M. bovirhinis* and *M. dispar)* are more commonly harboured in the upper respiratory tract in clinically healthy calves compared to *M. bovis* (Thomas et al., 2002a). This may have led to a low agreement between sampling methods. Notably, *T. pyogenes* was identified in five times as many NS samples as TW samples. This supports the fact that *T. pyogenes* is a commensal in the upper respiratory tract where it can often be isolated (Rzewuska et al., 2019). The prevalence of *P. multocida* and *M. haemolytica* was also higher in NS samples compared to TW samples. As the bacteria adheres to the nasal mucosa and subsequently can be inhaled into the lower respiratory tract (Griffin et al., 2010), it was expected to find the bacteria in both the upper and lower respiratory tract, but with a majority in the upper tract.

The agreement for IDV has, to the knowledge of the authors, never been investigated. In this study, moderate agreement was found. Another study detected IDV in both the upper and lower respiratory tract (Ferguson et al., 2016). Unfortunately, BRSV and BCV were not detected in enough samples to calculate the agreement between the sampling methods. However, BCV was only found

in NS samples and BRSV was only found in TW samples, which can indicate what sampling method to use. A study from 2017 compared the sampling methods NS, nasopharyngeal swab and bronchoalveolar lavage to transtracheal wash in clinically sick calves. The results support the findings in this thesis as BCV was found more frequently in samples from the upper respiratory tract and BRSV was found more frequently in the lower respiratory tract (Doyle et al., 2017). A study also found it to be possible to detect BRSV in the nasal mucosa, but concluded that samples from the cranioventral part of the lungs are more sensitive (Larsen et al., 1999).

Overall, the agreement between the two sampling methods was low. None of the interpretations of agreement reached strong agreement as proposed by McHugh (2012), meaning that in general NS could not predict the pathogens detected by TW.

4.3 Data quality

The target population of this thesis was Danish calves from 0 days to 4 months old in both dairy and veal herds. Herd selection was based on convenience cluster sampling and willingness of the farmers to participate, although random sampling would have been more representative. However, the 36 herds represented different housing and management types, breeds and locations which increased the representativity for the rest of the Danish calf population. The optimal sample size for this thesis was not calculated, since it was already decided upon as part of the Robust Calves project. The sample size was fairly large with 864 and 237 observation units for each objective respectively. However, *the model* for investigating Cq cut-offs involved stratifying observation units into groups based on herd and age group and some of these groups had relatively few observation units. A second model was tested where a cut-off was to be calculated with the ordinal variable “clinical score” as outcome. However, it failed to run correctly, possibly due to too few observation units in the groups.

Finally, treatment and vaccination status of the calves could have affected the clinical scores for the observation units as well as the qPCR results for the nine pathogens. Unfortunately, it was not possible to pay regard to this in the investigations, as they were unknown to the authors.

4.4 Clinical scoring and sampling

To determine clinically relevant Cq cut-offs, the observation units had to be classified as being either sick with respiratory disease or healthy. It should be noted that although the term “healthy” is generally used in this thesis, what is meant is that the observation unit does not meet or exceed the case definition of 5 points.

The two validated scoring systems developed at the University of Wisconsin-Madison (McGuirk, 2008) and the University of California-Davis (Love et al., 2014) were not applicable to the clinical assessments registered by the Robust Calves project. For this reason, a simple clinical scoring system was developed for this study by the authors. It included four clinical signs with few severity categories, with the aim of making the scoring more objective.

Another aim was to make the clinical scoring system as specific to identifying respiratory disease as possible, and thereby reducing the risk of classifying calves sick of other diseases (for instance diarrhoea) as sick with respiratory disease. This was attempted by adding an additional point if an observation unit had both nasal and ocular discharge, as the combination of these symptoms are considered rather specific for respiratory disease. Furthermore, four points were designated to mucopurulent nasal discharge, due to purulent material being indicative of infection. Coughing at least once was given a score of three points as it is a sign specific for respiratory disease. In the field study, coughs elicited in the process of rounding up animal were not registered, as it could be due to exertion, as well as dust and foreign bodies. Although increased temperature is not specific to respiratory disease, it is a common sign of BRD, is relatively objective, and is also included in the beforementioned validated scoring systems. However, all calves were handled during clinical assessment, and occasionally prior to, which might have increased their body temperature. Because rectal temperature is not specific for respiratory disease it was given only a maximum score of two. In this way, it was not singly decisive for the classification but would need to be accompanied by signs more specific to respiratory disease.

To improve the clinical scoring system, it would have been relevant to score respiratory rate and/or breathing difficult, as these are relatively objective and specific clinical signs for respiratory disease, when scored prior to entering the pen. These clinical signs were also included in the clinical scoring protocol developed at University of California (Love et al., 2014). In addition, the use of thoracic ultrasonography could have supported the clinical scoring system in this thesis as a study has shown that it increased the sensitivity of detecting respiratory disease when used in combination with clinical scoring (Buczinski et al., 2014). However, as the data collection was predetermined by the Robust Calves protocol, it was not possible for the authors to influence the on-farm clinical assessment to improve the clinical scoring. Furthermore, it was not possible to influence the sampling procedures. Several studies describe wiping the nostrils prior to sampling (Doyle et al., 2017; Godinho et al., 2007; Van Driessche et al., 2017), but this was not carried out in the project. Wiping the nostrils of the calves before collecting NSs might have decreased the number of commensal

organisms sampled. Another limitation regarding the sampling procedure arises from the fact that TWs were performed blindly, which makes the location sampled uncertain and possibly varying, thereby decreasing repeatability.

Because several persons were scoring, there was a risk of observer bias as well as variation in sampling technique. However, the individual persons scoring were qualified veterinarians and had previously partaken in a calibration session in order to decrease observer bias.

4.5 Real-time PCR

Using the high-throughput qPCR Fluidigm system to analyse samples presents several advantages, such as being able to test many samples for numerous pathogens at the same time, being able to use smaller volumes of material and being both a quantitative and qualitative method. From an economic point of view, the method is equally advantageous, as the cost could be reduced in comparison to regular PCR methods, which might encourage farmers and veterinarians to carry out more diagnostics. A limitation in this study is caused by the pre-amplification step prior to the qPCR, as it greatly influences the output values making them lower compared to other qPCR systems. This makes it impossible to compare the Cq values with previously published literature.

When running the qPCR, positive controls with known Cq values were included. Every time an analysis was carried out, it was checked that the positive control was within two Cq values of the expected value. If not, it was run again (N. B. Goecke, personal communication, 07-02-2020). This means, that there is an uncertainty of ± two Cq values in this study, which is important knowledge to have when interpreting qPCR results and applying the cut-offs determined in this thesis.

4.6 Statistical considerations

The statistical method which was given the most value when estimating a clinically relevant Cq cut-off was *the model*. This was because it contained a mixed-effects logistic regression and an optimization step. These steps made the analyses more objective than the visual estimation by the scatter plots which was a subjective method, making the validity debatable. However, the scatter plots were included in the study as it gave an indication of where the cut off values could be placed and thereby also supported *the model*. *The model* was more appropriate than the chi-squared test and Fisher's exact test used to test unadjusted associations, as it was possible to determine the optimal Cq cut-off while also adjusting for the possible effects of age and herd. Furthermore, for the tests for unadjusted associations, all tested cut-offs had a significant relationship with being classified as sick (with the exception of *T. pyogenes*) and therefore it was not possible to determine specific cut-offs.

The *p*-values tended to be less significant with decreasing Cq values, but this was most likely due to small ratios of positive samples at the lower Cq cut-offs. A limitation of the chi-squared test is that it simply tests associations between variables but does not describe the relationship, e.g. whether it is positive or negative, which means that this test should not stand alone.

Based on *the model*, a log-likelihood graph was created. The ideal graph would have had a normal distribution, but several of the graphs presented with multiple peaks, meaning they were difficult to interpret and often no clear cut-off was visible. The graph thereby also illustrated how difficult it was to place the cut-offs.

The data in this study was used due to the need for individually tested samples, as opposed to pooled samples. A disadvantage to this was that the data contained repeated measurements, as calves were sampled up to four times. This bears the risk of having a confounding effect, as some animals may be more or less prone to disease and recovery. However, it was not possible to account for this in the analyses. A further limitation was that the potential confounding effects of sex and season were not accounted for.

Multiple statistical methods exist to determine the level of agreement between observers or methods. For the second objective in this study, the authors were interested in the agreement between sampling methods simply in regard to whether a pathogen was detected or not detected, the reason being the lack of a normal distribution of data. The Cohen's Kappa test was found to be the most appropriate test for determining agreement of this qualitative outcome.

4.7 Biological and diagnostic considerations

An important limitation of this thesis was that it was not possible to investigate cut-offs for combinations of pathogens. Thus, the clinically relevant Cq cut-offs were calculated without accounting for the central interactions between the respiratory pathogens. Bovine respiratory disease often has a polymicrobial aetiology and it is possible that the presence of some pathogens influences the presence and/or growth of other pathogens (Taylor et al., 2010). Thereby the complex interactions of pathogens can affect the Cq values, complicating the determination of Cq cut-offs.

A recent study describes the dynamics of *H. somni*, *P. multocida* and *M. haemolytica* in healthy beef calves. Generally, the calves had high carriage rates for *H. somni* (26.8%) and especially *P. multocida* (75.9%) but lower carriage rates of *M. haemolytica* (5.7%). Not all calves became colonised with the bacteria even though they were placed same environment. Furthermore, the carriage rates decreased over time which can be explained by the calves getting immunologically more mature and thereby better at clearing pathogens (Thomas et al., 2019). A disease course is

equally dynamic and the pathogens which initiate BRD are not necessarily the same throughout the disease and in the later stages or at post-mortem (Taylor et al., 2010). In this thesis, 28% of the observation units which were scored as sick with respiratory disease did not have any respiratory pathogens detected in their NSs. Likewise, 38% of the observation units classified as healthy did have NS samples in which respiratory pathogens were detected. While there can be many explanations for this, including the detection of commensals, a clear limitation of this study was that samples and clinical assessments were taken and registered with intervals of at least two weeks. Therefore, a possible disease course could not be followed over time but were rather snapshots. It is therefore possible that a calf presenting with a low clinical score, but a high microbial load (low Cq value) could present with more severe clinical signs in the days following assessment and sampling. The inability to follow the possible disease course and pathogenic dynamics was a definite limitation. Another explanation for when a calf was scored with a low clinical score and a low Cq value can be due to PCR detecting DNA material from inviable organisms that are still present in the respiratory tract (Thomas et al., 2019). This means that some disease-causing infectious agents could still be detected in the respiratory tract after the respiratory disease has passed, and no symptoms would be present.

5. Conclusion

Based on NS samples paired with clinical scores from 864 observation units, it was possible to determine significant qPCR cut-offs associated with respiratory disease for the pathogens *H. somni*, *P. multocida,* and *M. bovis*. Using an optimisation algorithm with a mixed effects logistic regression in combination with tests for unadjusted associations and visual evaluation of scatterplots, log-likelihood graphs and predicted probability graphs, it was possible to estimate that the Cq cut-off ≤ 21 is applicable for *M. bovis*, *H. somni* and *P. multocida*. The Cq value ≤ 23 could be applied as a cut-off for *M. haemolytica* but with caution as this cut-off was based on a different case definition. The same statistical methods were used to investigate a cut-off for *T. pyogenes*. However, this was not possible, as the detection of *T. pyogenes* was generally not associated with respiratory disease in this study.

The occurrence of the nine respiratory pathogens in NS and TW samples was investigated by calculating the prevalence as well as the agreement beyond chance between the two sampling methods. Agreement ranged from κ=0.16 to 0.58 for the pathogens *M. bovis,* IDV*, M. haemolytica, Mycoplasma* spp.*, P. multocida* and *T. pyogenes*. The highest agreement was reached for *H. somni* at κ=0.70, but this was not considered strong enough to conclude that NSs can predict what can be found in TW samples. The virus BRSV was only found in TW samples and BCV in NS samples, and only in a limited number of samples. For this reason, it was not possible to estimate an agreement, but the results may suggest where in the respiratory tract the viruses occur. Except for BRSV and *M. bovis,* the prevalence of the pathogens was higher in NS compared to TW samples.

In conclusion, it has been shown that for some pathogens isolated by NS there is significant association between pathogenic load, thereby Cq value, and clinical signs of respiratory disease. As these pathogens may also exist as commensals in calves, these cut-offs could be utilized to discern between commensals and pathogens and thereby target treatment and preventive measures. However, as it was shown in this thesis, there was little agreement between the occurrence of pathogens in samples from the upper and lower respiratory tract. It is therefore possible that NSs cannot stand alone when investigating the composition of respiratory pathogens. As the qPCR Fluidigm system is still on a trial basis, further research is needed to fully utilize the potential of the qPCR Fluidigm system in relation to clinically relevant cut-offs.

6. Perspectives

Bovine respiratory disease is a considerable problem in the cattle sector, both in Denmark and internationally, and there is still much to be investigated for this multifactorial disease. Based on this thesis it would be interesting to investigate if Cq cut-offs vary between age groups as well as between dairy and veal herds. Investigating Cq cut-offs while accounting for combinations of pathogens would also be of high interest, as BRD is often not a single-aetiology disease. In relation to this, it would be preferable to also include *Salmonella* and BPIV-3 in future investigations.

Bovine respiratory disease is a problem on a group-level, as calves are housed together and sometimes mixed from different origins, and it is reasonable to expect an exchange of respiratory pathogens between animals (Callan and Garry, 2002). Therefore, it would be useful to investigate the potential of the high-throughput qPCR system to be used on group-level and determine relevant cut-offs for group-level testing. In groups, calves are typically at different stages of infection at any given time. Therefore, testing at group-level might be more representative of which pathogens are associated with disease than individual animal testing. Individual animal testing might be more affected by snapshots making it less representative.

In a study by Allen et al. (1991) they achieved higher agreement between lower and upper respiratory sampling methods by pooling samples compared to on individual animal-level. Even though Allen et al. (1991) identified pathogens by culture, it would still be relevant to investigate agreement between the sampling methods on a group-level and see if it is possible to achieve a higher agreement if samples are pooled. Furthermore, it would also be useful to investigate the agreement for BRSV and BCV further, as it was not possible in this study.

6.1 Considerations regarding application

The advantage of this high-throughput qPCR system is that multiple samples can be analysed for multiple relevant viruses and bacteria in the same setup, which makes it potentially more affordable, and suitable for screening as well as diagnostics of sick animals. It could be valuable to utilize the PCR system for diagnostic monitoring on herd-level in order to follow the dynamics of pathogens in a herd over time. This would enable the veterinarian and farmer to identify causative agents during disease outbreaks, if a herd baseline had been established prior to disease outbreaks or increased disease occurrence. The screenings might further be used to establish an appropriate vaccination programme for the individual herd.

When evaluating the qPCR Fludigm system as a diagnostic tool for BRD, it is important to contemplate whether the result will affect therapeutic or preventive measures. There is great focus on decreasing antimicrobial usage in livestock productions to prevent the development of antimicrobial resistance. In relation to this, one of the work packages of the Robust Calves project is aimed at lowering antimicrobial usage in the Danish calf production. To achieve this goal, diagnostic testing needs to be used more frequently and precede treatment decisions. Therefore, the authors believe that this qPCR system could become a valuable, and possibly cost-effective tool for preventive and treatment decisions. Many of the pathogens involved in BRD also exist as commensals, and therefore their mere presence may not be indicative of disease. However, if clinically relevant cut-offs were established for all respiratory pathogens, it would be possible to differentiate harmless commensals from disease causing pathogens. This knowledge would help the veterinarian tailor the therapeutic response, and thereby avoid excessive or needless use of antimicrobials.

In this study, it was found that the clinically relevant cut-off Cq $\leq$ 21 could be used for the pathogens *H. somni, M. bovis* and *P. multocida*. However, the system is newly established and due to the lack of investigations regarding clinical cut-offs for all relevant pathogens, as well as for combinations of pathogens and on group-level it is still mainly used on a trial-basis. Furthermore, the found cut-offs are based only on NS samples, and as shown in this thesis, NSs are generally not predictive of what is found in the lower respiratory tract. Cut-offs should also be found for TW samples, as NSs cannot stand alone if a full overview of the involved pathogens is desired.

Though, when faced with bovine respiratory disease in a herd, it is essential to remember that pathogens and the diagnostics for these are one side of a complex issue, as BRD is a product of stressors, which means that management and environmental factors are crucial to handle simultaneously.

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Appendix

Appendix A – Number of animals in participating herds

Table A-1 shows the total number of animals in each of the nine participating veal herds and Table A-2 shows the total number of milking cows in the 27 participating dairy herds. In Figure A-1 the geographical distribution of the 36 participating herds is illustrated.

Table A-1. The total number of animals in the nine participating veal herds are given. The data was extracted from The Central Husbandry Register (CHR) the 25th of January 2020.

Herd number	Number of veal calves
1	833
2	1,137
3	1,364
4	984
5	968
6	1,388
7	1,629
8	512
9	1,014
Total	9,828

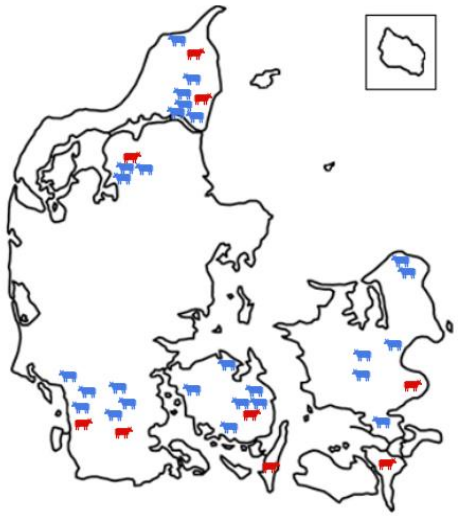


Figure A-2. Geographical distribution of all herds participating in the present study. The red mark each represent one veal herd and the blue mark each represent a supplying dairy herd.

Table A-2. The total number of milking cows in the 27 participating dairy herds are given. The data was extracted from The Central Husbandry Register (CHR) the 25th of January 2020.

Herd number	Number of cows
10	650
11	633
12	420
13	705
14	190
15	147
16	357
17	236
18	203
19	271
20	310
21	242
22	476
23	519
24	242
25	313
26	428
27	568
28	267
29	329
30	193
31	280
32	154
33	114
34	488
35	303
36	446
Total	9484

Appendix B – The Robust Calves project protocol

In the following an excerpt of the protocol for the Robust calves project relevant for this thesis is given.

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.

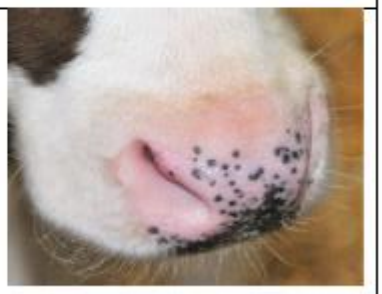
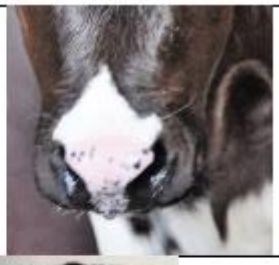
AU, Foulum 31. august 2018

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.

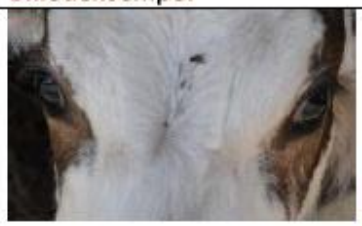
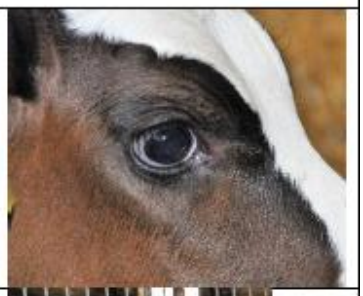
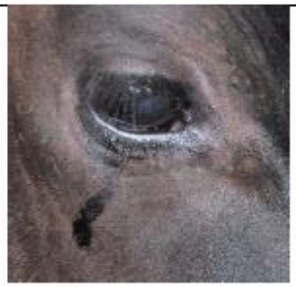
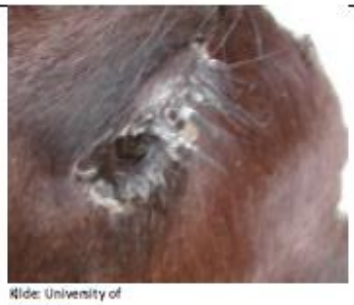
Score	Beskrivelse	Billede eksempel
0	**Normal** Ingen flåd eller pus i næseborene *Normal, no signs of fluid, exudate or pus in the nostrils*	 
1	**Serøst** Klart, vandigt (serøst) flåd i et eller begge næsebor. Ingen pus eller pus-tilblanding. *Clear, serous fluid/exudate in one or both nostrils. No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

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

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*	

23. Temperatur – *Rectal temperature*

Temperaturen måles med rektalt termometer. Angives i celsius med 1 decimal.

Body temperature measured rectally. Numeric value with one digit.

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.

Appendix C – Protocol for the tracheal wash procedure

In the following the protocol for the TW procedure, used in the Robust Calves project is given.

MSD Animal Health

Kvægfagdyrlæge Niels Peter Jensens

Vejledning til trachealskylleprøver på kalve

En kort, praksisorienteret gennemgang af undersøgelsesmetode fra hovedopgave udarbejdet i forbindelse med kvægfagdyrlægekursus.

Til skylningen anvendes et til formålet modificeret hoppeskyllekateter (uden sidehuller), 120 cm (vigtigt), afrundet i enden, enkeltvis steriliseret og pakket (Kruuse, vnr. 230755, ca. 25 kr.), 50/60 ml engangssprøjte, isotonisk NaCl, engangsvaskeklud af skumgummi, sterilt venojectglas plain samt, især i starten, lidt tålmodighed.

Undersøgelse foretaget på stående/liggende, evt.sederet kalv. Brug 0.25 ml xylaxin til ca. 80 kg kalv. Hjælperen holder kalven fikseret i det ene øre og under den modsatte underkæbegren.
Med et dobbelt lag skumgummiklud fatter man om tungen og trækker den, med et fast tag, lige fremad. Pas på at undersiden af tungen ikke læderes på fortænderne i undermunden. Med forsigtighed kan man trække tungen så langt frem at svælget danner en slags "tragt" som letter indføringen af katetret. Det faste træk fremad fastholdes i 20 -30 sekunder til kalven slapper af eller til man kan føle at stemmeridsen står åben.

"Kapitulationen" signaleres ofte med et lille brøl. Det er herefter muligt, efter et par forsøg, at føre kateteret ned i trachea. Det er sjældent muligt at se ned i svælget på kalven, så kateteret føres i stedet tæt ned over tungeroden til larynx. Man får et godt fingerpeg om, at man er i trachea, når kalven giver et lille host fra sig.

Det er af hensyn til minimal læsion af trachea vigtigt, at anvende den afrundede ende af kateteret og især når man anvender den nye type, stivere kateter, at anbringe kalven så trachea er så ret som muligt.

Under den videre indføring af kateteret kan man mærke luften fra eksspirationen samt et andet sikkert tegn på rigtig position, nemlig følelsen af at "hoppe" nedad den ene trachealring efter den anden. Kateteret føres forsigtigt til en anslået position ved bifurkaturen. Det er vigtigt at komme helt frem til ned i lungen for at kunne genopsuge skyllevæsken.

Ved hjælp af sprøjten infunderes, med rolig bevægelse, 50 ml isotonisk NaCl. Det vil herefter sædvanligvis være muligt at genopsuge 30 -35 ml skyllevæske, som derefter overføres til 1 - 2 venojectglas og forsendes (gerne på køl) til laboratorium. Det ene glas sedimenterer/-centrifugeres og bundfaldet anvendes til egen dyrkning. Det er vigtigt at der er min. 15 ml skyllevæske i sprøjten ("residualrum" i kateter) og at der er "snot" i væsken.

Hvis man overvejer anvendelse af metoden, melder der sig en naturlig skepsis overfor sikkerheden m.h.t. eventuel overlast/død blandt undersøgte kalve. Det kan i den forbindelse nævnes at der i forbindelse med projektet er skyllet over 100 kalve uden dødsfald endsige forværring af kliniske symptomer. Andre undersøgelser med samme metode bekræfter dette.

Praktiske spørgsmål: e-mail MSD Animal Health/Katja Hornbæk Mikkelsen (Katja.mikkelsen@merck.com, tlf. 28686321) eller Niels Peter Jensen (npjensen@nordfynsdyreklinik.dk eller tlf. 64 85 11 11 (Nordfyns Dyreklinik))

Appendix D – Cut-off for *M. haemolytica* with case definition ≥ 9

In Table D-1 the results of the logistic regression performed in *the model* by using the interval 14 to 26 and case definition ≥ 9 for *M. haemolytica*. A significant cut-off at Cq value ≤ 22.9 was found.

Table D-1. Results of the *model* for analysis of the optimal qPCR Cq cut-off for *M. haemolytica* using a case definition of ≤ 9 classifying if an observation unit is either sick or not. The estimates describe the log-transformed fixed effects: Cq cut-off and age group. Standard error (SE) and *p*-value *(P*) were calculated for the estimates. Variance (σ^2) and standard deviation (SD) were calculated for the random effect, GroupID. * = p-value < .05, ** = p-value < .05, ** = p-value < .01, ***=p-value < .001

Variable	Case definition	# calves sick/healthy		Estimate	SE	*P*	σ^2	SD
		In total	Positive for MH					
	9	41/823	18/96					
Fixed effects								
Intercept				-5.1116	0.7294	***		
Best Cq cut-off ≤22.9				1.0906	0.3794	**		
Age group 3				0.7107	0.9283	-		
Age group 5				1.6929	0.8297	*		
Age group 7				3.0427	0.7810	***		
Random effect								
Group ID							0.185	0.4299

Appendix E – Scatterplots of qPCR output for NS against TW samples

In Figure E-1 the scatterplots created to illustrate the Cq values of the paired NS and TW samples for each of the nine pathogens are illustrated. Below the scatterplots, Table E-1 and E-2 are shown with summaries of the number of the nine pathogens detected in each of the veal and its supplying dairy herds.

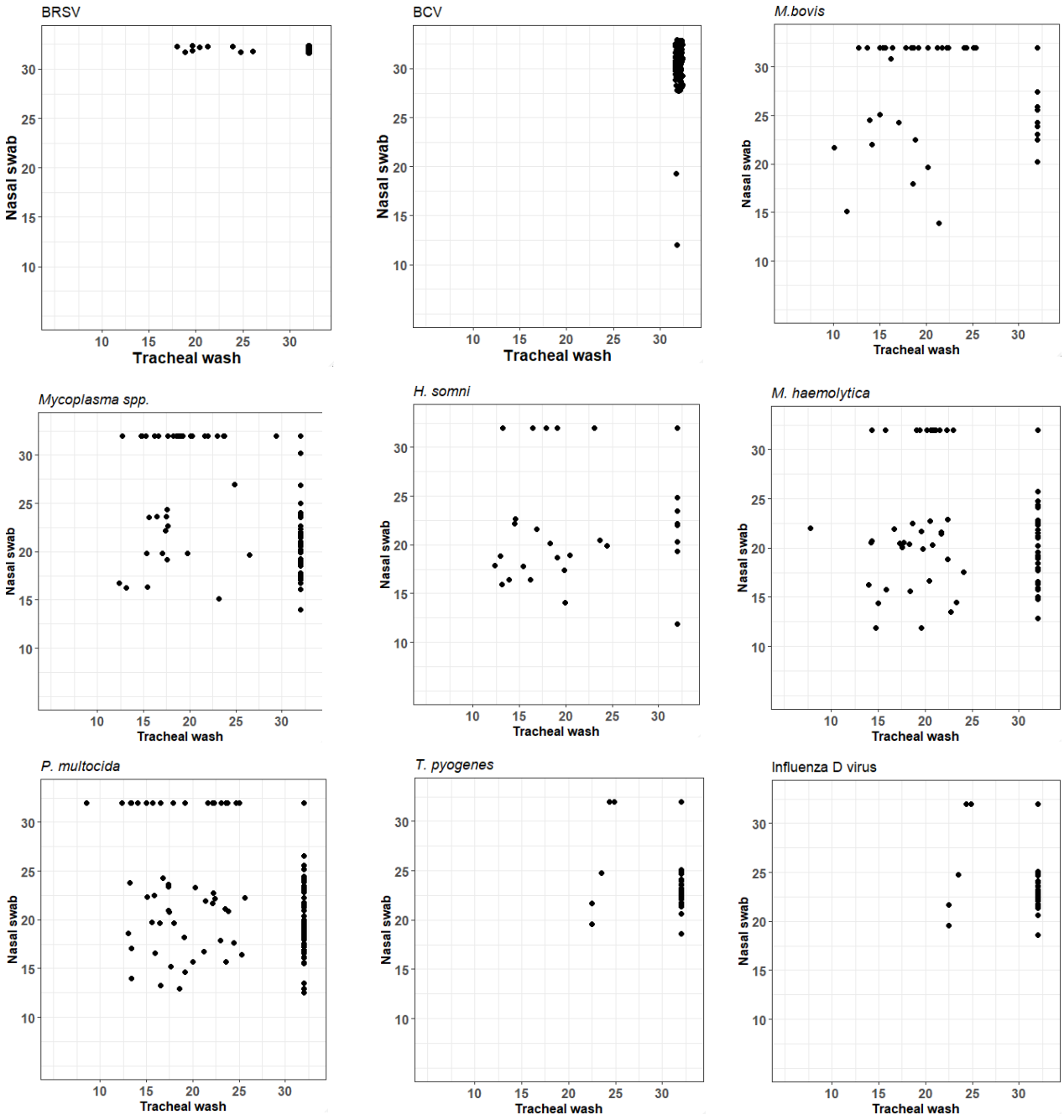


Figure E-1. An overview of the distribution of Cq values from NS and TW paired samples per pathogen tested.

Table E-1. The number of observation units (*n)* included from each of the three veal herds and a summary of the number of each of the nine pathogens detected in NS (N) and TW (T) samples respectively, in each of the herds. BRSV = Bovine respiratory syncytial virus, BCV= Bovine corona virus, MB = *M. bovis*, M spp. = *Mycoplasma* spp., MH = *M. haemolytica*, HS = *H. somni*, IDV= Influenza D virus, PM = *P. multocida*, TP = *T. pyogenes*.

Herd		BRVS		BCV		MB		M spp.		MH		HS		IDV		PM		TP	
number	*n*	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T
1	34	0	2	0	0	6	9	7	10	6	5	1	0	0	0	11	8	0	0
2	37	0	6	0	0	4	9	10	11	25	15	3	3	0	0	11	6	9	2
3	28	0	0	1	0	2	6	11	7	13	14	16	16	13	7	16	16	2	0
Total	99	0	8	1	0	12	24	28	28	44	34	20	19	13	7	38	30	11	2

Table E-2. The number of observation units (*n)* included from each of the nine dairy herds and a summary of the number of each of the nine pathogens detected in NS (N) and TW (T) samples respectively, in each of the herds. BRSV = Bovine respiratory syncytial virus, BCV= Bovine corona virus, MB = *M. bovis*, M spp. = *Mycoplasma* spp., MH = *M. haemolytica*, HS = *H. somni*, IDV= Influenza D virus, PM = *P. multocida*, TP = *T. pyogenes*.

Herd		BRVS		BCV		MB		M spp.		MH		HS		IDV		PM		TP	
number	*n*	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T	N	T
10	12	0	0	0	0	2	5	2	5	0	0	0	0	0	0	3	6	1	0
11	20	0	0	0	0	0	0	1	0	0	0	0	0	0	0	7	2	4	2
12	16	0	0	0	0	0	0	9	1	6	2	0	1	0	0	9	4	0	0
13	18	0	0	0	0	0	1	0	1	2	1	1	0	0	0	7	1	2	0
14	26	0	0	0	0	1	0	7	0	3	1	0	0	0	0	6	2	1	0
15	17	0	0	0	0	0	0	6	1	2	0	1	0	0	0	7	1	0	0
16	13	0	0	1	0	0	0	0	0	0	0	1	1	0	0	2	1	2	0
17	9	0	0	0	0	0	0	2	0	0	0	0	0	0	0	3	1	4	1
18	7	0	0	0	0	0	0	1	0	0	1	0	0	0	0	4	1	0	0
Total	138	0	0	1	0	1	1	28	18	13	5	3	2	0	0	48	19	14	3

Appendix F – Agreement between sampling methods at Cq value ≤ 21

In Table F-1 the calculations for the agreement between the sampling methods NS and TW by a cut-off Cq value of ≤ 21 defining whenever a sample was considered positive (≤) or negative (>) is given.

Table F-1 Agreement between the TW and NS samples for the identification of the nine respiratory pathogens from 237 paired samples, using Cq cut off value ≤ 21, calculated using Cohen's Kappa.

	# Calves with each combination (TW/NS)					
Pathogen	+/+	+/-	-/+	-/-	Kappa (95% CI)	Kappa interpretation
BRSV	0	5	0	232	0 (-1.1^{-10}-1.1^{-10})	-
BCV	0	0	1	236	0 (0–0)	-
M. bovis	3	19	2	213	0.19 (-0.01-0.4)	Slight
Mycoplasma spp.	7	22	30	178	0.09 (-0.06-0.2)	Slight
M. haemolytica	15	12	20	190	0.41 (0.24-0.58)	Moderate
H. somni	11	7	5	214	0.62 (0.42-0.82)	Substantial
IDV	6	1	6	224	0.62 (0.36-0.88)	Substantial
P. multocida	15	17	40	165	0.2 (0.07-0.35)	Slight
T. pyogenes	0	0	3	234	0 (-2.2^{-10}-2.2^{-10})	Slight