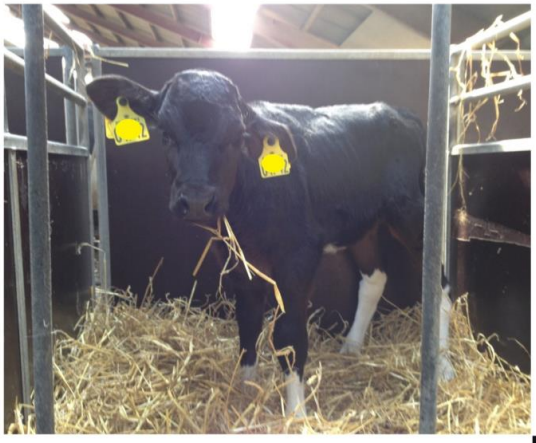
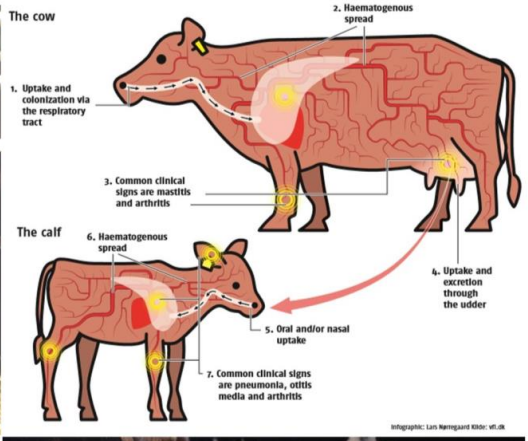
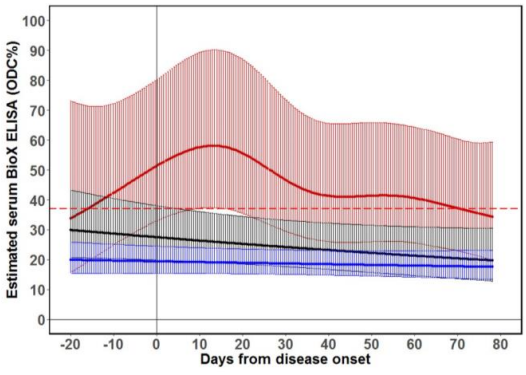
UNIVERSITY OF COPENHAGEN
FACULTY OF HEALTH AND MEDICAL SCIENCES

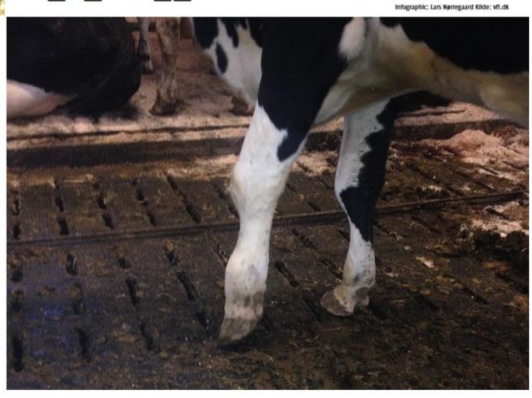
Mycoplasma bovis in dairy cattle

Clinical epidemiology and antibody measurements for decision making









PhD Thesis 2018 · Mette Bisgaard Petersen

DEPARTMENT OF VETERINARY AND ANIMAL SCIENCES
FACULTY OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN
PHD THESIS 2018 · ISBN 978-87-7209-172-3

METTE BISGAARD PETERSEN

Mycoplasma bovis in dairy cattle

Clinical epidemiology and antibody measurements for decision making

SL grafik

Mycoplasma bovis in dairy cattle

Clinical epidemiology and antibody measurements for decision making

PhD Thesis 2018

Mette Bisgaard Petersen

This thesis has been submitted to the Graduate School of Health and Medical Sciences, University of Copenhagen June 4th 2018

Principal supervisor: Professor Liza Rosenbaum Nielsen
Department of Veterinary and Animal Sciences,
Faculty of Health and Medical Sciences, University of Copenhagen,
Denmark

Co-supervisors: Kaspar Krogh, DVM, Cattle Herd Health specialist
Technical Services Manager Livestock, Nordics,
Ceva Animal Health

Associate Professor Matthew James Denwood
Department of Veterinary and Animal Sciences,
Faculty of Health and Medical Sciences, University of Copenhagen,
Denmark

Assessment Committee: Professor Hans Houe (Chairman)
Department of Veterinary and Animal Sciences,
Faculty of Health and Medical Sciences, University of Copenhagen,
Denmark

Professor Päivi Rajala-Schultz
Department of Production Animal Medicine,
University of Helsinki, Finland

Research Scientist Inna Lysnyansky
Department of Avian and Aquatic Diseases,
Kimron Veterinary Institute, Israel

ISBN 978-87-7209-172-3.
Printed by SL grafik, Frederiksberg, Denmark (www.slgrafik.dk)

Table of contents

Preface and Acknowledgements

This Ph.D. project was funded by the Faculty of Health and Medical Sciences, University of Copenhagen and was conceived to supplement an on-going data collection in December 2013. These data were made available from a study funded by the Milk and Cattle Levy Funds in 2013-2015, as a result of increased awareness of *Mycoplasma bovis* since 2011 when a series of severe disease outbreaks in Danish dairy herds happened and this data collection in 39 Danish dairy herds was initiated in 2013. During the PhD-project the Pesticide and Cattle Levy Funds also funded a smaller data collection in an additional four dairy herds. I would like to thank all the funding bodies for making this PhD-project possible and my supervisors for giving me the opportunity of conducting this PhD-project.

The past 4 ½ years have been a fantastic journey and I have enjoyed every part of it. However, without many dedicated and kind people it would have been much more frustrating and less rewarding.

First of all, a big thank you to my supervisors, a fantastic team! **Liza Rosenbaum Nielsen**, your extreme dedication and involvement in the project is infectious and has made me raise the bar to meets this. I have learned so much more from you than I could have ever imagined! **Kaspar Krogh,** thanks for your honest and practical advice, and despite a minor break I really appreciated you help and effort during the end of the project. **Matthew Denwood,** thanks for your patience with my many stupid R related questions, but mostly for raising the level and interpretation of the different statistical analysis.

A big thanks to all the **farmers (and their cows and calves)**, for agreeing to participate in the two data collections. Without your cooperation and willingness to open your farms, observational research like this is not possible to perform. In this context, **Ulla Torpe** and family is thanked for providing food and even my own room at our numerous herd visit trips around Jutland. It has been a pleasure working with you!

A bunch of great Master students all working on different *M. bovis* topics; Lene Jensen, Tobias Volhøj, Bolette Troldborg Rafn, Camilla Eskerod Kristensen, and in particular **Dinah L. Holm** and **Jeanette Pedersen**, who assisted with the data collection, laboratory work and also performed the first steps in the data analysis for Manuscript II.

Several people outside University of Copenhagen have shown interest in and provided help for this project. I would like to thank **Annette Kjær Ersbøll** for hosting my external research stay at National Institute of Public Health (SIF) – University of Southern Denmark and for you valuable contribution to Manuscript IV. Thanks to everyone at SEGES, Cattle for providing data and showing interest in the project and the results. In particular, **Lars Pedersen** for discussions, herd-visits and pushing for the practical application of the findings, even when they were hard to find. **Jørgen Katholm** for good discusions, both about *M. bovis* and PCR, and especially for testing some milk samples. **'*Mycoplasma bovis* project group'** (which unfortunately not really exists any more) for project-meetings and discussions in the beginning of the project. **Nadeeka Wawegama** and **Glenn**

Browning for opening our eyes to the MilA ELISA test, performing the laboratory work and contributions to Manuscript III. **Eurofins Steins Laboratory** and staff, especially **Dorthe Thanning Lavritsen** for storage and handling of samples.

All my great colleagues at G8 and G2 are thanked for providing an inspiring and pleasant working environment. In particular thanks to **Anne Marie Michelsen,** and my PhD-fellows **Karina Bech Gleerup** and **Amanda Brinch Kruse** for sharing frustrations and victories, as well as all the other stuff, and of course **Jeanne Oakman**, who knows the answer to all possible questions, small as large. A special thanks to the 'Julefrokost 2015' organising committee ☺ The **DTU PhD-students** (Camilla, Carolina and Per), I still remember our first Epi-course at Nils' office when we were just started and really did not know what we were going into.

A special thanks to my family. My parents for unconditional support for as long as I can remember. Without you I wouldn't have got this far. The biggest thanks I owe **Mark** and **Theo** for being my safe base and just being who you are.

Frederiksberg, May 2018

Mette Bisgaard Petersen

List of manuscripts and other scientific work

List of manuscripts included in the thesis

I. Mette B. Petersen, Kaspar Krogh and Liza R. Nielsen (2018)
Factors associated with variation in bulk tank milk *Mycoplasma bovis* antibody-ELISA results in dairy herds.
The manuscript has been published in *Journal of Dairy Science*, 2016, 99:3815-3823.

II. Mette. B. Petersen, Jeanette Pedersen, Dinah L. Holm, Matthew Denwood and Liza R. Nielsen (2018)
A longitudinal observational study of the dynamics of *Mycoplasma bovis* antibodies in naturally exposed and diseased dairy cows.
The manuscript has been published in *Journal of Dairy Science,* 2018, doi.org/10.3168/jds.2017-14340

III. Mette. B. Petersen, Nadeeka. K. Wawegama, Matthew Denwood, Philip F. Markham, Glenn F. Browning and Liza R. Nielsen.
***Mycoplasma bovis* antibody dynamics in naturally exposed dairy calves according to two diagnostic tests.**
The manuscript has been submitted to BMC Veterinary Research

IV. Mette B. Petersen, Annette K. Ersbøll, Kaspar Krogh and Liza R. Nielsen
Increased incidence rate of undesired early heifer departure in *Mycoplasma bovis*-antibody positive Danish dairy cattle herds.
The manuscript has been submitted to Epidemiology and Infection

Additional scientific work

Scientific work that is not included in the thesis, but were produced during the PhD-project period. Shown in chronological order

A. Mette B. Petersen, Matthew Denwood and Liza R. Nielsen (2018)
Ny testmetode kan redde danske malkekøer.
(in Danish). https://videnskab.dk/naturvidenskab/ny-testmetode-kan-redde-danske-malkekoeer
Paper for public science communication

B. Mette B. Petersen, Matthew Denwood and Liza R. Nielsen (2018)
Diagnostic opportunities for use of *Mycoplasma bovis* antibody measurements in serum and milk.
Presentation at the Nordic workshop on *Mycoplasma bovis* – research and diagnostics at Technical University of Denmark 2018.
Oral presentation

C. Mette B. Petersen, Matthew Denwood and Liza R. Nielsen (2017)
Diagnostic opportunities for use of *Mycoplasma bovis* antibody measurements in serum and milk.
In: Proceedings of the CPH Cattle Seminar "Up to date with Cattle Research", November 15, 2017. http://cphcattle.ku.dk/dokumenter/seminar-mat/
Abstract and oral presentation

D. Per K. Nielsen, Mette B. Petersen, Liza R. Nielsen, Tariq Halasa and Nils Toft (2015)
Latent class analysis of bulk tank milk PCR and ELISA testing for herd level diagnosis of *Mycoplasma bovis*.
Preventive Veterinary Medicine, No. 121, 2015, p. 338-342.

E. Mette B. Petersen, Kaspar Krogh and Liza R. Nielsen (2015)
***Mycoplasma bovis* – hvad ved vi egentlig?**
(in Danish). Dansk Veterinaertidsskrift 2, pages 14-18.
Scientific paper

F. Mette B. Petersen and Liza R. Nielsen (2015)
Detection of *Mycoplasma bovis* outbreaks by antibody testing of bulk tank milk from dairy herds.
In: Proceedings of the International Mycoplasma Meeting, Progress in human and animal mycoplasmosis, 2015
Abstract and oral presentation

G. Lene Jensen, Mette B. Petersen, Mikkel B. Svendsen, Kaspar Krogh and Liza R. Nielsen* (2015)
A study of actors associated with *Mycoplasma bovis* outbreaks in Danish dairy cattle herds 2010-2014.
In: Proceedings of the International Mycoplasma Meeting, Progress in human and animal mycoplasmosis, 2015
*Abstract and oral presentation**

H. Kaspar Krogh*, Mette B. Petersen and Liza R. Nielsen (2014)
Interpretation of *Mycoplasma bovis* tests in bulk tank and DHI-samples from cattle herds.
In: Proceedings of the World Buiatrics Conference, 2014.
*Abstract and oral presentation**

Summary

Mycoplasma bovis (*M. bovis*) is an opportunistic pathogen that can cause disease and production losses in cattle of all ages. It has primarily been associated with mastitis and arthritis in cows, and respiratory disease, arthritis and otitis media in calves. Over the last two decades, *M. bovis* has gained more attention due to its apparent increasing prevalence, but also due to increasing antibiotic resistance in recovered *M. bovis* isolates. Little attention has been paid to *M. bovis* in Denmark since its first detection in 1981, and during the 1980's and 1990's it was primarily associated with outbreaks of a mastitis syndrome that had not previously been seen in Denmark. However, new types of severe outbreaks of *M. bovis*-associated disease appeared in 2011, characterised by many arthritis cases and swollen legs in addition to the clinical signs that had been more traditionally recognised. A longitudinal field study involving 39 Danish dairy herds was initiated in 2013 as an industry-driven initiative with the aim of clarifying the importance and limiting the impact of *M. bovis* infections at both individual animal and herd level. However, difficulties with the interpretation of the available diagnostic test results led to requests for more research, and this PhD project was conceived to provide interpretation and recommendations based on the on-going data collection activities.

Enzyme-linked immunosorbent assays (ELISA) are diagnostic tests that are relatively inexpensive and easy to perform. In Denmark, ELISAs are routinely used for diagnostic purposes as well as in control and eradication programmes at both animal and herd level. However, there are few studies describing the use of *M. bovis* antibody measurements as a diagnostic tool, most of which describe the test as being useful for group-level rather than individual diagnosis. The published literature mainly reports on experimental studies that do not necessarily reflect the antibody response patterns that would be observed in naturally infected herds. Field studies investigating antibody measurements at animal and herd level would improve the understanding of how to use antibody detecting ELISAs as diagnostic tools, but might also lead towards a better understanding of the infection dynamics of *M. bovis* within affected herds.

The aim of the work presented in this thesis was to improve the understanding and interpretation of antibody measurements in relation to the clinical epidemiology of *M. bovis,* with the end goal being understanding of how to apply this diagnostic test method for control of *M. bovis* infections. Data were available from the longitudinal field study mentioned above in which the 39 Danish dairy herds were visited four times each at approximately 3-month intervals, with blood samples collected from young stock and milk samples from lactating cows (sampling activity #1), as well as a clinical epidemiology study in four Danish dairy herds with new outbreaks of *M. bovis*-associated disease. These four herds were visited five times at approximately 3-week intervals and clinical signs were registered and blood and milk samples were collected from groups of diseased and non-diseased cows and calves (sampling activity #2).

The first study that was carried out based on sampling activity #1 identified herd-level risk factors that were associated with bulk tank milk (BTM) ELISA optical density measurements (ODC%) using the commercially available BioX K302 ELISA kit (BioX). A reasonably strong association was found between BTM ELISA ODC% and the apparent prevalence of antibody-positive lactating cows based

on individual milk sample test results, with the BTM results increasing on average 9 ODC% for every 10% increase in the prevalence of antibody-positive lactating cows. However, it became obvious that clinical signs consistent with *M. bovis* were reported by farmers even in herds during periods with low ODC%-values measured in BTM, and it was decided to pursue explanations for the apparent lack of test accuracy.

The patterns in antibody responses in naturally infected animals with different clinical signs of *M. bovis*-associated disease were analysed by modelling antibodies directed against *M. bovis* in serum and milk from cows and serum from calves from sampling activity #2. To analyse the antibody response relative to the time since clinical signs started, all cows were divided into disease groups based on clinical examinations. The antibody response measured by the BioX ELISA was generally very dynamic, short-lived and dependent upon the observed clinical signs. Even in diseased cows, the mean estimated ELISA ODC% was below the recommended cut-off 60-70 days after clinical signs were observed. The ODC% in serum was not elevated for all the cows with clinical signs of systemic *M. bovis*-associated disease, while the ODC% in milk was mostly elevated in cows with PCR-positive *M. bovis* mastitis. The findings relating to the BioX ELISA results in calves differed from the cows. The clinical signs were not associated with antibody ODC%, but herd and time of expected *M. bovis* exposure were. Calves exposed to *M. bovis* at a young age did not appear to rise in antibody level until the age of 3 months, on average.

An alternative ELISA (known as the MilA ELISA) developed at University of Melbourne was applied to the samples from sampling activity #2 for comparison with the BioX ELISA. The MilA ELISA detected antibodies in calves shortly after birth, but were not associated with clinical signs. This suggests that the MilA ELISA is a sensitive test that can demonstrate exposure in calves older than 3 weeks. It was not possible to analyse the MilA ELISA results from the cows with statistical modelling, which means that the results are less generalizable than for the BioX ELISA. All serum MilA ELISA results were above the recommended cut-off in cows both with and without clinical signs of *M. bovis*-associated disease, whereas for milk, only cows with *M. bovis* mastitis seemed to have high reactions in the MilA ELISA. Although the sensitivity of MilA seems promising, an evaluation of the specificity in herds without *M. bovis*-infections is warranted.

Finally, the knowledge gained from the above-mentioned studies was used to evaluate the association between antibody-positivity to *M. bovis* using the BioX ELISA and the incidence rate of undesired early departures (i.e. slaughter, euthanasia or death) in cohorts of 636 heifers from 36 of the dairy herds included in sampling activity #1. The used Poisson regression model accounted for confounders and clustering effects at both animal and herd level. Increasing seroprevalence in the group of calves 3-12 months old was the primary factor found to increase the risk of undesired early departures of heifers, along with a weak association with the heifer's own animal-level ODC% corrected for age. This indicates that *M. bovis* has a prolonged effect on the health and performance of young stock.

In conclusion, the results of this PhD project have contributed new knowledge that improve our understanding of the antibody responses to *M. bovis* in dairy herds. This allows some updated recommendations to be made regarding the use of ELISAs for diagnosing *M. bovis*-associated disease. The antibody response to *M. bovis* is very dynamic and short-lived, excretion in milk and serum depends on the clinical signs in adult cows, and is primarily found in diseased cows, which

means that using antibody testing for detecting of *M. bovis* infections in dairy herds should be based on group testing rather than individual testing of cattle. It also limits the usefulness of BTM ELISA testing for *M. bovis* as this primarily reflects the presence of *M. bovis* udder infections in the herd. Antibody measurements in young calves are even more challenging due to a lack of or a slowly developing antibody response using the BioX ELISA. The MilA ELISA is apparently sensitive for measuring exposure to *M. bovis,* and can be applied from 3 weeks of age, but the specificity could not be determined with the samples available for the project. Infection with *M. bovis* in young stock seems to be associated with decreased heifer survival, so *M. bovis* infections in dairy herds should be regarded as serious, and control measures should be applied if diseased or antibody-positive animals are found.

Sammendrag

Mycoplasma bovis (*M. bovis*) er et opportunistisk patogen, der kan forårsage sygdom og produktionstab hos kvæg i alle aldre. De typiske kliniske tegn hos køer er yverbetændelse og ledbetændelse, mens kalve ofte får luftvejsinfektioner, ledbetændelse og mellemørebetændelse. I løbet af de seneste to årtier er der kommet mere fokus på *M. bovis* dels på grund af en tilsyneladende stigende prævalens, men også på grund af øget antibiotikaresistens i de isolerede stammer. Siden 1980´erne har der ikke været meget fokus på *M. bovis* i Danmark, som op gennem 1980'erne og 1990'erne mest var associeret med en række udbrud af atypisk yverbetændelse. I 2011 opstod en række alvorlige *M. bovis*-relaterede sygdomsudbrud, som ud over yverbetændelse, også var karakteriseret ved ledbetændelse og hævede ben. Et longitudinelt feltstudium i 39 danske malkekvægsbesætninger blev igangsat i 2013 med det formål at afklare betydningen og begrænse konsekvenserne af *M. bovis* infektioner på individ- og besætningsniveau. Fortolkningen af de tilgængelige diagnostiske tests var dog ikke lige til, hvilket gjorde det nødvendigt at gennemføre mere forskning, og dette ph.d.-projekt blev tilknyttet den igangværende dataindsamling.

'Enzyme-linked immunosorbent assays' (ELISA) er en relativt billig diagnostisk testmetode, der er let at udføre. I Danmark bruges ELISA rutinemæssigt til diagnostik i besætninger, samt i kontrol og bekæmpelses-programmer, både på individ- og besætningsniveau. Brugen af antistofmålinger til diagnostik af *M. bovis* er dog sparsomt beskrevet i litteraturen. Oftest beskrives testmetoden som ikke anvendelig til individuel diagnostik, men brugbar til diagnostik på gruppeniveau. Dog er de fleste studier lavet på eksperimentelt inficerede dyr, og det observerede antistofrespons er ikke nødvendigvis sammenligneligt med det, man ville observere i naturligt inficerede besætninger. Det var forventningen, at undersøgelser af antistofresponset både på individ- og gruppeniveau i naturligt inficerede besætninger kunne forbedre fortolkningen af ELISA-resultater til brug ved diagnostik og kan medvirke til at øge forståelsen af smittedynamikken i smittede besætninger.

Formålet med dette ph.d.-projekt, var at forbedre forståelsen af, hvordan antistofmålinger kan bruges til at fortolke den kliniske epidemiologi med henblik på at anvende ELISA som diagnostisk metode som led i bekæmpelsen af *M. bovis* infektioner i kvægbesætninger. Udover det før nævnte longitudinelle studium, hvor 39 danske malkekvægsbesætninger blev besøgt 4 gange med ca. 3 måneders mellemrum, og fik udtaget blodprøver fra ungdyr og mælkeprøver fra lakterende køer (Dataindsamling #1), blev data fra et klinisk epidemiologisk studium i fire udbrudsbesætninger tilvejebragt i løbet af ph.d. projektperioden. Disse fire malkekvægsbesætninger havde alle akut udbrud af *M. bovis*-relateret sygdom og blev besøgt fem gange med ca. 3 ugers mellemrum, hvor kliniske tegn blev registreret på dyreniveau, samt blod- og mælkeprøver fra grupper af syge og ikke-syge dyr blev udtaget (Dataindsamling #2).

Den første undersøgelse baseret på Dataindsamling #1 undersøgte hvilke besætningsfaktorer, der var associeret med tankmælks ELISAs optiske densitetsmålinger (ODC%) målt med BioX K302 ELISA testkit (BioX). Der blev fundet en fornuftig sammenhæng mellem tankmælks ELISA ODC% og prævalensen af antistofpositive køer i mælk. Tankmælksværdien steg med 9 ODC% for hver 10 % stigning i prævalensen af antistofpositive lakterende køer. Det viste sig dog, at landmændene fortalte, at de havde oplevet kliniske tegn forenelige med *M. bovis* i deres malkekvægsbesætninger i

perioder, hvor tankmælkens antistofværdier lå lavt. Det blev derfor besluttet at forsøge at undersøge denne tilsyneladende mangel på diagnostisk sikkerhed nærmere.

Dette blev gjort ved at undersøge *M. bovis*-antistofdynamikken i serum og mælk hos køer og serum fra kalve fra Dataindsamling #2. Ved at opdele køerne i grupper efter deres kliniske tegn, kunne antistofresponset relateres til tiden siden de kliniske tegn startede. Antistofresponset målt med BioX ELISA'en var meget fluktuerende og kortvarigt, og afhang af kategorien af kliniske tegn hos køerne. Selv hos køer med kliniske tegn på sygdom var den estimerede gennemsnitlige ODC% under den anbefalede grænseværdi allerede 60-70 dage efter de kliniske tegn startede. I serum var ODC% kun høj hos køer med tegn på systemisk spredning af *M. bovis*, mens ODC% i mælk primært var høj hos køer med yverbetændelse forårsaget af *M. bovis*. BioX ELISA'en opførte sig noget anderledes hos kalve. Her var de kliniske tegn ikke associeret med antistofresponset, men det var besætning og tidspunkt for forventet eksponering for *M. bovis* til gengæld. Kalve der blev eksponeret mens de var meget unge, steg ikke i antistof niveau før de i gennemsnit var omkring 3 måneder gamle, selvom de sandsynligvis blev eksponeret før den alder.

Prøverne fra Dataindsamling #2 blev også testet med en ELISA (kaldet MilA ELISA) udviklet på University of Melbourne, for sammenligning med BioX ELISA' en. Hos kalve kunne MilA ELISA'en måle antistoffer kort tid efter fødslen, men ligesom for BioX ELISA'en var der ikke forskel på om kalven havde kliniske tegn eller ej. Dette indikerer, at MilA ELISAen kan være en sensitiv test, der kan påvise eksponering for *M. bovis* blandt kalve > 3 uger. Resultaterne fra MilA ELISA'en hos køerne kunne ikke analyseres med en statistisk model, så resultaterne er mindre generaliserbare end resultaterne fra BioX ELISA'en. Alle antistof målinger i serum hos køer var over den anbefalede grænseværdi, både hos køer med og uden kliniske tegn på sygdom, mens det i mælk primært var køer med *M. bovis* yverbetændelse, der lå højt i antistofniveau. På trods af at MilA ELISA'en er en lovende følsom test, så mangler der en evaluering af specificiteten i besætninger uden *M. bovis* infektion.

Til sidst blev resultaterne af de ovenfor nævnte studier brugt til at undersøge sammenhængen mellem antistofpositivitet (ved brug af BioX ELISA'en) og incidensraten for tidlig afgang fra besætningen (dvs. til slagtning, aflivning eller død) i en kohorte på 636 kvier fra 36 af malkekvægsbesætningerne, der indgik i Dataindsamling #1. Der blev taget højde for konfunderende variable og cluster-effekter både på individ- og besætningsniveau. Af de undersøgte faktorer havde seroprævalensen blandt gruppen af ungdyr på 3-12 måneder størst betydning for tidlig udsætning, mens den individuelle ODC%, der var afhængig af alder, var mindre betydningsfuld. Dette indikerer, at *M. bovis* har en langvarig effekt på kviernes sundhed og præstation.

Dette ph.d.-projekt har bidraget med ny viden til at øge forståelse af *M. bovis* antistofresponset og har medført opdaterede retningslinjer for brug af ELISA ved diagnostik af *M. bovis*-relateret sygdom. Antistofresponset er meget fluktuerende og kortvarigt, og udskillelsen i serum og mælk afhænger af, hvilke kliniske tegn koen udviser og findes primært hos syge køer, hvilket medfører at antistofmålinger til påvisning af *M. bovis*-infektioner ved sygdom i malkekvægsbesætninger bør baseres på gruppediagnostik snarere end enkeltdyrsdiagnostik. Det betyder også, at anvendelsen af ELISA ODC% i tankmælk er af begrænset diagnostisk værdi, da det kun afspejler tilstedeværelsen af *M. bovis* yver-infektioner i besætningen. Antistofmålinger med BioX ELISA'en i kalve er udfordrende på grund af manglende antistofrespons særligt i helt unge kalve. MilA ELISA'en er potentielt en

følsom alternativ test, der reagerer på eksponering for *M. bovis* i dyr > 3 uger, men specificiteten for denne test er endnu ikke undersøgt. Infektion med *M. bovis* kan reducere overlevelsen hos kvier, og derfor skal *M. bovis* infektioner i malkekvægsbesætninger håndteres bedst muligt, og kontrolforanstaltninger bør iværksættes, hvis der findes syge eller antistofpositive dyr i besætningen.

List of abbreviations and terms

BC	bacterial culture
BioX	ELISA 'BIO K 302' from Bio X Diagnostics
BRD	bovine respiratory disease
BTM	bulk tank milk
BVDV	bovine viral diarrhoea virus
Ct	cycle threshold
DCD	Danish Cattle Database
ELISA	enzyme-linked immunosorbent assay
GAMM	generalised additive mixed model
Ig	immunoglobulin
M. bovis	*Mycoplasma bovis*
MilA	in-house ELISA developed at the University of Melbourne
ODC	optical density coefficient
PCR	polymerase chain reaction
PCI	posterior credibility interval
SampAct#1	sampling activity #1
SampAct#2	sampling activity #2
SEGES	name of the Danish farmer-owned knowledge, consultancy and technology centre that provides services to all Danish farmers[1]
UED	undesired early departure
Vsp	variable surface protein

[1] https://www.seges.dk/en

1 Introduction

1.1 Background and motivation

A series of severe outbreaks of *Mycoplasma bovis (M. bovis)*-associated disease emerged in 2011 in Danish cattle herds. Prior to this, animals with clinical signs of *M. bovis* were only sporadically seen in Denmark, and mostly before 2002. Visits to ten herds with suspected *M. bovis* outbreaks in 2011 and a repeated serological testing in a pilot project in 3 case and 3 control herds in 2012 were inconclusive regarding causal factors and underlying mechanisms for the observed varying clinical patterns (Aalbæk et al., 2012).

Since the first isolation in the USA in 1961, *M. bovis* has been recognised as a pathogen primarily responsible for mastitis (Fox, 2012). It has since spread to many countries and is now endemic in Europe (Nicholas and Ayling, 2003). At the same time, awareness of *M. bovis* has increased, which may lead to an apparent increase in reporting that may not reflect a true underlying increased prevalence. *M. bovis* was first isolated in Denmark in 1981 from lung tissue from cattle (Friis and Krogh, 1983). A few years later, 80-100 animals from 15 herds experienced a previously unknown mastitis syndrome and *M. bovis* was found to be the causative agent (Friis, 1984). During the 1990s, *M. bovis* was not considered a major pathogen, and was only found in 1 of 51 lung samples from calves with pneumonia submitted to the Danish Veterinary Laboratory for diagnostic purposes in 1993-94 (Tegtmeier et al., 1999). By 1997-99, the occurrence had apparently increased, as 12 out of 50 lung samples from calves with pneumonia were positive for *M. bovis* (Kusiluka et al., 2000). These samples were also submitted the Danish Veterinary Laboratory, but no further reports about either outbreaks or general disease status were published.

The *M. bovis* situation in Denmark dramatically changed in 2011 when cattle herds began to experience severe disease outbreaks (Worsøe, 2011). In addition to the known clinical manifestations (such as mastitis in cows and pneumonia and otitis media in calves), these outbreaks were characterised by arthritis as well as more diffusely swollen legs in both adults and young stock. Some herds were severely affected in all age groups, leading to loss of animal welfare, frustrations with lack of knowledge about how to manage infected herds and large insurance claims from farmers who had to cull many sick animals. Knowledge of many aspects of *M. bovis* was lacking and farmers, veterinarians and cattle consultants were requesting more information about diagnostics, prevention, treatment and control options.

The extent of *M. bovis* infections was not known, and difficulties in interpreting the diagnostic tests led to requests for more research. A longitudinal field study involving a relatively large collection of data from 39 Danish dairy herds was initiated in 2013 by the Danish cattle industry in collaboration with two universities. The aim of the project was to clarify the importance of *M. bovis* infections in joints, udders and lungs, and limit the impact of the infection at individual animal and herd level. If possible, a control programme for *M. bovis* would be developed to limit the further spread of infection. This PhD project was later connected to the field project that provided access to this data collection allowing for epidemiological analyses to improve the interpretation of diagnostic test results in the hope of supporting the development of a control programme.

In Denmark, other control and eradication programmes for cattle diseases rely on antibody measurements in serum and/or milk, e.g. the bovine viral diarrhoea virus (BVDV) and *Salmonella* Dublin eradication programmes (Houe et al., 2006; Warnick et al., 2006). ELISAs are easy to perform, provide additional information for some infections and (at least in Denmark) are relatively inexpensive per test compared to e.g. polymerase chain reaction (PCR) or bacterial culture (BC). The general convenience of ELISA tests warranted investigations into the usefulness of available *M. bovis* ELISAs as a diagnostic tool. However, antibody measurements used as a diagnostic tool for *M. bovis* are sparsely described in the published literature. They are mostly described as not being useful for individual diagnosis, but useful for group-level diagnostics (Maunsell and Donovan, 2009). However, this statement is based on little published literature, which mainly reports on experimental studies and does not necessarily reflect the situation in naturally infected farms.

National bulk tank milk (BTM) screenings involve testing all Danish dairy herds four times a year for the surveillance of BVDV and *Salmonella* Dublin. In 2013 and 2014, four national screenings for antibodies against *M. bovis* in BTM from all Danish dairy herds were also performed. However, it became apparent that the interpretation was unclear and there were no field studies to clarify whether the recommended individual animal level cut-off of the used ELISA was useful for interpretation of the BTM-result.

In order to develop guidelines for the use of diagnostic tests in outbreak situations or for a control programme relevant to Danish farmers, commercially available tests needed to be investigated. Increased knowledge about the characteristics of the measured antibody responses to *M. bovis* infection contracted under natural conditions and an interpretation of ELISA test results in individual animals with different disease syndromes as well as in BTM would improve our understanding of the limitations and merits of the ELISA as a diagnostic tool. Furthermore, antibody responses combined with other diagnostic test results and clinical investigations might lead to a better understanding of the pathogenesis of the disease and the infection dynamics within affected herds.

1.2 Aim and objectives

The aim of the work presented in this thesis was to improve our understanding of how to interpret antibody measurements in relation to the clinical epidemiology of *M. bovis* and as a diagnostic test method of relevance for the control of *M. bovis* infection in the Danish cattle population. Under the hypothesis that cattle will respond to *M. bovis* infections by raising an antibody response, the following specific objectives were pursued:

1. To improve the interpretation of herd-level diagnostics by investigating the correlation between bulk tank milk antibody test results and the within-herd prevalence of antibody-positive and clinically ill animals
2. To investigate the dynamics of antibodies against *M. bovis* in serum and milk from dairy cows with different clinical signs of *M. bovis*-associated disease
3. To investigate the dynamics of antibodies against *M. bovis* in serum from dairy calves with different clinical signs of *M. bovis*-associated disease

4. To compare the dynamics of antibody responses in serum and milk as measured by two different ELISAs
5. To determine the association between antibody test-positivity to *M. bovis* and heifer survival in dairy herds

1.3 Outline of the thesis

The rest of this thesis contains the following chapters:

Chapter 2 contains a literature review that presents current knowledge about *M. bovis*, with an emphasis on diagnostic methods – in particular antibody measurements and knowledge important for the interpretation of these.

Chapter 3 gives an overview of the sampling activities and other information sources that provided the data for analysis in this thesis. An overview of the statistical methods used is also presented.

Chapter 4 presents the main findings.

Chapters 5 and 6 contain a general discussion, conclusions and perspectives of the presented work.

Chapter 7 contains the references.

Chapter 8 contains the following four manuscripts:

Manuscript I: Factors associated with variation in bulk tank milk *Mycoplasma bovis* antibody-ELISA responses in dairy herds.

Manuscript II: A longitudinal observational study of the dynamics of *Mycoplasma bovis* antibodies in naturally exposed and diseased dairy cows.

Manuscript III: *Mycoplasma bovis* antibody dynamics in naturally exposed dairy calves according to two diagnostic tests.

Manuscript IV: Increased incidence rate of undesired early heifer departure in *Mycoplasma bovis*-antibody positive Danish dairy cattle herds.

Chapter 9 includes the appendices, with the questionnaire used for the first field sampling activity (SampAct#1) and the clinical protocol used for the second field sampling activity (SampAct#2).

2 Literature review

2.1 *Mycoplasma bovis* – an overview

The purpose of this literature review is to provide an overview of the pathogenesis and relevant diagnostic methods of *M. bovis*-associated disease of relevance for diagnosis and control, in particular antibody measurements and knowledge important for the interpretation of these.

2.1.1 Aetiology and pathogenesis

Mycoplasmas are microorganisms in the class *Mollicutes*, and are the smallest prokaryotic cells capable of self-replicating. They do not possess a cell wall and are therefore innately resistant to antibiotics that interfere with the synthesis of the bacterial wall, such as penicillin (Nicholas and Ayling, 2003). *Mycoplasma bovis* (*M. bovis*) was formerly known as *Mycoplasma agalactiae* subsp. *bovis,* but the name was changed in 1976 when it was concluded that *M. bovis* and *Mycoplasma agalactiae* should be regarded as two different species (Askaa and Ernoe, 1976).

Mycoplasmas inhabit mucosal surfaces, and their relationship with the host varies between commensal, primary and secondary pathogen (Maunsell and Donovan, 2009). Due to the small genome of *M. bovis*, the bacterium is dependent on the host to provide essential substances such as amino acids, lipoproteins and nucleotides (Burki et al., 2015). Adhesion to the host's epithelium cells is therefore very important for successful invasion of the host, and the membrane proteins are essential for colonising and survival within the host (Adamu et al., 2013). Modification of adhesion properties could potentially alter the mycoplasma from a 'local' to a 'systemic' stage capable of producing different clinical disease manifestations. Some mycoplasmas can invade non-phagocytic host cells, and thereby have the opportunity to resist host defences and selective antibiotic treatment, and establish chronic infections (Rosengarten et al., 2000). The interaction between *M. bovis* and the host is complex, and despite much attention being paid, many mechanisms are still to be determined. The variable surface protein antigens (Vsp) are probably the most well-characterised immune modulatory effects of *M. bovis.* The Vsp are highly variable and can maintain diversity in a *M. bovis* population within the host. This mechanism challenges the host's recognition and elimination of the bacterium, and allows *M. bovis* to evade the immune system, contributing to the chronic nature of *M. bovis* infections (Buchenau et al., 2010). Some Vsp have been shown to interfere with adhesion to the host cell, while others are involved in biofilm production. However, the disease severity, site of infection and bacterial genotype have not been associated with Vsp (Maunsell et al., 2009). The precise role of the Vsp therefore remains to be elucidated. Overall, *M. bovis* can interfere with the immune system in different ways in order to enhance survival within the host.

Uptake and spread of the bacteria in the host

The uptake of *M. bovis* occurs through the respiratory tract, oral ingestion or ascending via the teat end canal into the udder. Figure 2.1 gives an overview of the uptake and spread of *M. bovis* in calves and cows.

M. bovis is considered to be a contagious mastitis pathogen that spreads from udder to udder e.g. in the milking parlour. Many experimental studies have produced clinical signs of mastitis through the inoculation of *M. bovis* in the udder (Bennett and Jasper, 1978a; Biddle et al., 2003; Byrne et al., 2005) and after intra-mammary inoculation, *M. bovis* was isolated from different areas such as various lymph nodes, uterus, synovial fluid etc., which suggests some systemic involvement (Jain et al., 1967). Dissemination from one quarter to another one or several quarters is a typical clinical sign in *M. bovis* mastitis (Biddle et al., 2003) and possibly occurs via haematogenous or lymphogenic routes. However, it has not been determined whether haematogenous spread from the udder to other body sites is important in the epidemiology of *M. bovis* infections.

Uptake of the bacterium through the oropharynx can spread via the Eustachian tubes to the middle ear, with development of otitis media possible. Oral uptake seems to predispose for colonisation of the tonsils, which contributes to the development of otitis media. This was observed in comparison to transtracheal inoculation, which caused lower respiratory infection, but not upper respiratory tract infection (Maunsell et al., 2012). A recent study showed the development of typical lung lesions after exposure to an aerosolised culture of *M. bovis*, suggesting that nebulisation can be used to produce *M. bovis* respiratory infections (Kanci et al., 2017). Dissemination to the joints occurs via the haematogenous route, and both calves and cows can develop arthritis after intravenous administration of *M. bovis* (Chima et al., 1981; Stalheim and Page, 1975). However, it is not known whether the respiratory or oral infection route predisposes to arthritis development, or if arthritis can also develop after an infection ascending through the udder.

Excretion of bacteria from the host

Mycoplasmas typically inhabit mucosal surfaces, and the respiratory tract and the udder seem to be the most important sites for colonisation and shedding of *M. bovis* (Maunsell et al., 2011).

In experimental studies, *M. bovis* was shed in milk 3-4 days after inoculation in the inoculated quarters, and all four quarters shed *M. bovis* 17-19 days after inoculation into one quarter of the udder (Bennett and Jasper, 1978a). The duration of shedding *M. bovis* in milk after mastitis is not clear. Shedding of the bacteria in milk has been reported to last for up to 12 months after naturally occurring mastitis (Jasper et al., 1966; Ruhnke et al., 1976). However, other authors were not able to detect *M. bovis* in repeated milk samples from cows within 75 days (+/- 20) after the cows had had clinical mastitis and had been tested positive using PCR on milk samples (Aebi et al., 2015). The duration of shedding might be influenced by unknown host factors and may differ between naturally occurring and experimentally induced infections. Cows with mycoplasma mastitis tend to have an inconsistent daily shedding pattern, where the organism is not isolated on some days, and is isolated in high numbers on others (Biddle et al., 2003). This indicates that it might be beneficial to use composite milk samples or to test the cow repeatedly in order to increase the likelihood of mycoplasma detection at individual animal level.

M. bovis can be detected in the nasal cavity and respiratory tract of cattle of all ages, but the duration and causes of continued shedding have not been elucidated. Both clinically affected and asymptomatic carrier animals can harbour and shed the bacteria from the nasal cavity (Bennett and Jasper, 1977; Punyapornwithaya et al., 2010; Soehnlen et al., 2012).

Although *M. bovis* can be detected from other areas of the body such as the conjunctiva and the vulvovaginal tract (Fox et al., 2008; Punyapornwithaya et al., 2010), the duration and shedding patterns from these anatomical sites have not been elucidated. Information about possible shedding routes in faeces and urine is lacking. Bennett and Jasper (1978a) state that, 'occasionally, isolations were made from… bladder urine', but did not elaborate further.

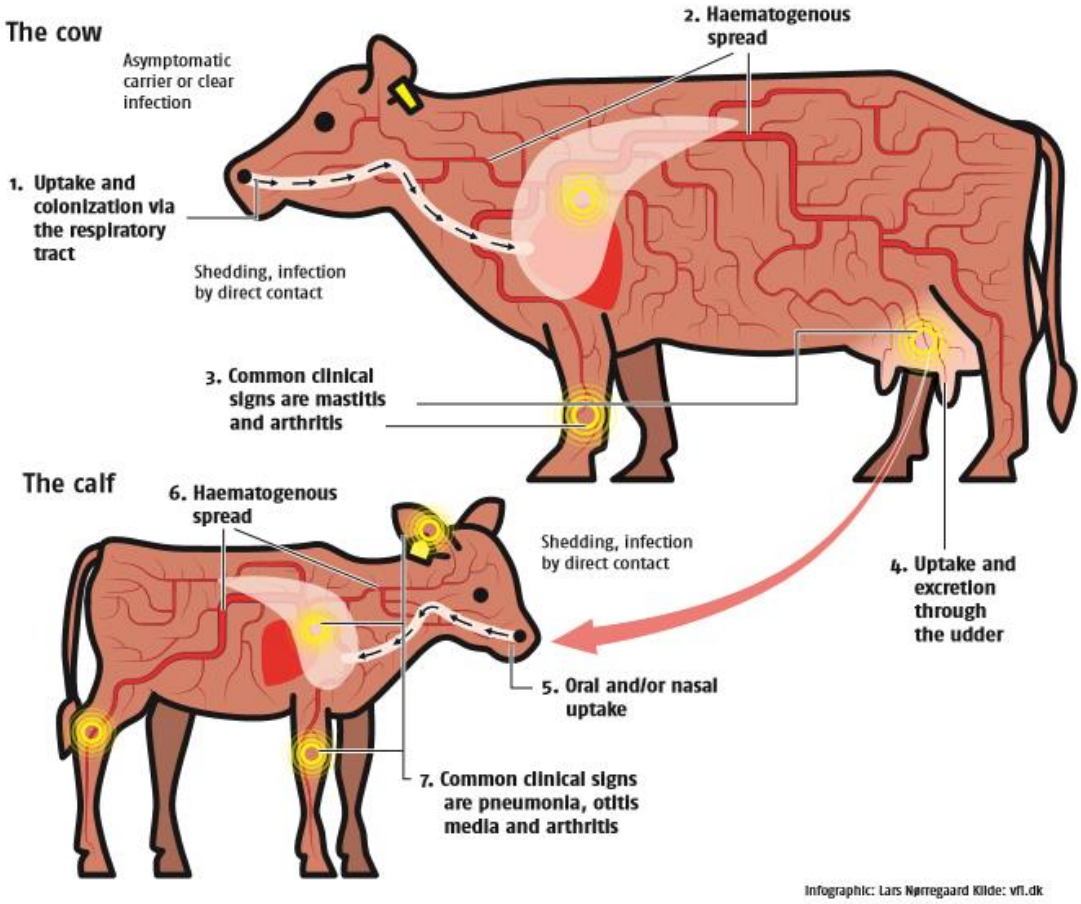


Figure 2.1: Overview of the uptake, transmission and clinical signs of *Mycoplasma bovis* in calves and cows. Clinically ill or asymptomatic carriers may clear the infection or continue to shed. (Petersen et al. Mycoplasma bovis – hvad ved vi egentlig. Dansk Veterinærtidskrift. 2, 2015, pages 14-18, with permission)

Clinical signs

Cows

In adult cattle, *M. bovis* primarily manifests as mastitis, arthritis and pneumonia (Maunsell et al., 2011; Pfutzner and Sachse, 1996).

M. bovis mastitis can be clinical or subclinical. The clinical signs are not specific, but a lack of response to treatment, with more than one quarter affected, sometimes all four, is typical (Biddle et al., 2003; Gonzalez and Wilson, 2003; Pfutzner and Sachse, 1996). Cows in all lactation stages, heifers and dry cows can all be affected (Bicknell et al., 1978; Fox et al., 2008). After experimental inoculation with 70 cell forming units/ml of *M. bovis* into the udder, the first clinical changes (including watery milk) were observed 5-6 days after inoculation, all four quarters showed clinical signs 19-21 days after inoculation, and 11 out of 16 cows still had clinical signs 56 days after inoculation (Bennett and Jasper, 1978a).

In the past, *M. bovis* was primarily associated with mastitis in adult cows, but more reports of joints and surrounding tissues being affected have been published over the last decade. *M. bovis* has been associated with arthritis (Gharagozlou et al., 2004; Henderson and Ball, 1999; Holzhauer and Engelen, 2016; Houlihan et al., 2007; Stalheim and Page, 1975; Szacawa et al., 2015), diffuse subcutaneous oedema and lameness (Wilson et al., 2007). The primarily affected joints seem to be the carpal and fetlock joints in the front legs (Henderson and Ball, 1999; Holzhauer and Engelen, 2016; Wilson et al., 2007). The reason for this apparent change in the clinical picture and predilection for joints in the front legs is not clear.

M. bovis has also been associated with keratoconjunctivitis (Alberti et al., 2006; Kirby and Nicholas, 1996), postsurgical seromas in beef cattle (Gille et al., 2016) and genital disorders (Pfutzner and Sachse, 1996). However, these associations are rarely reported, and their extent and importance are not clear.

Calves

In young calves, *M. bovis* is commonly associated with pneumonia, arthritis, otitis media or any combination of these (Maunsell and Donovan, 2009; Nicholas and Ayling, 2003).

M. bovis is part of the complex of bacteria and viruses that cause bovine respiratory disease (BRD) in calves, but can also be the primary pathogen in pneumonia (Thomas et al., 1986). Pneumonia and otitis media often co-occur, probably because bacteria from the oropharynx proliferate though the Eustachian tubes to the middle ear (Francoz et al., 2004; Lamm et al., 2004; Maunsell et al., 2012). Arthritis can be the sole clinical sign of *M. bovis* infection in calves, but often follows pneumonia and/or otitis media. The carpus and the stifle are often affected (Mahmood et al., 2017).

M. bovis has also been isolated from calves with keratoconjunctivitis (Levisohn et al., 2004; Schnee et al., 2015), decubital abscesses (Kinde et al., 1993) and from brain tissue in calves with clinical signs associated with *M. bovis* (Ayling et al., 2005; Maeda et al., 2003). As with cows, however, these clinical signs are rarely reported and the extent and importance are not clear.

There are no pathognomonic clinical signs for cows or calves, and this combined with other factors described below makes the recognition and diagnosis of *M. bovis* infection challenging.

Interaction with other pathogens

The relationship between mycoplasmas and the host varies from commensal to opportunistic or primary pathogens (Maunsell and Donovan, 2009). *M. bovis* has been isolated as the only pathogen from pneumonic lung lesions, but has also been isolated from healthy lungs and pneumonic lung lesions associated with other bacterial or viral causes. *M. bovis* can be isolated from arthritic as well as non-arthritic joints, suggesting that isolation does not always imply a causal relationship (Gagea et al., 2006). In natural respiratory infections, *M. bovis* is often detected with other pathogens, suggesting a degree of synergy. *M. bovis* is often found in combination with the common respiratory pathogens *Pasteurella multicida, Histophilus somni, Mannheimia haemolytica,* bovine respiratory syncytial virus, bovine herpes virus 1 or parainfluenza virus type 3. In respiratory infections, *M. bovis* can predispose to more severe symptoms in calves that are subsequently infected with *Pasteurella multocida* (Gourlay and Houghton, 1985), and can also cause lesions in tissue that has previously been damaged by *Pasteurella multocida*, *Histophilus somni* and *Mannheimia haemolytica* (Gagea et al., 2006). These interactions are thought to be responsible for the more severe and chronic lung lesions seen in older calves with BRD compared to younger calves affected only by *M. bovis* (Burki et al., 2015). These unclear causalities between *M. bovis* being present without causing disease and being either a primary or a secondary pathogen might also contribute to the difficulties faced in diagnosing *M. bovis*, as well as the unrewarding effect of treatment of *M. bovis*-associated disease, as described in Section 2.1.3.

2.1.2 Immune response

Basic concepts of the immune system

The immune system is complex and it is beyond the scope of this thesis to review all aspects of it, but the following sections contain a brief description of some basic concepts relevant to understanding the development of humoral immunity related to the objectives of this thesis.

The immune system consists of two primary components: the innate (also called non-specific) immune system and the acquired (also called specific) immune system. The two systems can work independently, but usually work together to combat invading bacteria and viruses. The innate immune system is available all the time or induced rapidly, but has no immunogenic memory. The immunity that develops based on antibodies is called the humoral immunity and is part of the acquired immune system. In short, when the immune system is presented with a foreign antigen (e.g. a bacterium), B-cells proliferate, differentiate to plasma cells and produce antibodies specific to the antigen presented. Antibodies are also called immunoglobulins (Ig) and are divided into five subclasses: IgA, IgG, IgM, IgD and IgE. IgG are the most abundant Ig class found in serum and are a major component in passive maternal antibody transfer. IgA are primarily found in different secretions such as milk or saliva. After a bacterium or virus has entered the body, the production of specific measurable antibodies can be expected in serum and other secretions.

The development and maturation of the immune system takes time, and the immune response in young animals can be different from that in adult animals. During pregnancy, the immune system of the calf develops and when the calf is born, it is capable of responding to various antigens, though not as many as when it is fully mature. The immune response in the new-born calf is usually slower, with low concentrations of immunoglobulins being produced (Barrington and Parish, 2001).

Protection of the young calf occurs primarily through the transfer of maternal antibodies from the dam through ingestion of colostrum, and maternal antibodies are measurable in serum after uptake.

Antibody response to Mycoplasma bovis

The antibody response against *M. bovis* in serum and milk, as well as the association between serostatus and disease are described in this section.

Antibodies such as IgG directed against *M. bovis* can be detected in serum 1-2 weeks following inoculation through the respiratory route of young calves (Howard et al., 1986; Kanci et al., 2017; Nicholas et al., 2002; Zhang et al., 2014), and 1-2 weeks after intramammary inoculation with *M. bovis* (Boothby et al., 1987; Byrne et al., 2005). In a structured literature review, the authors were able to find just four studies on the time to seroconversion. They reported a median time to seroconversion of 21 days and a median time to peak IgG levels in serum of 28 days for *M. bovis*-associated respiratory disease (Grissett et al., 2015).

Little is known about the duration of high levels of antibodies against *M. bovis* in serum post infection. Vaccinated calves had high ELISA titres (155% of the positive control) 6 months after vaccination without any challenge (Nicholas et al., 2002), but these vaccinated calves did not necessarily mimic the situation for calves infected under natural conditions. The challenged calves were only followed for 21 days, but the antibody titres remained high during this period. In another study, serum antibody levels remained elevated from the baseline for 18 weeks (Boothby et al., 1987), and IgG in both milk and serum remained above the positive cut-off for at least 45 days after intramammary inoculation (Byrne et al., 2005). Nicholas and Ayling (2003) stated that, "serological detection of antibodies by ELISA against *M. bovis* remains high for many months", but there was no clarification as to how many months or what was considered high.

Howard and Gourlay (1983) compared the immune response of calves of varying ages to *M. dispar* and *M. bovis* following subcutaneous injection of killed vaccines. They found that the youngest calves, injected at around 16 days of age, reached a lower antibody maximum and it that took a longer time to reach the maximum than for older calves injected at around 150 days of age (Howard and Gourlay, 1983). This was particularly evident for *M. dispar*, but was also found to be the case for *M. bovis*, suggesting that the immune system might have difficulty producing antibodies against mycoplasmas in young calves. This is in agreement with Virtala et al. (2000), who found that calves less than 3 months old often failed to seroconvert to pathogens commonly associated with BRD, including mycoplasmas.

Cows shed large amounts of antibodies in milk after both experimental (Bennett and Jasper, 1978b; Boothby et al., 1987) and natural *M. bovis* infection (Byrne et al., 2000). Antibodies against *M. bovis* can be detected in milk 1-2 weeks after intramammary inoculation (Boothby et al., 1987; Byrne et al., 2005). Antibodies have been detected in milk samples from both infected and uninfected quarters 9 weeks after initial isolation, but after 20 weeks, antibodies were only detected in milk samples from quarters that were previously infected with *M. bovis* (Byrne et al., 2000). Is not known whether cows with other clinical signs of *M. bovis*-associated disease (e.g. arthritis) also excrete antibodies in milk. However, this would be of particular interest if an ELISA were to be applicable as a diagnostic test for *M. bovis*-associated disease.

The extent to which maternal antibodies against *M. bovis* are important in calves and whether they have a protective effect against *M. bovis*-associated disease remains unclear. One study found low titres for *M. bovis* in young calves from dairy herds, indicating either low exposure or failure of passive transfer. They also found that there was no association between antibodies against *M. bovis* in the first 2 weeks of life and the occurrence of pneumonia in colostrum-fed dairy calves (Van Donkersgoed et al., 1993), which indicates little or no protective effect of maternally derived antibodies in calves.

In conclusion, studies of serology under field conditions are sparse, and the findings of experimental studies are not directly comparabe to natural infections.

Studies on the association between serostatus and *M. bovis*-associated disease in calves and cows are limited and the findings are not clear. Martin et al. (1990) found an association between the incidence risk of *M. bovis* seroconversion and BRD incidence risk at group level, but not at individual calf level, over the first 4 weeks after entry to Canadian feedlots. In contrast, the individual *M. bovis* serostatus upon arrival in a veal calf setting was not a predictor for the development of BRD in two-week-old veal calves (Pardon et al., 2015). The herd serum prevalence of *M. bovis* in heifers and cows did not have an impact on the incidence of respiratory disease in calves (Raaperi et al., 2012). It is also unclear whether the serostatus is associated with production losses in calves. A tendency for lower weight gain has been found in seropositive weaned beef calves (Hanzlicek et al., 2011), while another study found no association between seroconversion and weight gain (Martin et al., 1989). There have been no studies on the association between serostatus and longevity in cows or calves, but information about the possible long-term consequences of *M. bovis* infection would be valuable in advising farmers both during and after an outbreak of *M. bovis*-associated disease.

2.1.3 Treatment options

Treatment of *M. bovis*-associated disease is difficult. In general, it is mostly unresponsive to antimicrobial treatment, and culling is often the last resort (Nicholas et al., 2016). This makes correct diagnosis and control measures even more important.

M. bovis bacteria are innately resistant to antibiotics that interfere with the cell wall, such as penicillins and cephalosporins. They do not synthesise folic acid and are therefore also resistant to sulphonamides (Heuvelink et al., 2016). Several studies have reported the *in vitro* sensitivity of *M. bovis* to different types of antimicrobials such as enrofloxacin, tulathromycin and oxytetracycline (Ayling et al., 2000; Ayling et al., 2014; Klein et al., 2017). However, *in vivo* studies of the treatment of pneumonia and otitis media with, among others, *in vitro* sensitive compounds have found varying results with little or no effect, or treatment had to be continued for a long period to be effective (Allen et al., 1991; Francoz et al., 2004; Gosselin et al., 2012; Poumarat et al., 2001). This difference is probably due to the fact that animals in experimental studies are often treated early in the disease process and naturally acquired respiratory infections are often mixed infections identified later in the disease development, when tissue damage has already occurred. This is supported by the findings of Romváry et al. (1977b), who concluded that that to be effective antibiotic treatment should be initiated at the onset of the febrile course preceding the symptoms of pneumonia and

always with high doses. In addition, failure to treat *M. bovis* infection has increasingly been attributed to antimicrobial resistance (Lysnyansky and Ayling, 2016). In Denmark, antibiotics containing florfenicol, enrofloxacin, doxycycline, tulathromycin, tilmicosin and tylosin are approved for the treatment of cattle with mycoplasma-associated disease (Anonymous, 2017a). However, in some countries (including Denmark), several of these antibiotics are not recommended as first choice so that they can be reserved for use in humans, and proof of the infectious agent being resistant to other antimicrobials is required before they can be prescribed (Anonymous, 2013). In practice, there is a lack of effective antibiotics on the market, and the required duration of treatment and the lack of treatment effect can often result in antibiotic treatment of *M. bovis* infections being futile.

2.1.4 Molecular epidemiology

Different molecular typing methods can be used to study genetic diversity among *M. bovis* bacteria isolated from e.g. different time periods, geographical locations, anatomical sites, as well as diseased versus non-diseased carrier animals. This would improve our understanding of the pathogenesis and bacterial determinants of the disease, as well as its resistance to treatment. The degree of diversity can also be used to determine and understand pathogen transmission and spread of the disease.

Whole genome sequencing recently indicated that 78 *M. bovis* isolates collected between 1981 and 2014 from Denmark could be divided into two clusters. Isolates collected before 2010 were genetically different from isolates collected after 2010, suggesting a shift in the dominant strain during the 2000s (Strube and Lindegaard, 2018). A similar pattern was found in France, where different sub-typing methods found that isolates collected over the last 35 years consisted of two clusters – one with isolates collected before 2000 and the other with isolates collected after 2000 (Becker et al., 2015). Multilocus sequence typing analysis found two major lineages of *M. bovis* in Switzerland and Austria, which consisted of isolates before and after 2007 (Burki et al., 2016). The shift in the *M. bovis* population from the old to the recent subtype occurred at the same time as or following the development of increased antimicrobial resistance (Khalil et al., 2017). Based on these studies, it is likely that the *M. bovis* disease outbreak that started in Denmark in 2011 was caused by either the introduction of a new strain or alterations in the existing strain. It could not be determined whether there was a difference between isolates from different anatomical sites in the Danish material, as the anatomical site of origin was not recorded for a sufficient number of the 78 samples (Strube and Lindegaard, 2018). Parker et al. (2016) investigated this connection further using whole genome sequencing for 94 Australian *M. bovis* isolates collected between 2005 and 2015 from different body sites including the nose, prepuce, semen, vagina, joints, milk, lungs and lymph nodes. They found minimal variation in gene content between isolates collected several years apart, isolates collected from different anatomical sites, between animals with different disease status, or across different geographical locations. This suggests that host and environmental factors are responsible for the diverse disease pattern and difference in outbreak severity among herds, although it is still possible that the expression of genes responsible for virulence and/or antibiotic resistance might play a role.

2.1.5 Transmission pathways

Transmission pathways can be divided into within-herd and between-herd transmission. The within-herd transmission is important once *M. bovis* is present in the herd, and understanding the infection dynamics can help prevent further dissemination of disease in the herd. Understanding how the between-herd transmission occurs is important in reducing the spread of *M. bovis* to or from other herds and for deciding upon external biosecurity actions for infected herds in control programmes.

Transmission between animals within the herd occurs through an exchange of respiratory secretions, udder-to-udder spread during milking and the ingestion of *M. bovis*-contaminated milk. Apparently healthy animals can harbour the organism in the upper respiratory tract for long periods of time, acting as a reservoir for infection in the herd (Bennett and Jasper, 1977; Pfutzner and Sachse, 1996). If segregation and biosecurity is not adequate then close contact between animals or indirect contact via feed or water can occur both between age groups and between animals of the same age group that are housed together. *M. bovis* is considered a contagious mastitis pathogen that can spread from udder to udder. Clinical changes in the milk were not observed until 5-6 days after inoculation (Bennett and Jasper, 1978a), and this delay between shedding mycoplasma and clinical changes in the milk could represent an important source of cow-to-cow spread that is difficult to control.

A major transmission route of *M. bovis* from cows to calves is thought to be ingestion of contaminated milk. Colonisation of the respiratory tract has been shown to occur more often in calves fed milk infected with *M. bovis* compared to calves fed non-infected milk (Bennett and Jasper, 1977). In addition, clinical disease was found to follow feeding with *M. bovis*-contaminated waste milk (Butler et al., 2000; Walz et al., 1997). Vertical transmission is rarely reported, although *M. bovis* has been isolated from vaginal secretions of cows at calving (Feenstra et al., 1991), from aborted foetuses and placentae (Hassan and Dokhan, 2004; Langford, 1975), and has been associated with congenital disease in calves (Bocklisch et al., 1986; Stipkovits et al., 1993). The role of vertical transmission in the spread of *M. bovis* is therefore unknown.

Introduction of asymptomatic carriers is thought to be the main risk associated with *M. bovis* being introduced into cattle herds (Gonzalez and Wilson, 2003; Maunsell et al., 2011). It is challenging to identify these asymptomatic carrier animals and control the between-herd spread of *M. bovis*.

M. bovis has been cultured from bull semen, and antibodies against *M. bovis* have been detected in sera from aborted cows inseminated with semen containing *M. bovis* (Stipkovits et al., 1983). Semen and bulls was recently proposed as a potential way of introducing *M. bovis* into a naïve herd (Haapala et al., 2018; Hazelton et al., 2018a). However, this route of infection must be supported by further studies to determine its role in transmission.

2.1.6 Control measures

The primary recommended control measure for *M. bovis* is to maintain a closed herd and not introduce potentially infected carrier animals (Maunsell et al., 2011). If purchase cannot be avoided, the *M. bovis* status of the herd from which new animals are purchased should be established. This can be challenging, and no well-documented recommendations are available to classify source herds before purchase. Therefore, the best method may be to combine different diagnostic tests and take a clinical history of all age groups in the herd.

Herd-management practices are important in the control of *M. bovis.* Cow-to-cow transmission can be reduced by improving milking hygiene and culling or at least segregating cows known to be diseased (Maunsell et al., 2011). Calves should also be fed milk free from *M. bovis* – either by pasteurising the milk (Butler et al., 2000) or feeding milk replacer. As *M. bovis* is shed from the nares, good sectioning between groups of housed calves, and segregation of diseased calves should be performed in order to reduce nose-to-nose transmission. In addition, maintaining a low stocking density will reduce the spread of the bacterium (Nicholas, 2011).

Development of an effective vaccine against *M. bovis* is a possible way to control the disease, yet vaccination against *M. bovis* has shown varying results. Zhang et al. (2014) found a protective effect of vaccination for cattle aged 5-6 months. In contrast, Prysliak et al. (2013) found no protection against clinical disease when vaccinating calves aged 6-8 months, and an increased incidence of otitis media was seen in one herd after vaccination with a commercial vaccine (Maunsell et al., 2009). This study also found that vaccination stimulated a systemic antibody response, but most clinical disease occurred prior to this response. Development of a vaccine is therefore challenging, and a better understanding of antigens and the immune response to *M. bovis* (especially in young calves) is needed if vaccination is to be an effective way of controlling *M.bovis*-associated disease (Maunsell and Donovan, 2009).

2.2 Diagnostic tests

2.2.1 Enzyme-linked immunosorbent assay (ELISA)

Two commercial ELISA kits (BioX Bio K 260 and Bio K 302) for the detection of antibodies (IgG) against *M. bovis* are available from BioX Diagnostics in Belgium. This section covers what is known about the performance of these tests at both individual animal and herd level according to the available literature.

Another company, BioVet in Canada, have a commercial *M. bovis* ELISA test and other research groups have developed in-house ELISAs (Fu et al., 2014; Wawegama et al., 2014). However, only the BioX tests are commercially available in Europe, which is why they are used for the projects presented in this thesis.

Animal level

The antigen used in the two BioX ELISAs is a recombinant *M. bovis* protein expressed by *E. coli*, but the identity of the specific antigen(s) has been kept secret. BioX K260 has a negative control for each sample well, which should reduce the number of false positives, while the BioX K302 has one negative control on each plate. The BioX K302 uses the recommended cut-off of 37 ODC%, and the sensitivity and specificity are reported to be 100% by the manufacturer, based on 14 experimentally exposed and 16 non-infected animals (Anonymous, 2017b).

In experimentally infected animals, the sensitivity and specificity of BioX K260 has been found to be 0.28 (95% CI: 0.01-0.92) and 1 (95%CI: 0.93-1), respectively, measured 24-68 days after experimental infection (Schibrowski et al., 2018). The BioX K260 has been shown to have little correlation with the occurrence of disease and with PCR and bacterial culture results (Szacawa et al., 2015; Szacawa et al., 2016). However, those studies were cross-sectional, with the aim of comparing results of different diagnostic tests, and did not provide sensitivity and specificity estimates.

Wawegama et al. (2016) estimated the sensitivity of BioX K302 to be 0.37 (95% CI: 0.22-0.54) and the specificity to be 0.95 (95% CI: 0.83-0.99) in experimentally infected animals at the cut-off value 37 ODC%, as recommended by the manufacturer. This was in line with the results of Schibrowski et al. (2018), who found the sensitivity and specificity to be 0.47 (95% CI: 0.10-0.87) and 0.96 (95% CI: 0.87-0.99), respectively. These studies are based on experimentally infected animals and because they lack independence between observations and there is variation in the immunological responses of experimentally and naturally infected animals, they do not necessarily reflect the situation in naturally infected animals (Schibrowski et al., 2018). In addition, no animal-level field study evaluations of cut-off values are available in the literature, and the cut-off at 37 ODC% is generally used in practice despite a lack of substantial documentation of the validity of this threshold for the dichotomisation of test results. Field studies of ELISA performance in terms of detecting antibodies against *M. bovis* are therefore needed.

Bulk tank milk

Little is known about antibodies in BTM for herd-level diagnosis. The manufacturer of the BioX 302 ELISA does not provide any guidelines for its use in BTM, but some authors have investigated

different aspects of this (Nielsen et al., 2015; Parker et al., 2017a). By using latent class analysis, Nielsen et al. (2015) found that in order to lower the number of false positive herds in prevalence estimations at a national level, it might be beneficial to raise the cut-off for herd-level diagnosis in BTM to 50 ODC%. This resulted in a sensitivity of 0.44 (95% posterior credibility interval (PCI): 0.21-0.93) and a specificity of 0.996 (95% PCI: 0.99-1) compared with 0.60 (95% PCI: 0.38-0.96) and 0.97 (95% PCI: 0.94-0.998) when applying 37 ODC% as the cut-off. However, the optimal cut-off value depends on the purpose of testing.

Parker et al. (2017a) found that an increased time since the start of the calving period and increased time since the initial *M. bovis* outbreak were associated with decreasing BTM ODC%, while the time since the most recent positive *M. bovis* culture or PCR and herd size did not affect the BTM ODC%. The time since the start of the calving period could be explained by the fact that *M. bovis*-associated disease is often observed post-partum, due to increased stress around calving, and therefore the BTM was generally higher around calving season when collected in dairy herds with seasonal calving patterns. It is likely that the lack of association between BTM ODC% and *M. bovis* culture, PCR or herd size is due to the difference between the presence of *M. bovis* and a disease outbreak – i.e. *M. bovis* may be present in the herd without causing disease.

In conclusion, the use of antibody measurements in BTM for herd diagnosis or surveillance of *M. bovis* needs further investigation.

2.2.2 Other diagnostic tests

BC has been used for culturing samples from both individual animals as well as BTM samples, and is still considered the gold standard. However, culturing mycoplasmas requires special media and equipment, and can take up to 7-10 days. Careful sampling, handling and transportation is essential for optimal culture conditions (Wawegama and Browning, 2017) and in Denmark, BC is expensive and not always readily available, making the diagnosis of *M. bovis* by BC cumbersome.

Compared to culture, PCR can amplify viable as well as non-viable DNA and is less time consuming. Several PCR assays have been described in the literature as being useful for detecting *M. bovis* in milk and other body fluids. The sensitivity of PCR has been reported at 0.77-0.89 and the specificity at 0.96-1, which is equivalent to or better than BC (Cai et al., 2005; Clothier et al., 2010; Justice-Allen et al., 2011; Parker et al., 2017b; Pinnow et al., 2001).

Common to both BC and PCR is the difficulty in obtaining the diagnostic material used for isolation, especially from calves that require swabs, transtracheal wash and/or joint fluid. This also applies to cows with arthritis, since it is not known whether they excrete *M. bovis* in measurable amounts in milk or the respiratory system. This results in some limitations in the use of BC and PCR for diagnosis in practice.

3 Materials and Methods

3.1 Data collection

Data available for the studies presented in this thesis were collected as part of two longitudinal observational sampling activities originating from separate projects performed as a collaboration between SEGES, the Technical University of Denmark and the University of Copenhagen. This chapter provides an overview of the activities and the data that were made available to meet the objectives addressed in this thesis.

3.1.1 Sampling of suspected case and control herds – sampling activity #1

Sampling activity #1 (SampAct#1) was initiated in spring 2013 by SEGES, which is a farmer-owned knowledge, consultancy and technology centre that provides services to all Danish farmers. This was before the start of this PhD project (December 2013). The overall purpose of the project behind SampAct#1 was to clarify the importance of *M. bovis* infections in Denmark and to limit the impact of the disease by characterising bacterial isolates, increasing the understanding of diagnostic test results, providing knowledge about risk factors and reducing the spread of infection within and between herds. This PhD project was connected to the main project, focusing on studying the within-herd dynamics of *M. bovis* mainly based on antibody responses directed against *M. bovis.* The microbiological characterisation and between-herd spread of infection were covered by other researchers.

Herd selection

The recruitment of herds was conducted by a veterinarian at SEGES. Selecting the study herds was not an easy task due to differences in the perceptions and reporting of clinical signs by local veterinarians and farmers, use of different diagnostic tests and difficulties in the interpretation of the results.

The aim was to include 40 dairy herds that would represent a wide distribution of herds with different histories of *M. bovis*-associated disease (suspected case herds) and some with no history of *M. bovis*-associated disease (control herds). No attempt was made to include a representative sample of Danish dairy herds, because this would have resulted in the inclusion of too few herds with an ongoing or recent *M. bovis* infection. Information about clinical disease and previously performed diagnostic tests were obtained from the local herd veterinarian, who in many cases was the first to contact SEGES about a possible disease outbreak. In addition, the veterinarian from SEGES had prior knowledge of the herds from national BTM screening rounds, where all Danish dairy herds were tested for antibodies against *M. bovis* with the BioX Bio K 302 ELISA kit (BioX Diagnostics, Rochefort, Belgium) and the PathoProof Major-3 PCR kit (Thermofischer Scientific, Waltham, USA) in 2011-12 and 2013. The national prevalence of PCR-positive dairy herds was 1.8% in January 2012 (Aalbæk et al., 2012). In the autumn of 2013, the prevalence of ELISA and PCR-positive herds was found to be 7.2% and 1.6%, respectively, using the recommended cut-offs (Nielsen et al., 2015). This corresponds to 245 ELISA-positive dairy herds and 58 PCR-positive dairy

herds in that screening round. To be included in the study, herds also had to be enrolled in the voluntary milk recording system RYK[2] (which includes approximately 90% of Danish dairy herds), and only herds with a least 100 cows were included to make sure the herd had enough young stock to sample.

SamtAct#1 included 39 Danish dairy herds. To ensure a diverse representation of clinical signs, infection status and test results, herds were classified for enrolment purposes as one of four predefined groups: 'Acute', 'Previously infected', 'BTM-positive' and 'Control'. The initial *M. bovis* diagnosis was made by the farmer and his veterinarian, and the *M. bovis* infection status was not further investigated before the sampling was initiated. The following farms met the basic inclusion criteria:

- 'Acute' (n=14): consisted of herds with recent suspected *M. bovis*-associated disease.
- 'Previously infected' (n=7): consisted of herds that previously had *M. bovis* test-positive, clinically ill animals, but currently had no acutely diseased animals.
- 'BTM-positive' (n=10): consisted of herds selected among those with the highest BTM ODC% (ranging from 68-110) in the most recent Danish national screening of all dairy herds in summer 2013.
- Control (n=8): consisted of herds with no history of clinical disease related to *M. bovis* for the past 3 years and were negative prior to inclusion in PCR, ELISA and BC.

Sampling and information collection procedures

Each of the 39 participating herds were visited four times approximately 3 months apart during the period March 2013 to December 2014 (Figure 3.1). Calves in the age groups 0-3, 3-6, 6-9 and 9-12 months old were blood sampled at each herd visit. At the first herd visit, a cohort of 20 calves between 0 and 3 months of age were sampled and these calves were followed with repeated blood samplings at subsequent herd visits (Figure 3.1). The cohort was selected randomly by the SEGES veterinarian before the first herd visit. At each herd visit, 15 calves in each of the other age groups were sampled. These calves were chosen at each herd visit by the sampling veterinarian and the farmer. Conjunctival swabs for bacteriological culture were collected from a varying number of young calves primarily at the first herd visit.

At the milk recording date closest to the herd visit, milk samples were collected from 50 lactating cows and a BTM sample was taken by the milk truck drivers as part of the milk control scheme. The 50 lactating cows were selected randomly by the SEGES veterinarian. All samples were analysed for antibodies against *M. bovis* at Eurofins Steins Laboratory in Vejen with the indirect ELISA test from BioX, as described in section 3.2.

As part of the sampling procedure, all herds were visited by the author of this thesis to get an overview of the farm, management procedures and details about the extent and duration of *M.* bovis-associated disease within the herd. The farmers were interviewed in a semi-structured way regarding the disease outbreak, diagnostics, duration, handling of the outbreak, number of dead and slaughtered animals, as well as some management questions primarily focusing on contact between animals, animal movement and feeding the calves. The questions are provided in Appendix I. In

[2] https://www.landbrugsinfo.dk/Kvaeg/RYK/Sider/Startside.aspx

almost all herds, the author and farmer talked about the questions while walking through the herd and attempting to see as much of the herd as possible.

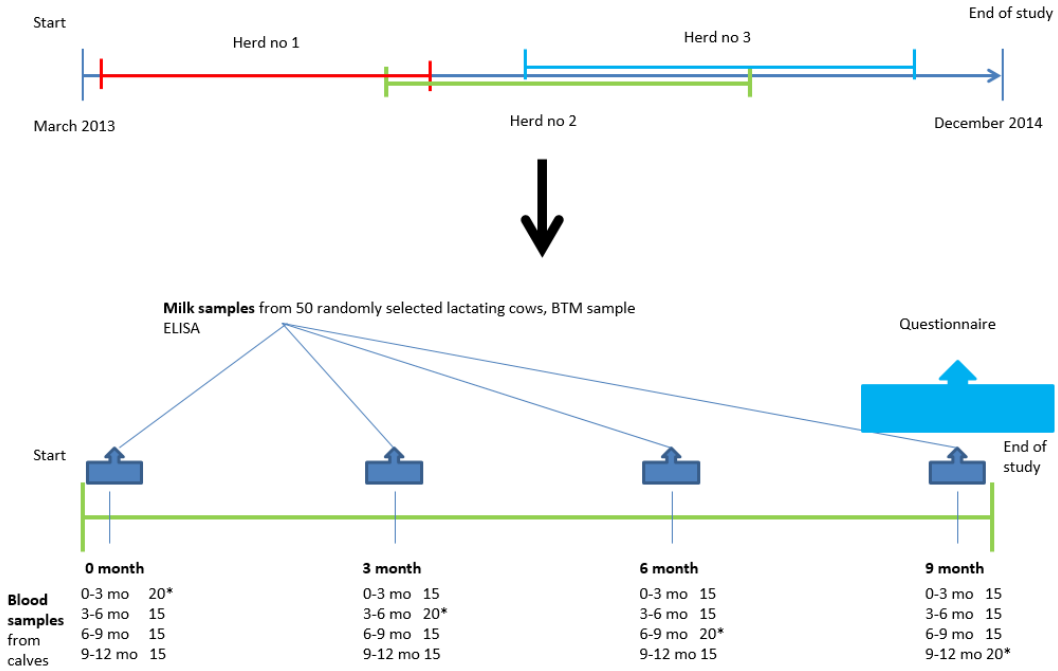


Figure 3.1: Timeline and sampling procedure for sampling activity #1. (* cohort calves, Mo=Months old). In addition to the samples shown here, eye swabs were collected from the youngest calves from all herds to obtain bacterial isolates for molecular investigation.

3.1.2 Clinical epidemiology study - sampling activity #2

It became evident during SampAct#1 that there was not enough information available at animal level to develop a sufficient understanding of the antibody responses at herd level. Furthermore, the intervals between herd visits in SampAct#1 were too long to capture the rapid dynamics of *M. bovis*. Therefore, another sampling activity was organised, in which animals showing clinical signs of *M. bovis*-associated disease and healthy animals in herds experiencing an acute disease outbreak were examined and sampled to investigate the antibody responses associated with the clinical signs expressed by the animals (SampAct #2). It was decided that the this sampling activity would give priority to thorough clinical examinations of the individual animals, rather than inclusion of a large number of animals and herds, taking into account the budget and resources available. Therefore, SampAct#2 involved a smaller number of farms and smaller number of animals within each farm than SampAct#1.

Herd selection

The herds were enrolled from July 2015 to January 2016. Cattle practitioners in Denmark were informed and asked to contact the author if they attended herds with signs of acute *M. bovis*-associated disease among the cows and/or calves. The presence of *M. bovis* in the herds was confirmed by ELISA and PCR testing before enrolment in the study. The aim was to visit the herds no longer than 3 weeks after clinical signs of *M. bovis*-associated disease started, and to follow the herds with five visits approximately 3 weeks apart. The information obtained about the farms prior to enrolment is shown in Table 3.1.

Table 3.1: Knowledge about the herds in sampling activity #2 prior to enrolment.

Herd no.	**Herd A**	**Herd B**	**Herd C**	**Herd D**
Herd size (cows)	177	174	182	391
Primary clinical signs in cows	Mastitis, pneumonia	Mastitis, arthritis	Arthritis	Mastitis, arthritis
Positive ELISA[a]	Yes	Yes	Yes	Yes
Positive PCR[b]	Yes	Yes	No	Yes
Primary clinical signs in calves	Pneumonia	Arthritis, otitis media	No clinical signs observed	Arthritis, otitis media, pneumonia
Outbreak start (according to the farmer)	Early June 2015	Early July 2015	Late November 2015	Mid December 2015

[a] ODC% values > 37 for *M. bovis* antibodies in BioX Bio K 302 or 260 (BioX Diagnostics, Belgium)
[b] Ct value ≤ 37 for *M. bovis* in PathoProof Mastitis Major-3 (ThermoFischer Scientific, USA)

The project budget allowed for the inclusion of approximately 80 cows and 80 calves in total, with each animal being tested five times. However, it is likely during an outbreak of *M. bovis*-associated disease that some animals would be culled due to clinical disease, so we attempted to compensate for this by either including more than 20 animals at the first visit or recruiting additional animals at consecutive visits. Accordingly, a minimum of 20 cows and 20 calves were identified from each farm during the first herd visit with the help of the farmer. The animals were sampled strategically while attempting to ensure that a sufficiently high number of animals both with and without suspected *M. bovis*-associated disease were included. New animals suspected of having *M. bovis*-associated disease at subsequent visits were also included. The rationale behind this targeted (non-random)

sampling strategy was to maximise the probability of including a sufficient number of animals to be able to compare the antibody responses between groups with and without different clinical signs indicating *M. bovis*.

Sampling procedure

At each herd visit, all selected animals were subject to a clinical examination with a focus on the respiratory, musculoskeletal, nervous system and, for cows, udders. See Appendix II for the clinical protocol used. Blood samples from all animals and composite milk samples from all lactating quarters of the cow were collected. All samples were transported to Eurofins Steins Laboratory in Vejen within 48 hours and analysed for antibodies against *M. bovis* with an indirect ELISA, and milk samples were also analysed for the presence of *M. bovis* DNA with a PCR test, both described in section 3.2.

All clinical examinations and sampling were performed by a veterinarian with assistance from veterinary students. The author of this thesis and a practicing veterinarian developed the clinical protocol and one or both of them were present at all herd visits. In addition, one other veterinarian performed the clinical examinations at two herd visits.

3.2 Laboratory procedures

All samples in the two data collection activities were transported to and analysed at Eurofins Steins Laboratory in Vejen, Denmark. The ELISA test used was the BioX Bio K 302 from BioX Diagnostics, Rochefort, Belgium (in the rest of the thesis referred to as BioX). It was performed according to the manufacturer's recommendation (Anonymous, 2017b) and the optical density coefficient (ODC%) was calculated as:

$$ODC\% = (OD_{sample} - OD_{negative\ control}) / (OD_{positive\ control} - OD_{negative\ control}) \times 100\%$$

where OD is the optical density measured by the ELISA reader relating to each test sample, as well as the positive and negative controls on the sample ELISA plate. An ODC% ≥ 37 was considered positive, as recommended by the manufacturer. The reported sensitivity and specificity of the test at this cut-off was estimated at 100% based on a small sample of experimentally infected calves and uninfected control calves (Anonymous, 2017b). However, as described in section 2.1.1, other authors have found the sensitivity and specificity to be lower, and it was anticipated that they would also be lower in naturally infected cattle.

The PCR test used was the PathoProof® Mastitis Major-3 kit from ThermoFischer Scientific, Waltham, MA. It was performed according to the manufacturers' recommendation and a cycle threshold (Ct) ≤ 37 was considered positive. Bacterial DNA is detected in high quantities when Ct < 22; in moderate quantities when Ct is 22-32 and in low quantities when Ct is 32-37 (Anonymous, 2017c). This translates to Ct 30≈1,400 genome copies in qPCR reaction and Ct 20≈1,400,000 genome copies in qPCR reaction. The genome numbers roughly translate to: Ct 30≈80,000 genomic copies/ml of milk and Ct 20≈80,000,000 genomic copies/ml of milk (Mika Silvennoinen, Finland, personal communication).

Both the BioX ELISA test and the PathoProof PCR test are commercially available tests routinely used in Danish cattle practice and by SEGES for surveillance.

After analysis, the samples from SampAct#2 were frozen and stored at Eurofins Steins Laboratory in Vejen. Unfortunately, samples from the first visit to Herd A were lost after initial analysis and therefore could not be re-analysed later. During autumn 2016, the samples were retested by Nadeeka Wawegama (University of Melbourne) using an in-house ELISA (known as the MilA ELISA) (Wawegama et al., 2014). The mean antibody concentration in antibody units (AU) was calculated by plotting the OD values on a standard curve derived from a set of known positive-control sera included on each plate. The sensitivity and specificity of this assay were estimated in feedlot cattle with BRD at 0.943 (95%CI: 0.899-0.996) and 0.944 (95%CI: 0.903-0.996), respectively, using 105 AU as the cut-off (Wawegama et al., 2016). However, the authors now recommend using AU > 135 as a cut-off for a test-positive interpretation, and this cut-off is therefore used in this thesis.

Bacterial culture

Eye swabs from calves and samples from diseased cows sampled during the sampling period were analysed at the Veterinary Institute at the Technical University of Denmark. Bacterial culture was performed with the special requirements necessary for *M. bovis* (Nicholas and Baker, 1998).

3.3 Databases

Animal characteristics and calving dates were collected from the Danish Cattle Database (DCD). Data registered in the DCD comes from different sources, e.g. farmers, veterinarians and laboratories (Bundgaard, 2005). The data used in this thesis included date of birth, dam's ID-number, date of departure from the herd, departure destination and calving dates. Data entry for these variables is done by the farmer and is required by law. An agreement was signed by the farmer of each participating herd to make these data available for this research.

3.4 Creation of datasets for analysis

All data management was carried out in the statistical program R (R Core Team, 2016). All data from the laboratory analysis were received in Excel spreadsheets that also contained information about sampling date, individual animal identification, herd identification number and birth date of the animal. Clinical data collected at herd visits for SampAct#2 were registered in pre-prepared registration forms (Appendix II). Data from these forms were entered to an Excel spreadsheet by the author of this thesis and continually checked for missing or implausible values.

Five datasets were generated based on SampAct#1 and SampAct#2 (Figure 3.2):

Three datasets were created from SampAct#1 for the 39 dairy herds:

1. Data from BTM samples
2. Individual milk samples from selected lactating cows and blood samples from selected calves
3. Blood samples from the cohort of calves

Two datasets were created from SampAct#2 for the four dairy herds:

4. Clinical recordings, milk test results and blood test results from the cows sampled
5. Clinical recordings and blood test results from the calves sampled

Relevant data from DCD were merged into all datasets.

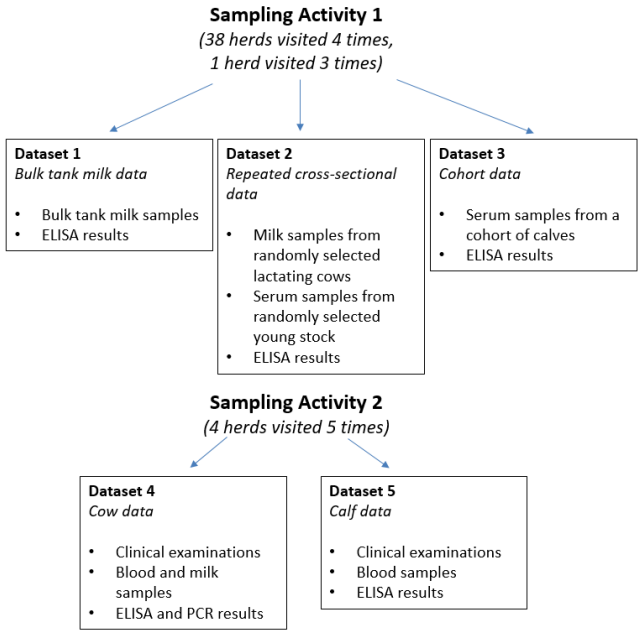


Figure 3.2: Overview of the datasets and their contents based on Sampling activities #1 and #2 created to meet the objectives of this thesis.

3.5 Statistical analyses

All statistical analyses were carried out in the statistical program R (R Core Team, 2016).
Table 3.2 provides an overview of the datasets used and the statistical analyses performed based on each specific objective. Details of the analyses can be found in Manuscripts I-IV.

Table 3.2: Overview of objectives, study designs, materials and methods used in this thesis. Cow-prevalence = milk prevalence of antibody-positive cows, YS-prevalence = seroprevalence of antibody-positive young stock.

Objective	Description and study design	Data sources and types	Methods of analysis	Comments
1	Factors influencing BTM ODC% *Study design:* Observational longitudinal	**Data:** ELISA BTM test results combined with individual blood and milk samples aggregated to herd level (Datasets 1 and 2) **Outcome variable:** BTM ODC% (continuous) **Predictors:** Cow-prevalence, YS-seroprevalence, herd size	Linear mixed model Random effect of herd ID	All details provided in Manuscript I and sections 3.1.1, 4.1 and 4.3
2	Dynamics of antibodies in cows *Study design:* Observational longitudinal with repeated sampling of individual animals	**Data:** ELISA blood test results combined with clinical data from cows (Dataset 4) **Outcome variable:** ODC% for the individual cow (continuous) **Predictor:** Days from disease onset	Generalised additive mixed models (GAMM) One model for each of the four disease groups: 'Mastitis', 'Systemic', 'Non-specific' and 'None' Random effect of animal ID and herd ID	All details provided in Manuscript II and sections 3.1.2 and 4.4
3	Dynamics of antibodies in calves *Study design:* Observational longitudinal with repeated sampling of individual calves	**Data:** ELISA blood test results combined with clinical data from calves (Dataset 5) **Outcome variable:** ODC% for the individual calf (continuous) **Predictors:** Days from disease onset, disease group, herd ID	Linear mixed model Random effect of animal ID	All details provided in Manuscript III and sections 3.1.2 and 4.5

4	Compare dynamics of two ELISAs in serum and milk *Study design*: Observational longitudinal with repeated sampling of individual animals	**Data:** ELISA blood and milk test results combined with clinical data from cows and calves (Datasets 4 and 5) **Outcome variable:** ODC% for the individual animal (continuous) **Predictor:** Days from disease onset, disease group, herd ID	Linear mixed models One model for each ELISA test Random effect of animal ID	All details provided in Manuscript III and sections 3.1.2 and 4.6
5	Association between antibody positivity to *M. bovis* and heifer survival in dairy herds *Study design:* Observational longitudinal with repeated sampling of individual animals	**Data:** ELISA blood test results from a cohort of calves combined with herd variables (Datasets 2 and 3) **Outcome variable:** Undesired early departure (death, euthanasia or slaughter) of a cohort of calves (dichotomous) **Predictors:** Age, individual antibody response, Cow-prevalence, YS- prevalence and calf mortality percentage	Poisson-regression model Random effect of herd ID	All details provided in Manuscript IV and sections 3.1.1 and 4.7

4 Results

The main results from the work conducted during this PhD project are presented in this section. Firstly, descriptive results from SampAct#1 and SampAct#2 are given, followed by results from the statistical analysis of factors influencing the BTM ELISA ODC% (Manuscript I). Thirdly, results from the investigation of antibody responses against *M. bovis* in individual cows and calves (Manuscripts II and III) are given. Results of antibody testing with the in-house MilA ELISA in calves (Manuscript III) and cows are then presented. Finally, knowledge on the use of diagnostic testing with the BioX ELISA obtained in Manuscripts II and III was used to investigate the association between antibody positivity and undesired early heifer departure from the herds in SampAct#1 (Manuscript IV).

4.1 Descriptive results from suspected case and control herds

The 39 dairy herds participating in SampAct#1 were included based on different inclusion criteria for each of the four groups described in Section 3.1.1.

At the time of inclusion in 2013, little was known about the development of *M. bovis*-associated disease outbreaks in Denmark or the interpretation of diagnostic test results, risk factors and management procedures needed to control an outbreak. There was no clear protocol to indicate which diagnostic tests should be used or how to confirm the presence of *M. bovis* in the herds, apart from bacteriological culture from infected body sites such as milk or joint fluid and BTM. A standardised set of *M. bovis* laboratory results was therefore not available from the herds before the project sampling was initiated. In addition, information about the disease outbreak had not been systematically collected at the time of enrolment, and a feasible way to obtain this information was through the farmer interviews. A detailed description of the duration of outbreaks and clinical signs as experienced and reported by the farmers in SampAct#1 are summarised in Table 4.1. The descriptive results of the ELISA testing performed in SampAct#1 in individual animals are summarised in Figure 4.1 as within-herd prevalence of positive milk samples (with error bars), and the same applies for serum samples from calves in Figure 4.2. The distribution of BTM samples from the herds stratified according to enrolment group is shown in Figure 1 in Manuscript I. There are more than four BTM samples per herd due to other sampling activities such as national screening rounds and extra farmer-requested BTM samples outside SampAct#1.

The distribution of ODC% in the BTM samples varied across enrolment groups and no clear pattern was seen (Figure 1, Manuscript I). Some herds had a very dynamic ODC% over time, while others showed a more stable pattern and in some herds, especially some of the 'Acute' herds, it appeared to decline over time.

The 'Acute' herds and 'Previously infected' herds were difficult to separate based on information about disease outbreaks from the farmer interview (Table 4.1). Nearly all reported having experienced an outbreak of diagnosed M. bovis-associated disease, but the time from onset of clinical signs to the first herd visit was more than 2 months in all but one herd, and was not clearly different between the two groups. The 'BTM-positive' herds were a mixture of herds that had a

disease outbreak and herds with farmers who reported not having experienced M. bovis-associated disease. Among the 'Control' herds, no farmers reported having had M. bovis-associated disease before enrolment, but one herd experienced a disease outbreak while enrolled in the study (Table 4.1). In addition, the duration of outbreaks differed markedly among the herds, ranging from 1 month to at least 18 months with an ongoing outbreak situation at the time of questioning. There was therefore no obvious distinction between the herds in terms of disease outbreak.

In addition, no clear distinction was seen between the enrolment groups when comparing the milk prevalence of antibody-positive lactating cows, as measured at each of the four herd visits. The prevalence in the 'Control' herds was generally lower than the other enrolment groups. However, no clear difference was seen among the other enrolment groups. A pattern of declining prevalence (as seen for Herds 2 and 29) and a more stable pattern (as seen for Herds 5, 20 and 26) were found across the three case enrolment groups (Figure 4.1).

In terms of the seroprevalence in calves there was even less difference between the herds in the three case groups than for the cows. Some of the 'Acute', 'Previously infected' and 'BTM positive' herds had stable pattern of seroprevalence (e.g. Herds 4, 20 and 28), while others had a very dynamic pattern (e.g. Herds 9, 16 and 29). The 'Control' herds seemed to have lower seroprevalence, with the exception of Herds 35 and 39 (Figure 4.2). Herd 39 will be discussed in more detail in section 5.1.1.

Overall, this indicates that there were subjective differences between the herds originally enrolled as suspected case herds and 'Control' herds, yet no clear, systematic difference was evident among the three suspected-case groups. It is also clear that there is substantial variation among herds within the same classification group. The presence of *M. bovis*-associated disease at some point in time and the ability of the herd and animals to clear the infection will influence the antibody response, but the exact relationship is not clear based on this data. There are also differences in the expression and duration of antibodies between cows and calves, at least when measured in milk and serum, and this warrants further investigation.

The descriptive results showed that while the enrolment groups were a useful way of ensuring a diverse representation of clinical signs, infection status and test pattern among the enrolled herds, there was little merit in using these classifications to further stratify the herds for the purposes of the studies presented in this thesis. Data arising from SampAct#1 and data from DCD were used analytically to meet Objectives 1 and 5 in Manuscripts I and IV, and the results are summarised in sections 4.3 and 4.7, respectively.

Table 4.1: Overview of the outbreak of *Mycoplasma bovis*-associated disease and clinical signs presented (as experienced by the farmer), bacterial cultures performed and herd size.

	Outbreak (+/-)	Duration of outbreak (months)	Approximate time between clinical disease onset and first herd visit (months)	Clinical signs in cows	Clinical signs in calves	Number of positive bacterial cultures/ samples collected	Herd size (number of cows)
'Acute' herds							
1	+	1	3	Arthritis	Arthritis, otitis, pneumonia	1 (eye swab)/13	324
2	+	10	2.5	Arthritis, some mastitis cases	Arthritis, otitis	5 (joint fluid and eye swabs)/30	380
3	+	> 18 [b]	3	Mastitis, drop in milk yield, arthritis, pneumonia	Arthritis and some otitis cases	0/9	500
4	+	> 18 [b]	2.5	Mastitis, arthritis	Arthritis, otitis	0/28	750
5	+	12	14	Mastitis, arthritis, pneumonia	Otitis, pneumonia	0/27	221
6	+	3-4	26	Mastitis, arthritis	Otitis, pneumonia	1 (eye swab)/24	641
7	- [a]			Some mastitis cases	Some cases of pneumonia and otitis	0/12	350
8	-			Eye problems	Eye problems	0/21	360
9	+	3	4	Arthritis, some cases of mastitis and pneumonia	Arthritis, otitis	0/24	133
10	+	10	1	Mastitis and some arthritis cases	Arthritis, otitis, pneumonia	1 (eye swab)/12	215
11	+	6	2	Mastitis, arthritis	Arthritis, pneumonia, eye problems	3 (joint fluid and eye swab)/19	400
12	+	4	2	Mastitis, arthritis, pneumonia	Otitis	0/16	500
13	+	3	12	Arthritis and some mastitis cases	Otitis	0/0	200
14	+	6	6.5	Some mastitis cases	Arthritis, otitis, pneumonia	0/24	436
'Previously infected' herds							
15	+	1-2	1	Mastitis, arthritis	Diarrhoea, apathy	0/17	110
16	+	4	26	Mastitis	Otitis, pneumonia	0/24	356
17	+	6	6	Mastitis, arthritis, pneumonia	Some otitis cases	0/24	220

18	+	7	9	Mastitis, arthritis	Arthritis, otitis, pneumonia	1 (eye swab)/24	1980
19	+	6	19	Arthritis, pneumonia and some mastitis cases	Arthritis, otitis, pneumonia	0/12	248
20	+	4	24	Mastitis, arthritis	Some cases of arthritis and otitis	0/15	280
21	+	1.5	9	Arthritis and some mastitis cases	Arthritis, otitis, some cases of pneumonia	0/30	510
'BTM-positive' herds							
22	-			Some arthritis cases	Some cases of otitis	0/28	220
23	+	4	5	Mastitis, arthritis	Otitis	0/30	213
24	+	2	3	Mastitis, arthritis	Otitis	0/23	318
25	+	4	42	Arthritis and drop in milk yield		0/12	520
26	-					1 (eye swab)/28	260
27	+	6	5		Arthritis, otitis, pneumonia	0/12	270
28	+	12	10	Decrease in milk yield	Otitis	1 (eye swab)/28	251
29	-					0/24	200
30	-				Otitis	1 (eye swab)/20	280
31	+	4, 12[c]	18	Arthritis, mastitis	Arthritis, otitis, pneumonia	2 (eye swab)/27	240
'Control' herds							
32	-					0/16	103
33	-					1(eye swab)/24	332
34	-					0/8	220
35	-					0/15	440
36	-					0/28	200
37	-					0/23	250
38	-					0/21	460
39	+	4[d]	2 months before clinical signs started	Arthritis and some mastitis cases	Some cases of otitis	0/20	200

[a] Farmer reported no definitive outbreak, but more animals died during an unstated period

[b] Clinical signs were still present at the time of questioning

[c] Two disease outbreaks separated by approximately 6 months

[d] Enrolled as a control herd, but experienced a disease outbreak after sampling was initiated

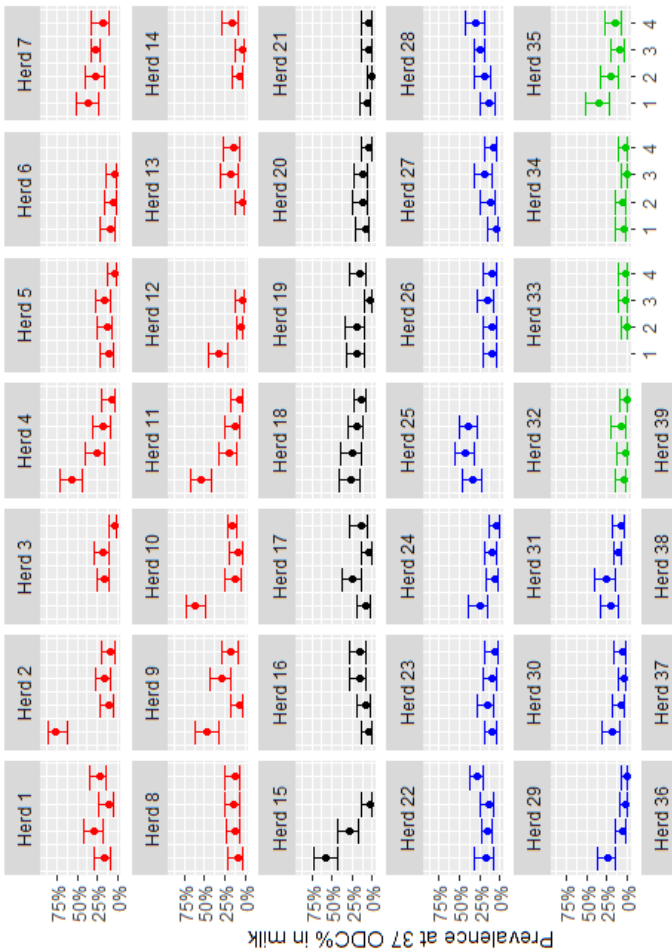


Figure 4.1: Prevalence of antibody-positive lactating cows (ODC% > 37) in milk in each of the four sampling rounds for the 39 Danish dairy herds from sampling activity #1. The graphs are coloured according to enrolment group. Error bars indicate lower and upper 95% confidence intervals for the prevalence obtained using a Bayesian approach. The number of samples per round varied between 29 and 203 (median 49) [Six of the herds were concurrently enrolled in another small study involving samples being taken from 200 cows on one of the sampling dates].

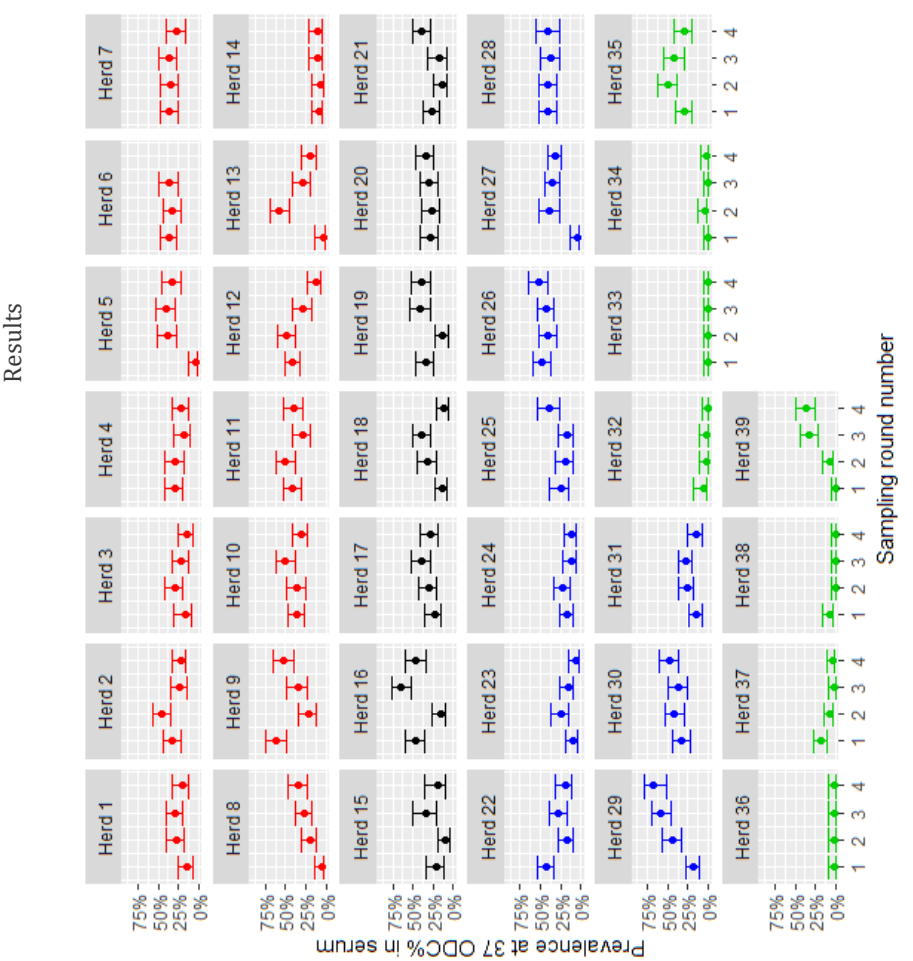


Figure 4.2: Seroprevalence in calves (ODC% > 37) in each of the four sampling rounds for the 39 Danish dairy herds from sampling activity #1. The graphs are coloured according to enrolment group. Error bars indicate lower and upper 95% confidence intervals for the prevalence obtained using a Bayesian approach. The number of samples per round varied between 34 and 117 (median 65).

4.2 Descriptive results from the clinical epidemiology study of outbreak herds

Data originating from SampAct#2 were used to investigate antibody responses in animals with and without different clinical signs of *M. bovis*-associated disease and healthy animals. An overview of the knowledge and data obtained by the investigators during the study period is given in Table 4.2. A detailed description of inclusion criteria and descriptive results for cows and calves can be found in the Results sections of Manuscripts II and III, respectively.

Table 4.2: Overview of knowledge about the four Danish dairy herds in sampling Activity #2, obtained by the investigators in the study period 1st July to 5th April 2016. Modified from Table 1, Manuscript II and Table 1, Manuscript III.

Herd no.	**Herd A**	**Herd B**	**Herd C**	**Herd D**
Data collection period	1st July 2015 - 16th September 2016	20th July 2015 - 6th October 2015	8th December 2015 - 23th February 2016	20th January 2016 - 5th April 2016
Primary affected age group	Cows	Cows and calves	Cows	Cows and calves
Number of animals/serum samples (milk samples)				
Cows	29/120(119)	25/98(87)	32/134(131)	34/95(94)
Calves	15/51	22/101	20/89	26/93
Primary clinical signs				
Cows	Mastitis	Mastitis, arthritis	Arthritis	Mastitis, arthritis
Calves	Pneumonia, otitis media	Arthritis, otitis media, pneumonia	Few cases of arthritis and otitis media	Arthritis, otitis media, pneumonia
Positive bacteriological cultures of *M.bovis* (necropsied calves)	0	1	0	2

4.3 Factors influencing the antibody response in bulk tank milk

The diverse and dynamic antibody responses in BTM from the 39 herds in SampAct#1 described in section 4.1 called for an investigation of the individual antibody responses in animals within these herds to clarify whether the fluctuations in BTM could be explained by within-herd milk prevalence of test-positive animals as an indicator of ongoing *M. bovis* infection. It was therefore important to investigate whether it was only cow-related factors that influenced the BTM ODC%, or if the infection level among young stock also played a role. This was investigated in Manuscript I.

Manuscript I revealed that for each 10% increase in the milk prevalence of antibody-positive lactating cows, the BTM OCD% rose by 9 ODC% and the seroprevalence of young stock was not associated with the BTM ODC%. Despite this fairly good correlation between predicted BTM ODC% and the apparent milk prevalence of antibody-positive lactating cows, there was some inconsistency when adding information on clinical signs observed in the herd when BTM samples were collected. Based on information from the farmer interviews, observations were colour-coded according to whether or not there were clinical signs at the time the samples were collected, or if it was less than 4 months since there had been observed clinical signs (Figure 4.3). Herds with high BTM ODC% and a high apparent milk prevalence of antibody-positive lactating cows were in general sampled within 4 months of clinical signs becoming evident, but samples from herds with low BTM ODC% and low apparent prevalence were taken from a mixture of herds with and without clinical signs. To gain a better understanding of how to interpret the observed dynamics of BTM ODC% and associations with underlying infection patterns, an investigation of the antibody responses in cows with different disease manifestations is warranted.

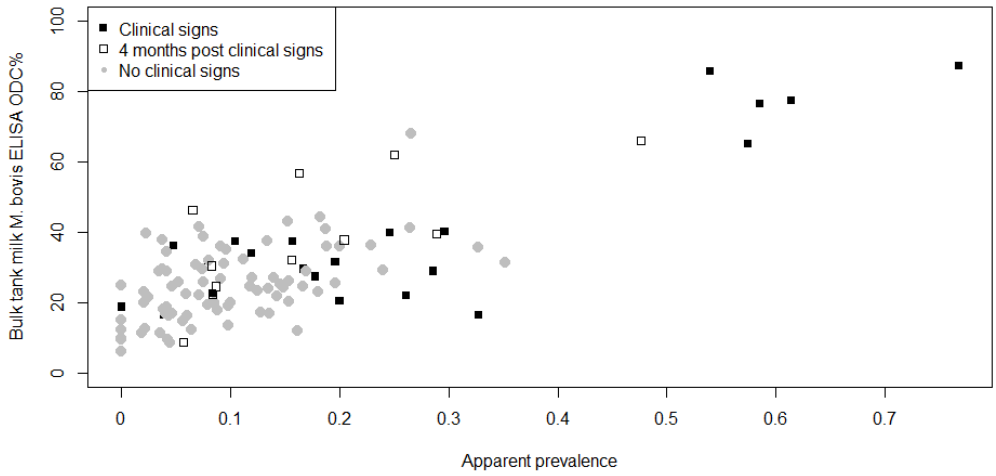


Figure 4.3: Descriptive statistics showing the bulk tank milk *Mycoplasma bovis* ELISA optical density measurement (ODC%) plotted against the apparent milk prevalence of antibody-positive lactating cows. Modified from Figure 2 in Petersen et al. 2016, Factors associated with variation in bulk tank milk *Mycoplasma bovis* antibody-ELISA results in dairy herds. J. Dairy Sci. 99:3815-3823 (included as Manuscript I in this thesis).

4.4 Antibody dynamics in individual cows

Data from SampAct#2 were used to study the dynamics of antibody responses in cows with different clinical signs of *M. bovis*-associated disease. In total, 120 cows were included and all cows were assigned to one or two of the four disease groups: 'Mastitis', 'Systemic', 'None' or 'Non-specific', as described in Manuscript II. Eight cows were included in both the 'Mastitis' and 'Systemic' group, because they had clinical signs of *M. bovis* mastitis and arthritis, these were referred to as the 'dual-syndrome' cows.

The ELISA response in serum differed among the four disease groups (Manuscript II). In particular, cows in the 'Systemic' group generally had a higher estimated antibody response than cows in the 'None' and 'Non-specific' groups (Figure 4.4). The confidence intervals for the 'Systemic' and 'None' groups did not overlap from disease onset to 70 days after disease onset. The 'Mastitis' group was too small to include in a model. However, the cows with *M. bovis* mastitis primarily had a rise in antibodies around and shortly after disease onset if they also had signs of systemic disease ('dual-syndrome' cows) (Figure 1, Manuscript II).

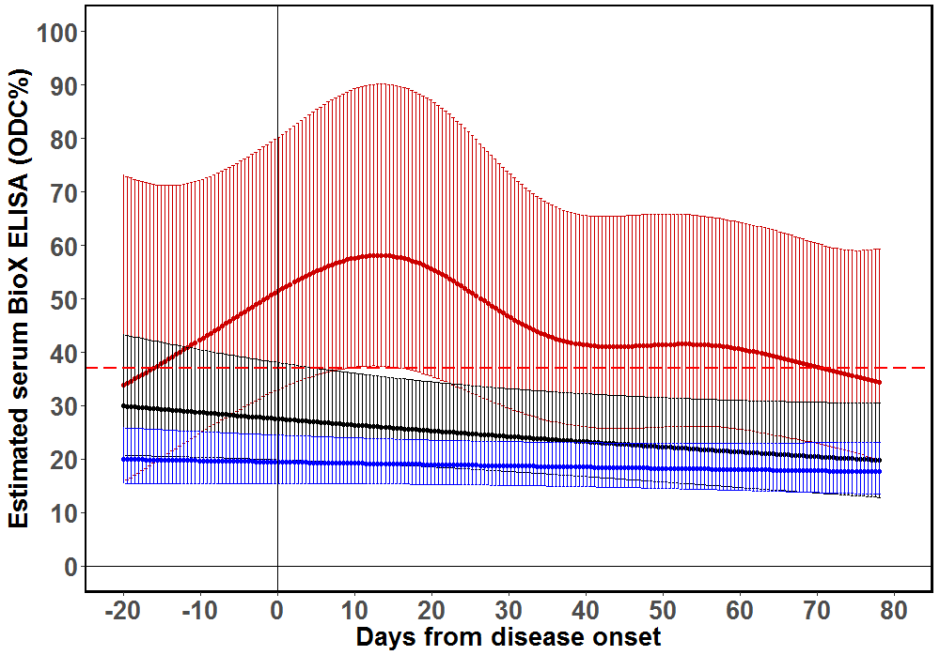


Figure 4.4: Estimated mean antibody response in serum (solid line) and 95% confidence intervals (shaded area) as measured by the BioX ELISA Bio K302. Red represents the 'Systemic' group, blue is the 'None' group and black is the 'Non-specific' group. The dotted red line shows the recommended ELISA cut-off (37 ODC%). Modified from Figure 3 in Petersen et al. 2018, A longitudinal observational study of the dynamics of *Mycoplasma bovis* antibodies in naturally exposed and diseased dairy cows. J. Dairy Sci. https://doi.org/10.3168/jds.2017-14340 (included as Manuscript II in this thesis).

The difference in the ELISA response in serum was greater than the response in milk among the four disease groups. Only cows with clinical *M. bovis* mastitis seemed to have increased ODC% in milk (Manuscript II). Cows with e.g. arthritis as the only clinical sign did not have an elevated ELISA ODC% in milk.

4.5 Antibody dynamics in individual calves

Data from SampAct#2 were also used to study the dynamics of the antibody response in calves with different clinical signs of *M. bovis*-associated disease. In total, 83 calves were included and assigned to one of the three disease groups: '*M. bovis*', 'Respiratory' and 'None', as described in Manuscript III.

The antibody responses in calves seemed to differ among herds, but not among calves with different clinical signs within the same herd. BioX ELISA rarely detected antibodies in calves under the age of 3 months (Manuscript III). The estimated mean ELISA ODC% response did not rise above the recommended cut-off for the entire study period in three of the four herds, with Herd 4 having a rise in mean ODC% at around day 100-120 (Figure 4.5). However, this apparent difference was driven by few observations, which is also evident by the broad 95% confidence interval.

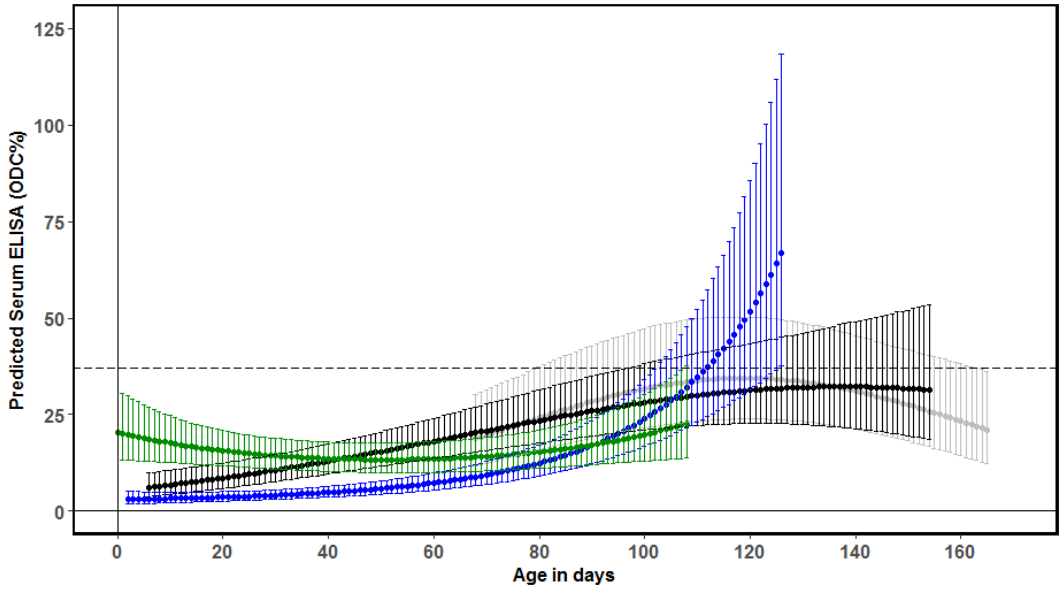


Figure 4.5: Estimated mean antibody response in serum (solid line) and 95% confidence intervals (shaded area) as measured by the BioX ELISA Bio K302 assay for the herd-specific age ranges for which observations were available. Herd 1 is grey, Herd 2 is black, Herd 3 is blue and Herd 4 is green. The dashed line shows the recommended individual animal ELISA cut-off (37 ODC%). From Petersen et al. *Mycoplasma bovis* antibody dynamics in naturally exposed dairy calves according to two diagnostic tests. Submitted to BMC Veterinary Research (included as Manuscript III in this thesis).

4.6 The MilA ELISA results

In addition to the commercial BioX ELISA, blood and milk samples from SampAct#2 were also analysed with the in-house MilA ELISA, as described in section 3.2. The following section presents the antibody dynamics measured by the MilA in cows (i.e. results that were not included in any of the manuscripts in this thesis) and in calves, as described in Manuscript III. The MilA ELISA antibody response was analysed in the same way as described for the BioX ELISA and the animals were divided into the same disease groups as described in Manuscripts II and III.

Graphs of the raw data of serum MilA ELISA measurements in cows are shown in Figure 4.6. There were very few negative samples when a cut-off of 135 AU was applied and the antibody response was rather dynamic – both within and between cows. No apparent differences were seen among disease groups or herds. Satisfactory model fit could not be achieved for serum MilA ELISA responses in cows due to the extreme variability within the raw data.

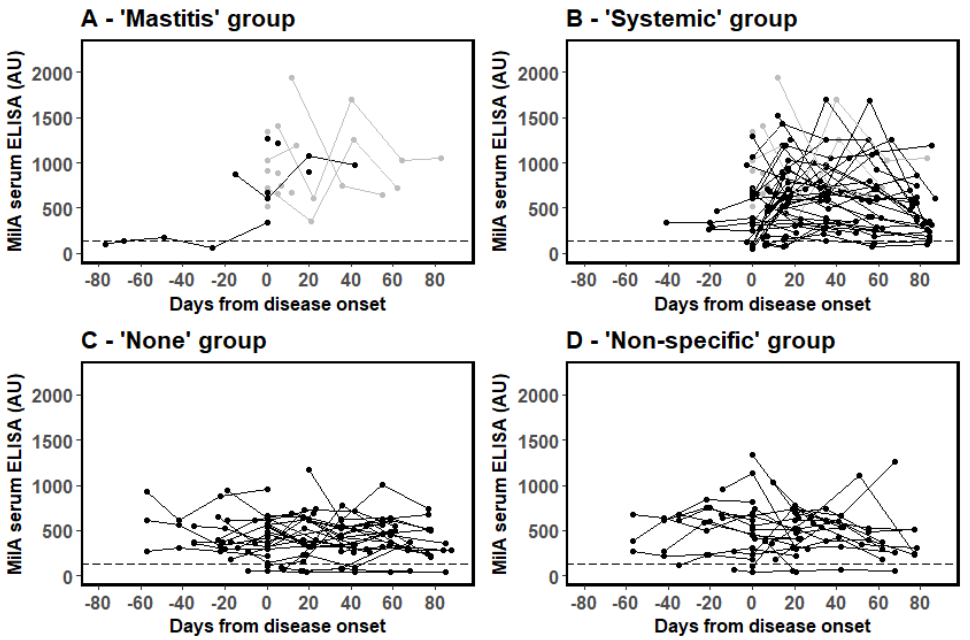


Figure 4.6: Distribution of serum MilA ELISA measurements for antibodies directed against *Mycoplasma bovis* in the four disease groups of dairy cows from four Danish herds: A: 'Mastitis', B: 'Systemic', C: 'None', D: 'Non-specific'. Grey dots represent the 'dual-syndrome' cows. Horizontal dotted lines show the recommended ELISA cut-off (135 AU). AU = antibody units. Results from the same cow are linked by lines.

The MilA antibody response was rather different in milk compared to serum (Figure 4.7). Many cows were below the cut-off throughout the study period, especially in the 'None' and 'Non-specific' groups. As for the BioX ELISA it seemed to show that only cows with clinical *M. bovis* mastitis secreted antibodies in milk.

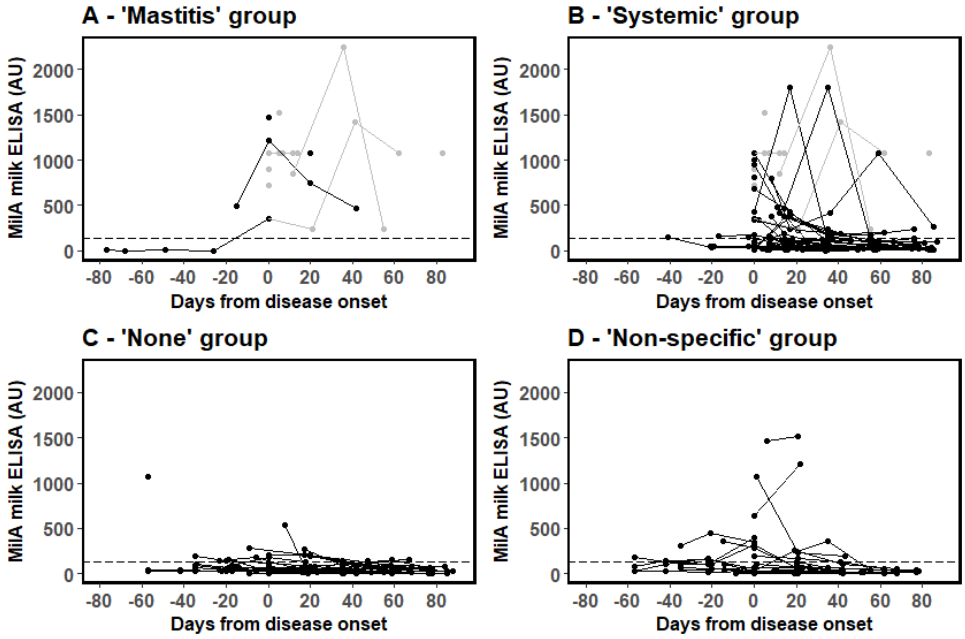


Figure 4.7: Distribution of milk MilA ELISA measurements for antibodies directed against *Mycoplasma bovis* in the four disease groups of dairy cows from four Danish herds: A: 'Mastitis', B: 'Systemic', C: 'None', D: 'Non-specific'. Grey dots represent the 'dual-syndrome' cows. Horizontal dotted lines show the recommended ELISA cut-off (135 AU). AU = antibody units. Results from the same cow are linked by lines.

In calves, there was no difference in antibody response in serum from calves with different clinical signs, but there was a difference when including herd as a fixed effect (Manuscript III). Based on the raw data shown in Figure 2 in Manuscript III, the results from Herds 2 and 4 showed that the MilA ELISA can be used to evaluate antibodies in calves shortly after birth. In addition, the results from Herd 3 showed that the MilA ELISA could be below the cut-off at 135 AU in several calves for more than one sampling. The estimated mean AU response increased rapidly to above the recommend cut-off for the entire study period in two of the four herds (Figure 4.8). The calves in Herd 3 stayed below the cut-off until approximately 80 days of age, while the calves in Herd 1 were older at sampling, so it was not possible to see the early antibody response in these calves. However, the shape of the curve seemed to mimic Herds 2 and 4, but at an older age (Figure 4.8).

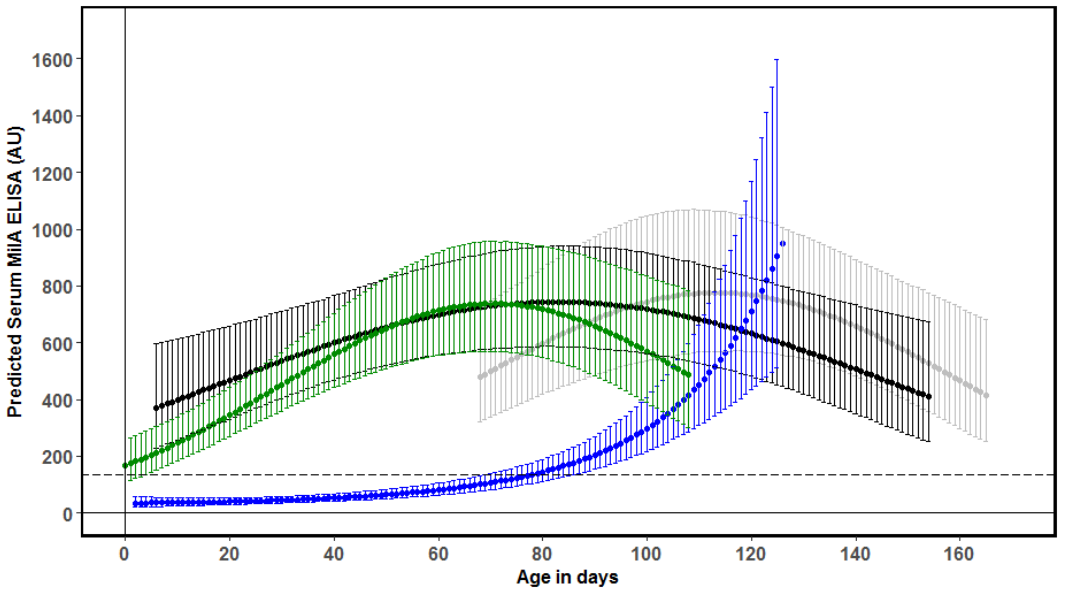


Figure 4.8: Estimated mean antibody response in serum (solid line) and 95% confidence intervals (shaded area) as measured by the in-house MilA ELISA for the herd-specific age ranges for which observations were available. Herd 1 is grey, Herd 2 is black, Herd 3 is blue and Herd 4 is green. The dashed line shows the recommended individual animal ELISA cut-off (135 AU). From Petersen et al. *Mycoplasma bovis* antibody dynamics in naturally exposed dairy calves according to two diagnostic tests. Submitted to BMC Veterinary Research (included as Manuscript III in this thesis).

4.7 The association between antibody-positivity for *M. bovis* and undesired early heifer departure

A cohort of 636 heifer calves from 36 of the 39 herds enrolled in SampAct#1 were used, along with antibody measurements from lactating cows and other calves in the herds, to study the association between antibody-positivity and undesired early departure (UED) in heifer calves as described in Manuscript IV.

The association between antibody-positivity to *M. bovis* using the BioX ELISA and the incidence rate of UED (i.e. slaughter, euthanasia or death) was performed using a Poisson regression model adjusting for the confounding effects of age and mortality percentage. Increasing seroprevalence in the group of calves 3-12 months old was the primary factor found to increase the risk of UED of heifers. For a 10% increase in seroprevalence the incidence rate of UED increased by 23%. A weaker association with the interaction between individual ELISA ODC% and age was significant, which means that effect of individual ELISA ODC% was different depending on age of the heifer. For a 10% increase in individual ELISA ODC%, the incidence rate of UED increased by 27% for heifers aged 9-12 months and decreased by 23% for heifers aged > 27 months (Table 3, Manuscript IV).

5 General discussion and conclusions

The main aim of this PhD project was to improve the use of antibody testing for animal- and herd-level diagnostics by improving our understanding of how to interpret antibody measurements in relation to the clinical epidemiology of *M. bovis* and control of the disease. Although antibody measurements in both individual animals and BTM are being used to screen herds for or diagnose animals with *M. bovis* infection, the interpretation of the results from available commercial ELISA kits have not been sufficiently substantiated by field studies. In addition, the question of how to manage antibody-positive animals in infected herds is often raised by farmers and veterinarians.

Results from this project show that there are limitations and therefore precautions that need to be taken when using BTM ODC% as a herd-level test mainly due to lack of sensitivity (Objective 1). The antibody responses measured by BioX ELISA in both milk and serum upon infection of cows with *M. bovis* are very dynamic and depend on which clinical signs the cow exhibits (Objective 2), while in calves, the antibody responses depend highly on age and exposure rather than disease (Objective 3). The MilA ELISA seems to be a relevant and more sensitive alternative diagnostic antibody test for use in both adult dairy cattle and calves (Objective 4). Finally, increased seroprevalence in calves below one year old was found associated with decreased survival of heifers, whereas the individual antibody response in the calves was only associated with early departure from the herd in some time-periods of the heifer's life (Objective 5).

More detailed discussion of the specific findings of the different studies can be found in Manuscripts I-IV. The findings can be used in different combinations to improve the diagnosis of *M. bovis* infections in dairy herds depending on the purpose of testing and the herd-specific situation. Defining the intended purpose of testing is very important when assessing the performance of a diagnostic assay (Nielsen et al., 2011). The study results will be discussed in the following sections, with focus on the applicability of *M. bovis* antibody measurements in individual animals and at group level as well as the clinical implications of the findings.

5.1 Applicability of bulk tank milk antibody testing

The following section addresses the antibody response against *M. bovis* measured in BTM as a herd-level screening tool to define the status of the herd, as well as the challenges associated with this.

The antibody response in BTM can provide information about the disease status of a dairy herd for diseases such as *Salmonella* Dublin, BVDV and *Coxiella Burnetii* (Beaudeau et al., 2001; Nielsen, 2003; Taurel et al., 2012). In Denmark, antibody measurements in BTM are part of several disease control programmes, e.g. for *Salmonella* Dublin and BVDV (Houe et al., 2006; Warnick et al., 2006), and there has been a desire from different stakeholders in the Danish cattle industry to use it for surveillance and pre-movement screening of cattle herds. For the ELISA response in a BTM sample to be useful (e.g. for classifying the herd into different risk groups), it needs to be representative of the herd and reflect the infection status of the animals within it.

The ELISA ODC% in milk from individual animals was only above the cut-off in cows with *M. bovis* mastitis (Manuscript II). As *M. bovis* can give rise to other clinical signs such as arthritis, this suggests that the antibody level in BTM mainly reflects the presence of udder infections associated with *M. bovis*, rather than *M. bovis*-associated disease in general. This is probably partly why there was some inconsistency between BTM antibody response and farmer-reported clinical signs in the herds in SampAct#1 (Figure 4.3). It is likely that herds with arthritis as the primary clinical sign of *M. bovis* reported clinical signs when the BTM ODC% was low. In fact, many of the *M. bovis* disease outbreaks reported in Denmark since 2011 were characterised by a high incidence of arthritis cases (Jensen, 2015). Another important issue is that as milk from cows with clinical mastitis must not be milked into the bulk tank, the BTM antibody response could merely reflect the presence of subclinical *M. bovis* udder infections within the herd.

Parker et al. (2017a) investigated the BTM *M. bovis* BioX ODC% as a biosecurity tool in Australian dairy herds. They found a significant association between the seroprevalence of lactating cows and BTM ODC%. However, their model explained less of the variation than the model in Manuscript I, where the milk prevalence of antibody-positive lactating cows was associated with BTM ODC%. This also suggests that the BTM ODC% mainly reflects udder infections and not other disease manifestations of *M. bovis*. In the Australian study, the hospital herd within a large dairy herd was also found to have a higher antibody level in the BTM than the milking herd (Parker et al., 2017a). This suggests that the antibody response in milk primarily comes from diseased or at least animals separated away from the herd milking for delivery of milk for human consumption. Different management, treatment and culling strategies can therefore influence which cows are milked into the bulk tank and have a large impact on the diagnostic value of a BTM sample from the milking herd. This reduces the sensitivity and hence validity of the BTM test method for surveillance purposes.

Another challenge with BTM testing is that the BTM ODC% for *M. bovis* seems to be more dynamic than for other cattle diseases such as *Salmonella* Dublin (Nielsen, 2003) or BVDV (Houe, 1999). In one study, the mean BTM BioX ODC% was found back below 37 ODC% less than 1 year after the disease outbreak started (Parker et al., 2017a). This is in line with Herd 39 from SampAct#1. This herd was unique as it was originally enrolled in the study as a 'Control herd' in November 2013, but clinical signs of *M. bovis*-associated arthritis began to present among the cows in February 2014. As a consequence of this opportunity, BTM samples were collected every second week to be able to follow the development of the antibody dynamics in BTM before, during and after an outbreak of *M. bovis*-associated disease. The BTM went from being negative to positive in both PCR and ELISA tests when clinical signs of *M. bovis* appeared in the herd (Figure 5.1). Within 6 months of clinical signs appearing, both PCR and ELISA in BTM were below the recommended cut-off again, and clinical signs were no longer present. The outbreak period was approximately 4 months. However, the BTM BioX ODC% was below 37 ODC% even before the clinical signs had ceased which further underpins the poor sensitivity of BTM testing for herd classification.

Parker et al. (2017a) studied BTM ELISA response as a biosecurity tool for detecting dairy herds with past exposure to *M. bovis*, and they were also challenged by the fact that the antibodies against *M. bovis* are dynamic. They found that the greatest probability of identifying a herd with past exposure was between 0 and 8 months after the initial disease outbreak. In addition, the BTM

ODC% was below the cut-off 12 months after disease outbreak, despite positive BC or PCR results for *M. bovis* being found in the herd.

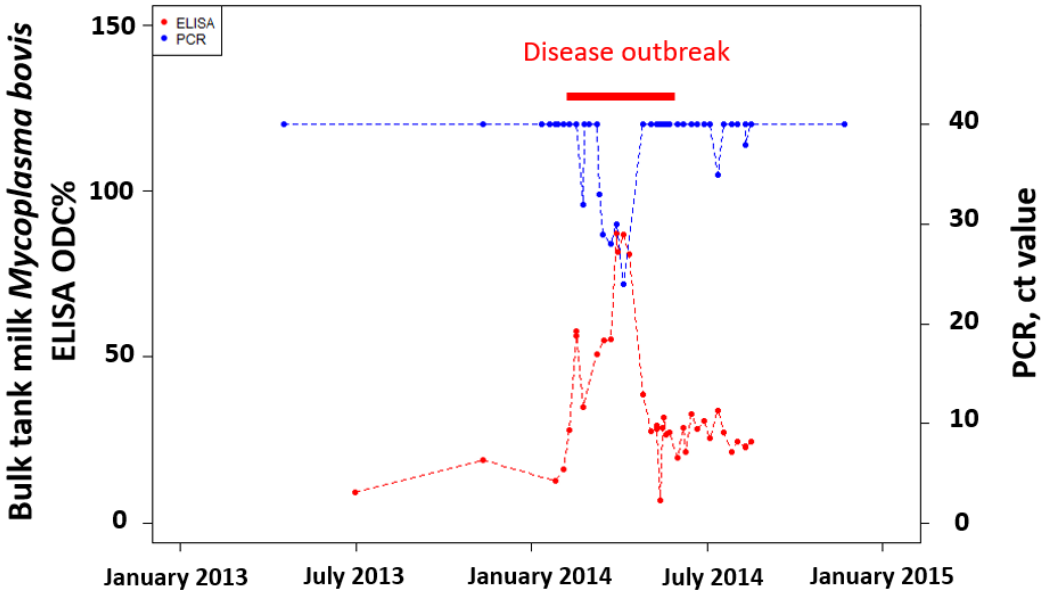


Figure 5.1: The dynamics of the bulk tank milk antibody responses against *Mycoplasma bovis* measured by the BioX K302 ELISA (red dots and red dashed line) and PCR ct values measured by PathoProof® Mastitis Major-3 (blue dots and blue dashed line) for Herd 39. The red solid bar indicates the time period during which the farmer reported observing clinical signs of *Mycoplasma bovis*-associated disease in the herd.

This implies that very frequent monitoring of the ODC% in BTM would be necessary in order to detect *M. bovis* infection emergence in a herd. However, more frequent testing would increase the probability of false-positive test results, because none of the available tests have perfect specificity (Nielsen et al., 2015; Schibrowski et al., 2018). Nielsen et al. (2015) estimated a sensitivity of 0.604 and a specificity of 0.973 at cut-off 37 ODC% and a sensitivity of 0.435 and a specificity of 0.997 at cut-off 50 ODC% for the BioX ELISA, compared with the PathoProof® Mastitis Major-3 PCR kit at cut-off 37 Ct in a latent class analysis in BTM. However, the estimates were quite uncertain (large Posterior Credibility Intervals) and based on the work presented in this theses, it is likely that the underlying disease definition for the herds covered mainly presence of *M. bovis* udder infections.

As the seroprevalence among young stock was not associated with the BTM level (Manuscript I), a group-level marker for this age group would also be needed if the herd testing procedure was to be improved.

In conclusion, the use of antibody testing in BTM for detecting *M. bovis* is questionable because: 1) not all clinical symptoms in cows will be reflected in the BTM; 2) the antibody response in BTM is

very dynamic, making continuous and frequent monitoring necessary in order to detect new infections in due time; 3) another test strategy must be implemented to monitor the status of the calves and young stock within the herd.

5.2 Interpretation of antibody responses in individual cows

The use of antibody responses in different diagnostic materials from individual animals can be used to confirm suspected clinical cases or to estimate the prevalence of infection or exposure . The target condition depends on the purpose of testing (Nielsen et al., 2011) and could include animals with clinical signs associated with *M. bovis* or infected animals without clinical signs. The target condition in the studies of individual animals (SampAct#2) in this thesis was an animal with clinical signs likely to be associated with *M. bovis* infection.

The usual way to evaluate diagnostic tests is to provide estimates of sensitivity and specificity, as done by e.g. Schibrowski et al. (2018). However, to the author's knowledge no information on the dynamics of *M. bovis* antibodies in individual animals under field conditions is available from the published literature, and without this, the estimates of sensitivity and specificity are difficult to understand and use. For antibodies against *M. bovis* to be used for diagnostic testing in either serum or milk, the first step is to know the dynamics of antibodies in the different diagnostic materials in animals with different clinical disease syndromes. The following section therefore contains a discussion of the dynamics of antibodies in serum and milk in cows for the purposes of diagnosing individual diseased animals, as well as being an indicator of disease at group-level.

5.2.1 Antibody response in serum

There were many sick cows with continuously low BioX ODC%, particularly in the group of cows with systemic disease, and reasons for this are discussed in Manuscript II. Whatever the reason, this indicates a low sensitivity of the BioX ELISA, which has also been reported by others, who found the sensitivity to be 0.47-0.50 in experimentally infected animals (Schibrowski et al., 2018; Wawegama et al., 2016). In order to improve the diagnostic sensitivity of the herd testing procedures using the BioX ELISA, it is therefore recommended to test a group of cows or combine antibody measurements with other diagnostic tests.

The antibody response in serum seems to be more dynamic than often seen for other infectious disease in cattle e.g. *Salmonella* Dublin (Nielsen, 2003) and BVDV, where the level of antibodies remains elevated for up to 3 years (Fredriksen et al., 1999). The antibody response in serum in the group of cows with systemic *M. bovis*-associated disease was significantly different from the group of cows with no clinical signs of *M. bovis* during the entire study period (Manuscript II), which indicates that it would be possible to differentiate diseased from non-diseased cows based on the BioX ELISA. However, this was only evident for 60-70 days after disease onset, following which the antibody level was already below the recommended cut-off. This means that the period during which it is possible to diagnose *M. bovis*-associated disease or recent disease with antibody tests in serum is short. This also has implications for group-level diagnostics, as it can make it challenging to identify groups of animals or herds with recent circulation of *M. bovis* infection that may still constitute a contact risk. Recently, Hazelton et al. (2018a) measured the seroprevalence of 50 cows

in four herds to evaluate the serological profile of the herd. They found the lowest seroprevalence in the herd with the longest history of *M. bovis*-associated disease, while the highest was found in the herd with the most recent history of disease.

The results of Manuscript II also indicate that differentiation between the clinical manifestations of *M. bovis* (such as mastitis, arthritis and respiratory disease or a combination of these) is essential in order to diagnose *M. bovis*-associated disease. It was difficult to form a conclusion on the 'Mastitis' group in Manuscript II since the majority of cows were euthanised after being identified with mastitis, so repeated samples were only available from seven cows. This also meant that it was not possible to run a statistical model for the 'Mastitis' group. However, the raw data (Figure 1 in Manuscript II) suggest that it is primarily the 'dual-syndrome' cows that have high antibody responses in serum in the 'Mastitis' group, so it is likely that it is the systemic component of the disease syndrome that causes a high level of antibodies in serum. This leaves us with unanswered questions regarding how *M. bovis* disseminates in the host. Mastitis might be mainly a local infection in the udder originating from uptake via the teat canal, whereas haematogenous spread to the joints is more likely to be a sequela to uptake via the respiratory route, as seen in calves (Mahmood et al., 2017; Romvary et al., 1977a). On the other hand, it cannot be ruled out that a sufficiently high infectious dose in the udder can lead to systemic disease, as suggested by Pfutzner and Sachse (1996). Arthritis has been seen after both intraarticular and intravenous inoculation of *M. bovis*, but arthritis did not develop after inoculation of *M. bovis in*to the udder (Pfutzner and Sachse, 1996; Stalheim and Page, 1975), and no published literature has been identified in which arthritis was reported to follow mastitis. This does not prove that arthritis cannot develop after uptake through the teat canal, but in combination with an apparently different antibody response in cows with mastitis and arthritis, it gives weight to different pathogeneses producing the different clinical syndromes.

In conclusion, to use the BioX ELISA for antibody measurements in serum, the following precautions should be considered: 1) only cows with systemic disease can be expected to have measurable antibodies in serum, 2) the time period during which high antibody response results in a positive diagnosis is relatively short – only 60-70 days after disease onset, 3) it is preferable to test groups rather than individual animals in order to increase the sensitivity of testing procedure to confirm suspected *M. bovis*-associated disease in a cattle herd. More work needs to be done to determine the most optimal testing strategies with regard to sample sizes and target groups depending on clinical signs and herd structures.

5.2.2 Antibody response in milk

The antibodies in milk seemed to be even more dynamic and short-lasting than in serum, making it a less suitable material for diagnosing *M. bovis*-associated disease in general, even though it is often more convenient to collect from lactating cows.

The BioX ELISA mainly detected antibodies in milk from cows with clinical *M. bovis* mastitis, and since the 'Mastitis' group was small it was not possible to analyse and conclude much about cows with *M. bovis* mastitis (Manuscript II). As mentioned above, repeated samples were only available from seven cows, mainly due to the euthanasia of affected cows, which could have introduced bias

to these estimates. If the more severely diseased cows were removed from the study, a potential underestimation of the antibody response might have been seen in the 'Mastitis' group. A reliable estimate of the antibody response in severely diseased animals might be impossible to obtain, as one of the recommendations for the control of *M. bovis* is culling affected animals (Maunsell et al., 2011; Pfutzner and Sachse, 1996), in addition to the welfare issues associated with keeping these animals alive.

A direct comparison between serum and milk BioX ODC% in cows has been performed by others. Approximately 200 cows in each of eight of the Danish dairy herds with recent *M. bovis*-associated disease occurrence also enrolled in SampAct#1 were sampled, with paired blood and milk samples taken on the same day and analysed with the BioX ELISA (Nielsen, 2014). The results of that study are shown in Figure 5.2. The milk ODC% was generally lower than the serum ODC% from the same cow, and a coefficient of determination (r^2) at 0.47 indicates disagreement among many observations. Combined with information from Manuscript II, this discrepancy can be explained by different underlying disease manifestations in the cows. The cows with very high ODC% in milk but not in serum are likely to have *M. bovis* udder infections, while the opposite scenario (high serum and low milk ODC%) is likely to represent a systemic *M. bovis* infection. In addition, the majority of sampled cows were low in ODC% in serum and milk, despite that herds had recent *M. bovis*-associated disease occurrence (Nielsen, 2014). This can be explained by the short lasting antibody responses and the fact that antibodies to *M. bovis* are primarily produced by diseased cows, as shown in Manuscript II. These findings highlight the importance of performing longitudinal studies including clinical recordings to improve the interpretation of diagnostic test results, as will be discussed further in Section 5.7.

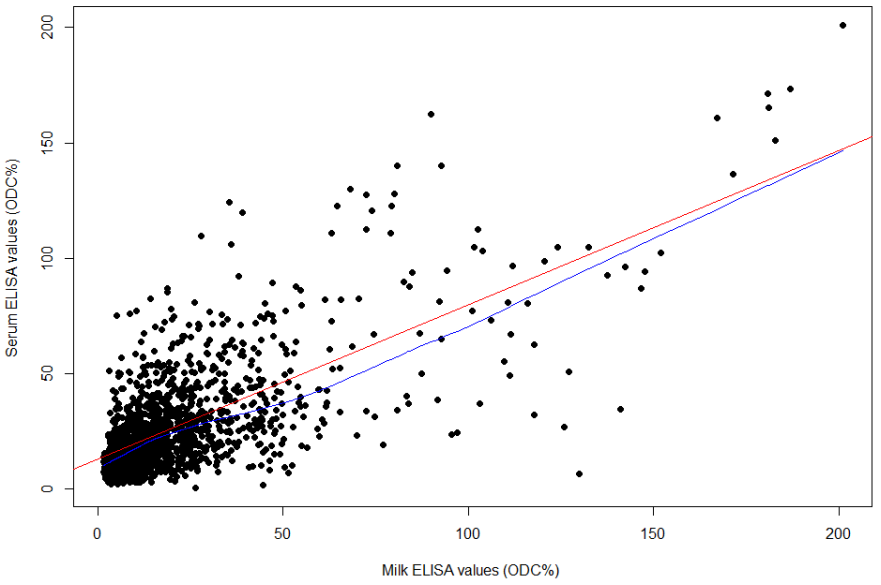


Figure 5.2: The correlation between BioX K302 ELISA values in serum and milk from cows in eight Danish dairy herds (serumELISA =2.5 + 0.7*milkELISA, r^2= 0.47), red line = regression line, blue line = lowess line (from Nielsen, 2014 with permission).

5.3 Interpretation of antibody responses in individual calves

Interpretation of the antibody responses seemed to be different in calves compared to cows. There was no apparent difference in antibody response between the different disease groups in the BioX ELISA, but differences were found between herds, which may be related to different ages at exposure to *M. bovis* (Manuscript III).

It is important to note that the use of the BioX ELISA was not useful for testing calves less than 3 months of age (Manuscript III). This deduction is primarily based on results from Herds 2 and 4. These were heavily infected herds with severe disease problems among the calves, likely associated with *M. bovis*, which was detected by PCR in necropsied calves from the herds. There was little or no segregation between cows and calves, and the calves were fed non-pasteurised whole milk, at least at the beginning of the disease outbreak. Despite this, the mean estimated ODC% was not above the recommended cut-off in either herd (Figure 4.5). In contrast, the estimated ODC% in calves from Herd 3 rose very sharply around the age of 3 months. There were few clinical signs suggestive of *M. bovis* among the young calves in this herd and they were separated from the cows, suggesting that they were not exposed to *M. bovis* until the age of around 90 days, when the sharp increase in antibodies was seen. At this age, calves are suspected to be more immunocompetent, which may influence their ability to respond to *M. bovis* (Barrington and Parish, 2001). This apparent age-related response to *M. bovis* exposure is important as the calves are thought to be a reservoir, maintaining *M. bovis* infection within the dairy herd (Maunsell et al., 2011). To diagnose a group of calves with *M. bovis*-associated disease, either the cut-off of the BioX ELISA should be adjusted to a lower ODC% level, or another diagnostic method should be applied for the young calves.

No difference was found between calves with different clinical signs of disease; the primary difference was seen between herds, this most likely relates to the age of exposure to *M. bovis.* This suggests that the BioX ELISA reflects exposure in a group of calves, rather than clinical disease in the individual calf. This is in line with the findings of Martin et al. (1990), who concluded that the antibody level in a group of calves (rather than individuals) was indicative of disease being present in the group.

In conclusion, to use and interpret BioX antibody measurements in serum from calves, one should bear in mind: 1) the age of the calves tested, 2) that the antibody response is not related to clinical signs in calves, and 3) that the antibody response is not a good indicator of disease in the individual calf, but is likely to be a good group-level test for exposure to *M. bovis.*

5.4 Comparison of the BioX and MilA ELISA results

Differences and similarities between the antibody responses measured by the BioX and MilA ELISAs in the samples available for in the work presented in this thesis will be discussed in the following section. A schematic overview of diagnostic opportunities for both tests in cows and calves will be also given.

As described in section 3.2, the samples from SampAct#2 were analysed with the in-house MilA ELISA, developed at the University of Melbourne (in addition to the BioX ELISA). Raw data are shown in Figures 4.6 and 4.7. An attempt was made to analyse the MilA ELISA measurements in cows with statistical models for each disease group, as was done for the BioX ELISA measurements. However, the statistical models did not converge, probably due to sparse and highly variable data indicating a dynamic antibody response, with the same cows changing from less than 500 AU to more than 1500 AU and back to 500 AU over three sampling rounds. The lack of evaluation based on statistical modelling is not optimal and means that evaluation of the MilA ELISA in cows cannot be generalised to the same degree as the BioX ELISA. The units and scales on which the two ELISAs are currently applied are very different. The BioX uses a back-ground corrected sample-to-positive control ratio, which results in a semi-quantitative scale, and the antibody units for the MilA ELISA are antibody concentrations calculated based on a series of standards included in the kit (Wawegama et al., 2016), which can lead to more dynamic results. To make more accurate comparisons, both ELISAs should be evaluated on similar scales.

As with the BioX ELISA, the MilA ELISA did not detect antibodies in milk from cows other than the ones with *M. bovis* mastitis. This supports the hypothesis that antibodies in milk are mostly produced locally and the extent of filtering from the serum is minimal. This is interesting, among other reasons because it is different from what is seen for other pathogens where it was found that the Ig in milk was primarily serum-derived (Caffin and Poutrel, 1988; Spier et al., 1991).

Nearly all cows were above the recommended cut-off in the MilA ELISA in serum, regardless of disease group (Figure 4.6). All cows in SampAct#2 were expected to be exposed to *M. bovis* since all herds experienced clinical signs of *M. bovis*-associated disease among cows, and *M. bovis* is an infectious pathogen that is widespread in infected herds (Maunsell and Donovan, 2009). This suggests that the MilA is a more sensitive test, detecting exposure to *M. bovis*. This is supported by others, who found the sensitivity to be 0.943 in feedlot cattle with BRD (Wawegama et al., 2016). However, it is important to question whether the MilA ELISA is sufficiently specific. As all of the herds in SampAct#2 were outbreak herds, the data were not appropriate to quantify the test specificity. In order to do this, *M. bovis*-free herds should be tested and specificity estimates deducted from those. However, the young calves in Herd 3 were nearly all low in antibodies for the first 60 days (Figure 2, Manuscript III), and there were few clinical signs suggestive of *M. bovis* among the calves in this herd, as described in Section 5.3. In combination with generally good management practices and pasteurisation of milk given to the calves, this probably contributed to lower exposure for the calves until around 80 days of age, when the antibody response in the MilA ELISA rose. This suggests that the MilA ELISA might have a reasonable specificity, as also suggested by Wawegama et al. (2016), who estimated the specificity at 0.944 in naturally infected feedlot cattle. Still, further investigationx of truly non-infected cattle populations must be performed.

In contrast to the BioX ELISA, the MilA ELISA detected antibodies in many calves from the two heavily infected herds from as early as around 20 days of age. It therefore seems as though the MilA ELISA can be applied to cattle > 3 weeks of age and used at herd level to classify herds. If the reported sensitivity of 0.90 (Wawegama et al., 2016) is also valid in field studies, fewer animals would be needed for testing in each group if the MilA ELISA were used as a group-level test. However, further studies including statistical modelling are needed to better understand the dynamics of the test results, especially in naïve herds, before it is applied in e.g. control programmes.

Based on the knowledge gained from Manuscripts I-III, Table 5.1 summarises the findings of antibody responses against *M. bovis* in cows and calves using the two different ELISAs, BioX and MilA.

5.5 Association between antibody-positivity and survival in heifers

The applicability of the BioX ELISA in cows and calves in field studies has now been evaluated. To assess the long-term effect of antibody positivity in calves, which is a question often raised by farmers and veterinarians, the following section contains a discussion of the association between *M. bovis* antibody positivity and dairy heifer survival.

The finding that it was primarily the serostatus of the group of young stock that was associated with UED is in agreement with the limited published literature, as described in Manuscript IV. The impact of the direct presence of *M. bovis* has been investigated by others. In a study of respiratory pathogens and their relationship with clinical status and weight gain in dairy calves, only isolation of *M. bovis* from the nasal cavity was associated with a higher odds of clinical signs and lower daily weight gain (Francoz et al., 2015). In a study in white veal calves, respiratory disease and arthritis were associated with higher mortality risk and lower carcass weight. Otitis media alone was not associated with high mortality or decrease in carcass traits in white veal calf production, but the simultaneous presence of arthritis and otitis was associated with lower carcass weight (Pardon et al., 2013). However, caution should be taken to interpret these results, since the number of arthritis and otitis cases were low in that study. Even though the study did not investigate the aetiology of the lesions, *M. bovis* is associated with BRD, arthritis and otitis. Together this suggest that there is a negative effect of having *M. bovis* circulating and causing disease among calves in dairy herds.

The herd-level variable included in the analysis to account for the infection level among the adult cows was the milk prevalence of antibody-positive lactating cows. As discussed in Manuscript IV, it would have been highly relevant to also include the seroprevalence of lactating cows, as this would have added a measure of systemic *M. bovis*-associated disease and not only accounting for the presence of udder-infections. Raaperi et al. (2012) have investigated the association between BRD in calves and different management factors, as well as presence of other infections. They did include the seroprevalence of *M. bovis* antibody positive heifers and cows, but found no association with BRD among the calves. One reason for not finding an association could be the number of animals chosen and sampled. A rather small sample of maximum 25 heifers and 10 cows were randomly sampled. If these were not sick cows, they are not expected to have an elevated antibody response in the BioX ELISA (Manuscript III). A specific description of which animals were sampled lacks, so it

Table 5.1: Summary of the advantages, disadvantages and usefulness of *Mycoplasma bovis* antibody measurements in different materials for diagnosing *Mycoplasma bovis* in dairy herds. The summary is based on results from the studies in this thesis combined with published experimental and observational studies by others as described in sections 5.1-5.4 of this thesis. BioX = BioX K 302, MilA = in-house ELISA developed at University of Melbourne.

	Advantages	**Disadvantages**	**BTM**	**Individual cows**		**Individual calves**
				Serum	***Milk***	***Serum***
BioX ELISA	Commercially available Good specificity Performance on individual and BTM level has been evaluated	Low sensitivity Short antibody response, even in diseased animals	Fairly good association between BTM ODC% and the prevalence of test-positive lactating cows in milk Mainly reflects the presence of *M. bovis* udder infections in the herd	Reflects systemic *M. bovis*-associated disease in the cow when seropositive Not useful for individual diagnosis Useful at group-level to confirm *M. bovis* infection	Reflects *M. bovis* udder infection in the cow when positive	Not useful for calves < 3 months old Not useful for individual diagnosis Useful at group level for calves > 3 months old Cut-off might need to be lowered for calf diagnosis
MilA ELISA	A sensitive test of exposure to *M. bovis*	Not commercially available (soon to be) Further studies are needed to determine the specificity	Studies are lacking	Likely reflects exposure to *M. bovis* and not disease Not useful for individual diagnosis Probably useful for group-level diagnosis	Reflects *M. bovis* udder infection in the cow when positive	Can detect antibodies in calves within 20 days after birth Useful at group level for exposure to *M. bovis*

is difficult to compare and conclude further on whether the sample is representative and indicative of *M. bovis* status of the herd and hence if this is the real association between circulating *M. bovis* among the cows and BRD in calves.

It could be discussed whether another method than the Poisson regression chosen would be more suitable, because there were few events in several of the age groups. Therefore, the association was also analysed using Cox regression, however, the assumption of proportional hazards was violated and is therefore not reported.

The findings of Manuscript IV support the findings of Manuscripts II and III and published literature (Martin et al., 1990; Maunsell et al., 2011; Maunsell and Donovan, 2009) that suggests that the antibody response to *M. bovis* is only useful for group-level diagnostics. In the individual animal the relationship between mycoplasmas and the host varies from commensal to opportunistic or primary pathogens (Maunsell and Donovan, 2009). This shifting between primary and secondary pathogen probably makes it difficult to isolate the effect of *M. bovis* in the individual animal, which supports that the ELISA response in individual animals provides limited information on the health of the tested animals. The co-infections with other pathogens are often seen in older calves and are thought to be responsible for severe and chronic lung lesions (Burki et al., 2015). If *M. bovis* is circulating in a group of animals some will have an antibody response and the seroprevalence will depend on the infection pressure of *M. bovis*, whereas the clinical disease and severity hereof, will also depend on which other pathogens are present.

5.6 Data quality

The epidemiological studies performed in this thesis were based on two sample activities, SampAct#1 and SampAct#2. The following sections will discuss the quality of the data obtained.

5.6.1 Sampling of suspected case and control herds

In SampAct#1, the herds were selected to represent a broad spectrum of clinical signs, infection status and test results. Less was known about *M. bovis* disease outbreaks and diagnostic testing in Denmark at the time when the field studies were initiated than is known today, so it was difficult to present specific inclusion criteria and unify representative groups of herds. However, diversity is also a characteristic of *M. bovis* infections and disease outbreaks. In fact, the diversity in herd type and *M. bovis* occurrence in the data from the 39 enrolled herds made them suitable for evaluating factors that affect the BTM ODC%, interpretation of diagnostic test results and decision-making based on antibody positivity in young stock in a way that would not have been achieved with more restrictive case definitions, while still being representative of the range of infections seen in the Danish cattle population.

Ideally, the BTM samples collected in SampAct#1 should have been collected on the same day as the randomly sampled lactating cows. However, this was not possible due to practicalities such as unpredictable milk truck arrival times. It is likely that this could have influenced the findings of Manuscript I, given the dynamic antibody response in individual cows found in Manuscript II. On

the other hand, narrowing the time span between individual samples and BTM from 30 to 14 days did not influence the results, as described in Manuscript I.

Based on the findings in Manuscript II, the conclusions in Manuscript IV might have been different had it been possible to include a herd-level variable measuring systemic *M. bovis*-associated disease in cows as a measure of infection pressure in the herd, instead of only including the level of *M. bovis* udder infections among the cows. The low number of events observed might also have influenced the result. More events and/or samples from more calves than the cohort of 20 calves would have been preferable.

5.6.2 Sampling in the clinical epidemiology study

The budget for SampAct#2 was limited, leading to either fewer herds being sampled, fewer animals sampled within more herds, or a reduced number of times each animal was sampled. It was considered to be important to follow the antibody dynamics in individual animals closely, as we expected that it was more dynamic than for other diseases. To be able to compare antibody responses among animals in the same herds, a reasonable number of animals from each herd was required. Altogether, given the budget available only four herds could be included, even though more herds would have been preferable. Having said this, due to the rather specific inclusion criteria and the rarity of major *M. bovis* herd outbreaks in Denmark, only six herds were considered for the project over the recruitment period of 9 months, and only four of these were found to be suitable. In addition, this kind of intensive, longitudinal sampling scheme is seldom seen in field studies, and there was limited information about antibody dynamics in the literature. From this perspective, the data available from SampAct#2 are deemed suitable for the performed investigations of antibody dynamics and can form the basis for further investigations of diagnostic test validity and interpretation.

Herd 1 from SampAct#2 differed from the other three herds. The farmer stated that the disease outbreak started 1 month prior to the first herd visit. However, the clinical signs observed by the veterinarians collecting the samples in this herd were less severe than those observed in the other three participating herds. It is possible that other management and health issues masked the true time of the outbreak, which is likely to have been earlier than that stated by the farmer. For this reason, we considered excluding Herd 1 from the analyses. However, after running all models without this herd, we drew the same overall conclusions as when it had been included, but with more uncertainty due to a lower number of samples and test results. We therefore decided to keep Herd 1 in the dataset.

An interesting additional finding from Manuscript II was that the antibody response in cows was apparently high at the time clinical signs were present (Figure 4.4). This could indicate that *M. bovis* was present in the herds for at least 1-2 weeks before causing clinical signs, because antibodies are developed 1-2 weeks after exposure (Byrne et al., 2005; Kanci et al., 2017). The variable 'Days from disease onset' could also have influenced this. Total accuracy could not be obtained because of the three-week intervals between herd visits, and the varying time from onset of the outbreak to the first herd visit. The onset of clinical signs might have been reported later than they first presented, especially if they were detected at the first herd visit, since the outbreak had already lasted for some

time. Despite this uncertainty, it remains likely that *M. bovis* is present for some time in the herd before clinical disease becomes evident.

The herd visits in both sampling activities were performed by a small number of individuals, the samples were delivered to the laboratory within 2 days of sampling, and in general, few samples were missing because of either laboratory or data errors. Although milk samples were not collected during six herd visits in SampAct#1, and the MilA ELISA was not performed on samples from the first herd visit to Herd 1 in SampAct#2, continual data editing resulted in comprehensive datasets that were generally suitable for their purposes.

5.7 Reflections on the clinical epidemiology of *Mycoplasma bovis*

Having worked intensively with many aspects of *M. bovis* over the last 4½ years through herd visits, reading the literature, working with Master thesis students, analysing and discussing data and results of epidemiological analyses, some general considerations worth sharing emerged across all these activities.

When diagnosing *M. bovis,* I generally find it necessary to distinguish between *M. bovis* being present in the herd and herds with disease problems caused by *M. bovis* (e.g. a disease outbreak among many animals in the herd in one or more age groups/sections). These are two very different situations. It is likely that *M. bovis* can be present in many herds without causing disease, while it causes severe disease problems in other herds. Having said this, it is unclear why this difference arises. It could be due to management factors in the herd (Aebi et al., 2015; Fox et al., 2003; Jensen, 2015), characteristics of the bacteria or the host, and most likely a combination of these. This distinction between the presence of bacteria and clinical disease is very important when evaluating diagnostic tests, and a specific purpose and target condition is always important. *M. bovis* is no exception, and a lack of knowledge about many aspects of its pathogenesis and epidemiology further complicates this distinction.

Arthritis and swollen legs were common clinical findings in herds with an outbreak of *M. bovis*-associated disease in both the clinical epidemiology study and in Denmark in general (Jensen, 2015). 'Dual-syndrome' cows (i.e. cows with both arthritis and mastitis) were particularly common in Herd 4 in the clinical epidemiological study. These combined clinical signs are perhaps not well recognised in cows, and the presence of one or a combination of these should alert veterinarians and farmers to investigate whether *M. bovis* might be the cause of disease. Similarly, diagnosis of *M. bovis* (or animals with antibodies against *M. bovis*) within a herd necessitates further investigation of possibly sick animals and additional diagnostics to confirm or rule-out *M. bovis*-associated disease.

M. bovis-associated disease is usually regarded as chronic, yet many cows with arthritis and swollen legs actually recovered over time, especially in Herd 3. The farmer culled cows where it was deemed necessary from a welfare perspective, but kept many of the less severe cases on soft bedding for a period, after which they were returned to the milking herd. The infectiousness of the cows was not assessed, and whether it is wise to let the cows remain in the herd in this respect is

unknown. However, from an animal welfare perspective, it seems as though the cows were capable of recovery.

Severe otitis media and arthritis were present among the calves in Herds 2 and 4. In the published literature, no experimental studies report otitis media and arthritis to the extent seen in natural infections. This may be a result of the different infection routes. Many of the experimental infections are produced by respiratory inoculation, which results in respiratory disease, while Maunsell et al. (2012) developed an oral inoculation model in which several calves developed otitis media, suggesting that this inoculation route might reflect natural exposure more accurately. Several calves in Herd 2 had more severe otitis media and arthritis, and as they were fed unpasteurised milk, it is likely that they were infected orally. One calf had more severe arthrosis in several joints at the age of 4 months, and according to the farmer, the calf was born with swollen joints. It is possible that this calf might have been infected *in utero*, considering the severity of lesions at this early age, the disease history and the fact that *M. bovis* have been isolated from aborted foetuses (Byrne et al., 1999; Hassan and Dokhan, 2004). In addition to pathogenesis considerations in cows (described in section 5.2.1), the pathogenesis in relation to different *M. bovis* disease syndromes should also be investigated in order to gain a better understanding of the diagnostic tests.

The very dynamic nature of the antibody response to *M. bovis* and the clear difference in antibody responses related to different clinical signs were previously unclear. This lack of basic knowledge hindered the author of this thesis during the data analysis for Manuscript I, as well as other authors (Hazelton et al., 2018a; Hazelton et al., 2018b; Nielsen, 2014; Szacawa et al., 2016), making the conclusions on their cross-sectional studies difficult to interpret. This demonstrates the value of basic longitudinal studies before more sophisticated epidemiologic analysis can be applied and interpreted appropriately. If a basic understanding of the material or diagnostic test is lacking, interpretation in different contexts and for different purposes will be challenging, and recommendations based on these might be incomplete.

5.8 Conclusions and recommendations for diagnosing *Mycoplasma bovis* with antibody measurements

The thesis was based on the hypothesis that cattle produce antibodies against *M. bovis,* but the conclusions of the thesis are that the observed antibody responses are dynamic and relatively short-termed and their dependence on clinical disease in cows make diagnosing *M. bovis*–associated disease based on antibody measurements challenging. As many animals with clinical signs of *M. bovis* do not test positive, the BioX ELISA cannot be recommended for individual diagnosis in calves or cows. However, use of the BioX ELISA for diagnosing *M. bovis* at group level for both cows and calves is feasible. However, this requires knowledge about clinical signs, age groups affected and management to be able to choose appropriate sampling materials and the age groups from which to sample. In terms of diagnosing cows using the BioX ELISA, ill cows should be chosen, but when interpreting the results of antibody laboratory results for calves, knowing the age and estimating the likely time of expected exposure is more important. Within each affected herd, the farmer and veterinarian must then decide which animals and diagnostic materials are suitable for diagnosing

the clinical problems specific to that herd. For example, if arthritis among cows is the major problem, serum samples from affected cows should be chosen, while suspected *M. bovis* udder infections require milk samples. This complicates the definition of a general sampling scheme (e.g. for a control programme based on antibody measurements), and it might not be economically feasible to implement if many age and disease syndrome groups must be tested.

The MilA ELISA might be a good alternative test. The ability to detect antibodies that are not maternally derived in very young calves and to detect exposure to *M. bovis* rather than clinical disease makes the MilA ELISA a promising candidate for surveillance purposes. However, further studies are needed to determine the sensitivity and specificity of non-diseased, exposed animals, as well as non-exposed animals and herds.

Table 5.2 summarises the recommendations for investigating whether *M. bovis* is the likely cause of disease in a herd experiencing clinical signs suspected of being associated with *M. bovis* using ELISA. This does not preclude that other diagnostic tests can be preferred in some or more of the below mentioned situations, but it is beyond the scope of this thesis to evaluate other diagnostic tests. The recommendations are based on the research presented in this thesis along with the author's experiences and other published literature. The recommendation for the farmer with seropositive young stock is to focus on initiatives for reducing the *M. bovis* infection among calves in the group, rather than focusing on the individual calf.

Table 5.2: Recommendations for investigating whether *Mycoplasma bovis* is the likely cause of disease in a herd experiencing clinical signs suspected of being caused by *Mycoplasma bovis*, using ELISA. The recommendations are based on the research presented in this thesis along with the author's experiences and other published literature.

Age group	How to sample	How to analyse	Interpretation
Cows	**Serum** samples from a group of cows with clinical signs of systemic disease, e.g. arthritis	BioX K302	**If mean ODC% ≥ 37** = likely *M. bovis*-associated systemic disease among the cows in the herd **If mean ODC% < 37** = not likely that *M. bovis*-associated systemic disease among the cows in the herd **If mean ODC% < 37, but one or more individual cows > 37 ODC%** = inconclusive, *M. bovis*-associated systemic disease might be in the herd, more samples from diseased cows are needed, or combine with alternative tests
	Milk samples from cows with mastitis	BioX K302	**If one or more is ≥ 37 ODC%** = likely *M. bovis* mastitis present in the herd **If all samples are < 37 ODC%** = not likely *M. bovis* mastitis present in the herd

Calves < 3 months of age	**Serum** samples from a group of calves, in which clinical signs are present or exposure is expected	MilA ELISA	**If mean AU ≥ 135** = likely *M. bovis* exposure among the calves in the group **If mean AU < 135** = not likely *M. bovis* exposure among the calves in the group
Calves > 3 months of age	**Serum** samples from a group of calves, in which clinical signs is present or exposure is expected	BioX K302	**If mean ODC% ≥ 37** = likely *M. bovis* exposure among the calves in the herd **If mean ODC% < 37, but calves are expected to be infected at a young age** = inconclusive, *M. bovis* might present among the calves, use another test.
		MilA ELISA	**If mean AU ≥ 135** = likely *M. bovis* exposure among the calves in the group **If mean AU < 135** = not likely *M. bovis* exposure among the calves in the group

6 Perspectives

The results of this thesis have contributed new knowledge about the clinical epidemiology of *M. bovis* and how antibody measurements can be useful in decision-making processes in dairy herds. A better understanding of antibody responses in both individual animals and BTM was gained. However, these are only small pieces in the big *M. bovis* puzzle. Many more questions require answers and new ones have emerged.

Having specific guidelines for testing with ELISA (including sample size considerations and combinations with other test methods) would be very helpful for farmers and veterinarians. In order to provide these, further studies are needed on the number of animals required for testing at group level, with optimisation of sensitivity and specificity at different underlying levels of prevalence of infection and maybe using different cut-off values than recommended today.

Further studies of the MilA ELISA would be interesting and relevant, especially because the ELISA will soon be made commercially available. From the data presented in this thesis, an insight into the short-term dynamics and the ability to differentiate between clinical signs modelled as for the BioX ELISA would aid in our understanding of the MilA ELISA in naturally infected animals. By calculating the ODC% instead of using AU as the unit for test-results, data might be less scattered and fluctuate less over time, and the statistical models explaining differences between disease groups and risk factors might work more effectively. As also mentioned above, further studies in truly non-infected herds are warranted to assess the specificity in under field conditions.

The work presented in this thesis was based on animals displaying clinical disease or measurable antibodies. *M. bovis* is thought to spread through asymptomatic carrier animals. Hence, another interesting research area would be to determine the best testing strategy to identify infectious animals. More knowledge is required on risk factors for development of subclinical carrier animals, determine of how long they are infectious and which factors influences the excretion of *M. bovis*. This would represent an important step in developing a test scheme to certify the risk at herd level and would, together with improved knowledge about the diagnostic tests, make herd-level risk factor studies easier to conduct. All with the aim of preventing spread of *M. bovis* within and among cattle herds and thereby reduce the consequences for animal welfare and farm economics.

The pathogenesis for different clinical syndromes in cows and calves needs more investigation. This would improve the understanding of diagnostic tests and aid in development of control measures.

Further studies are also warranted for decision making in dairy herds. Analysis of other outcomes, e.g. disease among cows is needed. These studies would aid in giving the farmer more advice about how to handle test-positive individual animals, as well as which animals to keep or cull/slaughter during a disease outbreak. This is important information for both economic and welfare reasons.

7 References

Aalbæk, B., B. Kokotovic, H. E. Jensen, J. Agerholm, N. B. Kristensen, R. Thøgersen, K. A. Nielsen, J. Katholm, and K. Krogh. 2012. RAPPORT OM MYCOPLASMA (In danish). Landbrug og Fødevarer.

Adamu, J. Y., N. K. Wawegama, G. F. Browning, and P. F. Markham. 2013. Membrane proteins of *Mycoplasma bovis* and their role in pathogenesis. Res. Vet. Sci. 95(2):321-325.

Aebi, M., B. H. Borne, A. Raemy, A. Steiner, P. Pilo, and M. Bodmer. 2015. *Mycoplasma bovis* infections in Swiss dairy cattle: a clinical investigation. Acta Veterinaria Scandinavica 57(10).

Alberti, A., M. F. Addis, B. Chessa, T. Cubeddu, M. Profiti, S. Rosati, A. Ruiu, and M. Pittau. 2006. Molecular and antigenic characterization of a *Mycoplasma bovis* strain causing an outbreak of infectious keratoconjunctivitis. J. Vet. Diagn. Invest 18(1):41-51.

Allen, J. W., K. G. Bateman, L. Viel, S. Rosendal, and P. E. Shewen. 1991. The microbial flora of the upper and lower respiratory tracts of feedlot calves with undifferentiated bovine respiratory disease. Bovine Practitioner 26:162-168.

Anonymous. 2013. Retningslinier for brug af antibiotika til kvæg i Danmark (In Danish). http://herd. ku. dk/documents/Brug_af_Antibiotika_i_Kvaeg_final_2013_09_05. pdf. Accessed 30-05-18.

Anonymous. 2017a. Medicin til dyr (In Danish), http://medicintildyr.dk/produkt.aspx?soeg=alt&dyr=14&i=768&p=&av=&aks=. Accessed 19-12-17.

Anonymous. 2017b. MYCOPLASMA BOVIS ELISA KIT, http://www.biox.com/en/bio-k-302-monoscreen-abelisa-mycoplasma-bovis-indirect-monowell-p-250/. Accessed 17-11-17.

Anonymous. 2017c. PathoProof® Mastitis Major -3 kit, https://www.thermofisher.com/order/catalog/product/PF0914L?SID=srch-srp-PF0914L. Accessed 05-12-17

Askaa, G., and H. Ernoe. 1976. Elevation of Mycoplasma agalactiae subsp. bovis to species rank: *Mycoplasma bovis* (Hale et al.) comb. nov. International Journal of Systematic Bacteriology 26(3):323-325.

Ayling, R., R. Nicholas, R. Hogg, J. Wessels, S. Scholes, W. Byrne, M. Hill, J. Moriarty, and T. O'Brien. 2005. *Mycoplasma bovis* isolated from brain tissue of calves. Vet. Rec. 156(12):391-392.

Ayling, R. D., S. E. Baker, M. L. Peek, A. J. Simon, and R. A. Nicholas. 2000. Comparison of in vitro activity of danofloxacin, florfenicol, oxytetracycline, spectinomycin and tilmicosin against recent field isolates of *Mycoplasma bovis*. Vet. Rec. 146(26):745-747.

Ayling, R. D., R. S. Rosales, G. Barden, and F. L. Gosney. 2014. Changes in antimicrobial susceptibility of *Mycoplasma bovis* isolates from Great Britain. Veterinary Record. Doi: 101136/vr.102303.

Barrington, G. M., and S. M. Parish. 2001. Bovine neonatal immunology. Veterinary Clinics of North America, Food Animal Practice 17(3):463-476.

Beaudeau, F., S. Assie, H. Seegers, C. Belloc, E. Sellal, and A. Joly. 2001. Assessing the within-herd prevalence of cows antibody-positive to bovine viral diarrhoea virus with a blocking ELISA on bulk tank milk. Veterinary Record 149:236-240.

Becker, C. A. M., F. M. Thibault, M. A. Arcangioli, and F. Tardy. 2015. Loss of diversity within *Mycoplasma bovis* isolates collected in France from bovines with respiratory diseases over the last 35 years. Infection, Genetics and Evolution 33:118-126.

Bennett, R. H., and D. E. Jasper. 1977. Nasal prevalence of *Mycoplasma bovis* and IHA titers in young dairy animals. Cornell Vet. 67(3):361-373.

Bennett, R. H., and D. E. Jasper. 1978a. Bovine mycoplasma mastitis from intramammary inoculation of small numbers of *Mycoplasma bovis*. I. Microbiology and pathology. Veterinary Microbiology 2:341-355.

Bennett, R. H., and D. E. Jasper. 1978b. Systemic and local immune responses associated with bovine mammary infections due to *Mycoplasma bovis*: resistance and susceptibility in previously infected cows. Am. J. Vet. Res. 39(3):417-423.

Bicknell, S. R., G. Jackson, E. Boughton, and C. D. Wilson. 1978. *Mycoplasma bovis* mastitis in the dry cow. Vet. Rec. 102(17):389.

Biddle, M. K., L. K. Fox, and D. D. Hancock. 2003. Patterns of mycoplasma shedding in the milk of dairy cows with intramammary mycoplasma infection. Journal of the American Veterinary Medical Association 223(8):1163-1166.

Bocklisch, H., H. Pfutzner, J. Martin, G. Templin, and S. Kreusel. 1986. [*Mycoplasma bovis* abortion of cows following experimental infection]. Arch. Exp. Veterinarmed. 40(1):48-55.

Boothby, J. T., D. E. Jasper, and C. B. Thomas. 1987. Experimental intramammary inoculation with *Mycoplasma bovis* in vaccinated and unvaccinated cows: effect on local and systemic antibody response. Can. J. Vet. Res. 51(1):121-125.

Buchenau, I., F. Poumarat, G. D. Le, H. Linkner, R. Rosengarten, and M. Hewicker-Trautwein. 2010. Expression of *Mycoplasma bovis* variable surface membrane proteins in the respiratory tract of calves after experimental infection with a clonal variant of *Mycoplasma bovis* type strain PG45. Res. Vet. Sci. 89(2):223-229.

Bundgaard, E. 2005. Registreringernes vej fra gården til Kvægdatabasen (In danish). Dansk Kvæg Kongres 2005:26-27.

Burki, S., J. Frey, and P. Pilo. 2015. Virulence, persistence and dissemination of *Mycoplasma bovis*. Veterinary Microbiology, 179:15-22.

Burki, S., J. Spergser, M. Bodmer, and P. Pilo. 2016. A dominant lineage of *Mycoplasma bovis* is associated with an increased number of severe mastitis cases in cattle. Veterinary Microbiology 196:63-66.

Butler, J. A., S. A. Sickles, C. J. Johanns, and R. F. Rosenbusch. 2000. Pasteurization of discard mycoplasma mastitic milk used to feed calves: thermal effects on various mycoplasma. J. Dairy Sci. 83(10):2285-2288.

Byrne, W., B. Markey, R. McCormack, J. Egan, H. Ball, and K. Sachse. 2005. Persistence of *Mycoplasma bovis* infection in the mammary glands of lactating cows inoculated experimentally. Vet. Rec. 156(24):767-771.

Byrne, W. J., H. J. Ball, N. Brice, R. McCormack, S. E. Baker, R. D. Ayling, and R. A. Nicholas. 2000. Application of an indirect ELISA to milk samples to identify cows with *Mycoplasma bovis* mastitis. Vet. Rec. 146(13):368-369.

Byrne, W. J., P. Brennan, R. McCormack, and H. J. Ball. 1999. Isolation of *Mycoplasma bovis* from the abomasal contents of an aborted bovine fetus. Vet. Rec. 144(8):211-212.

Caffin, J. P., and B. Poutrel. 1988. Physiological and Pathologocal Factors Influencing Bovine Immunoglobulin G2 Concentrations in Milk. Journal of Dairy Science 71(8):2035-2043.

Cai, H. Y., P. Bell-Rogers, L. Parker, and J. F. Prescott. 2005. Development of a real-time PCR for detection of *Mycoplasma bovis* in bovine milk and lung samples. J. Vet. Diagn. Invest 17(6):537-545.

Chima, J. C., B. N. Wilkie, K. H. Nielsen, H. L. Ruhnke, R. B. Truscott, G. Maxie, and B. Chick. 1981. Synovial immunoglobulin and antibody in vaccinated and nonvaccinated calves challenged with *Mycoplasma bovis*. Can. J. Comp Med. 45(1):92-96.

Clothier, K. A., D. M. Jordan, C. J. Thompson, J. M. Kinyon, T. S. Frana, and E. L. Strait. 2010. *Mycoplasma bovis* real-time polymerase chain reaction assay validation and diagnostic performance. J. Vet. Diagn. Invest 22(6):956-960.

Feenstra, A., M. E. Bisgaard, N. F. Friis, A. Meyling, and P. Ahrens. 1991. A field study of *Mycoplasma bovis* infection in cattle. Zentralbl. Veterinarmed. B 38(3):195-202.

Fox, L. K. 2012. Mycoplasma mastitis: causes, transmission, and control. (Special Issue: Mastitis in dairy cows.). Vet. Clin. North Am. Food Anim Pract. 28:225-237.

Fox, L. K., D. D. Hancock, A. Mickelson, and A. Britten. 2003. Bulk tank milk analysis: factors associated with appearance of Mycoplasma sp. in milk. Journal of Veterinary Medicine B 50:235-240.

Fox, L. K., F. J. Muller, M. L. Wedam, C. S. Schneider, and M. K. Biddle. 2008. Clinical *Mycoplasma bovis* mastitis in prepubertal heifers on 2 dairy herds. Can. Vet. J. 49(11):1110-1112.

Francoz, D., S. Buczinski, A. M. Belanger, G. Forte, O. Labrecque, D. Tremblay, V. Wellemans, and J. Dubuc. 2015. Respiratory pathogens in Quebec dairy calves and their relationship with clinical status, lung consolidation, and average daily gain. Journal of Veterinary Internal Medicine 29:381-387.

Francoz, D., G. Fecteau, A. Desrochers, and M. Fortin. 2004. Otitis media in dairy calves: a retrospective study of 15 cases (1987 to 2002). Can. Vet. J. 45(8):661-666.

Fredriksen, B., T. Sandvik, T. Loken, and S. A. Odegaard. 1999. Level and duration of serum antibodies in cattle infected experimentally and naturally with bovine virus diarrhoea virus. Veterinary Record, 144:111-114.

Friis, N. F. 1984. Mycoplasma-bovis-induced mastitis in cattle in Denmark. Nord. Vet. Med. 36(9-10):324-325.

Friis, N. F., and H. V. Krogh. 1983. Isolation of mycoplasmas from Danish cattle. Nord. Vet. Med. 35:74-81.

Fu, P., Z. Sun, Y. Zhang, H. Zhang, D. Su, F. Jiang, and W. Wu. 2014. Development of a direst competitive ELISA for the detection of *Mycoplasma bovis* infection based on a monocloncal antibody of P48 protein. BMC Veterinary Research 10(42).

Gagea, M. I., K. G. Bateman, R. A. Shanahan, D. T. van, B. J. McEwen, S. Carman, M. Archambault, and J. L. Caswell. 2006. Naturally occurring *Mycoplasma bovis*-associated pneumonia and polyarthritis in feedlot beef calves. J. Vet. Diagn. Invest 18(1):29-40.

Gharagozlou, M. J., J. Najafi, A. H. Tabatabayi, and P. Khazrainia. 2004. A pathologic and microbiologic study on bovine arthritis associated with Mycoplasma spp. Archives of Razi Institute 58:97-104.

Gille, L., P. Pilo, B. R. Valgaeren, L. v. Driessche, H. v. Loo, M. Bodmer, S. Burki, F. Boyen, F. Haesebrouck, P. Deprez, and B. Pardon. 2016. A new predilection site of *Mycoplasma bovis*: postsurgical seromas in beef cattle. Veterinary Microbiology 186:67-70.

Gonzalez, R. N., and D. J. Wilson. 2003. Mycoplasmal mastitis in dairy herds. Vet. Clin. North Am. Food Anim Pract. 19(1):199-221.

Gosselin, V. B., D. Francoz, M. Babkine, A. Desrochers, S. Nichols, E. Dore, C. Bedard, J. Parent, J. H. Fairbrother, and G. Fecteau. 2012. A retrospective study of 29 cases of otitis media/interna in dairy calves. Can. Vet. J. 53(9):957-962.

Gourlay, R. N., and S. B. Houghton. 1985. Experimental pneumonia in conventionally reared and gnotobiotic calves by dual infection with *Mycoplasma bovis* and Pasteurella haemolytica. Res. Vet. Sci. 38(3):377-382.

Grissett, G. P., B. J. White, and R. L. Larson. 2015. Structured literature review of responses of cattle to viral and bacterial pathogens causing bovine respiratory disease complex. Journal of Veterinary Internal Medicine 29:770-780.

Haapala, V., T. Pohjanvirta, N. Vähänikkilä, J. Halkihlati, H. Simonen, S. Pelkonen, T. Soveri, H. Simojoki, and T. Autio. 2018. Semen as a source of *Mycoplasma bovis* mastitis in dairy herds. Veterinary Microbiology 216:60-66.

Hanzlicek, G. A., B. J. White, D. G. Renter, D. E. Anderson, and R. L. Larson. 2011. Associations between the prevalence of Mollicutes and *Mycoplasma bovis* and health and performance in stocker calves. Vet. Rec. 168(1):21.

Hassan, N. I., and K. Z. Dokhan. 2004. Isolation of Mycoplasma from bovine aborted fetuses and weak calves, with reference to histopathological findings and detection of the causative species in fresh frozen tissue sections, using immunofluorescent tests. Assiut Veterinary Medical Journal 50(103):148-167.

Hazelton, M. S., J. M. Morton, K. L. Bosward, P. A. Sheehy, A. M. Parker, C. J. Dwyer, P. G. Niven, and J. K. House. 2018a. Isolation of *Mycoplasma* spp. and serological responses in bulls prior to and following their introduction into *Mycoplasma bovis*-infected dairy herds. Journal of Dairy Science 101:1-13.

Hazelton, M. S., P. A. Sheehy, K. L. Bosward, A. M. Parker, J. M. Morton, C. J. Dwyer, P. G. Niven, and J. K. House. 2018b. Short communication: Shedding of *Mycoplasma bovis* and antibody responses in cows recently diagnosed with clinical infection. Journal of Dairy Science 101(1):584-589.

Henderson, J. P., and H. J. Ball. 1999. Polyarthritis due to *Mycoplasma bovis* infection in adult dairy cattle in Northern Ireland. Vet. Rec. 145(13):374-376.

Heuvelink, A., C. Reugebrink, and J. Mars. 2016. Antimicrobial susceptibility of *Mycoplasma bovis* isolates from veal calves and dairy cattle in the Netherlands. Veterinary Microbiology 189:1-7.

Holzhauer, M., and E. v. Engelen. 2016. Mycoplasma - the Dutch experience of an emerging disease? Proceedings of the Cattle Lameness Conference 2016:19-22.

Houe, H. 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Veterinary Microbiology 64(2-3):89-107.

Houe, H., A. Lindberg, and V. Moennig. 2006. Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. Journal of Veterinary Diagnostic Investigation 18:427-436.

Houlihan, M. G., B. Veenstra, M. K. Christian, R. Nicholas, and R. Ayling. 2007. Mastitis and arthritis in two dairy herds caused by *Mycoplasma bovis*. Vet. Rec. 160(4):126-127.

Howard, C. J., and R. N. Gourlay. 1983. Immune response of calves following the inoculation of Mycoplasma dispar and *Mycoplasma bovis*. Vet. Microbiol. 8(1):45-56.

Howard, C. J., K. R. Parsons, and L. H. Thomas. 1986. Systemic and local immune responses of gnotobiotic calves to respiratory infection with *Mycoplasma bovis*. Vet. Immunol. Immunopathol. 11(3):291-300.

Jain, N. C., D. E. Jasper, and J. D. Dellinger. 1967. Experimental bovine mastitis due to Mycoplasma. Cornell Veterinarian 59:10-28.

Jasper, D. E., N. C. Jain, and L. H. Brazil. 1966. Clinical and laboratory observations on bovine mastitis due to Mycoplasma. Journal of the American Veterinary Medical Association 148(9):1017-1029.

Jensen, L. 2015. Outbreak characteristics and identification of risk factors for *Mycoplasma bovis* outbreaks in Danish dairy herds 2010-2014 (in danish). Veterinary master thesis, University of Copenhagen.

Justice-Allen, A., J. Trujillo, G. Goodell, and D. Wilson. 2011. Detection of multiple Mycoplasma species in bulk tank milk samples using real-time PCR and conventional culture and comparison of test sensitivities. J. Dairy Sci. 94(7):3411-3419.

Kanci, A., N. K. Wawegama, M. S. Marenda, P. D. Mansell, G. F. Browning, and P. F. Markham. 2017. Reproduction of respiratory mycoplasmosis in calves by exposure to an aerolised culture of *Mycoplasma bovis*. Veterinary Microbiology 210:167-173.

Khalil, D., C. A. M. Becker, and F. Tardy. 2017. Monitoring the decrease in susceptibility to ribosomal RNAs targeting antimicrobials and its molecular basis in clinical *Mycoplasma bovis* isolates over time. Microbial Drug Resistance 23(6):799-811.

Kinde, H., B. M. Daft, R. L. Walker, B. R. Charlton, and R. Petty. 1993. *Mycoplasma bovis* associated with decubital abscesses in Holstein calves. J. Vet. Diagn. Invest 5(2):194-197.

Kirby, F. D., and R. A. Nicholas. 1996. Isolation of *Mycoplasma bovis* from bullocks' eyes. Vet. Rec. 138(22):552.

Klein, U., A. d. Jong, H. Moyaert, F. El-Garch, R. Leon, A. Richard-Mazet, M. Rose, D. Maes, A. Pridmore, J. R. Thomson, and R. D. Ayling. 2017. Antimicrobial susceptibility monitoring of Mycoplasma hyopneumoniae and *Mycoplasma bovis* isolated in Europe. Veterinary Microbiology 204:188-193.

Kusiluka, L. J., B. Ojeniyi, and N. F. Friis. 2000. Increasing prevalence of *Mycoplasma bovis* in Danish cattle. Acta Vet. Scand. 41(2):139-146.

Lamm, C. G., L. Munson, M. C. Thurmond, B. C. Barr, and L. W. George. 2004. Mycoplasma otitis in California calves. J. Vet. Diagn. Invest 16(5):397-402.

Langford, E. V. 1975. Mycoplasma recovered from bovine male and female genitalia and aborted foeti. Proceedings of the 18th Annual Meeting of the American Association of Veterinary Laboratory Diagnosticians 18:221-232.

Levisohn, S., S. Garazi, I. Gerchman, and J. Brenner. 2004. Diagnosis of a mixed mycoplasma infection associated with a severe outbreak of bovine pinkeye in young calves. J. Vet. Diagn. Invest 16(6):579-581.

Lysnyansky, I., and R. D. Ayling. 2016. *Mycoplasma bovis*: mechanisms of resistance and trends in antimicrobial susceptibility. Frontiers in Microbiology 7:595.

Maeda, T., T. Shibahara, K. Kimura, Y. Wada, K. Sato, Y. Imada, Y. Ishikawa, and K. Kadota. 2003. *Mycoplasma bovis*-associated suppurative otitis media and pneumonia in bull calves. J. Comp Pathol. 129(2-3):100-110.

Mahmood, F., A. Khan, R. Hussain, I. A. Khan, R. Z. Abbas, H. M. Ali, and M. Younus. 2017. Patho-bacteriological investigation of an outbreak of *Mycoplasma bovis* infection in calves - emerging stealth assault. Microbial Pathogenesis 107:404-408.

Martin, S. W., K. G. Bateman, P. E. Shewen, S. Rosendal, and J. E. Bohac. 1989. The frequency, distribution and effects of antibodies, to seven putative respiratory pathogens, on respiratory disease and weight gain in feedlot calves in Ontario. Can. J. Vet. Res. 53(3):355-362.

Martin, S. W., K. G. Bateman, P. E. Shewen, S. Rosendal, J. G. Bohac, and M. Thorburn. 1990. A group level analysis of the associations between antibodies to seven putative pathogens and respiratory disease and weight gain in Ontario feedlot calves. Can. J. Vet. Res. 54(3):337-342.

Maunsell, F., M. B. Brown, J. Powe, J. Ivey, M. Woolard, W. Love, and J. W. Simecka. 2012. Oral inoculation of young dairy calves with *Mycoplasma bovis* results in colonization of tonsils, development of otitis media and local immunity. PLoS. One. 7(9):e44523.

Maunsell, F. P., and G. A. Donovan. 2009. *Mycoplasma bovis* Infections in young calves. Vet. Clin. North Am. Food Anim Pract. 25(1):139-77.

Maunsell, F. P., G. A. Donovan, C. Risco, and M. B. Brown. 2009. Field evaluation of a *Mycoplasma bovis* bacterin in young dairy calves. Vaccine 27(21):2781-2788.

Maunsell, F. P., A. R. Woolums, D. Francoz, R. F. Rosenbusch, D. L. Step, D. J. Wilson, and E. D. Janzen. 2011. *Mycoplasma bovis* infections in cattle. J. Vet. Intern. Med. 25(4):772-783.

Nicholas, R. A. 2011. Bovine mycoplasmosis: silent and deadly. Vet. Rec. 168(17):459-462.

Nicholas, R. A., and R. D. Ayling. 2003. *Mycoplasma bovis*: disease, diagnosis, and control. Res. Vet. Sci. 74(2):105-112.

Nicholas, R. A., R. D. Ayling, and L. P. Stipkovits. 2002. An experimental vaccine for calf pneumonia caused by *Mycoplasma bovis*: clinical, cultural, serological and pathological findings. Vaccine 20(29-30):3569-3575.

Nicholas, R. A. J., and S. Baker. 1998. Recovery of mycoplasmas from animals. Pages 37-44 in Mycoplasma protocols. R. J. Miles and R. A. J. Nicholas, ed. Humana Press, Totowa.

Nicholas, R. A. J., L. K. Fox, and I. Lysnyansky. 2016. Mycoplasma mastitis in cattle: to cull or not to cull. Veterinary Journal 216:142-147.

Nielsen, L. R. 2003. *Salmonella* Dublin in dairy cattle: Use of diagnostic tests for investigation of risk factors and infection dynamics. PhD Thesis PhD Thesis, The Royal Veterinary and Agricultural University, The Royal Veterinary and Agricultural University.

Nielsen, L. R. 2014. Report on investigations aiming to improve the interpretation and use of *Mycoplasma bovis* diagnostics in cattle herds by use of the PathoProof for PCR-testing and Bio-X Diagnostics *Mycoplasma bovis* ELISA kit for antibody testing. Internal report, University of Copenhagen.

Nielsen, P. K., M. B. Petersen, L. R. Nielsen, T. Halasa, and N. Toft. 2015. Latent class analysis of bulk tank milk PCR and ELISA testing for herd level diagnosis of *Mycoplasma bovis*. Preventive Veterinary Medicine, 121:338-342.

Nielsen, S. S., N. Toft, and I. A. Gardner. 2011. Structured approach to design of diagnostic test evaluation studies for chronic progressive infections in animals. Veterinary Microbiology 150:115-125.

Pardon, B., J. Alliet, R. Boone, S. Roelandt, B. Valgaeren, and P. Deprez. 2015. Prediction of respiratory disease and diarrhea in veal calves based on immunoglobulin levels and the serostatus for respiratory pathogens measured at arrival. Preventive Veterinary Medicine 120:169-176.

Pardon, B., M. Hostens, L. Duchateau, J. Dewulf, K. d. Bleecker, and P. Deprez. 2013. Impact of respiratory disease, diarrhea, otitis and arthritis on mortality and carcass traits in white veal calves. BMC Veterinary Research 9(79).

Parker, A. M., S. Ankit, J. K. House, M. S. Hazelton, K. L. Bosward, B. Kokotovic, and P. A. Sheehy. 2016. Genetic characterization of Australian *Mycoplasma bovis* isolates through whole genome sequencing analysis. Veterinary Microbiology 196:118-125.

Parker, A. M., J. K. House, M. S. Hazelton, K. L. Bosward, J. M. Morton, and P. A. Sheehy. 2017a. Bulk tank milk antibody enzyme-linked immunosorbent assay as a biosecurity tool for detecting dairy herds with past exposure to *Mycoplasma bovis*. Journal of Dairy Science, 100:1-14.

Parker, A. M., J. K. House, M. S. Hazelton, K. L. Bosward, and P. A. Sheehy. 2017b. Comparison of culture and a multiplex probe PCR for identifying Mycoplasma species in bovine milk, semen and swab samples. PLoS One 12(3):e0173422.

Pfutzner, H., and K. Sachse. 1996. *Mycoplasma bovis* as an agent of mastitis, pneumonia, arthritis and genital disorders in cattle. Rev. Sci. Tech. 15(4):1477-1494.

Pinnow, C. C., J. A. Butler, K. Sachse, H. Hotzel, L. L. Timms, and R. F. Rosenbusch. 2001. Detection of *Mycoplasma bovis* in preservative-treated field milk samples. J. Dairy Sci. 84(7):1640-1645.

Poumarat, F., G. D. Le, S. Philippe, D. Calavas, F. Schelcher, P. Cabanie, P. Tessier, and H. Navetat. 2001. Efficacy of spectinomycin against *Mycoplasma bovis* induced pneumonia in conventionally reared calves. Vet. Microbiol. 80(1):23-35.

Prysliak, T., J. van der Merwe, and J. Perez-Casal. 2013. Vaccination with recombinant *Mycoplasma bovis* GAPDH results in a strong humoral immune response but does not protect feedlot cattle from an experimental challenge with M. bovis. Microb. Pathog. 55:1-8.

Punyapornwithaya, V., L. K. Fox, D. D. Hancock, J. M. Gay, and J. R. Alldredge. 2010. Association between an outbreak strain causing *Mycoplasma bovis* mastitis and its asymptomatic carriage in the herd: a case study from Idaho, USA. Prev. Vet. Med. 93(1):66-70.

R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Raaperi, K., S. Bougeard, A. Aleksejev, T. Orro, and A. Viltrop. 2012. Association of herd BHV-1 seroprevalence with respiratory disease in youngstock in Estonian dairy cattle. Res. Vet. Sci. 93(2):641-648.

Romvary, J., J. Rozsa, L. Stipkovits, and J. Meszaros. 1977a. Incidence of diseases due to *Mycoplasma bovis* in a cattle herd. I. Pneumo-arthritis syndrome in calves. Acta Vet. Acad. Sci. Hung. 27(1-2):29-37.

Romvary, J., J. Rozsa, L. Stipkovits, and J. Meszaros. 1977b. Incidence of diseases due to *Mycoplasma bovis* in a cattle herd. II. Experimental therapy of the pneumo-arthritis syndrome of calves. Acta Vet. Acad. Sci. Hung. 27(1-2):39-45.

Rosengarten, R., C. Citti, M. Glew, A. Lischewski, M. Droeße, P. Much, F. Winner, M. Brank, and J. Spergser. 2000. Host-pathogen interactions in mycoplasma pathogenesis: Virulence and survival strategies of minimalist prokaryotes. International Journal of Medical Microbiology 290(1):15-25.

Ruhnke, H. L., D. Thawley, and F. C. Nelson. 1976. Bovine mastitis in Ontario due to Mycoplasma agalactiae subsp. bovis. Can. J. Comp Med. 40(2):142-148.

Schibrowski, M. L., T. S. Barnes, N. K. Wawegama, M. E. Vance, P. F. Markham, P. D. Mansell, M. S. Marenda, A. Kanci, J. Perez-Casal, G. F. Browning, J. S. Gibson, and T. J. Mahony. 2018. The

Performance of Three Immune Assays to Assess the Serological Status of Cattle Experimentally Exposed to *Mycoplasma bovis*. Veterinary Sciences 5(27).

Schnee, C., M. Heller, E. Schubert, and K. Sachse. 2015. Point prevalence of infection with Mycoplasma bovoculi and Moraxella spp. in cattle at different stages of infectious bovine keratoconjunctivitis. Veterinary Journal 203:92-96.

Soehnlen, M. K., A. Aydin, K. S. Murthy, E. J. Lengerich, A. L. Hattel, B. A. Houser, G. D. Fenton, H. R. Lysczek, C. M. Burns, A. M. Townsend, J. W. Brooks, D. R. Wolfgang, and B. M. Jayarao. 2012. Epidemiology of *Mycoplasma bovis* in Pennsylvania veal calves. Journal of Dairy Science 95(1):247-254.

Spier, S. J., B. P. Smith, J. S. Cullor, H. J. Olander, L. Da Roden, and G. W. Dilling. 1991. Persistent experimental Salmonella dublin intramammary infection in dairy cows. Journal of Veterinary Internal Medicine 5(6):341-350.

Stalheim, O. H., and L. A. Page. 1975. Naturally occurring and experimentally induced mycoplasmal arthritis of cattle. J. Clin. Microbiol. 2(3):165-168.

Stipkovits, L., J. Meszaros, B. Pazmany, and Z. Varga. 1983. Isolation of mycoplasmas from bull semen and serological examination of aborted cows sera for presence of mycoplasma antibodies. Archiv fur Experimentelle Veterinarmedizin 37:429-433.

Stipkovits, L., M. Rady, and R. Glavits. 1993. Mycoplasmal arthritis and meningitis in calves. Acta Vet. Hung. 41(1-2):73-88.

Strube, M. L., and M. Lindegaard. 2018. Whole genome comparisons of *Mycoplasma bovis* isolates in Denmark. Internal report, Technical University of Denmark.

Szacawa, E., K. Niemczuk, K. Dudek, D. Bednarek, R. Rosales, and R. Ayling. 2015. *Mycoplasma bovis* infections and co-infections with other Mycoplasma spp. with different clinical manifestations in affected cattle herds in eastern region of Poland. Bulletin of the Veterinary Institute in Pulawy, 59:331-337.

Szacawa, E., M. Szymanska-Czerwinska, K. Niemczuk, K. Dudek, D. Bednarek, and R. D. Ayling. 2016. Comparison of serological, molecular and cultural diagnostic methods for the detection of *Mycoplasma bovis* infections in cattle. Animal Science Papers and Reports, 60:391-397.

Taurel, A. F., R. Guatteo, A. Joly, and F. Beaudeau. 2012. Relationship between the level of antibodies in bulk tank milk and the within-herd seroprevalence of Coxiella burnetii in cows. Epidemiology and Infection 140:1710-1713.

Tegtmeier, C., A. Uttenthal, N. F. Friis, N. E. Jensen, and H. E. Jensen. 1999. Pathological and microbiological studies on pneumonic lungs from Danish calves. Zentralbl. Veterinarmed. B 46(10):693-700.

Thomas, L. H., C. J. Howard, E. J. Stott, and K. R. Parsons. 1986. *Mycoplasma bovis* infection in gnotobiotic calves and combined infection with respiratory syncytial virus. Vet. Pathol. 23(5):571-578.

Van Donkersgoed, J., C. S. Ribble, L. G. Boyer, and H. G. Townsend. 1993. Epidemiological study of enzootic pneumonia in dairy calves in Saskatchewan. Can. J. Vet. Res. 57(4):247-254.

Virtala, A. M. K., Y. T. Grohn, G. D. Mechor, H. N. Erb, and E. J. Dubovi. 2000. Association of seroconversion with isolation of agents in transtracheal wash fluids collected from pneumonic calves less than three months of age. Bovine Practitioner; 34(2):77-80.

Walz, P. H., T. P. Mullaney, J. A. Render, R. D. Walker, T. Mosser, and J. C. Baker. 1997. Otitis media in preweaned Holstein dairy calves in Michigan due to *Mycoplasma bovis*. J. Vet. Diagn. Invest 9(3):250-254.

Warnick, L. D., L. R. Nielsen, J. Nielsen, and M. Greiner. 2006. Simulation model estimates of test accuracy and predictive values for the Danish Salmonella surveillance program in dairy herds. Preventive Veterinary Medicine 77:284-303.

Wawegama, N. K., and G. F. Browning. 2017. Improvements in diagnosis of disease caused by *Mycoplasma bovis* in cattle. Animal Production Science, 57(7):1482-1487.

Wawegama, N. K., G. F. Browning, A. Kanci, M. S. Marenda, and P. F. Markham. 2014. Development of a recombinant protein-based enzyme-linked immunosorbent assay for diagnosis of *Mycoplasma bovis* infection in cattle. Clinical and Vaccine Immunology 21(2):196-202.

Wawegama, N. K., P. F. Markham, A. Kanci, M. Schibrowski, S. Oswin, T. S. Barnes, S. M. Firestone, T. J. Mahony, and G. F. Browning. 2016. Evaluation of an IgG enzyme-linked immunosorbent assay as a serological assay for detection of *Mycoplasma bovis* infection in feedlot cattle. Journal of Clinical Microbiology 54(5):1269-1275.

Wilson, D. J., R. T. Skirpstunas, J. D. Trujillo, K. B. Cavender, C. V. Bagley, and R. L. Harding. 2007. Unusual history and initial clinical signs of *Mycoplasma bovis* mastitis and arthritis in first-lactation cows in a closed commercial dairy herd. J. Am. Vet. Med. Assoc. 230(10):1519-1523.

Worsøe, T. 2011. "Køerne døde, og vi anede ikke hvad de fejlede" (In Danish). Landbrugsavisen http://www.lvk.dk/upload/fckeditor/file/Koerne_doede_og_vi_anede_ikke.pdf. Accessed 30-05-18.

Zhang, R., X. Han, Y. Chen, R. Mustafa, J. Qi, X. Chen, C. Hu, H. Chen, and A. Guo. 2014. Attenuated *Mycoplasma bovis* strains provide protection against virulent infection in calves. Vaccine 32:3107-3114.

8 Manuscripts

Manuscript I
Petersen, M. B., Krogh, K. and Nielsen, L. R. 2016. Factors associated with variation on bulk tank milk *Mycoplasma bovis* antibody-ELISA results in dairy herds.
Journal of Dairy Science. 99:3815-3823.

Manuscript II
Petersen, M. B., Pedersen, J., Holm, D. L., Denwood, M. and Nielsen, L. R. 2018. A longitudinal observational study of the dynamics of *Mycoplasma bovis* in naturally exposed and diseased dairy cows.
In press: doi.org/10.3168/jds.2017-14340

Manuscript III
Petersen, M. B., Wawegama, N. K., Denwood, M., Markham, P. F., Browning, G. F. and Nielsen, L. R. *Mycoplasma bovis* antibody dynamics in naturally exposed dairy calves according to two diagnostic tests. Submitted to BMC Veterinary Research.

Manuscript IV
Petersen, M. B., Ersbøll, A. K., Krogh, K. and Nielsen, L. R. Increased incidence rate of undesired early heifer departure in *Mycoplasma bovis*-antibody positive Danish dairy cattle herds.
Submitted to Epidemiology and Infection.

8.1 Manuscript I

Factors associated with variation in bulk tank milk *Mycoplasma bovis* antibody-ELISA results in dairy herds

Mette B. Petersen*, Kaspar Krogh† and Liza R. Nielsen*

*University of Copenhagen, Faculty of Health and Medical Sciences, Department of Large Animal Sciences, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark
†Kaspar Krogh, Veterinary Cattle Specialist, 8560 Kolind, Denmark

Corresponding author: Mette Bisgaard Petersen, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark, Phone +45 35 32 06 94, Fax +45 35 28 30 22, e-mail mbp@sund.ku.dk.

The manuscript has been published in *Journal of Dairy Science*, 2016, 99:3815-3823.

Abstract

The relevance and limitations for using measurements of antibodies against *Mycoplasma bovis* (*M. bovis)* in bulk tank milk (BTM) as a potentially cost-effective diagnostic tool for herd classification has not been evaluated before. Assuming that an increasing or high sero-prevalence is a result of on-going or recent spread of *M. bovis* in a dairy herd, we tested the hypothesis that increasing prevalences of antibody positive cows and young stock are associated with increasing BTM antibody ELISA values against *M. bovis* in Danish dairy herds with different courses of *M. bovis* infection. Furthermore, we tested whether herd size was associated with variations in the BTM responses.

Thirty-nine Danish dairy herds selected to represent four different herd level infection groups (8 control herds, 14 acute outbreak herds, 7 herd with previous outbreaks and 10 herds with elevated BTM ELISA-values directed against *M. bovis* (>64 ODC%)) were visited 4-5 times approximately 3 m apart. At each visit 65 young stock were blood sampled. At the milk recording date closest to the herd visit date, 50 milk recording samples from individual lactating cows were randomly selected. In addition a BTM sample was collected as a representative sample directly from the bulk tank by the dairies' milk truck drivers as part of the mandatory milk quality control scheme. Blood and milk samples were tested for antibodies against *M. bovis* with a commercially available ELISA test (Bio-X BIO K 302, Bio-X Diagnostics, Rochefort, Belgium).

A linear mixed effects model was used to analyse the effects of the prevalence of antibody positive lactating cows and young stock and herd size on the BTM *M. bovis* ELISA results. Herd was included as a random effect to account for clustering of BTM samples originating from the same herd.

Increasing prevalence of antibody positive lactating cows was the only variable associated with increasing *M. bovis* BTM ELISA optical density measurement (ODC%). In contrast, the prevalence of antibody positive young stock did not correlate with the BTM ODC%.

In conclusion, some *M. bovis* associated herd infections are detectable by BTM ELISA-testing, but there are limitations and further investigations of the effect of different clinical disease expressions in the herds are warranted.

Key words: *Mycoplasma bovis*, enzyme-limked immunosorbent assay (ELISA), bulk tank milk, antibody.

Introduction

Mycoplasma bovis (*M. bovis*) can cause severe disease and production losses in both dairy and beef producing cattle herds. In adult cattle *M. bovis* infection is often associated with mastitis, but also arthritis and pneumonia can be seen. In calves the typical disease manifestations are otitis media, pneumonia and/or arthritis (Maunsell et al., 2011). *M. bovis* seems to be an emerging pathogen in countries all over the world, and even though *M. bovis* was first isolated in Denmark in 1981 (Friis, 1984), it has not been considered a major pathogen in Danish cattle prior to 2011. However, the Danish cattle industry has had increased focus on this infection over the last couple of years due to an increase in the number of severe outbreaks of *M. bovis* associated disease on herd-level.

Traditionally *M. bovis* has been detected by bacteriological culture (BC) from either individual milk samples or bulk tank milk (BTM) samples. In recent years detection by polymerase chain reaction (PCR) has become more widely used, since it is less time consuming and apparently can produce similar sensitivity and specificity to conventional BC methods (Pinnow et al., 2001; Cai et al., 2005). At individual level, antibodies directed against *M. bovis* can be detected in serum and milk 1-2 weeks after uptake of the bacteria (Boothby et al., 1987; Byrne et al., 2005), but the use for diagnosis in individual animals is not always straight forward (Maunsell et al., 2011). *M. bovis* can also be isolated from asymptomatic carrier animals (Punyapornwithaya et al., 2010), but it is not known how the antibody response in these animals reacts compared to clinically ill animals. However, in beef cattle, group-level antibody titers and seroconversion can be associated with active infection (Martin et al., 1990), and spread of the disease in a dairy herd could therefore be expected to lead to a marked increase in seroprevalence. Except for Nielsen et al. (2015), who evaluated the performance of an antibody detecting enzyme linked immunosorbent assay (ELISA) against PCR for BTM for national screening purposes, the use of antibodies in BTM for diagnosing either disease or presence of *M. bovis* in specific dairy herds has not been addressed in published literature. Antibody measurements on BTM have been used as a diagnostic tool for the control of other infectious diseases, because it can be easy and inexpensive to use in national surveillance programs (Lindberg and Alenius, 1999; Nielsen, 2013). But in order to use antibodies against *M. bovis* in BTM for surveillance purposes it is essential to know which factors influence the antibody level in BTM.

The use of ELISA on BTM samples to classify or monitor dairy herds for *M. bovis* infection will, in a setting such as the Danish, be of interest since the sampling can be automated via a mandatory milk quality control scheme, and is inexpensive compared to BC and PCR. A requirement for BTM antibody testing to be useful is that there must be a good correlation between the BTM antibody level and the prevalence of infection in individual cattle in the herd. In the case of other infectious diseases, such as Salmonella Dublin, bovine virus diarrhea virus and Q-fever, it has been shown that the level of antibodies in the BTM correlates well with the within-herd prevalence of antibody positive cows (Nielsen and Ersbøll, 2005; Muskens et al., 2011; Taurel et al., 2012). Increasing herd size has been shown to be a risk factor for presence of *M. bovis* infection (Thomas et al., 1981; Pinho et al., 2013). On the other hand, antibodies might be diluted in herds with a large number of cows contributing milk to the BTM (Nekouei et al. 2015). Hence, herd size may have to be taken into account when evaluating BTM testing for herd diagnosis.

The objective of this study was to test the hypothesis that increasing within-herd prevalence of antibody positive lactating cows and increasing seroprevalence in young stock increases the BTM antibody ELISA values against *M. bovis* in Danish dairy herds. Furthermore, we wanted to test whether herd size affected the level of antibodies in BTM.

Materials and Methods

Populations

The target population was all Danish dairy herds enrolled in the voluntary milk recording system (https://www.landbrugsinfo.dk/Kvaeg/RYK/Sider/RYK_English.aspx), which at the beginning of the study period consisted of approximately 3000 (90% of all) Danish dairy herds. Their average annual milk yield per cow was 9,663 kg milk and the average herd size was 166 lactating cows. The study population consisted of herds about which the Knowledge Centre for Agriculture (now SEGES, Aarhus, Denmark) had prior knowledge about *M. bovis* associated disease either from farmers or veterinarians. SEGES is the merger of the former Knowlegde Centre for Agriculture and the Danish Pig Research Centre, effective as per 1 January 2015. The company is owned by the farmers and provides knowledge, consultancy and technology to all Danish farmers (https//www.seges.dk/en).

Only herds with more than 100 dairy cows were included. More than 100 cows were needed to make sure the herd had enough young stock to sample. The study population consisted of 39 dairy herds selected by a veterinarian at SEGES during the period March 2013-February 2014. The veterinarian at SEGES had prior knowledge about the herds from national screenings in 2012 and 2013, where BTM from all dairy herds were tested for antibodies against *M. bovis* and with PCR, as well as information provided by the local consulting veterinarian in the herds. To ensure collection of data from herds with different severity and duration of disease, the following criteria were used to select herds to fit into 1 of 4 groups prior to enrollment in the field data collection part of the study:

Control herds: negative in diagnostic tests (PCR, ELISA and BC), no history of clinical signs that could be related to *M. bovis* over the past 3 years; 8 herds.

Case herds – acute: Recent clinical suspicion of disease associated with *M. bovis,* 14 herds. In these 14 herds, the presence of *M. bovis* was confirmed by positive *M. bovis* PCR [PathoProof, Thermo Fisher Scientific, Waltham, MA: with a cycle threshold (Ct) <37] in milk samples from individual cows and/or BTM. In 4 herds the confirmation was performed in BTM, in 5 herds at individual cow level, and in 5 herds at both individual cow level and in BTM. In addition, 5 of the 14 herds were positive for *M. bovis* in BC of samples from individual animals.

Case herds – previous: Previous clinical suspicion of disease associated with *M. bovis;* 7 herds. This group included herds with former *M. bovis* test positive clinically ill animals, but that no longer had any acutely diseased animals. In these 7 herds, the presence of *M. bovis* was confirmed by positive PCR [PathoProof PCR (Ct < 37)] in milk samples from individual cows and BTM in 3 herds, and in BTM in 4 herds.

Case herds – BTM: High ELISA value against *M. bovis* in BTM (Bio-X Bio K 302, Bio-X Diagnostics, Rochefort, Belgium; ELISA value >64 optical density measurement) in a national screening in summer 2013; 10 herds.

The selection of farms was done as described above to ensure representation of all types of clinical signs, infection and test patterns in the study herds so that the full scale of BTM and seroprevalences were represented in the data set for analysis. The allocation to groups was not used in the analyses.

The distribution of BTM optical density (ODC%) measurements from the different herds over time, divided into the abovementioned 4 categories are shown in Figure 1. We aimed to include herds of different sizes and geographical locations. However, systematic stratification according to these factors was not used. More than 90 % of the Danish dairy cattle are located on the peninsula of Jutland, and all herds enrolled in this study were located in Jutland. Because the prevalence of *M. bovis* infection is low, the selection criteria were used to ensure inclusion of herds with evidence of disease and/or spread of *M. bovis*.

Each herd was visited 4-5 times approximately 3 mo apart. At each visit 65 young stock equally distributed in the age group 0-12 mo old were blood sampled. At the milk recording date closest to the herd visit date, 50 milk recording samples from individual lactating cows were randomly selected. A BTM was sampled as a representative sample while the bulk tank was emptied by the dairies' milk truck drivers as part of the mandatory milk quality control scheme.

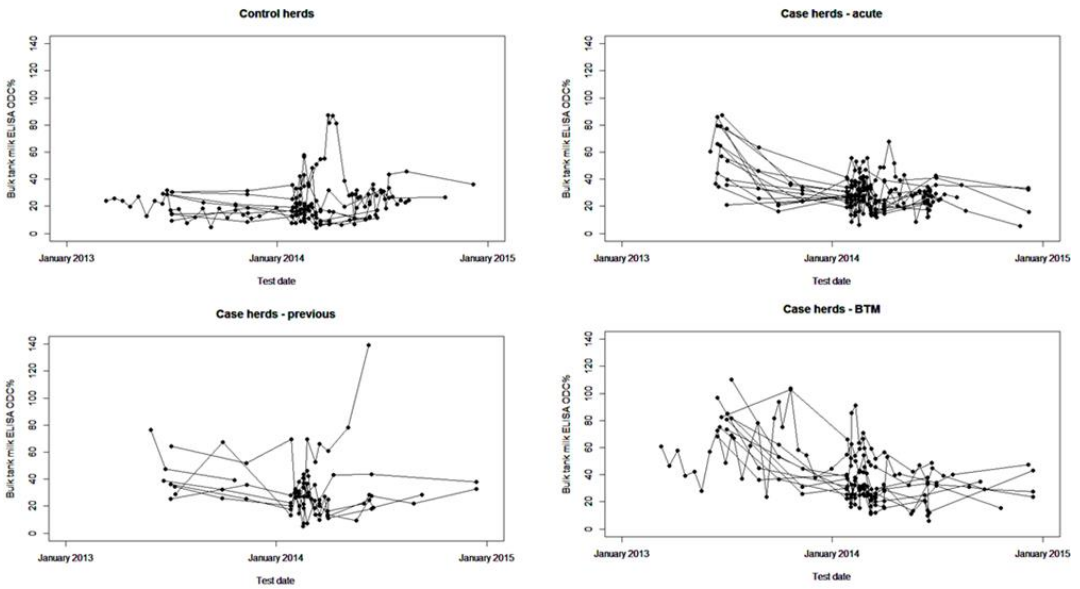


Figure 1. Distribution of bulk tank milk (BTM) ELISA *optical density measurements* (ODC%) of antibodies against *Mycoplasma bovis* in herds initially selected as control herds, case herds with acute outbreaks, case herds with previous outbreak and case herds with high BTM. The lines connect results from the same herd.

Detection of Antibodies

Milk samples from both individual animals and BTM, and serum samples from the young stock were analyzed for antibodies against *M. bovis* using the commercial kit Bio-X BIO K 302 *Mycoplasma bovis* ELISA kit at Eurofins-Steins Laboratory (Holstebro and Vejen, Denmark). A sample coefficient was calculated as: ODC% = (OD sample - OD negative control)/(OD positive control - OD negative control) x 100 %, where OD is the optical density measured by the ELISA reader for each test sample, and negative and positive control samples on the sample ELISA plate. For animal-level testing a sample coefficient ≥ 37 ODC% was considered positive, and a sample coefficient < 37 ODC% was considered negative according to the recommendations of the manufacturer of the ELISA kit. The test has to the authors' knowledge not been evaluated with regard to sensitivity (Se) and specificity (Sp) for animal level diagnosis in the field.

It has been evaluated for use on BTM in national screening of dairy herds for national or regional prevalence estimation by Nielsen et al. (2015). The Se and Sp at cut-off 37 ODC% were 60.4 and 97.3, respectively. At a cut-off of 50 ODC% the Se 43.5 and the Sp was 99.6.

Description of Variables

The outcome variable was the continuous *M. bovis* BTM ODC%. Four explanatory variables were tested as potential explanatory variables of the *M. bovis* BTM ODC%.

- *The apparent prevalence of antibody positive lactating cows:* This variable was calculated as the proportion of cows with individual-ELISA ODC% ≥ 37 in milk out of all tested cows in the herd on the sampling d.
- *The apparent prevalence of antibody positive young stock*: This variable was calculated as the proportion of young stock with individual-ELISA ODC% ≥ 37 in blood out of all tested young stock in the herd on the sampling d.
- *The apparent prevalence of antibody positive lactating cows > 50 ODC%:* To assess if there was an effect of the ELISA cut-off used for apparent prevalence calculations, the apparent prevalence was also calculated as the proportion of cows with individual-ELISA ODC% > 50 (ELISA50) in milk.
- *Herd Size:* Herd size was calculated as the average number of cows in the herd, in the quarter of the yr where the BTM sample was collected.

An observation was excluded if it was not possible to match the date of the apparent prevalence with a BTM sample within ±30 days or if the number of animals for the prevalence calculations was low (n < 30).

Statistical Analysis

Scatter plots of all the explanatory variables plotted against each other were assessed in order to evaluate whether there were linear relationships between the variables. Variables which were highly correlated ($\rho > 0.8$) were not included in the same model.

Two linear mixed effects models were created. The models were built by backwards stepwise elimination of non-significant variables and their 2-way interactions. The criteria for keeping a variable in the model was $p < 0.05$, and the model fit was assessed by Akaike´s Information Criteria (AIC), the lower AIC the better model. The *p*-values were calculated as an ANOVA comparison

between a model with all variables and a model without the specific variable and its interaction terms.

Herd was included as a random effect to account for clustering of BTM samples originating from the same herd. The explanatory degree of the model was assessed by calculation of the ratio: (R_e-R_{fm}/R_e), where R_e is the residual variance of the model only containing the random effect of herd and R_{fm} is the residual variance of the final model. Data management and analyses were made using "R: A language and environment for statistical computing" version 3.0.2 (www.r-project.org).

Results

Descriptive Statistics

Data selection yielded 113 observations distributed on 37 herds with 2-5 observations per herd, on average 3 observations per herd. Descriptive statistics of the outcome, *M. bovis* BTM ODC% and explanatory variables are shown in Table 1. A visual presentation of the raw data is provided in Figure 2, where the BTM ELISA ODC% is plotted against the apparent prevalence of antibody positive lactating cows.

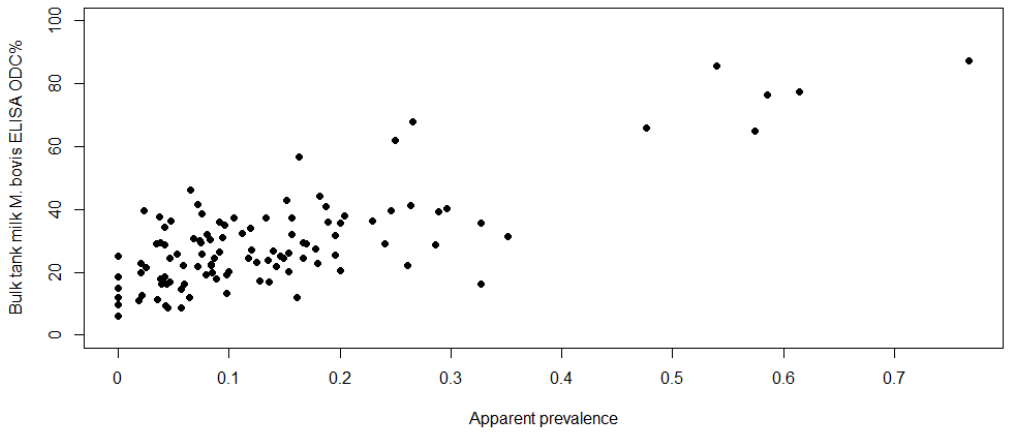


Figure 2. Descriptive statistics showing the bulk tank milk *Mycoplasma bovis* (*M. bovis*) ELISA *optical density measurement* (ODC%) is plotted against the apparent prevalence of antibody positive lactating cows.

When adding the prevalence of antibody positive young stock to the dataset, many observations were lost when limiting the prevalence calculation to ±30 days from the BTM date. Therefore another dataset was created that only contained the prevalence of antibody positive young stock and the BTM samples closest to the date of the prevalence calculation (n=116). Descriptive statistics of the young stock prevalence are shown in Table 2. From Figure 3 it is apparent that the prevalence

of antibody positive young stock did not correlate well with the BTM *M. bovis* ELISA ODC%, and the variable was therefore not included in further analysis.

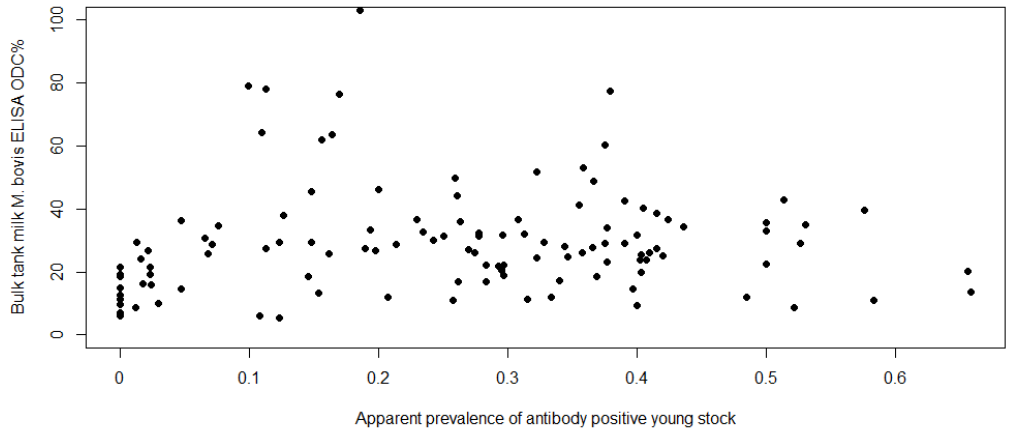


Figure 3. Bulk tank milk *Mycoplasma bovis* (*M. bovis*) ELISA *optical density measurement (*ODC%) plotted against the apparent prevalence of antibody positive young stock.

Table 1. Descriptive statistics of bulk tank milk (BTM) *Mycoplasma bovis* (*M. bovis*) ELISA *optical density measurement* (ODC%) and the explanatory variables tested in models for BTM M. bovis ELISA ODC% in 37 herds (113 observations).

	Min	Q1	Median	Q3	Max
BTM *M. bovis* ELISA ODC%					
	6	19	26	36	87
Prevalence of antibody positive lactating cows (≥37 ODC%)					
	0	0.04	0.1	0.18	0.77
Prevalence of antibody positive lactating cows (>50 ODC%)					
	0	0.02	0.05	0.1	0.49
Herd size					
	76	201	273	367	779

Table 2. Descriptive statistics of the prevalence of *Mycoplasma bovis* antibody positive young stock (≥37 *optical density measurement* (ODC%)) in 39 herds (116 observations).

Clinical signs	Min	Q1	Median	Q3	Max
Prevalence of antibody positive young stock (≥37 ODC%)					
	0.00	0.12	0.28	0.38	0.66

Analytical Statistics

Collinearity was found between the apparent prevalence of antibody positive lactating cow and ELISA50, which were consequently not tested simultaneously, but with the same explanatory variables in different models.

The resulting final model included only the apparent prevalence of antibody positive lactating cows. The model had the AIC closest to 0 and showed the best prediction when evaluating the plots of predicted vs. observed values visually. The final model explained 54% of the variation (Table 3).

The predicted *M. bovis* ELISA ODC% in BTM is plotted against the observed values in Figure 4. Overall the model predicted the BTM values well, eventhough be a tendency towards overestimation of the high values, and underestimation of the low BTM values may have occured.

Table 3. Results of the final model describing explanatory variables and random effects of bulk tank milk (BTM) ELISA *optical density measurement (*ODC%) for *Mycoplasma bovis*

Variables (Explains 54% of the variation)			
Random effects	Variance		S.D.
Herd	19		4
Residuals	80		9
Fixed effects	Estimate	S.E.	P-value
BTM ELISA ODC% (intercept)	17	1.4	-
Prevalence of AB positive lactating cows (per 10% increase)	9	0.7	< 0.001

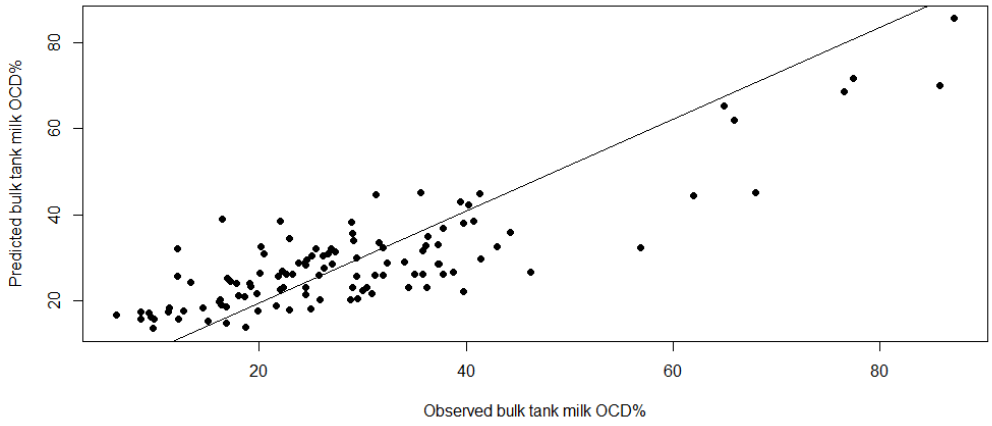


Figure 4. Predicted bulk tank milk *Mycoplasma bovis* ELISA *optical density measurement* (ODC%) values plotted against observed values for the model (n=113). The line shows the regression line between observed and predicted values.

Discussion

Our objective was to test the associations of different factors with the variation in BTM antibodies against *M. bovis* in Danish dairy herds. We found that a rather large proportion of the variation could be explained by the apparent prevalence of antibody positive lactating cows.

The prevalence of antibody positive lactating cows was positively associated with the BTM ODC%. Each time the prevalence increased by 10% the BTM ODC% increased by 9 ODC%. This means that with increasing number of antibody positive cows in the herd, indicative of recent spread of *M. bovis* bacteria, we can expect the BTM ODC% to increase. This association is in agreement with other studies on other infectious diseases in dairy herds (Nielsen and Ersbøll, 2005; Muskens et al., 2011; Taurel et al., 2012). For *Salmonella* Dublin, Nielsen and Ersbøll (2005) in addition found that the degree of explanation increased when including the prevalence or number of high ELISA-responders and whether or not the herd had had a positive BC for *Salmonella* Dublin. In our study, the prevalence of high ELISA-responders could not be included in the same model as the prevalence and unfortunately we did not have sufficient BC-results for *M. bovis* from all farms or comprehensive and consistant systematic recordings of clinical disease associated with *M. bovis* in individual animals, which would have been interesting to study the effect of.

Eventhough the prevalence of antibody positive cows is associated with the BTM ODC%, it is more ambiguous than seen with other diseases. In our dataset and according to our final model, the prevalence of antibody positive cows was above 30% before the BTM on average went above the cut-off of 37 ODC% (Table 3 and Figure 2) indicating that a large proportion of the cows had to have been exposed to *M. bovis* to make the BTM antibody testing able to detect it with reasonably Se and

Sp (Nielsen et al., 2015). This hampers the ability to classify herds based on a BTM sample. A more persistent pattern has been found for *Coxiella burnetii* measurements in BTM where all samples above the cut-off value had a within-herd prevalence of at least 20% (Muskens et al., 2011). The discrepancy may arise because many *M. bovis* clinically diseased and medically treated cows do not contribute to the bulk tank. The apparent prevalence in our study stems from samples from individual cows at milk recording. Most of these cows would have contributed to the BTM on the day they were sampled. A minor part of medically treated cows could also have been part of milk recording, but the milk from those cows would not have entered the BTM due to procedures for preventing antibiotic residues entering the milk for consumption.

As mentioned in the introduction, the use of antibodies to detect disease among individual animals is not straight forward, and clinical disease is not always followed by a rise in antibodies (Maunsell et al., 2011). Unfortunately, evaluation of antibody reactions in individual animals in field studies is sparse. On group level, however, antibody titers show correlation with disease in beef cattle (Martin et al., 1990), which would suggest that the same could be the case for dairy herds. There is also a lack of investigations of the correlation between antibodies in milk and serum in the literature, but the manufacturer of the used ELISA test states in a data-sheet about the test that the correlation is 0.59 (http//www.biox.com/Default.aspx?tabid=64&udtid=215). In an unpublished field study (L. Nielsen, University of Copenhagen, Frederiksberg, Denmark) from Denmark 1442 paired serum and milk samples from 8 dairy herds had a correlation of 0.7. When considering the different clinical manifestations of *M. bovis* disease, it could be that antibodies in milk are not a good measure of on-going disease in a dairy herd. A better understanding of the correlations between different clinical signs, extretion of bacteria and serum and milk antibodies would help interpret the BTM antibody response.

Herd size was not associated with the BTM ODC% in herds in this study. Other studies have found an increasing probability of isolating *M. bovis* by BC from the BTM with increased number of lactating cows (Thomas et al., 1981; Pinho et al., 2013). This is probably related to the different outcomes in the studies, and the fact that in our model the presence of *M. bovis* is already taken into account by the within-herd prevalence. Our study investigated the factors associated with variance in BTM ODC%, while the other studies have investigated risk factors for a BC-positive BTM. With increasing herd size there is a risk that the contribution of antibodies to the BTM by 1 cow becomes diluted *(Nekouei et al. 2015)*. For *Salmonella* Dublin a better explanation of the BTM ODC% was found when using the mean yield-corrected ODC%, also indicating a dilution effect in the BTM (Nielsen and Ersbøll, 2005). This was not the case in our study.

As mentioned earlier, *M. bovis* can give rise to a variety of clinical signs in different age groups; thus a BTM sample may or may not be able to detect all types of disease manifestations in a herd. Two questions arise from this: (1) is it possible to detect disease among young stock in the BTM, and (2) is it possible to detect all types of disease manifestations among cows in the BTM. We included the prevalence of antibody positive young stock as an explanatory variable to partially clarify this issue. The prevalence of antibody positive young stock did not correlate with the BTM ELISA ODC%, indicating that the status of young stock is not reflected in the BTM. Hence, to determine the status of the young stock, samples from individual animals are probably needed. Further studies on this matter are definitely warranted.

The other part of this question is whether or not disease among cows manifested primarily as e.g. arthritis will be detectable in a BTM sample. Unfortunately, we do not have systematically recorded information about the prevalence of the different disease manifestations in the different herds, so this issue cannot be further elucidated in this study. Further studies where the distinction in the expression of clinical disease can be made are warranted.

Another model with the prevalence of lactating cows based on ELISA50 as the explanatory variable instead of the prevalence at the recommended cut-off at 37 ODC% was tried. This did not change the model fit when the other explanatory variables were the same (results not shown). The reason for exploring the effect of changing the cut-off is that there is a lack of evidence for the optimal ELISA cut-off at animal-level with regard to detection of infected or infectious animals within infected herds. A higher cut-off might detect more truly infected animals as opposed to previously exposed animals, and hence the ELISA50-prevalence might be better correlated with the BTM-antibody level. However, this did not seem to be the case. We did not try with high cut-off values, because there were few cows with higher ELISA-responses.

To the best of the authors' knowledge no studies have evaluated antibodies in BTM as a diagnostic tool for *M. bovis* in relation to the underlying disease manifestation in dairy herds. Nielsen et al. (2015) evaluated the overall performance of the BTM-test method for national or regional screening purposes and provided stimates of Se, Sp and predictive values. However, the estimates were associated with much uncertainty due to few test-positive herds in the dataset. The results of that study and the present study complement each other. Our study illustrates that the lack of Se may be due to the fact that quite high prevalences of affected animals are required for the BTM antibody level to increase. As discussed above the results from our study are in overall agreement with similar studies about other infectious diseases such as *Salmonella* Dublin, bovine viral diarrhea virus and *Coxiella burnetii* infections (Nielsen and Ersbøll, 2005; Muskens et al., 2011; Taurel et al., 2012). However, we also found some challenges that have to be addressed in order to use BTM-ELISA testing as a tool in herd level *M. bovis* diagnosis of dairy herds.

In most instances, the prevalence estimates were not based on the same date of sampling, but within ±30 d of the BTM sample. Hence, we cannot be certain that milk from all the individual cows used for calculating the prevalence was present in the BTM sample. To evaluate the limitation of this, a dataset consisting of 87 of the observations (75%) sampled within ±14 d of the BTM sample were used to rerun the final model. This rerun model yielded approximately the same estimates as the model based on the larger dataset, and did not make the predictions for the model better. Hence, our final model appeared to be robust to the uncertainties in the prevalence estimation related to the time of BTM sampling. In individual animals the antibody response can persist for at least 6 month (Nicholas et al. 2002). Nontheless, from our data it seems to be important to realize that the BTM antibody level is actually quite dynamic, and a high response in BTM does not necessarily persist for long time (Figure 1).

The repeated measurements in theory have a temporal structure, but this was ignored and a simple random effect used because any temporal effects from such a small number of repeated measurements were considered to be uninteresting and to have a small effect on the data. In addition, our primary interest was not to describe the nature of the dependency between the BTM-

measurements, so the random effect was merely included to take potential dependencies into account in order not to overestimate the effect of the explanatory variables in the final model.

Conclusions

The objective was to identify factors that influence the variation in BTM ELISA ODC% against *M. bovis* in Danish dairy herds. Increasing prevalence of antibody positive cows was associated with increasing *M. bovis* BTM ELISA ODC%. In contrast, the prevalence of antibody positive young stock did not correlate with the BTM ODC%. Herd size was not associated with M. bovis BTM ELISA ODC%. A combination with distinction between different clinical signs would be very interesting, but the available data did not support such investigation. More studies to investigate risk factors for variance in BTM ELISA ODC% for *M. bovis* and potential combinations of test-procedures to use for herd classifications are warranted before this method can be deemed useful for disease control purposes.

Acknowledgements

Thank you to all the farmers participating in the *M. bovis* project. Thanks to veterinarians Ulla Torpe (Dyrlægerne Egtved, Egtved, Denmark), Johanne Juul (DanHatch, Vrå, Denmark) and Camilla Eskerod Kristensen (Kvægdyrlægerne Djursland, Grenå, Denmark) for contribution to the *M. bovis* project including collection of samples and outbreak information from the herds. We also take the opportunity to thank two peer-reviewers for comments that helped us improve the manuscript. The Danish Milk and Cattle Levy Funds provided funding for the sampling and laboratory testing. The funding source was not involved in the study design, analyses and interpretation, nor in the decision to publish the study.

References

Boothby, J. T., D. E. Jasper, and C. B. Thomas. 1987. Experimental intramammary inoculation with *Mycoplasma bovis* in vaccinated and unvaccinated cows: effect on local and systemic antibody response. Can. J. Vet. Res. 51(1):121-125.

Byrne, W., B. Markey, R. McCormack, J. Egan, H. Ball, and K. Sachse. 2005. Persistence of *Mycoplasma bovis* infection in the mammary glands of lactating cows inoculated experimentally. Vet. Rec. 156(24):767-771.

Cai, H. Y., P. Bell-Rogers, L. Parker, and J. F. Prescott. 2005. Development of a real-time PCR for detection of *Mycoplasma bovis* in bovine milk and lung samples. J. Vet. Diagn. Invest 17(6):537-545.

Friis, N. F. 1984. Mycoplasma-bovis-induced mastitis in cattle in Denmark. Nord. Vet. Med. 36(9-10):324-325.

Lindberg, A. L. E., and S. Alenius. 1999. Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. Vet. Microbiol. 64:197-222.

Martin, S.W., K. G. Bateman, P. E. Shewen, S. Rosendal, J. G. Bohac and M. Thorburn. 1990. A Group Level Analysis of the Associations Between Antibodies to Seven Putative Pathogens and Respiratory Disease and Weight Gain in Ontario Feedlot Calves. Can J Vet Res. 54:337-342.

Maunsell, F. P., A. R. Woolums, D. Francoz, R. F. Rosenbusch, D. L. Step, D. J. Wilson and E. D. Janzen. 2011. *Mycoplasma bovis* infections in cattle. J. Vet. Intern. Med. 25(4):772-783.

Muskens, J., E. v. Engelen, C. v. Maanen, C. Bartels, and T. J. G. M. Lam. 2011. Prevalence of *Coxiella burnetii* infection in Dutch dairy herds based on testing bulk tank milk and individual samples by PCR and ELISA. Vet. Rec. 168:79.

Nicholas, R. A. J., R. D. Ayling and L. P. Stipkovits. 2002. An experimental vaccine for calf pneumonia by *Mycoplasma bovis*: clinical, culture, serological and pathological findings. Vaccine. 20:3569-3575.

Nekouei, O., H. Stryhn, J. VanLeeuwen, D. Kelton, P. Hanna and G. Keefe. 2015. Predicting within-herd prevalence of infection with bovine leukemia virus using bulk-tank milk antibody levels. Prev. Vet. Med. Article in press.

Nielsen, L. R. 2013. Review of pathogenesis and diagnostic methods of immediate relevance for epidemiology and control of *Salmonella* Dublin in cattle. Vet. Microbiol. 162:1-9.

Nielsen, L. R., and A. K. Ersbøll. 2005. Factors associated with variation in bulk-tank-milk *Salmonella* Dublin ELISA ODC% in dairy herds. Prev. Vet. Med. 68:165-179.

Nielsen, P. K., M. B. Petersen, L. R. Nielsen, T. Halasa and N. Toft. 2015. Latent class analysis of bulk tank milk PCR and ELISA testing for herd level diagnosis of *Mycoplasma Bovis.* Prev. Vet. Med. 121(3-4):338-342.

Pinho, L., G. Thompson, M. Machado, and J. Carvalheira. 2013. Management practices associated with the bulk tank milk prevalence of *Mycoplasma* spp. in dairy herds in Northwestern Portugal. Prev. Vet. Med. 108(1):21-27.

Pinnow, C. C., J. A. Butler, K. Sachse, H. Hotzel, L. L. Timms, and R. F. Rosenbusch. 2001. Detection of *Mycoplasma bovis* in preservative-treated field milk samples. J. Dairy Sci. 84(7):1640-1645.

Punyapornwithaya, V., L. K. Fox, D. D. Hancock, J. M. Gay, and J. R. Alldredge. 2010. Association between an outbreak strain causing *Mycoplasma bovis* mastitis and its asymptomatic carriage in the herd: a case study from Idaho, USA. Prev. Vet. Med. 93(1):66-70.

Taurel, A. F., R. Guatteo, A. Joly, and F. Beaudeau. 2012. Relationship between the level of antibodies in bulk tank milk and the within-herd seroprevalence of *Coxiella burnetii* in cows. Epidemiol. Infect. 140:1710-1713.

Thomas, C. B., P. Willeberg, and D. E. Jasper. 1981. Case-control study of bovine mycoplasmal mastitis in California. Am. J. Vet. Res. 42(3):511-515.

8.2 Manuscript II

A longitudinal observational study of the dynamics of *Mycoplasma bovis* antibodies in naturally exposed and diseased dairy cows

Mette B. Petersen,[1] Jeanette Pedersen,[2] Dinah L. Holm,[3] Matthew Denwood, and Liza R. Nielsen

Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark

[1] Corresponding author: Mette Bisgaard Petersen, Grønnegårdsvej 8, 1870 Frederiksberg C, Denmark, Phone +45 35 32 06 94, Fax +45 35 28 30 22, e-mail mbp@sund.ku.dk.
[2] Present affiliation: Øster Jølby Dyreklinik, Udvejen 3, 7950 Erslev, Denmark
[3] Present affiliation: Dyrlæge Center Vest, Herningvej 74 A, 6950 Ringkøbing, Denmark

The manuscript has been published in *Journal of Dairy Science,* 2018, doi.org/10.3168/jds.2017-14340

Abstract

In cattle, *Mycoplasma bovis (M. bovis)* is an important pathogen causing disease and substantial economic losses. However, knowledge of the dynamics of antibody responses in individual cows in the face of an outbreak is currently extremely limited. The use of commercial antibody tests to support clinical decision making and for surveillance purposes is therefore challenging.

Our objective was to describe the dynamics of *M. bovis* antibody responses in four Danish dairy herds experiencing an acute outbreak of *M. bovis* associated disease, as well as to compare the antibody dynamics between dairy cows with different disease manifestations. A total of 120 cows were examined using a standardized clinical protocol and categorized into four disease groups: 'Mastitis', 'Systemic', 'Non-specific' and 'None'. Paired blood and milk samples were collected and tested using a commercial *M. bovis* antibody detecting ELISA. Plots of raw data and generalized additive mixed models with cow and herd as random effects were used to describe serum and milk antibody dynamics relative to the estimated time of onset of clinical disease.

Cows with mastitis had high optical density measurement (ODC%) of antibodies in both milk and serum at disease onset. The estimated mean ODC% in milk was below the manufacturer's cut-off for the other groups for the entire study period. The estimated mean serum ODC% in the 'Systemic' group was high at onset of disease and stayed above the cut-off until 65 d after disease onset. However, the lower 95% confidence interval (CI) for the mean ODC% was only above the manufacturers' cut-off between 7 to 17 d after onset of disease. The CI of the 'Systemic' and 'None' groups did not overlap at any time between the d of disease onset and 65 d after disease onset, and the estimated mean ODC% for both the 'Non-specific' and 'None' groups were generally below the cut-off for the majority of the study period. In conclusion, the serum antibody responses were highly dynamic and showed a high level of variation between individual cows. This strongly suggests that serology is unlikely to be useful for individual diagnosis of *M. bovis* associated disease in dairy cows. However, it might still be useful for herd- or group-level diagnosis. Antibodies in milk were only increased in cows with *M. bovis* mastitis, indicating that milk antibody measurements only have diagnostic utility for cows with mastitis.

Keywords: *Mycoplasma bovis*, ELISA, antibody, BioX Bio K 302, dairy cow

Introduction

Mycoplasma bovis (*M. bovis*) is an infectious disease of cattle that is associated with a diverse spectrum of clinical signs and substantial production losses worldwide (Nicholas, 2011). In cows, *M. bovis* typically causes mastitis, arthritis and pneumonia (Maunsell et al., 2011), whereas pneumonia, otitis media and arthritis are more commonly seen in calves (Maunsell and Donovan, 2009). The preferred method of diagnosing *M. bovis* has historically been bacteriological culture of body fluids (e.g. milk and joint fluids) or swabs (e.g. eye and nasal swabs) from individual animals, as well as bacteriological culture of bulk tank milk for herd-level diagnosis. Even though PCR is becoming more frequently used due to reductions in cost and processing time (Wawegama and Browning, 2017), these methods can be poorly sensitive in practice due to intermittent shedding patterns, and difficulties in obtaining the best sample materials from the infected animals on which to apply these tests. Therefore there is an increasing interest in using serology for *M. bovis* diagnostics.

In Denmark, serological assays such as ELISA are frequently used for testing dairy cows, because they are inexpensive and convenient, especially if applicable to milk samples routinely collected for other purposes. Two commercial ELISA kits are available from BioX Diagnostics in Belgium; one of these (BioX Bio K 260) has been shown to have little correlation with the occurrence of disease and with PCR and bacterial culture results (Szacawa et al., 2015; Szacawa et al., 2016). However, these studies aimed mainly to compare antibody measurements to other diagnostic tests using cross-sectional study designs that are not suitable for the assessment of dynamics and persistence of antibodies. Nielsen et al (2015) found that for the BioX Bio K 302 ELISA it might be beneficial to raise the cut-off for herd-level diagnosis to 50 ODC% in bulk tank milk to increase the specificity, but no field study evaluations of cut-off values at animal level have been published. Therefore, the cut-off of 37 ODC% as recommended by the manufacturer is used in practice despite the lack of substantial documentation for the validity of this threshold.

Appropriate interpretation of ELISA test results requires knowledge of the dynamics and duration of excretion of antibodies against *M. bovis* relative to the time of infection and onset of associated disease. However, as recently pointed out by Hazelton et al. (2018), there is currently a limited understanding of the dynamics of antibodies directed against *M. bovis* in terms of time to seroconversion and longevity in naturally exposed cattle. The antibody response has been shown to remain high in both milk and serum for as long as 20 weeks after both experimental inoculation in the udder and naturally occurring *M. bovis*-associated mastitis (Boothby et al., 1987; Byrne et al., 2000). However, the *M. bovis* antibody response to systemic disease such as arthritis has not yet been fully described. Work in calves vaccinated against *M. bovis* with an experimental vaccine at the age of three weeks showed that the animals appeared to seroconvert within 14 d, and that a high IgG level was maintained for at least 42 d in serum (Nicholas et al., 2002). Apart from this work in individual animals, serology has been suggested to be useful for herd-level diagnostics (Le Grand et al., 2002; Martin et al., 1990).

Further investigation of ELISA test result patterns in milk and serum from naturally exposed and diseased dairy cows is therefore warranted. In particular to understand how the ELISA response can be expected to develop over time in animals with varying clinical signs of *M. bovis*-associated disease compared to exposed animals without overt clinical signs. The objective of this study was therefore to describe the temporal dynamics of antibody responses to *M. bovis* in serum and milk

taken from individual dairy cows in herds experiencing a *M. bovis* disease outbreak, with a particular emphasis on differences in these patterns between groups of animals exhibiting different disease manifestations.

Materials and Methods

Study Design

This study was a longitudinal observational study in Danish dairy cattle herds. To fulfill the objective of describing dynamics of antibody responses in exposed animals, only herds experiencing acute outbreaks of *M. bovis*- associated disease within the study period were eligible for inclusion. This was based on the likely presence of *M. bovis*-associated disease as diagnosed by the herd advisory veterinarian based on positive PCR or ELISA test results, and an outbreak was defined as having several animals with clinical signs of either mastitis (Maunsell et al., 2011), arthritis and/or subcutaneous swelling of the limbs (Henderson and Ball, 1999; Wilson et al., 2007) and/or pneumonia (Maunsell et al., 2011) and positive *M. bovis* ELISA or PCR tests.

Four herds were identified as matching these criteria and permission was obtained from each herd to undertake an outbreak investigation. Each herd was visited five times, at approximately three-week intervals, during the period 1st July 2015 to 5th April 2016. The herd visits were initiated as closely as possible following the presumed date of onset of the disease outbreak. At all visits, the aim was to assess the clinical status using a standard protocol and to collect paired blood and milk samples from selected individual cows. Where possible, the same animals were sampled at each visit. As many repeated samples as possible were obtained from as large a number of individual animals as the project budget would allow. Where it was not possible to resample the same animals, new animals were sampled.

Study Population

All four of the identified herds had a history of sudden onset of *M. bovis* related clinical signs in the cows and/or calves, and several strongly positive ELISA and/or PCR test results for *M. bovis* (Table 1). During the study period, one or more cows from all study herds tested positive at least once in ELISA or PCR. Detailed farm information obtained before and after enrolment is given in Table 1.

Although bacterial cultures were not included in the study design due to financial constraints, a few calves were euthanized due to severe disease and autopsies were performed. A calf from Herd 2 had chronic degenerative arthrosis in several joints, and bronchopneumonia with overlying pleuritis. *M. bovis* was cultured from joint fluid, and joint fluid and lung tissue were found positive for *M. bovis* by PCR. Two calves autopsied from Herd 4 had chronic omphalitis, bronchopneumonia, synovitis in several joints and bilateral otitis media. *Mycoplasma spp.* were cultured from both calves, and typed by PCR to be *M. bovis.* From Herd 3, two calves were autopsied and arthritis and otitis media were found in both animals, but no bacterial pathogens were isolated.

Table 1. Summaries of the four Danish dairy herds in the study and results of different diagnostic tests performed prior to and during the study period 1st of July 2015 to 5th of April 2016.

Herd no.	**Herd 1**	**Herd 2**	**Herd 3**	**Herd 4**
		Knowledge prior to enrolment		
Number of cows	177	174	182	391
Primary clinical signs in cows	Mastitis, pneumonia	Mastitis, arthritis	Arthritis	Mastitis, arthritis
Positive serum ELISA[1]	Yes	Yes	Yes	Yes
Positive PCR[2]	Yes	Yes	No	Yes
Outbreak start (according to the farmer)	Early June 2015	Early July 2015	Late November 2015	Mid December 2015
		Knowledge after enrolment		
Data collection period	1st July 2015 - 16th September 2016	20th July 2015 - 6th October 2015	8th December 2015 - 23th February 2016	20th January 2016 - 5th April 2016
Primary affected age group	Cows	Cows and calves	Cows	Cows and calves
Primary clinical signs				
Cows	Mastitis	Mastitis, arthritis	Arthritis	Mastitis, arthritis
Calves	Pneumonia, otitis media	Arthritis, otitis media, pneumonia		Arthritis, otitis media, pneumonia
Diagnostic tests				
Positive ELISA[3] (total)				
Serum	35 (120)	35 (98)	52 (134)	48 (95)
Milk	41 (119)	24 (87)	20 (130)	14 (94)
Positive PCR[2] (total)	8 (119)	15 (87)	6 (131)	30 (94)

[1] ODC% values ≥ 37 for *Mycoplasma bovis* antibodies in BioX Bio K 302 or 260 (BioX Diagnostics, Belgium)

[2] composite milk samples, Ct value ≤ 37 for *M. bovis* in PathoProof Mastitis Major-3 (Thermo Scientific, USA).

[3] ODC% values ≥ 37 for *Mycoplasma bovis* antibodies in BioX Bio K 302 (BioX Diagnostics, Belgium)

Sample Collection

The project budget allowed for the inclusion of approximately 80 cows in total, with each being tested five times. However, during an outbreak of *M. bovis* it is likely that some cows would be culled due to clinical disease, so we therefore allowed for more cows to be included at the first or consecutive visits. Therefore, a minimum of 20 cows were identified from each farm during the first herd visit. The cows were sampled strategically to ensure that a sufficient number of cows with clinical suspicion of *M. bovis*-associated disease were included along with a sample of cows without such clinical disease suspicion. New cows suspected of having *M. bovis*-associated disease at subsequent visits were also included. The rationale behind this non-random sampling strategy was to maximize the chances of including sufficient numbers of animals with and without different clinical signs.

At each visit, the selected cows were subject to a clinical examination focusing on the respiratory system, udder, and musculoskeletal system using a standardized clinical protocol (Appendix). The clinical examination was done by one of three veterinarians, of whom two had collaboratively developed the clinical protocol. At least one of these was present at each visit, and the use of the clinical protocol was demonstrated for the benefit of the third veterinarian that had not helped to develop the protocol.

In addition to the clinical examination, a venous blood sample was collected in a 10 ml Vacutainer plain tube (Kruuse, Denmark), and one composite milk sample was collected in a bronopol coated tube from each cow. Samples were stored in a cool environment, and delivered to Eurofins Steins Laboratory (Vejen, Denmark) within 36 hours.

Laboratory Test Methods

All samples were analyzed for antibodies directed against *M. bovis* using the commercial kit BioX Bio K 302 (BioX Diagnostics, Rochefort, Belgium). The assay was performed according to the manufacturer's instructions for use. The optical density coefficient was calculated as:

$$ODC\% = (OD_{sample} - OD_{negative\ control}) / (OD_{positive\ control} - OD_{negative\ control}) \times 100\%$$

where OD is the optical density measured by the ELISA reader of each test sample and the positive and negative control on the sample ELISA plate. An ODC% ≥ 37 was considered positive, as recommended by the manufacturer. The manufacturer has reported a sensitivity and specificity of the test at 100% based on a small sample of experimentally infected calves and negative control calves (Anonymous, 2017). However, lower accuracy has been reported under field conditions (Hazelton et al., 2018; Wawegama et al., 2016).

Milk samples were analyzed for presence of *M. bovis* DNA with the commercial PCR kit PathoProof Mastitis Major-3 assay (Thermo Scientific, Waltham, USA) at Eurofins Steins Laboratory. A cycle threshold (Ct) value ≤ 37 was considered positive in the PCR, as recommended by the manufacturer.

Grouping of Cows for Analysis

Following the completion of the data collection period, individual animals were categorized into disease groups to facilitate stratification of descriptive statistics and modeling of antibody response dynamics. The three primary groups were as follows:

1) Likely *M. bovis*-associated mastitis ('Mastitis')

2) Likely *M. bovis*-associated systemic disease ('Systemic')

3) No disease believed to be associated with *M. bovis* ('None')

In addition, a fourth group was defined for the remaining animals that could not be reliably assigned to any of the above groups 1-3:

4) Disease that is not typically associated with *M. bovis,* but where *M. bovis* could not reasonably be excluded ('Non-specific').

Classification was based on the recorded clinical signs and photographs taken of the selected cows during the herd visits, according to the specific inclusion criteria as follows:

- **1) Mastitis:** cows with recordings of mastitis, defined as any visual abnormality in the milk and a *M. bovis* positive milk PCR at one or more herd visits;
- **2) Systemic:** cows with clinical signs indicating systemic spread of *M. bovis.* All cows in this group had arthritis and/or subcutaneous edema in the limbs at one or more herd visits. In addition to these clinical signs from the musculoskeletal system, 3 cows had clinical signs of respiratory disease, 2 had drooping ears and 12 had mastitis.
- **3) None:** cows with no clinical signs, and cows with only clinical signs that are not likely to be associated with *M. bovis*. These included hock lesions, wounds, claw lesions, cows with a dry quarter and lameness without joint swelling. However, due to the presence of clinical signs in co-managed animals, it is assumed that these animals were exposed to the pathogen.
- **4) Non-specific:** cows that had clinical signs that are not typical for *M. bovis*, but where *M. bovis* could not be excluded. Clinical signs in this group were minor, short term limb swellings, and/or mastitis (without an *M. bovis* PCR positive test), mild/short term respiratory signs, keratoconjunctivitis and abscesses.

All four groups were modeled separately in order to facilitate the focus on the differences of the temporal dynamics between groups. Twelve cows fulfilled the criteria for being in both the 'Mastitis' and the 'Systemic' group and they are subseqently referred to as 'dual-syndrome' cows. They are included in both the 'Mastitis' and the 'Systemic' group for analysis of antibody dynamics.

Primary Explanatory Variable of 'Days from disease onset'

The primary explanatory variable for the longitudinal analysis was the number of d between the estimated disease onset in the individual cow and the sample date for the relevant ELISA result. It was not possible to determine this exactly, but was estimated from the first date of observed clinical signs in each cow. To make this as accurate as possible, records from the farmers' electronic disease recording system and written records from the consulting veterinarians were used where available. In 39% of the cows, a similar diagnosis as the one given at the herd visit was recorded by farmer or

veterinarian within one week prior to the herd visit, and this d was then set as the d of disease onset. In 61% of the cows the d of disease onset was recorded as the date of the herd visit at which the clinical sign was detected for the first time. To be able to make comparisons between all groups, cows in the 'Non-specific' group were assigned a representative imputed date that was sampled from the distribution of estimated disease onset dates from the cows in the 'Mastitis' and 'Systemic' groups within the same herd.

Modeling of Antibody Responses

To investigate the potentially non-linear temporal patterns of antibody responses, we selected an explorative approach to avoid imposing a pre-specified functional form to the antibody dynamics over time. Four separate generalized additive mixed models (GAMM) were used for the two outcomes (serum and milk) in each of the four disease groups, i.e. eight models in total. ODC% was log-transformed to improve the normality of the residuals. To allow log-transformation of a small number of ELISA-values that were recorded as numerically 0, a fixed constant of 1 was added to all ODC%. The data used for the models were restricted to the most relevant time periods of between 21 d prior to disease onset up to 80 d after disease onset (Figure 1). As well as reflecting the most relevant time period in relation to the presumed date of onset of clinical signs, this procedure also ensured that time periods were limited to those with observations from a minimum of three cows.

A thin plate regression spline was used to fit the non-linear effect of d from disease onset, and random effects of cow identification number (Cow) and herd identification number (Herd) were included to account for repeated samples from the same cow and clustering of cows within herds. Confidence intervals for the predicted mean ODC% values (with random effects of herd and cow set to zero) were calculated and plotted for each disease group and outcome. Model fit was assessed for each model by evaluating the distribution of the residuals. All data management and analyses were done in R version 3.2.2 (R Core Team, 2016), using the gamm function in the mgcv package to implement the GAMM (Wood, 2011).

Results

Descriptive Statistics

In total, 120 cows were enrolled in the study, and 447 blood samples and 431 milk samples were collected. See Table 2 for detailed information about number of cows and samples stratified by herd.

Table 2. Number of cows and samples in each disease group, stratified by herd.

Herd no.	Total		'Mastitis'		'Systemic'		'Non-specific'		'None'	
	Serum	Milk	Serum	Milk	Serum	Milk	Serum	Milk	Serum	Milk
Herd 1										
Cows	29	29	2	2	0	0	16	16	11	11
Samples	120	119	7	7	0	0	62	62	51	50
Herd 2										
Cows	25	24	3	3	14	13	0	0	10	10
Samples	98	87	9	7	54	46	0	0	43	40
Herd 3										
Cows	32	32	1	1	19	19	6	6	6	6
Samples	134	131	5	5	74	75	27	26	28	25
Herd 4										
Cows	34	34	12	12	21	21	5	5	6	6
Samples	95	94	16	16	53	52	19	19	21	21
Total										
Cows	120	119	18	18	54	53	27	27	33	33
Samples	447	431	37	35	181	173	108	107	143	136

Cows were sampled an average of 3.7 times, with Herd 4 having the lowest average number of observations per cow (2.8) (Table 3). This reflects the fact that many cows with mastitis were slaughtered or culled soon after disease onset in Herd 4. It was not possible to collect milk samples from cows that were dried off during the study period, but clinical examination and blood samples continued to be collected during the dry period. Reasons for loss from the study were culling, slaughter or movement to another property for drying-off.

Table 3. Average number of samples per cow and the distribution of samples per cow, stratified by herd.

	Average	One sample	Two samples	Three samples	Four samples	Five samples
Herd 1	4.1	0	6	1	5	17
Herd 2	4	4	1	2	4	14
Herd 3	4.2	4	0	1	8	19
Herd 4	2.8	13	3	3	8	7
Total	3.7	21	10	7	25	57

All but one of the cows in the 'Mastitis' group had a serum result that was well above the manufacturer's cut-off on the day of disease onset, and most of the cows declined rapidly in ODC% following the date of onset (Figure 1A).

A total of 27 out of 54 (50%) of the cows with systemic *M. bovis*-associated disease had serum results that were above the cut-off for the entire study period (Figure 1B). A total of 15 out of 54 (28%) cows with systemic disease were not above the cut-off at any point in time, and 12 of the 54 (22%) cows had some samples above and some below the cut-off during the study period. The antibody responses slowly declined for most of the cows, ending up below the cut-off before or at the last visit.

A total of 22 out of 33 (67%) of the cows in the 'None' group had serum results that were below the cut-off for the entire study period (Figure 1C). One out of 33 (3%) cows was above the cut-off for the entire study period, and 10 out of 33 (30%) cows had some samples above and some below the cut-off during the study period.

A total of 2 out of 27 (7%) of the cows in the 'Non-specific' group had serum results that were above the cut-off for the entire study period (Figure 1D). A total of 8 out of 27 (30%) cows were below the cut-off at all points in time, and 17 of the 27 (63%) cows had samples above and below the cut-off during the study period. On average, the ODC% values in the 'Non-specific' group were lower than in the systemically diseased group.

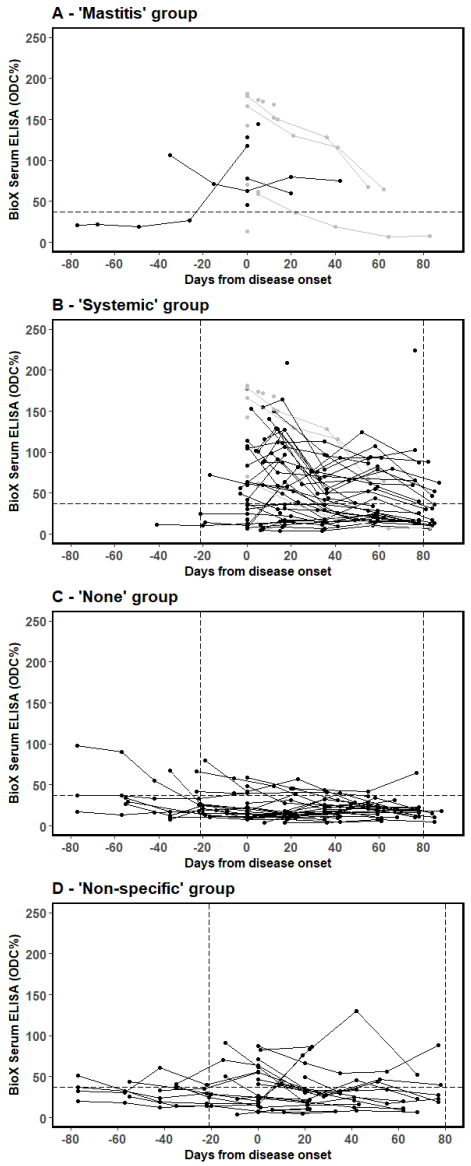


Figure 1. Distribution of serum ELISA measurements for antibodies directed against *Mycoplasma bovis* in the four disease groups of dairy cows from four Danish herds. 'Dual-syndrome' cows are colored in grey. Horizontal dotted lines show the recommended ELISA cut-off (37 ODC%). Vertical dotted lines indicate the temporal limits for inclusion in the modeling of antibody response dynamics (d -21 and 80 relative to disease onset). ODC% = sample coefficient. Results from the same cow are linked by lines.

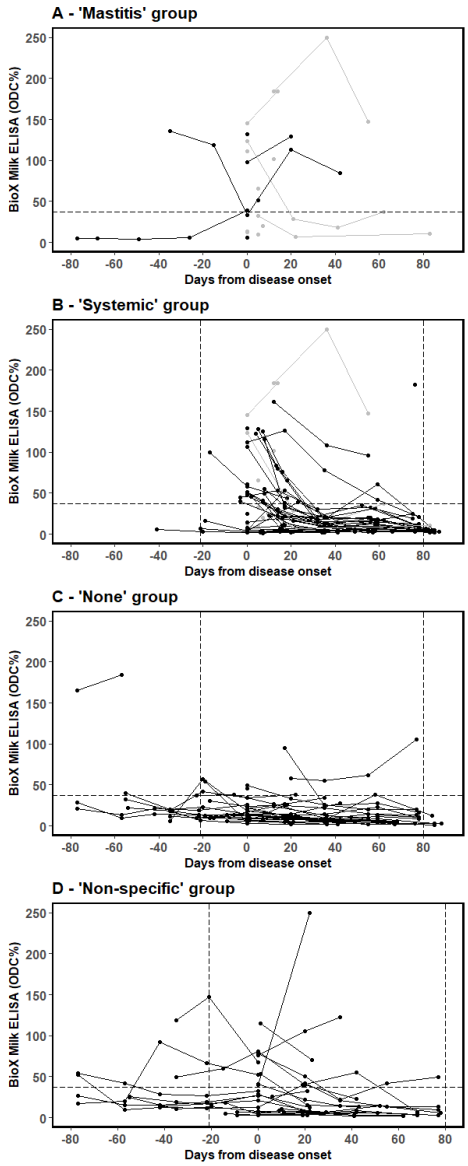


Figure 2. Distribution of milk ELISA measurements for antibodies directed against *Mycoplasma bovis* in the four disease groups of dairy cows from four Danish herds. 'Dual-syndrome' cows are colored in grey. Horizontal dotted lines show the recommended ELISA cut-off (37 ODC%). Vertical dotted lines indicate the temporal limits for inclusion in the modeling of antibody response dynamics (d -21 and 80 relative to disease onset). ODC% = sample coefficient. Results from the same cow are linked by lines. Three observations with ODC% > 250 are shown as 250 ODC%.

The overall pattern was less consistent for milk samples than for serum in the 'Mastitis' group. A total of eight out of 18 (44%) cows with *M. bovis* mastitis had milk results that were above the cut-off for the entire study period. A total of seven out of 18 (39%) cows had milk results that were below the cut-off for the entire study period and two of the 18 (11%) cows had milk results above and below the cut-off during the study period (Figure 2A).

A total of 11 out of 53 (20%) cows with systemic *M. bovis* disease had milk results that were above the cut-off for the entire study period (Figure 2B) and five of these cows also had *M. bovis* mastitis. A total of 22 out of 53 (42%) cows had milk results that were not above the cut-off at any time, and 20 of the 53 (38%) cows had milk results above and below the cut-off during the study period.

A total of 23 cows out of 33 (70%) cows in the 'None' group had milk results that were below the cut-off for the entire study period (Figure 2C). A total of three out of 33 (9%) cows had milk results above the cut-off for the entire study period and 7 of the 33 (21%) cows had milk results above and below the cut-off during the study period.

A total of 4 out of 27 (15%) cows in the 'Non-specific' group had milk results that were above the cut-off for the entire study period (Figure 2D), whereas 14 out of 27 (52%) cows had milk results that were below the cut-off at all times, and 9 of the 27 (33%) cows had milk results both above and below the cut-off during the study period.

Modeling Results

A total of eight separate models were used for the two outcomes (log-transformed milk and serum ODC%-values) and four disease groups, although meaningful results could not be obtained for the 'Mastitis' group models of either milk or serum samples due to sparse and highly variable data (Figures 1A and 2A). Visual inspection of residual plots indicated an acceptable model fit for the remaining six models, results of which are presented below.

The estimated number of degrees of freedom (edf) for the smooth term describing the effect of d from disease onset indicates the complexity of the function, with values of 1 indicating a simple linear function. Based on the edf, substantial non-linearity was evident in the 'Systemic' group for both milk and serum and in the 'None' group for milk. The standard deviation associated with the random effect of Cow was larger than the residual standard deviation in all the models, and the random effect attributed to Herd was generally smaller than that attributed to Cow. However, the random effect of Herd is likely to be underestimated due to the small number of herds included in the study, so some of the true differences between herds are likely included in the Cow random effect estimate. We therefore consider only the combined effect of Cow and Herd relative to the residual standard deviation as a meaningful comparison (Table 4).

Table 4. Results of the final models with antibody optical density measurement (ODC%) for *Mycoplasma bovis* in serum and milk as outcome in different disease groups.

Disease group	**'Systemic'**		**'None'**		**'Non-specific'**	
	Serum	**Milk**	**Serum**	**Milk**	**Serum**	**Milk**
Random effect (SD)[1]						
Combined Cow/Herd level	0.88	1.14	0.49	0.80	0.62	1.08
Residuals	0.43	0.65	0.35	0.47	0.46	0.53
Smooth term (edf)[2]						
Days from onset of disease	4	2.3	1	1.6	1	1

[1] SD, standard deviation

[2] edf, estimated degrees of freedom

The estimated mean ODC% in serum in the 'Systemic' group was estimated to be high at the time of disease onset, and remained above the manufacturers' cut-off until 65 d after disease onset (Figure 3, red line). The 95% confidence intervals (CI) follow the same pattern, but indicate that the mean ODC% was clearly above the cut-off only between 7 - 17 d after disease onset in this group. The estimated mean ODC% with 95% CIs in the 'None' group were below the cut-off for most of the time period, and there was no overlap of CI between the 'Systemic' and 'None' group between one week before d of disease onset and approximately 68 d after disease onset (Figure 3). The CI of the 'Non-specific' group overlapped with the 'None' group during the whole study period, and only differed markedly from the 'Systemic' group according to the CIs between 7-20 d after disease onset.

Estimated ODC% in milk was below the cut-off for all disease groups for the entire time period, with wide 95% CIs that overlapped substantially between disease groups throughout the study period (Figure 4).

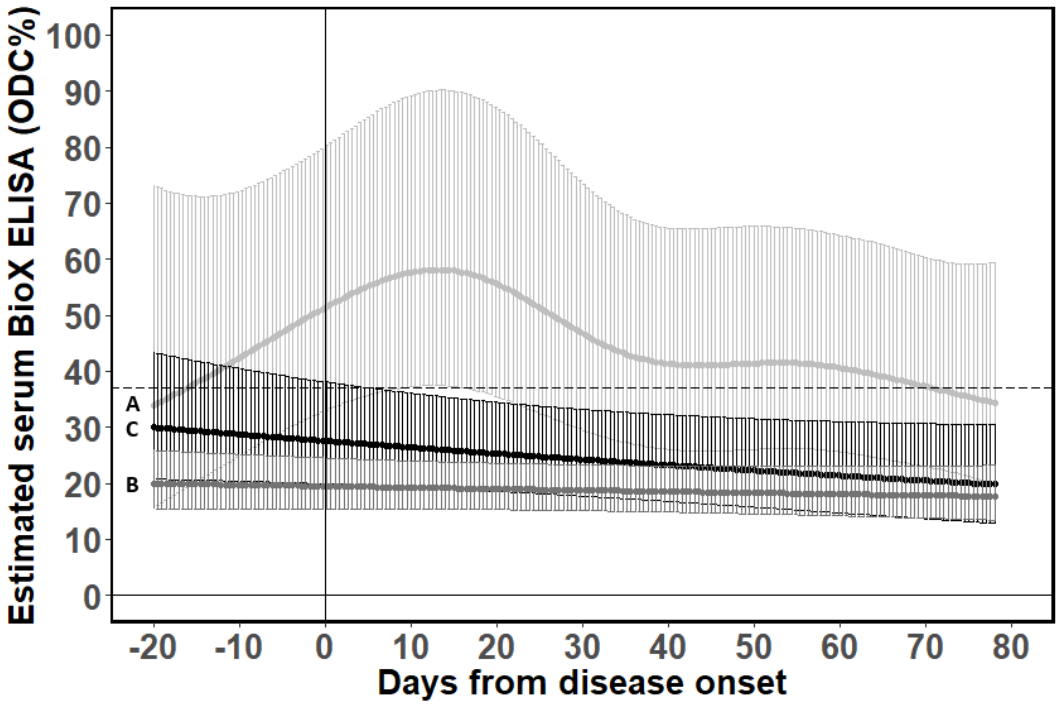


Figure 3. Estimated mean antibody response in serum (solid line) and 95% confidence intervals (shaded area) as measured by the BioX ELISA Bio K302. A (light grey) = 'Systemic' group (likely *Mycoplasma bovis*-associated systemic disease), B (dark grey) = 'None' group (no disease believed to be associated with *M. bovis*), C (black) = 'Non-specific' group (disease not typically associated with *M. bovis*, but where *M. bovis* could not be ruled out). The dashed black line shows the recommended ELISA cut-off (37 ODC%).

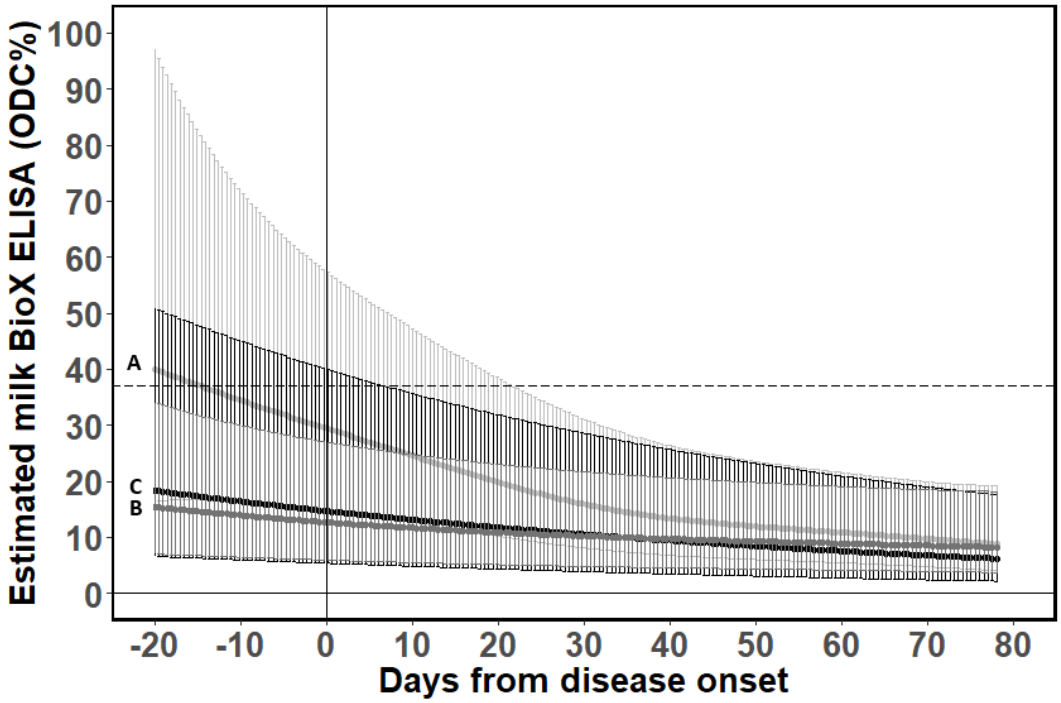


Figure 4. Estimated mean antibody response in milk (solid line) and 95% confidence intervals (shaded area) measured by the BioX ELISA Bio K302. A (light grey) = 'Systemic' group (likely *Mycoplasma bovis*-associated systemic disease), B (dark grey) = 'None' group (no disease believed to be associated with *M. bovis*), C (black) = 'Non-specific' group (disease not typically associated with *M. bovis*, but where *M. bovis* could not be ruled out). The dashed black line shows the recommended ELISA cut-off (37 ODC%).

Discussion

This observational study is the first to illustrate and analyze the dynamics of the antibody response directed against *M. bovis* from milk and serum in naturally exposed and diseased dairy cows. The key findings were that the antibody responses varied markedly between cows and were very dynamic within individual cows. The serum antibody level generally declined within two months following the onset of clinical disease, even in cows with clear clinical signs of systemic *M. bovis* disease. The antibody level in milk was only high if cows had mastitis.

In calves, the serum IgG-level has been found to rise between one and two weeks after vaccination (Nicholas et al., 2002) and intra-tracheal inoculation of *M. bovis* bacteria (Howard et al., 1986). The antibody responses observed in this study from cows that responded to infection were high from the estimated day of disease onset, which indicates either that the cows had been infected for a week or two before becoming sick, or that antibody responses in adult cows are faster and stronger than in young calves. In experimentally inoculated cows, the antibody responses in serum and milk have been found to increase within 7-10 d from inoculation (Boothby et al., 1986; Byrne et al., 2005), indicating that the antibody responses in cows might be slightly faster than in calves. This also fits with the increasing pattern in estimated mean ODC% that can be observed in Figure 3 between 0 to 14 d after disease onset.

Antibodies against M. bovis in serum

This study suggests that the level of serum antibodies generally increases in cows with *M. bovis*-associated disease around the time of disease onset (Figures 1A, 1B and 3), which to our knowledge has not previously been described in naturally infected cows. For experimentally induced mastitis the same tendency has been found (Boothby et al., 1987), but no such studies about arthritis are published. It appears that ODC% in the 'Mastitis' group declined more quickly than the 'Systemic' group, but it is difficult to compare with so few samples in the 'Mastitis' group. Although we were not able to follow all cows until their antibody response declined below the cut-off, a faster decline in antibodies was seen for *M. bovis* relative to other diseases for which antibody measurements are commonly used in cattle, such as *Salmonella* Dublin (Nielsen, 2003) and bovine virus diarrhea virus (Fredriksen et al., 1999).

The serum model for the 'Systemic' group was repeated excluding the 12 'dual-syndrome' cows (with both mastitis and systemic disease) to assess the sensitivity of our results to the data from these animals. The shape of the curve was qualitatively similar, and the time period during which the confidence intervals did not overlap was the same, although the highest estimated mean declined by approximately 5 ODC% when excluding the 12 'dual-syndrome' cows. Unfortunately it was not possible to repeat this procedure for the 'Mastitis' group, because excluding the 'dual-syndrome' cows left us with a sample size of only 6 animals in this group. However, we acknowledge that further investigation into specific features of antibody responses in 'dual-syndrome' cows exhibiting both sets of clinical signs, and how this differs from cows exhibiting only signs of mastitis, would be relevant for future studies.

Some of the cows in the 'Systemic' group did not exhibit an antibody response (Figure 1B). This included one of the 12 'dual-syndrome' cows in this group, but that animal was only tested once. One explanation could be that the clinical signs in the non-responding cows were not due to *M. bovis*

infection, but some other pathogen/disease. We did not have the opportunity to diagnostically rule out other pathogens in this study. Another explanation for the lack of antibody responses could be that the ability to produce antibodies against *M. bovis* infection varies between individual cattle. It is unknown whether this difference between host responses is due to host (e.g. genetics) or pathogen factors, or a combination of the two. The antibody response to *M. bovis* has not been found to be correlated with treatment of bovine respiratory disease in calves (Rosendal and Martin, 1986), but no such studies have been conducted in cows. The antigenic variation in *M. bovis* bacteria is large and alterations of membrane surface lipoproteins occur during an outbreak, even between different sub clones of the same strain (Bürki et al., 2015). This might result in different antibody responses in cows with similar clinical signs. Despite the lack of a clear explanation for the fact that some cows did not produce measurable antibodies, this finding is in agreement with others who found little correlation between antibody measurements and clinical signs in cows (Szacawa et al., 2016). However, in that study the cows were not followed over time, so it was not possible to explain the missing correlation.

Other authors have warned against using serology for individual *M. bovis* diagnosis (Maunsell et al., 2011). Martin et al. (1990) suggested that antibodies directed against *M. bovis* are not correlated with disease in individual calves, but could be useful at group level. Our results also indicate that the ODC% are not well correlated with clinical disease in individual cows within herds with circulating *M. bovis* (Figure 1B). However, the estimated mean ODC% with corresponding 95% confidence intervals suggests that it is possible to use the average serum ODC% of a group of diseased animals to assess whether or not the clinical signs in the herd are likely to be associated with *M. bovis* infection for a period of up to 65 d after disease onset in the affected animals (Figure 3). It is beyond the scope of this study to identify optimal testing scenarios for group or herd diagnostics, but the results of the raw data for mastitis (despite the fact that a model could not be fit to these data) indicate that such a screening tool would also be relevant for outbreaks with mastitis cases, and not only systemic disease.

Antibodies against M. bovis in Milk

The level of antibodies against *M. bovis* in milk from cows other than those with *M. bovis* mastitis was low. Even if a cow had clear clinical signs of systemic spread of *M. bovis,* for example arthritis with antibodies detectable in serum, almost no antibodies were detectable in the milk. In contrast, the cows with mastitis had high levels of antibodies in both milk and serum, and the cows in the 'Systemic' group with high milk antibodies were primarily cows with both clinical signs of systemic disease and mastitis. Byrne et al. (2000) found that antibodies in milk remained high for a longer period in quarters infected with *M. bovis* compared to quarters not infected in the same cow, suggesting that the antibody response to *M. bovis* in the udder is primarily a local immune response, and to a lesser extent filtering of antibodies from serum to milk. Antibodies in milk can therefore only be used for diagnosis of cows with mastitis, and not for cows with systemic disease without mastitis. In mastitis cases in other studies, antibodies in milk lasted for at least 40 d (Boothby et al., 1987; Byrne et al., 2000). We were not able to confirm this, as it was not possible to determine the duration of high antibody levels in milk in cows with mastitis due to culling/euthanasia of many of the cows with mastitis.

Our results explain why others have found limitations in the use of antibody measurements in bulk tank milk for diagnosis *M. bovis* at herd level (Nielsen et al., 2015; Parker et al., 2017; Petersen et al., 2016). Usually cows with clinical signs of mastitis will not be milked into the bulk tank for delivery to the dairy and therefore do not contribute to the bulk tank milk antibody response. However, since the antibody level in milk after mastitis seem to last for at least 20 weeks (Boothby et al., 1987; Byrne et al., 2000), they might contribute after clinical signs/treatment have ended. Different management factors, treatment and segregation strategies can then be the cause of variations in bulk tank milk antibody levels in herds experiencing an outbreak of *M. bovis*-associated disease. For this reason, bulk tank milk sampling is less suitable for identification of *M. bovis* infected herds than for other pathogens, which elicit stronger antibody responses in subclinically infected cattle.

Cows with non-specific or no M. bovis associated disease

To be as certain as possible that the cows in the 'Systemic' group represented *M. bovis* infected animals, and that cows in the 'None' group were not infected with *M. bovis*, an intermediate group was necessitated. Cows in this group may be a mix of cows that are infected with *M. bovis* without showing clear clinical signs, cows that have been infected earlier and not showing clinical signs anymore and cows that are not infected. It is therefore difficult to make too many firm conclusions regarding the dynamics in this group, and we note that the ODC % was generally estimated by the model as being somewhere between the 'None' and 'Systemic' groups (although closer to the former).

Herd 1 was overrepresented in the 'Non-specific' group. This could be because herd visits were initiated for Herd 1 later in the outbreak than the other herds, so some cows might have shown clinical signs earlier and were recovering at the time we visited the herd. This could explain the intermediate antibody responses in many cows in this disease group and the slightly declining mean predicted ODC% (Figure 3).

Two cows from the 'None' and 'Non-specific' group stand out as having high ODC% in milk (Figures 2C and 2D). The most likely explanation for these must be undiagnosed or subclinical mastitis (Fox, 2012), since records in the electronic herd recording system did not reveal former treatment of mastitis. Given the fact that the mean for the 'Non-specific' group was closer to the mean of the 'None' group than the 'Systemic' group, it is more likely that these cows were either not infected or previously infected with *M. bovis.*

Study Design and Limitations

The aim of this study was purely to investigate the dynamics of antibodies directed against *M. bovis* under field conditions.

Advice about how to handle the disease outbreak or specific cows was not given by any of the project veterinarians, and decisions regarding treatment and herd-specific disease control strategies were solely made by the farmer and his consulting veterinarian. We cannot be sure that our presence and interest in sampling did not influence the decisions about strategy, but it is not our impression that this has happened to a great extent. However, the culling strategies varied a lot between herds due to different local veterinary advice and differences in clinical signs and severity of disease.

In addition, we note that the predefined test-positive cut-off value of the BioX ELISA (37 ODC%) has not yet been substantially documented in naturally infected cattle. To evaluate which cut-off is meaningful, an overview of the variation between individual cows, as provided here, is needed as the first step. However, the proportion of test results that would be expected to be over a given arbitrary threshold based on our results can be calculated as follows:

$$P(+|\,\mu_{g,d}, \sigma_a, \sigma_r, t) = 1 - \Phi\left(\frac{\log(t+1) - \log(\mu_{g,d})}{\sqrt{{\sigma_a}^2 + {\sigma_r}^2}}\right)$$

where $\mu_{g,d}$, is the estimated mean value for group g at time d (Figures 3 and 4), σ_a& σ_r are the standard deviation associated with cow/herd and residual for the same group g (Table 4), t is the desired test threshold (for example 37 ODC%), and Φ is the standard normal distribution.

Conclusions

To our knowledge this is the first longitudinal observational field study of naturally infected herds in which the dynamics of the IgG response directed against *M. bovis* in dairy cows are described and compared between groups of animals with different disease manifestations. We conclude that antibody measurements in milk are only useful for differential diagnosis of mastitis, and that serum antibody responses are highly dynamic and variable between individual cows. Consequently, serology based on the BioX Bio K 302 ELISA is not likely to be of useful for individual diagnosis of *M. bovis* associated disease in dairy cows. However, this does not preclude use of the test for herd or group level diagnosis, which is supported by the fact that the estimated mean of the measured antibody response in the group of cows with *M. bovis* associated systemic disease was markedly higher than in cows without *M. bovis* associated disease between 7 and 17 d after disease onset in particular. Further studies are therefore warranted to identify optimal herd-level testing strategies based on the information provided here.

Acknowledgements

The authors would like to thank veterinarians Ulla Torpe and Martin Lund (Dyrlægerne Egtved), Lars Pedersen (SEGES, Aarhus, Denmark) and veterinarians/veterinary students Annie Nielsen (Dyrlægerne Optivet), Eva Elisabeth Toft-Petersen (Tinglev Dyrehospital), Franziska Helene Pedersen (University of Copenhagen) and Signe Rejnhardt Olsen (Skovbjerg Dyrlæge Team) for assistance at the herd visits. The Danish Milk and Cattle Levy Funds provided funding for the sampling and laboratory testing. The funding source was not involved in the study design, analyses, and interpretation, nor in the decision to publish the study.

References

Anonymous. 2017. MYCOPLASMA BOVIS ELISA KIT, http://www.biox.com/en/bio-k-302-monoscreen-abelisa-mycoplasma-bovis-indirect-monowell-p-250/. Accessed 17-11-2017.

Boothby, J. T., D. E. Jasper, and C. B. Thomas. 1986. Experimental intramammary inoculation with *Mycoplasma bovis* in vaccinated and unvaccinated cows: effect on the mycoplasmal infection and cellular inflammatory response. Cornell Vet. 76(2):188-197.

Boothby, J. T., D. E. Jasper, and C. B. Thomas. 1987. Experimental intramammary inoculation with *Mycoplasma bovis* in vaccinated and unvaccinated cows: effect on local and systemic antibody response. Can. J. Vet. Res. 51(1):121-125.

Burki, S., J. Frey, and P. Pilo. 2015. Virulence, persistence and dissemination of *Mycoplasma bovis*. Veterinary Microbiology, 179:15-22.

Byrne, W., B. Markey, R. McCormack, J. Egan, H. Ball, and K. Sachse. 2005. Persistence of *Mycoplasma bovis* infection in the mammary glands of lactating cows inoculated experimentally. Vet. Rec. 156(24):767-771.

Byrne, W. J., H. J. Ball, N. Brice, R. McCormack, S. E. Baker, R. D. Ayling, and R. A. Nicholas. 2000. Application of an indirect ELISA to milk samples to identify cows with *Mycoplasma bovis* mastitis. Vet. Rec. 146(13):368-369.

Fox, L. K. 2012. Mycoplasma mastitis: causes, transmission, and control. (Special Issue: Mastitis in dairy cows.). Vet. Clin. North Am. Food Anim Pract. 28:225-237.

Fredriksen, B., T. Sandvik, T. Loken, and S. A. Odegaard. 1999. Level and duration of serum antibodies in cattle infected experimentally and naturally with bovine virus diarrhoea virus. Veterinary Record, 144:111-114.

Hazelton, M. S., P. A. Sheehy, K. L. Bosward, A. M. Parker, J. M. Morton, C. J. Dwyer, P. G. Niven, and J. K. House. 2018. Short communication: Shedding of *Mycoplasma bovis* and antibody responses in cows recently diagnosed with clinical infection. Journal of Dairy Science 101(1):584-589.

Henderson, J. P., and H. J. Ball. 1999. Polyarthritis due to *Mycoplasma bovis* infection in adult dairy cattle in Northern Ireland. Vet. Rec. 145(13):374-376.

Howard, C. J., K. R. Parsons, and L. H. Thomas. 1986. Systemic and local immune responses of gnotobiotic calves to respiratory infection with *Mycoplasma bovis*. Vet. Immunol. Immunopathol. 11(3):291-300.

Le Grand, D., D. Calavas, M. Brank, C. Citti, R. Rosengarten, P. Bezille, and F. Poumarat. 2002. Serological prevalence of *Mycoplasma bovis* infection in suckling beef cattle in France. Vet. Rec. 150(9):268-273.

Martin, S. W., K. G. Bateman, P. E. Shewen, S. Rosendal, J. G. Bohac, and M. Thorburn. 1990. A group level analysis of the associations between antibodies to seven putative pathogens and respiratory disease and weight gain in Ontario feedlot calves. Can. J. Vet. Res. 54(3):337-342.

Maunsell, F. P., and G. A. Donovan. 2009. *Mycoplasma bovis* Infections in young calves. Vet. Clin. North Am. Food Anim Pract. 25(1):139-77.

Maunsell, F. P., A. R. Woolums, D. Francoz, R. F. Rosenbusch, D. L. Step, D. J. Wilson, and E. D. Janzen. 2011. *Mycoplasma bovis* infections in cattle. J. Vet. Intern. Med. 25(4):772-783.

Nicholas, R. A. 2011. Bovine mycoplasmosis: silent and deadly. Vet. Rec. 168(17):459-462.

Nicholas, R. A., R. D. Ayling, and L. P. Stipkovits. 2002. An experimental vaccine for calf pneumonia caused by *Mycoplasma bovis*: clinical, cultural, serological and pathological findings. Vaccine 20(29-30):3569-3575.

Nielsen, L. R. 2003. *Salmonella* Dublin in dairy cattle: Use of diagnostic tests for investigation of risk factors and infection dynamics. PhD Thesis PhD Thesis, The Royal Veterinary and Agricultural University, The Royal Veterinary and Agricultural University.

Nielsen, P. K., M. B. Petersen, L. R. Nielsen, T. Halasa, and N. Toft. 2015. Latent class analysis of bulk tank milk PCR and ELISA testing for herd level diagnosis of *Mycoplasma bovis*. Preventive Veterinary Medicine, 121:338-342.

Parker, A. M., J. K. House, M. S. Hazelton, K. L. Bosward, J. M. Morton, and P. A. Sheehy. 2017. Bulk tank milk antibody enzyme-linked immunosorbent assay as a biosecurity tool for detecting dairy herds with past exposure to *Mycoplasma bovis*. Journal of Dairy Science, 100:1-14.

Petersen, M. B., K. Krogh, and L. R. Nielsen. 2016. Factors associated with variation in bulk tank milk *Mycoplasma bovis* antibody-ELISA results in dairy herds. Journal of Dairy Science, 99:3815-3823.

R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Rosendal, S., and S. W. Martin. 1986. The association between serological evidence of mycoplasma infection and respiratory disease in feedlot calves. Can. J. Vet. Res. 50(2):179-183.

Szacawa, E., K. Niemczuk, K. Dudek, D. Bednarek, R. Rosales, and R. Ayling. 2015. *Mycoplasma bovis* infections and co-infections with other *Mycoplasma spp*. with different clinical manifestations in affected cattle herds in eastern region of Poland. Bulletin of the Veterinary Institute in Pulawy, 59:331-337.

Szacawa, E., M. Szymanska-Czerwinska, K. Niemczuk, K. Dudek, D. Bednarek, and R. D. Ayling. 2016. Comparison of serological, molecular and cultural diagnostic methods for the detection of *Mycoplasma bovis* infections in cattle. Animal Science Papers and Reports, 60:391-397.

Wawegama, N. K., and G. F. Browning. 2017. Improvements in diagnosis of disease caused by *Mycoplasma bovis* in cattle. Animal Production Science, 57(7):1482-1487.

Wawegama, N. K., P. F. Markham, A. Kanci, M. Schibrowski, S. Oswin, T. S. Barnes, S. M. Firestone, T. J. Mahony, and G. F. Browning. 2016. Evaluation of an IgG enzyme-linked immunosorbent assay as a serological assay for detection of *Mycoplasma bovis* infection in feedlot cattle. Journal of Clinical Microbiology 54(5):1269-1275.

Wilson, D. J., R. T. Skirpstunas, J. D. Trujillo, K. B. Cavender, C. V. Bagley, and R. L. Harding. 2007. Unusual history and initial clinical signs of *Mycoplasma bovis* mastitis and arthritis in first-lactation cows in a closed commercial dairy herd. J. Am. Vet. Med. Assoc. 230(10):1519-1523.

Wood, S. N. 2011. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. Journal of the Royal Statistical Society (B) 73(1):3-36.

Appendix

Herd no_____ Cow no_____ Date_____

	Levels
General condition	
Level of alertness	0/1 (0 = bright and alert, 1 = depressed to apathetic)
Body condition score	1 - 5 (1 = lean, 5 = fat)
Rectal temperature	Measured with a thermometer
Head	
CNS, head position	0/1 (0 = normal, 1 = head tilt)
Eye discharge	0/1 (0 = absent, 1 = visible ocular discharge)
Nasal discharge	0/1 (0 = absent, 1 = visible nasal discharge)
Cough	0/1 (0 = absent, 1 = present at examination)
Respiratory system	
Respiration characterisation	0/1 (0 = normal, 1 = abnormal breathing i.e. severe increase of frequency and intensity)
Pulmonary auscultation	0/1 (0 = normal, 1 = abnormal lung sounds, e.g. crackles, wheezes)
Cardiovascular system	
Auscultation	0/1 (0 = normal, 1 = abnormal heart sound/beat)
Musculoskeletal system	
Limb swelling	0/1 (0 = absent, 1 = present)
Skin on limbs	0/1/2 (0 = intact skin, 1 = hairless spots, intact skin, 2 = active/healed wounds)
Lameness	0/1/2 (0 = not lame, 1 = lame, 2 = severely lame)
Udder	
Inspection	0/1/3 (0 = symmetric, no wounds or swelling, 1 = asymmetric, wounds or swelling, 3 = cows with a dry quarter)
Palpation	0/1/2/3 (0 = soft normal udder tissue, 1 = oedema in one or more glands, 2 = soft swelling, consistent with acute inflammation, 3 = hard swelling, consistent with chronic changes)
Soreness	0/1 (0 = normal, 1 = sore when palpated)

8.3 Manuscript III

Mycoplasma bovis antibody dynamics in naturally exposed dairy calves according to two diagnostic tests

Mette Bisgaard Petersen[1*], Nadeeka K. Wawegama[2], Matthew Denwood[1], Philip F. Markham[2], Glenn F. Browning[2] and Liza Rosenbaum Nielsen[1]

[1] Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark
[2] Asia-Pacific Centre for Animal Health, Melbourne Veterinary School, Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria 3010, Australia

[*]**Correspondence:** Mette Bisgaard Petersen, Grønnegårdsvej 8, 1870 Frederiksberg, Denmark. mbp@sund.ku.dk

The manuscript has been submitted to BMC Veterinary Research

Abstract

Background

Inexpensive and convenient diagnostic tests for use in clinical work and for the surveillance of infection with *Mycoplasma bovis* are in demand. The objective of this longitudinal field study was to gain knowledge about the dynamics of antibodies against *M. bovis* in sera from naturally exposed calves with and without different clinical signs, measured by two different ELISA tests.

Results

A total of 83 calves were subject to between one and five blood samples and clinical examinations using a standard protocol during five herd visits to each of four outbreak dairy herds. The blood samples were analysed for the presence of antibodies against *M. bovis* using the commercial IgG ELISA test BioX K302 (BioX) and an in-house indirect IgG ELISA test (MilA ELISA).Linear mixed models were used to describe and compare the antibody dynamics as measured by the two tests in relation to the disease status and age of the animals.The BioX ELISA response was below the recommended cut-off (37 ODC%) for the entire study period in many of the calves. The estimated mean ODC% increased slowly but did not reach the recommended individual animal cut-off in three of the four herds. The highest estimated ODC% was not reached until the calf was approximately 120 days old. The MilA ELISA response rose above the recommended cut-off (135 antibody units (AU)) in almost all calves, and in two herds, the estimated mean was above the individual animal cut-off shortly after the birth of the calf. The highest estimated antibody concentration was reached when the calf was approximately 60 days old. Disease status of a calf was not significantly associated with the results of either test.

Conclusions

We conclude that the BioX ELISA cannot be recommended for use in calves below three month of age. The MilA ELISA was able to detect antibodies shortly after birth and is therefore a more sensitive test for *M. bovis* exposure in young calves. Neither ELISA seemed able to differentiate between calves with arthritis and/or otitis media, and respiratory disease.

Keywords: *Mycoplasma bovis*, ELISA, BioX BioK 302, MilA ELISA, dairy calves, antibody

Background

Mycoplasma bovis causes severe disease in cattle worldwide. The typical clinical manifestations in calves are pneumonia, otitis media and arthritis (Maunsell and Donovan, 2009). The primary diagnostic tool used in calves is bacterial culture of body fluid samples (Sachse et al., 2010), but this is too expensive and time-consuming for use in group or herd diagnostics or for surveillance purposes. Although bacterial DNA-detection tests (such as PCR assays) are becoming more popular, the diagnostic material used for this technique is more difficult to obtain and process than a blood sample. An ELISA for antibody-detection is easy to perform on serum samples and is often less expensive, and these assays are already commonly used for the diagnosis of other diseases in cattle. Knowledge about the dynamics of antibody response in infected animals in relation to disease and age is essential when using an ELISA as a diagnostic tool. This knowledge requires longitudinal studies of naturally exposed calves, involving repeated observations of clinical signs combined with samples being taken for laboratory testing. However, this is time-consuming, inconvenient and expensive, and therefore rarely implemented, meaning that our existing knowledge about antibody dynamics in calves comes mostly from experimental studies. Calves vaccinated with an experimental aerosol vaccine against *M. bovis* at three to four weeks and five to six months of age appeared to have a detectable immunoglobulin G (IgG) response against *M. bovis* within 14 days, and the antibody concentrations in serum remained at a high level for at least 42 and 30 days, respectively (Zhang et al., 2014).

However, neither the *M. bovis* antibody response to systemic disease syndromes, such as arthritis, or the dynamics of the antibody response over time in naturally infected calves is clear. It is also crucial to know how to interpret ELISA results in young calves, since maternally derived antibodies against *M. bovis* might be present in uninfected calves. Furthermore, very young calves may not be able to generate an antibody response to bacterial infections (Roden et al., 1992). Other authors have found the antibody titres in young dairy calves to be low, suggesting low levels of passive transfer of antibodies from the dam (Van Donkersgoed et al., 1993; Virtala et al., 2000). No correlation has yet been found between clinical signs and antibody response in individual calves, but seroconversion to *M. bovis* has been shown to be predictive of disease at a group level in feedlot cattle (Martin et al., 1990; Wawegama et al., 2016). To date, there have been few evaluations of the use and interpretation of different *M. bovis* ELISA tests under field conditions. Recently, the dynamics of the antibody response in cows was found to be very dynamic, short-lasting and dependent on clinical signs in the cow (Petersen et al., 2018), but similar studies in calves under different herd and disease conditions lacks.

An in-house IgG-detection ELISA (MilA ELISA) developed by Wawegama et al. (2014) has an estimated animal-level sensitivity and specificity of 94.3% and 94.4%, respectively using a cut-off of 105 antibody units (AU) (Wawegama et al., 2016). This study compared the results from the MilA IgG ELISA with those obtained from the BioX K302 and K260 ELISA assays in two small groups of experimentally infected calves, and found that both BioX tests had low sensitivity.

To the best of our knowledge, there has been no comparison of the responses measured using the MilA ELISA and the BioX K302 ELISA (BioX) in dairy calves, and knowledge about the generation of antibodies in serum in naturally exposed calves with and without different clinical signs is lacking. Therefore, the objective of this longitudinal field study was to describe and compare the dynamics

of antibody responses to *M. bovis* in the serum of dairy calves with different disease manifestations using two different ELISA tests.

Methods

Data for this study were collected from four dairy herds, from which both cows and calves were sampled. A description and an analysis of the data from cows are presented in Petersen et al. (Petersen et al., 2018), while analyses of the data from calves are presented here. The basic study design and herd selection were the same for the two studies. All farm owners were informed about the procedures in the study and gave written consent to use of their animals and farm data before study start. The study design was approved by the veterinary department of the agricultural advisory services, SEGES, before initiation.

Study design

A longitudinal observational study was carried out between 1st July 2015 and 5th April 2016 in four Danish dairy cattle herds with acute outbreaks of *M. bovis*-associated disease. Each herd was visited five times with an interval of approximately three weeks between each visit. The first visit was as close as possible to the onset of the disease outbreak. The clinical status of selected calves was assessed at each visit, and blood samples were collected from them. Where possible, the same animals were sampled again at each subsequent visit, allowing both between- and within-animal analysis over time.

Study population

The study herds were selected based on the detection of *M. bovis*-associated clinical signs by the herd advisory veterinarian and by diagnostic test results (positive in PCR assays on milk samples or in ELISA tests on sera from cows or calves). All herds had a recent history of sudden-onset of clinical signs indicative of *M. bovis* infection among the cows and/or calves, and several strongly positive ELISA and/or PCR test results for *M. bovis.*

All herds tested positive in both an ELISA and a PCR assay on at least one occasion during the study period, but the clinical signs present in the herds and the affected age groups differed. Information about the farms is presented in Table 1.

Table 1: Summary description of the four Danish dairy herds and diagnostic test results prior to and during the study period.

Herd no.	**1**	**2**	**3**	**4**
Prior to enrolment				
Herd size (No. of cows)	177	174	182	391
Estimated start of outbreak	Early Jun 2015	Early Jul 2015	Late Nov 2015	Mid Dec 2015
After enrolment				
Data collection	1 Jul 2015 - 16 Sep 2016	20 Jul 2015 - 6 Oct 2015	8 Dec 2015 - 23 Feb 2016	20 Jan 2016 - 5 Apr 2016
Age group primarily affected	Cows	Cows and calves	Cows	Cows and calves
Primary clinical signs				
Cows	Mastitis	Mastitis, arthritis	Arthritis	Mastitis, arthritis
Calves	Pneumonia, otitis media	Arthritis, otitis media, pneumonia	Few cases of arthritis and otitis media	Arthritis, otitis media, pneumonia
Diagnostic tests				
Positive samples[a]				
BioX[b]	16/51	26/101	14/89	13/93
MilA[c]	48/51	98/101	41/89	88/93
Positive cultures (necropsied calves)	0	1	0	2

[a] Number of seropositive samples out of all sera obtained during the study period
[b] ODC% values > 37 in BioX Bio K 302 ELISA (BioX Diagnostics, Belgium)
[c] AU > 135 in the MilA ELISA

Laboratory analysis of calves that were euthanised and necropsied outside the planned project activities revealed additional information about the study herds. One calf from Herd 2 had chronic degenerative arthrosis in several joints and bronchopneumonia with overlying pleuritis; *M. bovis* was cultured from joint fluid, and both joint fluid and lung tissue were PCR positive for *M. bovis*. Two calves in Herd 4 had chronic omphalitis, bronchopneumonia, synovitis in several joints and bilateral otitis media; *Mycoplasma spp.* were cultured from these calves and identified as *M. bovis* by PCR.

Bacterial culture was negative for two calves from Herd 3 that were necropsied, even though arthritis and otitis media were observed in both animals. However, the presence of typical clinical signs (including arthritis/swelling of the limbs, and very high serum antibody titres against *M. bovis*) in multiple cows, and the failure to detect any other pathogens suggest that it is highly likely that the clinical signs were associated with *M. bovis*.

No animals from Herd 1 were necropsied, but positive PCR results from milk from cows with mastitis were obtained before and during the study period.

Sample collection

During the first visit, the investigating veterinarian and the farmer selected 20 calves using the following standardised procedure. The farmer was told which clinical signs were considered indicative of infection with *M. bovis* (i.e. clinical signs consistent with arthritis, otitis media and pneumonia), and asked to identify ten calves that he believed were currently showing or had recently shown any of these clinical signs, and ten that he believed had not had any of these clinical signs. If ten calves with clinical signs could not be identified, additional calves were selected by the investigator to complete the cohort. All calves with the specified clinical signs at the first herd visit were included, even if there were more than ten. New calves suspected of having *M. bovis*-associated disease at subsequent visits were also included.

During the herd visits, each calf underwent a clinical examination with a focus on the respiratory and musculoskeletal system, using a standardised clinical protocol, which is available from the corresponding author. One of three veterinarians performed the clinical examinations, and at least one of the authors of the clinical examination protocol was present at each visit.

In addition, a venous blood sample was collected from each calf using a 10 ml plain Vacutainer tube (Kruuse, Denmark). Blood samples were stored in a cool environment and delivered to the Eurofins Steins laboratory (Vejen, Denmark) within 36 hours of collection. The serum samples were frozen at -18°C.

Detection of antibodies

The serum samples were thawed at the National Veterinary Institute, in the Technical University of Denmark (DTU), Copenhagen, and analysed for antibodies against *M. bovis* using the commercial kit BioX Bio K 302 ELISA (BioX Diagnostics, Rochefort, Belgium) and the in-house indirect IgG ELISA test (MilA ELISA) developed at the University of Melbourne, Australia, by Wawegama et al. (2014).

The BioX assay was performed according to the manufacturer's instructions. The test outcome was calculated as:

$$ODC\% = ((OD_{sample} - OD_{negative\ control}) / (OD_{positive\ control} - OD_{negative\ control})) \times 100$$

Where OD is the optical density. An ODC% > 37 was considered positive, as recommended by the manufacturer. The manufacturer reported the sensitivity and specificity of the test at this cut-off to be 100% in a small sample of experimentally infected and negative control calves (Anonymous, 2017). However, other authors have found poor sensitivity in experimentally infected animals (Schibrowski et al., 2018) and the a recent field study in cows has supported this (Petersen et al., 2018).

The MilA ELISA was performed as described by Wawegama et al. (2014). The mean antibody concentration in antibody units (AU) was calculated by plotting the OD values on a standard curve derived from a set of known positive-control sera included on each plate. In feedlot cattle with BRD the sensitivity and specificity of this assay have been estimated at 94.3% (95% confidence interval: 89.9-99.6%) and 94.4% (95% confidence interval: 90.3-99.6%), respectively, using 105 AU as cut-off (Wawegama et al., 2016). However, the authors recommend using AU > 135 as cut-off for a test positive interpretation.

Statistical analysis

Individual calves were categorised into the following disease groups:

1) Likely *M. bovis*-associated disease ('*M. bovis*')

2) Respiratory disease only ('Respiratory')

3) No clinical signs of disease ('Healthy')

This classification was based on the recorded clinical signs and photographs of each calf taken during the herd visits, using the following specific inclusion criteria:

- *M. bovis:* calves with clinical signs indicating infection with *M. bovis.* All calves in this group had arthritis and/or otitis media at one or more herd visits, and some calves had in addition signs of respiratory disease.
- Respiratory: calves with only clinical signs of respiratory disease that did not fall into the category given above. All calves had one or more of the following clinical signs at one or more herd visits: dyspnoea, abnormal lung sounds on auscultation, discharge from the nares or eyes and coughing.
- Healthy: calves with no clinical signs of disease.

Modelling of antibody responses

Separate linear mixed models were used, with BioX and MilA ELISA results as the outcome variables. The ODC% and AU measurements were log transformed to improve the normality of the residuals, and a fixed constant of 1 was added to all results to enable log transformation of all values, including the small number of zero values.

For each of the two outcomes, model selection was used to find the most parsimonious model based on the potential explanatory variables and their two-way interactions. The fixed effects to be tested were: the disease group (categorical variable), the herd (categorical variable) and the age (in days) of the calf on the sampling date (pseudo-continuous variable). An additional quadratic effect of age was included in order to allow for a non-linear relationship between age and ELISA response. The final model was obtained using backward stepwise elimination based on Akaike´s information criterion (AIC). A random effect of calf identification number was included in all models in order to account for repeated samples from the same calf. Confidence intervals for the predicted mean ODC% and AU values (for an "average" calf) were calculated for each herd using parametric bootstrapping.

Age intervals containing fewer than three observations from different calves in the same herd were removed. This was done because different age groups were sampled across the different herds, and to restrict the models to regions of parameter space with enough observations. As a result, herd-specific graphs of predicted ELISA responses span different age intervals for each herd. Dashed vertical lines in Figures 1 and 2 illustrate the herd-specific age ranges included in the modelling.

The goodness of fit of the model was estimated using marginal and conditional pseudo R^2 for mixed models, which was estimated using the method described by Nakagawa and Schielzeth (2013). Using this method, the marginal R^2 describes the variation explained by the fixed effects alone, and the conditional R^2 describes the variation explained by both fixed and random effects. All data management and analyses were done in R version 3.2.2 (R Core Team, 2016), with linear mixed models implemented using the lme4 package (Bates et al., 2015).

Results

Descriptive statistics

In total, 83 calves were enrolled in the study and 334 blood samples were collected. Table 2 shows the number of calves stratified by herd and disease group.

Of the 83 calves, 52 were sampled on five occasions, 16 on four occasions, 1 on three occasions, 6 on two occasions and 8 on one occasion. The primary reason for calves dropping out of the study was euthanasia (N=13), and eight calves were moved to another property during the study. Due to a laboratory error, samples from the first visit to Herd 1 were not analysed.

The BioX ELISA response was below the recommended cut-off of 37 ODC% for the entire study period for 48 of the calves, above the cut-off for the entire study period for eight calves, and 27 changed status (Figure 1). Few calves had an ODC% above the cut-off before they were 40-60 days old (Figure 1).

Table 2: Distribution of calves by disease group and herd in a Danish longitudinal field study of four dairy herds with an outbreak of *M. bovis*-associated disease.

Disease group		***'M. bovis'***	**Respiratory**	**Healthy**	**Total**
Herd 1					
	Calves	2	13	0	15
	Samples	8	43	0	51
Herd 2					
	Calves	7	14	1	22
	Samples	29	70	2	101
Herd 3					
	Calves	6	11	3	20
	Samples	23	51	15	89
Herd 4					
	Calves	15	10	1	26
	Samples	49	40	4	93
Total					
	Calves	30	48	5	**83**
	Samples	109	204	21	**334**

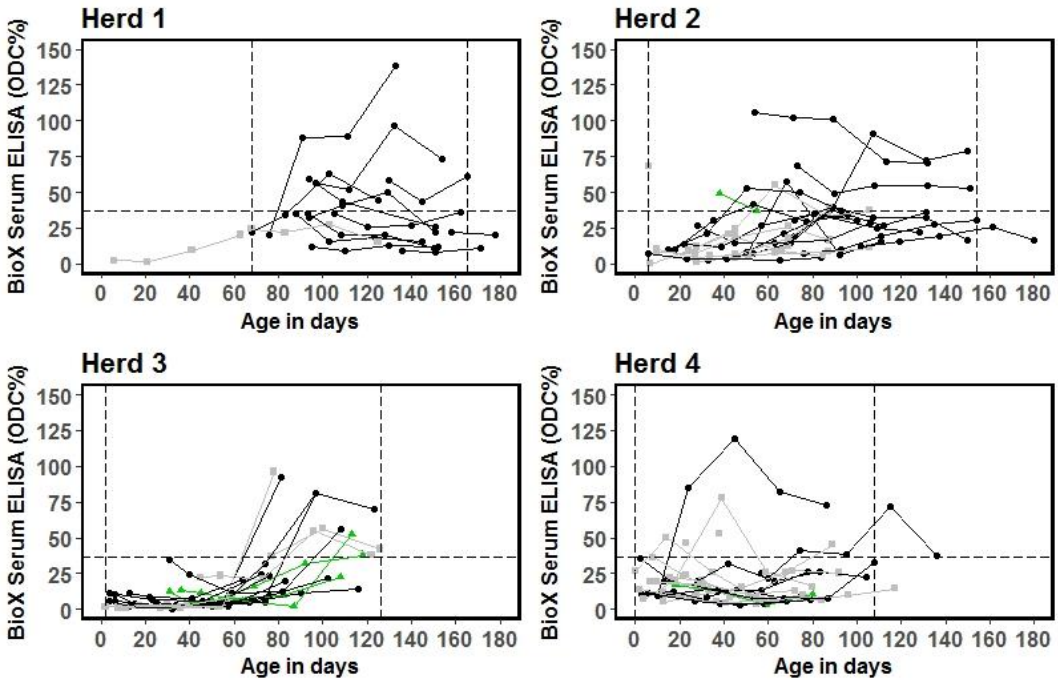


Figure 1: Distribution of ELISA measurements in the BioX ELISA Bio K302 assay (ODC% = sample coefficient) of serum antibodies against *M. bovis* in four Danish dairy herds. Grey squares = "*M. bovis"*; black dots = "Respiratory"; green triangles = "Healthy". Horizontal dashed lines show the recommended ELISA cut-off (37 ODC%). Vertical dotted lines indicate the limits for including observations in the modelling of antibody response dynamics. Results from the same calf are linked by lines.

The MilA ELISA response was above the recommended cut-off of 135 AU throughout the entire study period for the majority of the calves (Figure 2). Only one calf was below the cut-off at the end of the study period. The MilA ELISA detected antibodies soon after birth (i.e. at approximately 20 days of age), but as was evident in Herd 3, the antibodies did not increase quickly in all herds. The responses varied, but remained above the cut-off once it has been reached.

Based on the raw data plots, there seems to be no association between disease group and antibody responses for either ELISA test (Figures 1 and 2).

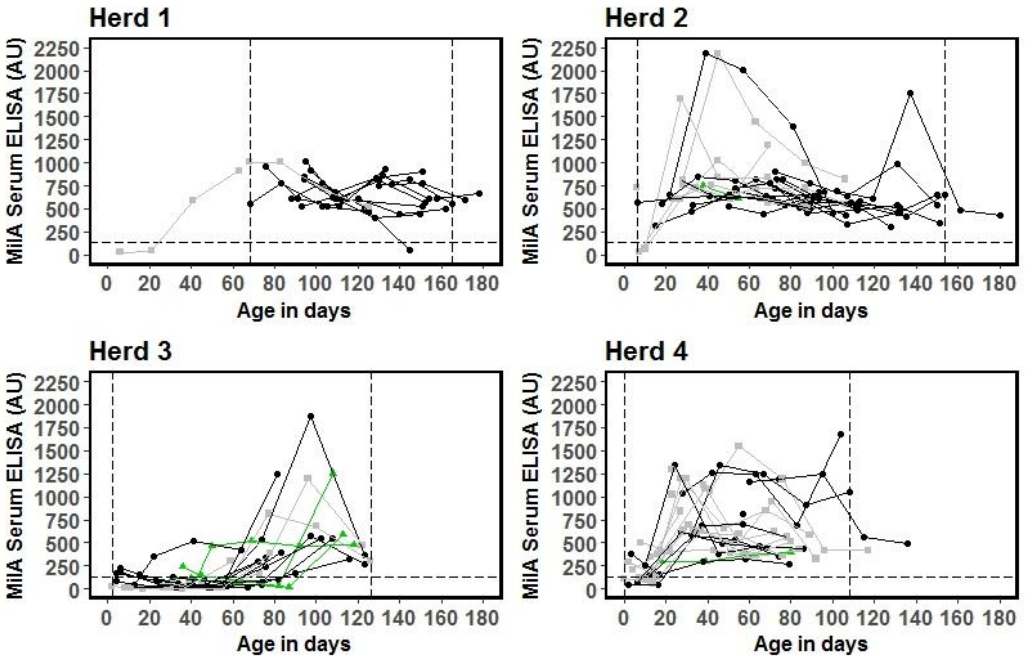


Figure 2: Distribution of serum ELISA measurements in the MilA assay (AU = sample antibody units) of serum antibodies against *M. bovis* in four Danish dairy herds. Grey squares = "*M. bovis*"; black dots = "Respiratory"; green triangles = "Healthy". Horizontal dashed lines show the recommended ELISA cut-off (135 AU). Vertical dotted lines indicate the limits for including observations in the modelling of antibody response dynamics. Results from the same calf are linked by lines.

Results of the statistical modelling

The final model with log transformed BioX ODC% as the outcome included the linear and quadratic effects of age, the fixed effect of herd, and the two-way interactions between herd and both the linear & quadratic effects of age (Table 3). The variance associated with the random effect of animal was considerable, although less so than the residual variance. There was a positive linear effect of age in all herds, but the sign of the quadratic effect was dependent on herd. Based on Figure 3, the mean estimate of ODC% in three of the four herds increased gradually with age and did not reach the recommended individual animal cut-off. In the remaining herd, the rate of increase in ODC% increased with the age of the calf. The highest mean estimate of ODC% was not reached until the calf was approximately 110-130 days old (depending on the herd), with some suggestion of a plateau and eventual decline above this age in three of the herds (Figure 3). However, a comparison of this relationship among herds is complicated by the difficulty in extrapolating the polynomial effect of age outside the observed parameter space, which was further compounded by the small differences in the ages of the calves among herds.

Table 3: Final model describing explanatory variables and random effects of log transformed BioX K302 ELISA optical density measurements (ODC%). The marginal R^2 was 39% and conditional R^2 was 61%.

Variables	**Variance**	**95% confidence interval**		
Random effects				
Animal	0.28	0.13 - 0.38		
Residuals	0.47	0.37 - 0.54		
		Estimate	**SE**	***P*-value**
Fixed effects				
Intercept		2.55	0.26	< 0.001
Age days (linear)		14.68	3.93	< 0.001
Age days (quadratic)		-8.16	2.27	< 0.001
Herd 1		0		-
Herd 2		0.30	0.30	0.311
Herd 3		-0.10	0.30	0.753
Herd 4		0.37	0.31	0.229
Age days (linear)*Herd 1		0		-
Age days (linear)*Herd 2		-5.93	4.27	0.165
Age days (linear)*Herd 3		7.11	4.59	0.123
Age days (linear)*Herd 4		-8.15	5.06	0.108
Age days (quadratic)*Herd 1		0		-
Age days (quadratic)*Herd 2		4.80	2.65	0.072
Age days (quadratic)*Herd 3		13.42	3.19	< 0.001
Age days (quadratic)*Herd 4		13.95	3.31	< 0.001

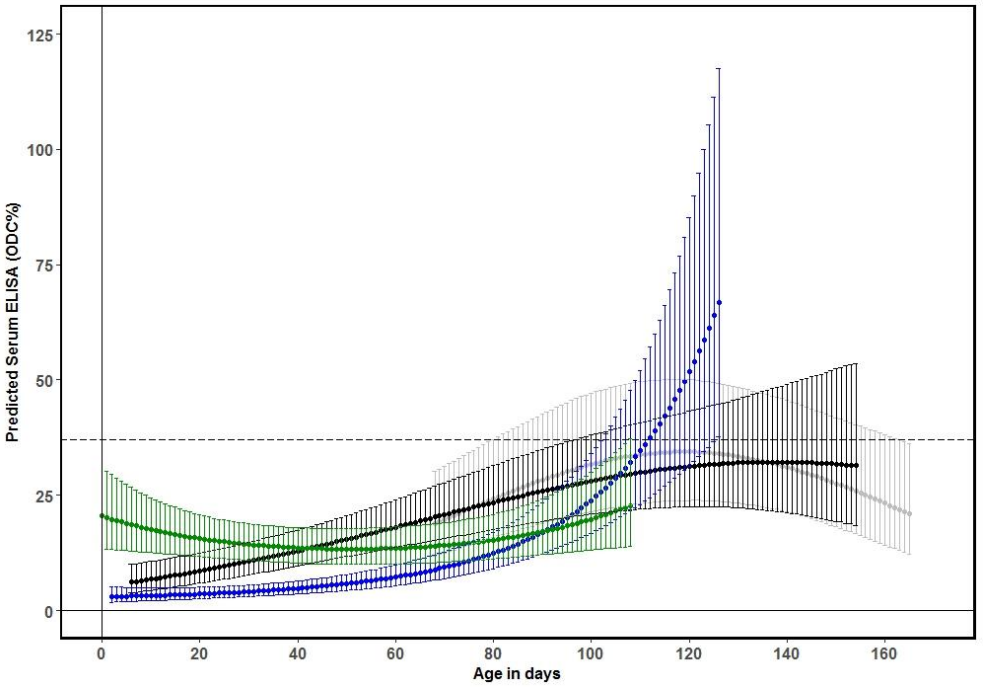


Figure 3: Estimated mean antibody response in sera (solid line) and 95% confidence intervals (shaded area) as measured by the BioX ELISA Bio K302 assay for the herd-specific age ranges for which observations were available. Herd 1 is grey, Herd 2 is black, Herd 3 is blue and Herd 4 is green. The dashed line shows the recommended individual animal ELISA cut-off (37 ODC%).

The final model with log transformed MilA AU as the outcome also included the linear and quadratic effects of age, the fixed effect of herd, and the two-way interactions between herd and both the linear & quadratic effects of age (Table 4). The variance associated with the random effect of animal was less than that estimated for the BioX ELISA. Again, there was a positive linear effect of age in all herds, but as for the BioX ELISA, the sign of the quadratic effect was dependent on herd. Based on Figure 4, it appears that the MilA ELISA detected antibodies in younger calves, and for two herds, the mean estimate of MilA AU was above the recommended animal cut-off value for animals less than ten days old. The overall shape of the relationship between age and estimated AU was similar in three of the four herds (Figure 4). It can be characterised by an initial phase of increase followed by a plateau and an eventual decrease, although the peak was reached at the older age of 110-120 days in Herd 1, compared to approximately 60-80 days in Herds 2 and 4. A significantly different pattern, which was more similar to an exponential increase from an initially low MilA AU value, was estimated for Herd 3 (Figure 4).

Table 4: Final model describing explanatory variables and random effects of log transformed MilA ELISA antibody units (AU). The marginal R^2 was 59% and conditional R^2 was 65%.

Variables	**Variance**	**95% confidence interval**		
Random effect				
Animal	0.09	0.007 – 0.150		
Residuals	0.57	0.463 – 0.655		
		Estimate	**SE**	***P*-value**
Fixed effects				
Intercept		5.77	0.22	< 0.001
Age days (linear)		12.91	3.53	< 0.001
Age days (quadratic)		-8.25	2.18	< 0.001
Herd 1		0		-
Herd 2		0.61	0.25	0.015
Herd 3		-0.83	0.25	0.001
Herd 4		0.32	0.26	0.231
Age days (linear)*Herd 1		0		-
Age days (linear)*Herd 2		-11.90	3.89	0.003
Age days (linear)*Herd 3		10.79	4.35	0.014
Age days (linear)*Herd 4		-15.88	4.84	0.001
Age days (quadratic)*Herd 1		0		-
Age days (quadratic)*Herd 2		4.13	2.64	0.118
Age days (quadratic)*Herd 3		14.71	3.29	< 0.001
Age days (quadratic)*Herd 4		-2.19	3.40	0.520

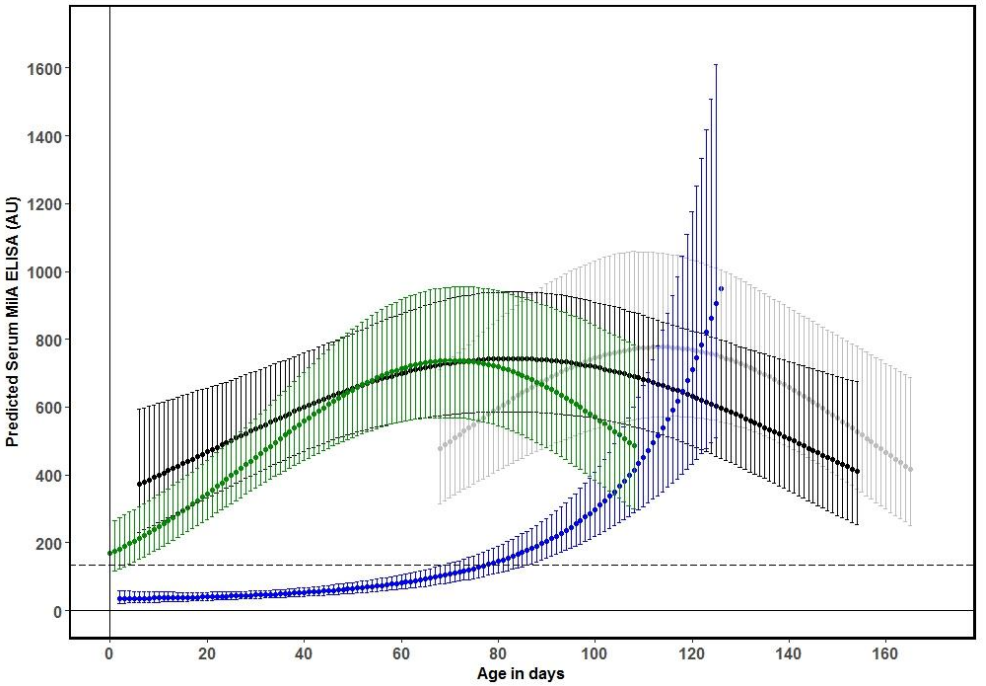


Figure 4: Estimated mean antibody response in serum (solid line) and 95% confidence intervals (shaded area) as measured by the in-house MilA ELISA for the herd-specific age ranges for which observations were available. Herd 1 is grey, Herd 2 is black, Herd 3 is blue and Herd 4 is green. The dashed line shows the recommended individual animal ELISA cut-off (135 AU).

Discussion

This is the first observational study to illustrate and analyse the dynamics of serum antibody responses against *M. bovis* in naturally exposed and infected calves. In addition to a large variation in antibody responses among calves both within and between herds, the key findings were that the BioX ELISA rarely detected antibodies in calves under the age of two months, while the MilA ELISA was able to detect antibodies in the same calves soon after birth. Since the calves in these herds were all very likely to be truly exposed, we conclude that the MilA ELISA is a potentially useful test of *M. bovis* exposure. However, neither ELISA could differentiate between calves with arthritis and/or otitis, and respiratory disease, which indicates that the tests may be less useful for distinguishing animals with *M. bovis*-related diseases from those that have just been exposed to the pathogen.

Antibody dynamics measured by the BioX ELISA

In general, few calves seroconverted to values above the recommended cut-off of 37 ODC%, and the majority remained below the cut-off for the entire study period the BioX ELISA, despite the fact that these calves either showed signs of *M. bovis*-associated disease or were housed with diseased calves during acute outbreaks of disease caused by *M. bovis*. In Herd 2, several calves had severe arthritis,

and *M. bovis* was cultured from one necropsied calf. Despite this, the BioX ELISA did not detect antibodies above the recommended cut-off in sera from these calves (Figure 1; grey triangles). It is possible that because these calves were very young when they were infected, their immune system was not yet able to produce antibodies against the *M. bovis* antigen used in the BioX ELISA. A similar phenomenon has been seen with *Salmonella* Dublin (Roden et al., 1992). Virtala et al. (2000) also found that young calves often fail to seroconvert to common respiratory pathogens including *M. bovis*, although this was assessed using a different assay. The BioX ELISA was also used in a vaccine challenge study by Dudek et al. (2016). In the positive control group, which consisted of five- to six-week-old heifers intratracheally challenged with *M. bovis*, the antibody response increased only slightly to a maximum of approximately 50 ODC% at four weeks after challenge, and thereafter declined slightly. By contrast, in the vaccinated group, which was inoculated subcutaneously with inactivated *M. bovis* mixed with two adjuvants, serum antibody concentrations rapidly increased within two weeks of vaccination and reached a maximum of around 200 ODC% in four weeks. This difference is likely to reflect the greater stimulation of the systemic immune response following inoculation with an adjuvanted whole cell vaccine compared to natural infection.

Our study is the first to evaluate the dynamics of the BioX ELISA in calves under field conditions and we have shown that it is not a suitable test for reliable diagnosis of *M. bovis* in calves below three months of age. However, this does not rule-out that it can be useful for group diagnostics in younger calves if the cut-off is adjusted. Further studies in larger number of herds with different disease occurrence are warranted to explore this.

Antibody dynamics measured by MilA ELISA

The MilA ELISA detected antibodies above the cut-off in calves as young as approximately 20 days of age, with antibody concentrations rising markedly over a short timeframe, and at the end of the study period, all but one of the calves were above the cut-off. However, the MilA ELISA did not detect antibodies early in all four herds. In Herd 3, the antibody levels did not start to rise until the calves were 60-80 days of age, and then increased rapidly. No *M. bovis*-associated disease was found among the calves in this herd during the first two herd visits, indicating that they had managed to prevent transmission between cows and calves and had kept the infection pressure low around the calves. In addition, the first visit to Herd 3 was only one week after the appearance of clinical signs in the herd, and the farmer did not feed any waste milk to the calves. The later response detected by the MilA ELISA in this herd could be a result of initial infection among the cows and later transmission to the calves (i.e. around 40-60 days after the outbreak had started). The other herds were visited three to four weeks after the outbreak had started, and transmission to the calves had already occurred by this time. This could explain the high serum antibody concentrations in young calves in these herds. It can therefore be concluded that the MilA assay does not detect young calves as positive in non-infected groups of calves, which supports a reasonable specificity and therefore the usefulness of this test for confirmation and surveillance purposes. To substantiate this finding, control herds with no known *M. bovis*-associated diseases should be assessed for comparison. The high antibody concentrations detected in most calves in this study, including the healthy calves, suggests that the MilA test is very sensitive and probably detects exposure to *M. bovis* rather than *M. bovis*-associated disease. Both experimental and field studies of the MilA ELISA also suggest that this test has a high level of sensitivity (Wawegama et al., 2016).

The use of antibodies to detect infection with *M. bovis*

Our study has shown that the *M. bovis* BioX and MilA ELISA tests give very different results for calves with the same exposure and disease status in calves younger than two months old indicating different immune reactions to the underlying infection (Herd 2 and 4 in Figures 1 and 2). In herds with clinical signs in older calves, the modelled antibody responses were similar in shape, indicating similar immune reactions to the underlying infection (Herd 1 and 3 in Figures 1 and 2). This is likely associated with the age-related development of immune competence in the calves, which is important for the interpretation of the test results for diagnosis.

Clinical disease did not seem to correlate with the antibody response detected by either ELISA assay. The effect of "Herd" was retained during model selection, indicating that the different ELISA responses were mostly influenced by differences among the herds, and not differences in the underlying disease status of individual animals. Although Martin et al. (1990) performed their study using an indirect haemagglutination assay, they also concluded that serum antibodies against *M. bovis* were not indicative of disease at an individual level, only at group level.

Only five calves were classified as being healthy, which is a small number to include in the models as a separate group, yet disease group was not found to be significant. To assess the robustness of the results, models were generated both without the 'Healthy' group, and with the healthy calves included in the 'Respiratory' group, and none of these variations altered the conclusions.

The 'Respiratory' group consisted of many calves, and it is not possible to know whether the disease in this group was caused by *M. bovis* alone or in conjunction with other respiratory pathogens. At the time of sampling, the calves were housed in herds with an active or recent spread of *M. bovis,* so it is likely that the disease seen was at least partly attributable to infection with *M. bovis*. In Herds 2 and 4, *M. bovis* was isolated from necropsied calves, and all herds were free of the likely differential diagnosis *Salmonella* Dublin throughout the study period, making it likely that the arthritis was caused by *M. bovis.*

Conclusions

This is the first study to evaluate the dynamics of antibody responses using the BioX ELISA in calves under field conditions and based on the data available for this study it cannot be recommended for use in calves below three month of age. The MilA ELISA was able to detect antibodies shortly after birth and is likely to be a good assay for detecting exposure to *M. bovis*. Neither ELISA could differentiate between calves with arthritis and/or otitis media, and respiratory disease.

List of abbreviations

AU	antibody units
BioX	BioX Bio K 302 ELISA test
ELISA	enzyme-linked immunosorbent assay
IgG	immunoglobulin G
M. bovis	*Mycoplasma bovis*
MilA	in-house indirect IgG ELISA test
OD	optic density
ODC%	corrected optic density measurement
PCR	polymerase chain reaction

Declarations

Ethics approval and consent to participate
All animals used in this study were handled in strict accordance with good clinical practice, and all efforts were made to minimise suffering. All animal owners gave written consent for their animals to be used in this study.

Consent for publication
Not applicable

Availability of data and material
The data that support the findings of this study are available from SEGES (Agro Food Park 15, 8200 Aarhus, Denmark), but restrictions apply to the availability of these data, which were used under license for the current study, and are therefore not publicly available. However, data are available from the authors upon reasonable request and with permission of SEGES.

Competing interests
The authors declare that they have no competing interests.

Funding
The study was funded by the Danish Milk Levy Fund, the Danish Cattle Levy Fund, the Pesticide Levy Fund and the University of Copenhagen. NKW's visit to Denmark to perform MilA ELISA was jointly funded by a Dyson Fellowship and a University of Melbourne Engagement and Partnerships grant from the Faculty of Veterinary and Agricultural Sciences, University of Melbourne.

Authors' contributions

MBP: Planned the study design and the clinical protocol, planned and performed the field sampling and clinical recordings, data management and statistical analysis, drafted the manuscript

NKW: Planned and developed the MilA ELISA, planned and performed the MilA ELISA testing in Denmark, lead all laboratory analyses and assisted with interpretation of laboratory results, commented on and approved the manuscript

MD: Supervised and assisted with the statistical analyses and interpretation of results, commented on and approved the manuscript

PFM: Planned and developed the MilA ELISA, commented on and approved the manuscript

GFB: Planned the development and assessment of the MilA ELISA, guided the interpretation of results, commented on and approved the manuscript

LRN: Planned the study design and the field study, supervised data management and statistical analysis, commented on and approved the manuscript

All authors approved the final version to be published and agreed to be accountable for all aspects of the work.

Acknowledgements

The authors would like to thank the veterinarians Ulla Torpe and Martin Lund (Dyrlægerne Egtved), Lars Pedersen (SEGES) and the veterinarians/veterinary students Annie Nielsen, Eva Elisabeth Toft-Petersen, Franziska Helene Pedersen and Signe Rejnhardt Olsen for their assistance with the herd visits and veterinarians Dinah Lerdahl Holm and Jeanette Petersen and the National Veterinary Institute at Technical University of Denmark for assistance and facilities for the laboratory testing of samples.

References

Anonymous. 2017. MYCOPLASMA BOVIS ELISA KIT, http://www.biox.com/en/bio-k-302-monoscreen-abelisa-mycoplasma-bovis-indirect-monowell-p-250/. Accessed 17-11-2017.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67(1):1-48.

Dudek, K., D. Bednarek, R. D. Ayling, A. Kycko, E. Szacawa, and T. A. Karpinska. 2016. An experimental vaccine composed of two adjuvants gives protection against *Mycoplasma bovis* in calves. Vaccine 34:3051-3058.

Martin, S. W., K. G. Bateman, P. E. Shewen, S. Rosendal, J. G. Bohac, and M. Thorburn. 1990. A group level analysis of the associations between antibodies to seven putative pathogens and respiratory disease and weight gain in Ontario feedlot calves. Can. J. Vet. Res. 54(3):337-342.

Maunsell, F. P., and G. A. Donovan. 2009. *Mycoplasma bovis* Infections in young calves. Vet. Clin. North Am. Food Anim Pract. 25(1):139-77.

Nakagawa, S., and H. Schielzeth. 2013. A general and simple method for obtaining R2 from generalized linear mixed-effects models. Methods in Ecology and Evolution 4(2):133-142.

Petersen, M. B., J. Pedersen, D. L. Holm, M. Denwood, and L. R. Nielsen. 2018. A longitudinal observational study of the dynamics of *Mycoplasma bovis* antibodies in naturally exposed dairy cows. Journal of Dairy Science.

Roden, L. d., B. P. Smith, S. J. Spier, and G. W. Dilling. 1992. Effect of calf age and Salmonella bacterin type on ability to produce immunoglobulins directed against Salmonella whole cells or lipopolysaccharide. American Journal of Veterinary Research 53(10):1895-1899.

Sachse, K., H. S. Salam, R. Diller, E. Schubert, B. Hoffmann, and H. Hotzel. 2010. Use of a novel real-time PCR technique to monitor and quantitate *Mycoplasma bovis* infection in cattle herds with mastitis and respiratory disease. Vet. J. 186(3):299-303.

Schibrowski, M. L., T. S. Barnes, N. K. Wawwgama, M. E. Vance, P. F. Markham, P. D. Mansell, M. S. Marenda, A. Kanci, J. Perez-Casal, G. F. Browning, J. S. Gibson, and T. J. Mahony. 2018. The Performance of Three Immune Assays to Assess the Serological Status of Cattle Experimentally Exposed to *Mycoplasma bovis*. Veterinary Sciences 5(27).

Van Donkersgoed, J., C. S. Ribble, L. G. Boyer, and H. G. Townsend. 1993. Epidemiological study of enzootic pneumonia in dairy calves in Saskatchewan. Can. J. Vet. Res. 57(4):247-254.

Virtala, A. M. K., Y. T. Grohn, G. D. Mechor, H. N. Erb, and E. J. Dubovi. 2000. Association of seroconversion with isolation of agents in transtracheal wash fluids collected from pneumonic calves less than three months of age. Bovine Practitioner; 34(2):77-80.

Wawegama, N. K., G. F. Browning, A. Kanci, M. S. Marenda, and P. F. Markham. 2014. Development of a recombinant protein-based enzyme-linked immunosorbent assay for diagnosis of *Mycoplasma bovis* infection in cattle. Clinical and Vaccine Immunology 21(2):196-202.

Wawegama, N. K., P. F. Markham, A. Kanci, M. Schibrowski, S. Oswin, T. S. Barnes, S. M. Firestone, T. J. Mahony, and G. F. Browning. 2016. Evaluation of an IgG enzyme-linked immunosorbent assay as a serological assay for detection of *Mycoplasma bovis* infection in feedlot cattle. Journal of Clinical Microbiology 54(5):1269-1275.

Zhang, R., X. Han, Y. Chen, R. Mustafa, J. Qi, X. Chen, C. Hu, H. Chen, and A. Guo. 2014. Attenuated *Mycoplasma bovis* strains provide protection against virulent infection in calves. Vaccine 32:3107-3114.

8.4 Manuscript IV

Increased incidence rate of undesired early heifer departure in *Mycoplasma bovis*-antibody positive Danish dairy cattle herds

M. B. Petersen[1*], A. K. Ersbøll[2], K. Krogh[3] and L. R. Nielsen[1]

[1] Department of Veterinary and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark
[2] National Institute of Public Health, University of Southern Denmark, Copenhagen, Denmark
[3] Ceva Animal Health A/S, Ladegaardsvej 2, 7100 Vejle, Denmark

[*]**Correspondence:** Mette Bisgaard Petersen, Groennegaardsvej 8, 1870 Frederiksberg, Denmark, mbp@sund.ku.dk

The manuscript has been submitted to Epidemiology and Infection

Summary

Mycoplasma bovis infections cause disease and production losses in cattle worldwide. The long-term consequences are not well described despite being important for management decisions during and after disease outbreaks. We investigated the association between *M. bovis* antibody-positivity and undesired early departure (UED, i.e. death, euthanasia or slaughter) before first calving in a cohort of 636 heifers from 36 Danish dairy herds with and without a history of *M. bovis*-associated disease. The herds were visited 4 times at 3-month intervals and blood samples from young stock and milk samples from lactating cows were collected. Poisson regression was performed to examine the association with UED as outcome, logarithmic transformation of risk time as offset and herd as a random effect. Individual antibody measurements and group-level variables representing the infection level among young stock and cows, age and mortality variables were included in the model. The incidence rate ratio of UED increased by 1.23 times for every 10% increase in young stock seroprevalence, while the effect of individual antibody level was modified by age and influenced UED less. In conclusion, UED in heifers was associated with *M. bovis* antibody-positivity in young stock and should be controlled in dairy herds to reduce losses.

Introduction

Mycoplasma bovis (*M. bovis*) is a bacterium associated with severe, often untreatable disease in cattle of all ages and is associated with production losses and decreased animal welfare worldwide (Maunsell and Donovan, 2009). Clinical signs include pneumonia and arthritis in all age groups, otitis media in calves and mastitis in cows (Maunsell et al., 2011; Maunsell and Donovan, 2009) leading to increased mortality risk in both calves and cows, and to impaired growth and lower carcass weight in veal calves (3;4).

A proportion of cattle diseased from *M. bovis* will have a humoral immune response that can be measured using ELISA testing of antibodies in serum samples soon after the clinical signs are observed (Nicholas et al., 2002; Petersen et al., 2018a; Petersen et al., 2018b). Therefore, antibody measurements might be used to confirm or rule-out that *M. bovis* infection is associated with the observed disease syndromes during disease outbreaks, and a question often raised by farmers and veterinarians is whether to cull antibody-positive cattle. However, the long-term consequences of individual *M. bovis* antibody positivity in cattle housed in dairy herds with current or previous *M. bovis* infections are not well described

The associations between *M. bovis* antibody response, disease and production parameters have not been studied much in cattle and the findings are not clear. In one study, the seroconversion rate against *M. bovis* in a group of feedlot calves was associated with bovine respiratory disease (BRD) rates (Martin et al., 1990), whereas the *M. bovis* serostatus at arrival in a veal calf setting was not a predictor of development of BRD during the two weeks after arrival in another study (Pardon et al., 2015). In a risk factor study, the seroprevalence of *M. bovis* in heifers and cows was not found to be associated with the incidence of respiratory disease in the calves (Raaperi et al., 2012). With regard to production parameters, a tendency for lower weight gain in *M. bovis* seropositive weaned beef

calves was seen in one study (Hanzlicek et al., 2011), while Martin et al. (1990) found no association between serostatus and growth of feedlot calves at group level.

The above mentioned studies all focus on BRD and weight gain in beef calves, which implies that it is primarily concerning male calves, which usually have a shorter lifespan than heifer calves in dairy herds. The implications for calves in a dairy herd experiencing a severe *M. bovis*-associated disease outbreak could be different and, in this case, other herd factors should be taken into account such as active infection among the lactating cows, colostrum management and other biosecurity procedures. In addition, the long-term effect of the disease including potentially reduced growth in heifer calves has not been described in published literature. This information is needed in order to advice farmers about the impact of implementing proper management of heifer calves and young stock during an *M. bovis* outbreak in a dairy herd. Due to the economic losses associated with feeding and housing of heifers that will be culled later due to poor performance, it would be useful to be able to provide advice on which calves to keep in the herd and which to cull early. Under the hypothesis that infections with *M. bovis* result in antibody positivity in affected cattle and that *M. bovis* has long-term health effects on infected cattle, we aimed to investigate the association between individual and herd-level *M. bovis* antibody-positivity on undesired early departure (UED) before first calving in a cohort of dairy heifers.

Methods

Study design and study population

The present study was based on a cohort of heifers in Danish dairy herds enrolled in a field project aiming to provide a better understanding of factors related to *M. bovis*-associated disease and interpretation of diagnostic methods. For a detailed description of how the study herds were selected, please see Petersen et al. (2016). In short, 39 Danish dairy herds were selected for the study aiming to be representative of a distribution of herds with different histories of *M. bovis*-associated disease in terms of different outbreak sizes and durations as well as time since the outbreak ended, also including some with no history of *M. bovis*-associated disease. Data from 3 herds had to be excluded from this study: one went out of business during the project and two had complex, multisite facilities with the animals being moved around between different properties between visits, making it impossible to connect milk samples to the same site that the calves were housed at. The herds were visited four times approximately 3 months apart. At each visit, blood samples were collected from 65 calves distributed in four age groups (Figure 1). At the first herd visit, a cohort of approximately 20 heifer calves were selected from the 0-3 months old calves ("cohort calves") and followed with repeated sampling, if still in the herd at the consecutive herd visits. With 3 repeated visits this meant that at the last sampling round calves were 9-12 months old. The rest of the calves present were convenience sampled aiming for 15 calves being sampled per age group (i.e. 0-3, 3-6, 6-9, 9-12 months old). Fifty milk samples from randomly selected lactating cows in the same herd were collected at the milk recording date closest to the herd visit date, as also described in Petersen et al. (2016).

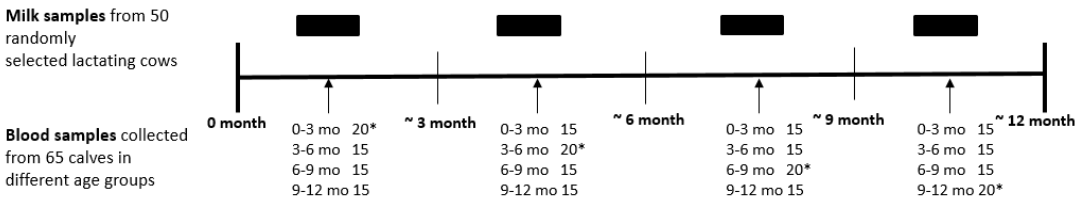


Figure 1: Overview of the four sampling rounds in each of the 36 Danish dairy herds included in this study. Black arrows indicate herd visit with blood sampling, black bars indicate milk sampling from lactating cows. At each sampling round, blood and milk samples were collected from the 20 cohort calves, 45 other calves and 50 lactating cows. Mo = months old, *= cohort calves.

Laboratory analysis

All samples were transported to Eurofins Steins Laboratory in Vejen, Denmark, and analysed for antibodies against *M. bovis* using the commercial ELISA BioX Bio K302 (BioX 302) (BioX Diagnostics, Rochefort, Belgium). It was performed according to the manufacturers recommendations (Anonymous, 2017) and the optical density coefficient (ODC%) was calculated as

$$ODC\% = (OD_{sample} - OD_{negative\ control}) / (OD_{positive\ control} - OD_{negative\ control}) \times 100\%$$

where OD is the optical density measured by the ELISA reader of each test sample and the positive and negative control on the sample ELISA plate. The manufacturer reported 100% sensitivity and specificity based on a small sample of experimentally infected calves and un-infected control calves (Anonymous, 2017). However, other experimental studies have estimated the sensitivity to be 0.37-0.47 and the specificity to be 0.95-0.96 (Schibrowski et al., 2018; Wawegama et al., 2016). No field study evaluations of cut-off values at animal level are available in the literature. However, the BioX 302 should not be used in calves younger than 3 months, because they are infrequently able to produce an antibody response measureable by this test and it might be worth considering the antibody response as positive at a lower cut-off (e.g. 20 ODC%) in calves than in adults (Petersen et al., 2018b).

Variables

Outcome

The outcome of interest was UED of individual cohort calves from the study herds due to either culling (slaughter or euthanasia) or death. Sold heifers were considered sold for profit (voluntary culling) and were censored on the day of departure. Information about date of birth, date of departure, departure destination and calving dates were collected from the Danish Cattle Database with written permission from the herd owners. Data entries for these variables were done by the farmer as required by law in Denmark. These entries are very reliable due to cross-check systems in place for EU-regulation determining agricultural subsidies.

Exposure

Test results from blood samples collected from calves younger than 90 days were excluded, as were observations from calves that did not survive until sampling round number two and therefore did not have any individual antibody measurements (Figure 2). Age of the calves was divided into 3-month intervals, with the first interval starting at date of birth plus 90 days (date of inclusion). Hence, the study period for analysis for each single calf started at date of inclusion and ended either at day of UED, or when censored due to first calving or being sold to another herd, whichever came first. Figure 2 shows examples of calves being included and excluded from the study.

Individual ELISA ODC%: The individual antibody responses in each heifer in each of the sampling rounds measured by the BioX 302 ELISA. An aggregated variable was created with the highest ODC% ever observed in heifers older than 90 days being assigned to all sampling rounds for each individual animal as an indicator of whether it was likely to have been infected with *M. bovis*. This was to account for the fact that antibody responses can be slow in calves.

Prevalence of antibody positive lactating cows (Cow-prevalence): This variable was calculated as the proportion of lactating cows with individual ELISA ODC% ≥ 37 in milk out of all cows tested in the herd at the day of sampling. An aggregated variable was created with the highest prevalence observed being assigned to all calves in the same herd as an indicator of the highest possible infection pressure in the adult cows during the study period.

Seroprevalence of antibody positive young stock (YS-seroprevalence): This variable was calculated as the proportion of calves with individual ELISA ODC% ≥ 37 in serum out of all calves tested in the herd at the day of sampling. An aggregated variable was created with the highest seroprevalence observed being assigned to all calves in the same herd as an indicator of the highest possible infection pressure in the young stock during the study period.

Potential confounders

Age group: Age was included as a confounder using 3-month intervals with the first interval being age group 3-6 months and the last being the 3-month interval where the last culling, calving or censoring was observed for each heifer, whichever came first.

Mortality-%: This variable was calculated as the average mortality risk for calves aged 0-14 days in the year before the fourth blood sampling rounds in the herd. It was categorized into the four categories, <1.3%, 1.3-2.6%, 2.7-4.2% and >4.2%, assuring an approximately equal distribution of events and time at risk in each category.

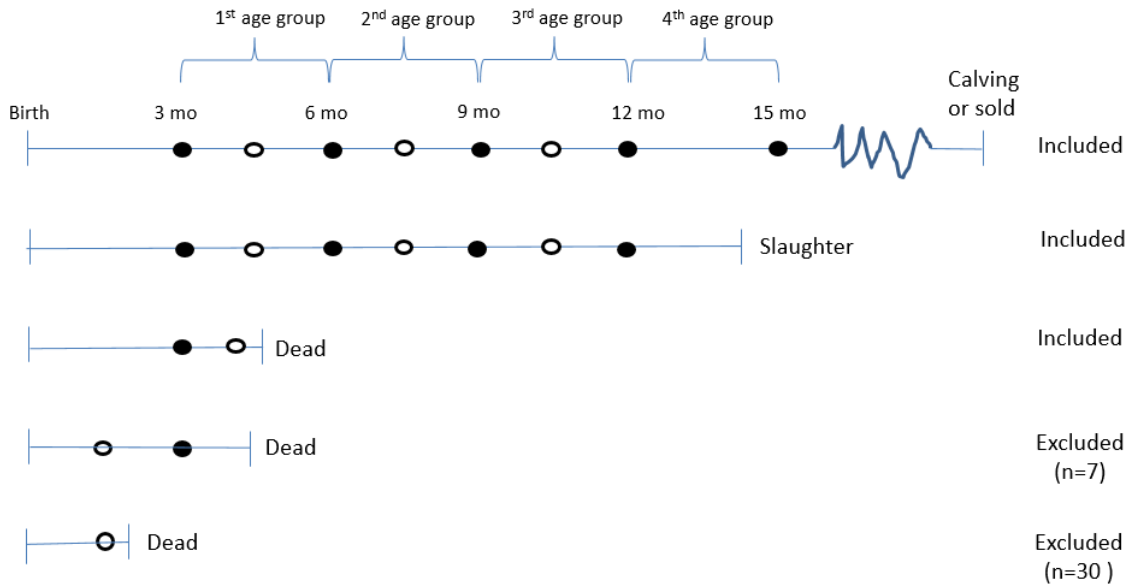


Figure 2: Schematic drawing showing examples of calves included in the study (not all types of observation patterns shown for included heifers) and the two possible sample combinations that led to exclusion of the heifer from the analysis dataset (n indicates the number of heifers excluded with a given exclusion pattern). Black circle = blood sample, black dot = age.

Statistical analysis

To examine the association between the exposure and confounder variables and incidence rate of UED, Poisson regression analyses were performed with UED as outcome and logarithmic transformation of time at risk as offset value. *M. bovis*-antibody-positivity as exposure was evaluated by including the continuous variables Individual ELISA ODC%, Cow-prevalence and YS-seroprevalence in the model as fixed effects. The analyses were adjusted for confounding effects of Age group and Mortality-%. To evaluate whether the association between Individual ELISA ODC% and the incidence rate of UED differed between age groups, the interaction between Individual ELISA ODC% and Age group was included in the model and evaluated with Individual ELISA ODC% and Age group included as main effects in the model. The incidence rate ratio (IRR) and 95% confidence intervals were calculated for the fixed effects. The IRR for the effect of a 10% increase in the Individual ELISA ODC% on the incidence rate of UED was calculated for each Age group. Herd identifiers were included as random effect to account for clustering of heifers within herds. Follow-up time was split by age resulting in approximately constant incidence rates. The assumption about linearity between each of the continuous variables and the outcome was evaluated by including the effect of the quadratic term in the model. A non-significant quadratic term was interpreted as linearity. Furthermore, the continuous variables were categorized and the parameter estimates were checked for rising/declining tendency. Data management and analyses were carried out in Statistical Analysis System (SAS version 9.4) and R version 3.2.2 (R Core Team, 2016).

Results

Descriptive statistics

In total 636 heifer calves were included of which 40 calves were sampled four times, 460 calves were sampled 3 times, 108 calves twice and 28 calves only once.

Sampling of lactating cows was missing in the third sampling round in one herd, and the prevalence of lactating positive cows at the third sampling round was then calculated as the average of same variable in rounds 2 and 4.

The mean seroprevalence of antibody-positive young stock was higher in the UED calves compared to censored calves, the mean individual ELISA ODC% was slightly higher in UED than censored calves, while the prevalence of antibody-positive lactating cows almost equal (Table 1). The incidence rate of culling was lowest from 9-18 months and increased from the age of 18-21 months, with a large increase from the age of 24 months and onwards. The time at risk for the age groups above 24 months was much lower than for the rest of the age groups mainly due to censoring of the heifers that calved. The incidence rate varied between the different Mortality-% groups (Table 2).

Table 1: Descriptive statistics of the continuous variables, Individual ELISA ODC%, prevalence of antibody positive lactating cows (Cow-prevalence) and seroprevalence of antibody positive young stock (YS-seroprevalence. Mean and standard deviation (SD)

Variable	Mean	SD
Individual ELISA ODC%		
Undesired early departure	45	35
Censored	43	30
Cow-prevalence		
Undesired early departure	0.25	0.14
Censored	0.24	0.17
YS-seroprevalence		
Undesired early departure	0.48	0.20
Censored	0.43	0.20

Table 2: Descriptive statistics of number of heifers with undesired early departures (UED), number of calf years and the incidence rate per 100 calf years overall, and stratified by mortality-% and age group.

Variable	Level	N (UED) (Euthanized or dead/ slaughter)	Time at risk in calf years	Incidence rate (UED per 100 calf years)
Overall		63 (20/43)	1090	5.8
Mortality-%	< 1.3%	14 (3/11)	302	4.6
	1.3-2.6%	19 (2/17)	266	7.2
	2.7-4.2%	11 (2/9)	285	3.9
	> 4.2%	19 (13/6)	238	8.0
Age group	3-6 months	6 (6/0)	156	3.8
	6-9 months	6 (6/0)	153	3.9
	9-12 months	3 (3/0)	152	2.0
	12-15 months	3 (1/2)	150	2.0
	15-18 months	3 (1/2)	148	2.0
	18-21 months	12 (0/12)	144	8.3
	21-24 months	9 (2/7)	120	7.5
	24-27 months	10 (1/9)	48	20.9
	> 27 months	11 (0/11)	19	59.2

Analytical statistics

Of the 3 variables measuring *M. bovis*-antibody-positivity, YS-seroprevalence and the interaction between Individual ELISA ODC% and Age group were significantly associated with UED. For a 10% increase in YS-seroprevalence, the incidence rate of UED increased by 23% (IRR=1.23, 95% CI: 1.02;1.48). Age significantly modified the association between Individual ELISA ODC% and the incidence rate of UED. For a 10% increase in Individual ELISA ODC%, the incidence rate of UED increased by 27% for age 9-12 months (IRR=1.27, 95% CI: 1.01; 1.59) and decreased by 23% for age> 27 months (IRR=0.77, 95% CI: 0.59; 0.99) (Table 3).

Table 3: Association between undesired early departure and *M. bovis*-antibody-positivity measured by individual ELISA ODC%, prevalence of antibody positive lactating cows (Cow-prevalence), seroprevalence of antibody positive young stock (YS-seroprevalence) and adjusted for differences in age (Age group), and mortality percentage with herd included as a random effect.

Fixed effects	**Incidence rate ratio**	**95% Confidence interval**	***P*-value (Overall p-value of fixed effects)**
YS-seroprevalence (per 10% increase)	1.23	1.02; 1.48	0.030
Cow-prevalence (per 10% increase)	0.95	0.77; 1.17	0.64
Individual ELISA ODC% (per 10 ODC% increase) by Age group			0.047#
3-6 months: ELISA ODC%	0.87	0.62; 1.22	
6-9 months: ELISA ODC%	0.63	0.38; 1.05	
9-12 months: ELISA ODC%	1.27	1.01; 1.59	
12-15 months: ELISA ODC%	0.47	0.19; 1.18	
15-18 months: ELISA ODC%	0.93	0.61; 1.42	
18-21 months: ELISA ODC%	1.09	0.94; 1.27	
21-24 months: ELISA ODC%	1.03	0.84; 1.27	
24-27 months: ELISA ODC%	0.95	0.76; 1.20	
> 27 months: ELISA ODC%	0.77	0.59; 0.99	
Age group			< 0.001
3-6 months	0.37	0.03; 4.79	
6-9 months	1.03	0.08; 13.44	
9-12 months	1	(ref)	
12-15 months	0.02	0.001; 0.53	
15-18 months	0.15	0.01; 3.00	
18-21 months	0.29	0.03; 3.14	
21-24 months	0.36	0.03; 4.22	
24-27 months	1.47	0.13; 16.71	
> 27 months	11.14	0.98; 126.5	
Mortality-%			0.27
< 1.3%	1	(ref)	
1.3-2.6%	1.47	0.65; 3.32	
2.7-4.2%	0.77	0.31; 1.92	
> 4.2%	1.70	0.73; 3.94	
Random effect	**Estimate**	**Standard error**	
Herd	0.18	0.18	

P-value for the interaction in a model including Individual ELISA ODC% and Age group as main effects

Discussion

This study showed an association between the UED (including euthanasia, death and slaughter) rate in heifers and increasing seroprevalence among young stock, when accounting for the underlying natural culling patterns in dairy herds by inclusion of age and mortality percentage. There was a weaker association between the individual antibody response depending on age group and UED. Together this suggests that exposure to and infection with *M. bovis* increases the risk that heifers will have to be removed from dairy herds before the first calving.

This is the first study to investigate the association between *M. bovis* antibody level and UED. However, other authors have investigated the association between the antibody response and disease and other production parameters. The findings of our study is in agreement with Martin et al. (1990) who found an association between the seroconversion rate against *M. bovis* in the group of feedlot calves and respiratory disease, which in itself might lead to death or euthanasia (Gulliksen et al., 2009). In contrast, Pardon et al. (2015) found no association between *M. bovis* serostatus at arrival at a feedlot and BRD. The difference between the conclusions can be due to difference in variables included and the timing of the serostatus measure in relation to disease development. Martin et al. (Martin et al., 1990) included the seroconversion rate in the first 28 days at the feedlot, while Pardon et al. (Pardon et al., 2015) only included the serostatus at arrival in a veal calf facility. Transmission from older calves to incoming calves is a source of infection with *M. bovis* (Maunsell et al., 2011) and it is likely that infection with *M. bovis* at least in some calves are seen after mixing with other calves at e.g. a rearing unit. In addition, the young age of the calves in Pardon et al. (Pardon et al., 2015) could have resulted in only few calves being seropositive and the serostatus might therefore not be representative for the infection status. The true effect of infection with *M. bovis* could therefore be masked in the study by Pardon et al. (Pardon et al., 2015). Both of these studies are conducted in feedlots and compared to the present study in dairy herds, there are probably differences in culling pattern and mortality that can have affected the conclusions.

The fact that age significantly modified the association between Individual ELISA ODC% and the incidence rate of UED, means that increasing individual ELISA ODC% had different effects in different age groups. Increasing ELISA ODC% was associated with increased UED at age 9-12 months, while in heifers > 27 months of age increasing ELISA ODC% was negatively associated with UED. Young calves are primarily euthanized/dies due to different diseases and management factors and the mortality percentage decreases with age (Gulliksen et al., 2009). An explanation for the positive association between ELISA ODC% and UED in age group 9-12 months can be that the calves in this age group are in general less vulnerable and less likely to die than younger calves, so the effect of *M. bovis* infection, which can be chronic and have a prolonged effect (Maunsell and Donovan, 2009), even if the heifers were infected as young, is then more evident than when confounded by other diseases or risk factors. Martin et al. (Martin et al., 1990) also included the individual antibody response, but did not find an association with disease. Their conclusion was that this could either be because *M. bovis* only has an effect at group level, or it may reflect the level of statistical control in the models. It could also be due to the fact that the calves were not followed for longer than 28 days and the effect of *M. bovis* was therefore not evident yet. The positive association between Individual ELISA ODC% and UED in heifers > 27 months old is more difficult to interpret biologically. One potential explanation could be that heifers that survived until around

calving were the ones not as severely affected by *M. bovis* as the ones that were culled/died earlier. Despite an elevated antibody response they were healthy and deemed capable of continuing in the herd as lactating cows, despite haven been exposed to *M. bovis* at a younger age.

Other authors have investigated the association between *M. bovis* and weight gain, and no association between seroconversion rate and weight gain was found in feedlot calves (Martin et al., 1990), while a tendency for lower weight gain in *M. bovis* seropositive weaned beef calves has been found (Hanzlicek et al., 2011). The reason for these rather inconsistent results could be that *M. bovis* is often harmless by itself but enhances the pathogenicity of other organisms (Martin et al., 1990). The impact of *M. bovis* serostatus is therefore dependent on other pathogens present in the included animals and this is impossible to unify or control for in field studies unless many pathogens are screened for simultaneously.

The outcome of interest, UED, consisted of both involuntary culling (slaughter or euthanasia) and death. As seen in Table 2, the distribution of specific events was different. Death/euthanasia happened mainly during early age, while slaughter mainly happened later. There were probably different underlying reasons for e.g. slaughter and death, with death/euthanasia usually happening in more severe cases of disease or injury, and the antibody response could have different impact on different reasons for UED of heifers. Euthanasia and slaughter of a heifer is a decision made by the farmer in contrast to death, and culling decisions are known to be affected by many different factors apart from poor health of the animals (Haine et al., 2017). An analysis of each of the specific events, slaughter, death and euthanasia separated would have been relevant, but it was not possible with the available data due to few events in each age-category. The decision of culling could in principle have been affected by the ELISA ODC% of the heifers, as these were made available to the farmer after the herd visits. However, during the study period no recommendations about use of the antibody results in relation to management were given, and farmers did not seem to know what to do with the results during the field study period. Furthermore, the last blood sample results were collected around the age of 1 year, and most of the heifers were slaughtered at least 6 months after. Taken together we find it not likely that the results of the blood samples have influenced farmer's choice of culling. If this is the case, it is more likely that it was in fact the health or production performance effect of *M. bovis* infection that was reflected in the results.

Samples taken when the calves were less than 90 days of age were excluded, because the interpretation of the ELISA response is uncertain in this age-group when using the BioX 302 ELISA (Petersen et al., 2018b). This means that calves that died before the age of 90 days were also excluded. That could have resulted in exclusion of young calves that actually died because of *M. bovis*-associated disease in the herd. This effect will primarily be seen in the herds with a recent history of *M. bovis*-associated disease outbreak. Many of the herds were more than 2 months past the disease outbreak when sampling was initiated, and therefore this is considered a minor issue. On the other hand, calves are proposed as being the reservoir for keeping the infection on-going in the herd (Maunsell et al., 2011), which suggests that on-going infection and disease caused by *M. bovis* can be expected in many of the included herds. Including the calves below 3 months old would have been preferable and would also have resulted in more events, which would have strengthened the statistical analyses.

To account for the *M. bovis*-associated disease status among all calves in the herd the maximum seroprevalence of all sampled calves observed in the herds was also included. This variable reflects the *M. bovis* status among calves aged 3-12 months in the herd. Despite antibodies against *M. bovis* are not recommended for individual diagnosis (Maunsell and Donovan, 2009), an increase in antibody titre occur after exposure to *M. bovis* (Kanci et al., 2017) and the seroprevalence in a group of calves is assumed to be a useful measure of exposure to *M. bovis*.

The level of *M. bovis* infection among the cows in the herds was accounted for by including the maximum prevalence of antibody positive lactating cows observed in the herds. The antibody level in milk samples reflects the presence of *M. bovis* infections in udders and no other clinical syndromes such as arthritis and pneumonia (Petersen et al., 2018a). To include a measure of systemic infections among the cows it would have been relevant to include the seroprevalence of cows. However, this was unfortunately not possible with the available data. In fact many of the Danish *M. bovis*-associated disease outbreaks happening from 2011-2014 were characterised by systemic disease (Jensen, 2015) and being able to take this into account in the model could possibly have had a significant effect on UED in the herds. The effect of living in a herd during a *M. bovis*-associated disease outbreak might therefore be underestimated because all disease manifestations among cows in the herd have not been taken into account.

The mortality percentage was included to account for the mortality in the young calves that could not have their serostatus evaluated due to lack of immune-competence. Death in young calves can have various causes e.g. acute diarrhoea and other infectious diseases, as well as physical and psychological stressors dependent on management (Gulliksen et al., 2009; Uetake, 2013). By including the mortality percentage in the young calves it was attempted to take different management in the herds into account, however, having said this we are aware that other factors than management also influence the calf mortality.

In conclusion, despite this study being limited in sample size and number of UED events per herd overall it points to a negative effect of having active *M. bovis*-infection causing disease among calves in dairy herds. A higher *M. bovis* seroprevalence in the group of calves and young stock increased the risk of heifers being culled, euthanized or dying before the first calving in this study, while the effect of the individual antibody responses was dependent on age. The long-term effect of *M. bovis* is most pronounced at group-level and management and preventive actions should be implemented to reduce the risk of infection and exposure in the group of calves, rather than focusing on the status of the individual calf.

Acknowledgements
The authors would like to thank veterinarians Johanne Juul og Ulla Torpe for conducting herd visits.

Financial support
The study was funded by the Danish Milk Levy Fund and the Danish Cattle Levy Fund.

Conflict of interest
None

Reference list

Anonymous. 2017. MYCOPLASMA BOVIS ELISA KIT, http://www.biox.com/en/bio-k-302-monoscreen-abelisa-mycoplasma-bovis-indirect-monowell-p-250/. Accessed 17-11-2017.

Gulliksen, S. M., K. I. Lie, T. Loken, and O. Osteras. 2009. Calf mortality in Norwegian dairy herds. Journal of Dairy Science 92:2782-2795.

Haine, D., R. Cue, A. Sewalem, K. Wade, R. Lacroix, D. Lefebvre, J. Rushton, J. Arsenault, E. Bouchard, and J. Dubuc. 2017. Culling from the actors' perspectives - decision-making criteria for culling in Quebec dairy herds enrolled in a veterinary preventive medicine program. Preventive Veterinary Medicine 148:1-9.

Hanzlicek, G. A., B. J. White, D. G. Renter, D. E. Anderson, and R. L. Larson. 2011. Associations between the prevalence of Mollicutes and *Mycoplasma bovis* and health and performance in stocker calves. Vet. Rec. 168(1):21.

Jensen, L. 2015. Outbreak characteristics and identification of risk factors for *Mycoplasma bovis* outbreaks in Danish dairy herds 2010-2014. Veterinary master thesis, University of Copenhagen.

Kanci, A., N. K. Wawegama, M. S. Marenda, P. D. Mansell, G. F. Browning, and P. F. Markham. 2017. Reproduction of respiratory mycoplasmosis in calves by exposure to an aerolised culture of *Mycoplasma bovis*. Veterinary Microbiology 210:167-173.

Martin, S. W., K. G. Bateman, P. E. Shewen, S. Rosendal, J. G. Bohac, and M. Thorburn. 1990. A group level analysis of the associations between antibodies to seven putative pathogens and respiratory disease and weight gain in Ontario feedlot calves. Can. J. Vet. Res. 54(3):337-342.

Maunsell, F. P., and G. A. Donovan. 2009. *Mycoplasma bovis* Infections in young calves. Vet. Clin. North Am. Food Anim Pract. 25(1):139-77.

Maunsell, F. P., A. R. Woolums, D. Francoz, R. F. Rosenbusch, D. L. Step, D. J. Wilson, and E. D. Janzen. 2011. *Mycoplasma bovis* infections in cattle. J. Vet. Intern. Med. 25(4):772-783.

Nicholas, R. A., R. D. Ayling, and L. P. Stipkovits. 2002. An experimental vaccine for calf pneumonia caused by *Mycoplasma bovis*: clinical, cultural, serological and pathological findings. Vaccine 20(29-30):3569-3575.

Pardon, B., J. Alliet, R. Boone, S. Roelandt, B. Valgaeren, and P. Deprez. 2015. Prediction of respiratory disease and diarrhea in veal calves based on immunoglobulin levels and the serostatus for respiratory pathogens measured at arrival. Preventive Veterinary Medicine 120:169-176.

Petersen, M. B., J. Pedersen, D. L. Holm, M. Denwood, and L. R. Nielsen. 2018a. A longitudinal observational study of the dynamics of *Mycoplasma bovis* antibodies in naturally exposed dairy cows. Journal of Dairy Science.

Petersen, M. B., N. K. Wawegama, M. Denwood, P. F. Markham, G. F. Browning, and L. R. Nielsen. 2018b. *Mycoplasma bovis* antibody dynamics in naturally exposed dairy calves according to two diagnostic tests. Submitted to BMC Veterinary Research.

Petersen, M. B., K. Krogh, and L. R. Nielsen. 2016. Factors associated with variation in bulk tank milk *Mycoplasma bovis* antibody-ELISA results in dairy herds. Journal of Dairy Science, 99:3815-3823.

R Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Raaperi, K., S. Bougeard, A. Aleksejev, T. Orro, and A. Viltrop. 2012. Association of herd BHV-1 seroprevalence with respiratory disease in youngstock in Estonian dairy cattle. Res. Vet. Sci. 93(2):641-648.

Schibrowski, M. L., T. S. Barnes, N. K. Wawwgama, M. E. Vance, P. F. Markham, P. D. Mansell, M. S. Marenda, A. Kanci, J. Perez-Casal, G. F. Browning, J. S. Gibson, and T. J. Mahony. 2018. The Performance of Three Immune Assays to Assess the Serological Status of Cattle Experimentally Exposed to *Mycoplasma bovis*. Veterinary Sciences 5(27).

Uetake, K. 2013. Newborn calf welfare: a review focusing on mortality rates. Animal Science Journal 84:101-105.

Wawegama, N. K., P. F. Markham, A. Kanci, M. Schibrowski, S. Oswin, T. S. Barnes, S. M. Firestone, T. J. Mahony, and G. F. Browning. 2016. Evaluation of an IgG enzyme-linked immunosorbent assay as a serological assay for detection of *Mycoplasma bovis* infection in feedlot cattle. Journal of Clinical Microbiology 54(5):1269-1275.

9 Appendices

Appendix I – Questions used for sampling activity #1

Appendix II – Clinical protocol used for sampling activity #2

Farmer interview regarding *Mycoplasma bovis*-associated disease

CHR nr:

Disease outbreak:

1. When did you become aware of *M.*bovis-associated disease in your herd?
    a. Start_____
    b. End_____
    c. There are still *M. bovis*-associated disease problems
    d. I think the actual outbreak has ended, but some animals with *M.* bovis-associated disease are occasionally found
    e. I have never seen *M. bovis*-associated disease in the herd

2. How did you detect the disease?
    a. Diseased animals, not confirmed by diagnostic testing
    b. Diseased animals, confirmed by diagnostic testing (PCR, antibody measurements, bacterial culture)
        Blood Joint fluid Milk
    c. Bulk tank milk samples (PCR, antibody measurements, bacterial culture)
    d. Individual samples (PCR, antibody measurements, bacterial culture)
    e. Accidental finding during……..

3. Who made the diagnosis of *M. bovis*-associated disease?
    Veterinarian Consultant Others

4. Which clinical signs have you experienced in the herd in the different age groups (note approximate number)?

	Clinical sign						
	Joint	**Respiratory**	**Ear/udder**	**Euthanised**	**Slaughtered**	**Treated**	**Recovered**
Calves							
Heifers							
Cows							

Other clinical signs_____

5. How did you treat diseased animals at the **beginning** of the disease outbreak?
    a. Culling of all clinically ill animals without medical treatment
    b. Culling of the worst cases
    c. Culling of PCR positive animals
    d. Culling of antibody-positive animals
    e. Medical treatment and culling if unresponsive
    f. Has medical treatment been attempted?
        a. If yes, on which criteria?

   b. Which age groups and clinical signs have been treated?
   c. Which drugs have you used?

   _____

6. Did the treatment strategy change during the disease outbreak?
   If yes:
   a. Culling of all clinically ill animals without medical treatment
   b. Culling of the worst cases
   c. Culling of PCR positive animals
   d. Culling of antibody-positive animals
   e. Medical treatment and culling if unresponsive

7. Have you received money from an insurance company?

8. Did the insurance company influence the treatment/culling strategy?

Questions about the herd:

9. How many different people work with milking, calf management, feeding and management in the herd?

10. Housing conditions:
    a. Tie-stall
    b. Loose housing with mattresses
    c. Loose housing with bedding

11. Do the cows go on pasture?

12. Do you use sick pens for cows, heifers and calves suspected of *M. bovis* infection?

    Yes, for cows Yes, for heifers Yes, for calves No

13. Did this change during the *M. bovis* outbreak?

14. How many cows are present in the calving area at the same time?

    1 2 More

15. Are there sick cows in the calving area?
    Never Occasionally Always

16. When is the calf removed from the cow?
    ≤ 1t 1 - 6t > 6t

17. Do you keep the bull calves to slaughter?
    Yes No, they are sold No, they are euthanised

    Did you change this routine during the *M. bovis* outbreak?

Milk-fed calves

18. Receives the calf colostrum < 6 hours after birth?

    Yes No

    a. How many litres?
    b. As much as they can drink
    c. Nasopharyngeal tube if they cannot drink the full amount
    d. Nasopharyngeal tube

19. How is the colostrum stored?
    a. Fed directly from the cow
    b. Coloquick-system
    c. Colostrum bank, quality tested
    d. Colostrum bank, not quality tested

20. Who is responsible for colostrum? _____

21. Is the colostrum pasteurised?

    Yes No

    If yes:

    a. Temperature:_____
    b. Duration:_____
    c. Is the quality checked?
        a. If yes, how?

22. Have there been changes in colostrum supply during the *M. bovis* outbreak?

23. How are the calves fed after colostrum is received?
    a. Waste milk e.g. milk from treated cows, cows with high SCC, colostrum
    b. Milk from the bulk tank
    c. Milk replacer
    d. Same management routines regarding housing and feeding for milk-fed heifer and bull calves?
    If no, what are the differences?

24. Is the milk pasteurised?
    Yes No

    If yes:

    d. Temperature:_____
    e. Duration:_____
    f. Is the quality checked?
        a. If yes, how?

25. Were there changes in milk supply during the *M. bovis* outbreak?

26. How and how often are the drinking trough/feeding bucket/automatic milk feeder cleaned?

a. Daily with clean water, always same equipment for the same calf
b. Changed daily
c. Weekly with clean water, always same equipment for the same calf
d. Changed weekly
e. Daily, with possibly contaminated water (e.g. milk and saliva from other animals)
f. Weekly, with possibly contaminated water (e.g. milk and saliva from other animals)
g. Cleaned when the calf is removed, always same equipment for the same calf
h. Not cleaned between calves

27. How is the calf pen/hutch cleaned before a new calf enters?
    a. Cleaned, washed, dried and disinfected
    b. Cleaned, washed, dried and disinfected for every 2nd or 3rd calf
    c. Cleaned, washed, dried and disinfected occasionally
    d. Cleaned, washed and dried, but not disinfected
    e. Cleaned, but not washed, dried or disinfected
    f. Cleaned occasionally

28. Were there any changes in management routines during the *M. bovis* outbreak?

Weaned calves

29. Are the heifers at a 'heifer-hotel'?
    Yes No Only heifers from my herd Heifers from more herds
    Which CHR?

30. How are the heifers' housing conditions?
    a. All in – all out, small groups (2-8), no contact between groups
    b. All in – all out, small groups (2-8), contact between groups (e.g. only separated by bars)
    c. All in – all out, large groups (> 8), no contact between groups
    d. All in – all out, large groups (> 8), contact between groups (e.g. only separated by bars)
    e. All heifers in the barn

31. Do the calves go on pasture?
    Yes No
    If yes, age groups: _____

32. Do calves and cows share pasture?
    a. Never
    b. Occasionally
    c. Yes

33. Were there changes in the heifer management during the *M. bovis* outbreak?

External biosecurity

34. Which of the following hygiene facilities are placed at the entrance to the cow barn?
    a. Visitors can wash their boots with warm water and soap
    b. No place for boot washing

c. Visitors can wash hands with warm water and soap
d. No place for hand wash

35. Are any hygiene steps required for visitors to enter the stables?
    a. Visitors wear protective clothing and boots made available by the herd
    b. Visitors have to wear clean clothing and boots
    c. Visitors have to wash and disinfect boots before entering the stables
    d. Nothing is required from visitors

36. Purchase of animals to the herd?
    a. No, never
    b. Yes, when?
        1. Only buy animals that are free of
        M. bovis *Salmonella* Dublin B-strep Para-tb

        2. Animals are bought without consideration of disease status

37. Were there any changes in the external biosecurity during the *M. bovis* outbreak?

Independent perception of the property

The flow of animal movements is drawn on a Google Maps picture of the herd, along with the number of pens and contact between groups of animals. Apply where the feeding truck, DAKA, manure truck and slaughter is driving.

Appendix B

Herd no_____ **Cow no_____** **Date_____**

	Levels	**Comments -** Describe any anomaly
General condition		
Level of alertness	0/1 (0 = bright and alert, 1 = depressed to apathetic)	
Body condition score	1-5 (1 = lean, 5 = fat)	
Rectal temperature	Measured with a thermometer	
Head		
CNS, head position	0/1 (0 = normal, 1 = head tilt)	
Eye discharge	0/1 (0 = absent, 1 = visible ocular discharge)	
Nasal discharge	0/1 (0 = absent, 1 = visible nasal discharge)	
Cough	0/1 (0 = absent, 1 = present at examination)	
Respiratory system		
Respiration characterisation	0/1 (0 = normal, 1 = abnormal breathing i.e. severe increase of frequency and intensity)	
Pulmonary auscultation	0/1 (0 = normal, 1 = abnormal lung sounds, e.g. crackles, wheezes)	
Cardiovascular system		
Auscultation	0/1 (0 = normal, 1 = abnormal heart sound/beat)	
Musculoskeletal system		

Limb swelling	0/1 (0 = absent, 1 = present)	
Skin	0/1/2 (0 = intact skin on limbs, 1 = hairless spots, intact skin, 2 = active/healed wounds)	
Lameness	0/1/2 (0 = not lame, 1 = lame, 2 = severely lame)	
Udder (only cows)		
Inspection	0/1/3 (0 = symmetric, no wounds or swelling, 1 = asymmetric, wound or swelling, 3 = cows with a dry quarter)	
Palpation	0/1/2/3 (0 = soft normal udder tissue, 1 = oedema in one or more glands, 2 = soft swelling, consistent with acute inflammation, 3 = hard swelling, consistent with chronic changes)	
Soreness	0/1 (0 = normal, 1 = sore when palpated)	