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# Comparison of clinical signs in newly *Salmonella* Dublin test-positive and test-negative dairy herds

**Master Thesis in Veterinary Medicine**
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Front page picture: From SEGES herd visit with acceptance of the respective owner.

# Preface

This master thesis was submitted as a part of the master's degree in Veterinary Medicine at the University of Copenhagen, Department of Veterinary and Animal Sciences, Section of Animal Welfare and Disease Control. The thesis work was carried out between November 2021 and March 2022 and corresponds to 30 ECTS points.

The dataset used in this thesis is a part of the data collected in a collaborative research project between the University of Copenhagen and SEGES which aims to further improve knowledge regarding introduction of *Salmonella* Dublin in naïve Danish dairy herds under Danish conditions.

Our sincere gratitude is given to our main supervisor, Professor Liza Rosenbaum Nielsen for all her dedicated guidance, support, and supervision. We would also like to thank our co-supervisor Lars Pedersen, for the many hours spent in the field, providing useful advice for this thesis, and discussing future veterinary dilemmas.

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# Abstract

There is a lot of variation in literature describing the clinical manifestations of *Salmonella enterica* subsp. *enterica* serovar Dublin (*S.* Dublin) in calves, and it is uncertain which clinical manifestations are most common under current conditions in Danish dairy herds. The aim of this study was to improve the knowledge of clinical disease seen during naturally acquired *S.* Dublin infections in calves when introduced into naïve dairy herds under Danish conditions. To achieve this, the objectives of this thesis were to 1) describe the distribution of clinical signs in calves in dairy herds with and without recent incursion of *S.* Dublin, and 2) analyse associations between clinical disease in calves and explanatory risk factors in calves in dairy herds with and without recent incursion of *S.* Dublin. The used data were collected in a collaborative research project between the University of Copenhagen and SEGES innovation. Data from 67 herds dispersed on 29 newly *S.* Dublin test-positive herds and 38 *S.* Dublin test-negative herds were included. In total, 1799 calves were randomly selected and subjected to a clinical assessment and separated into three groups (Group A: 0-10 days old, Group B: 14-28 days old and Group C: 100-130 days old). Group A and Group C were blood sampled. For the first objective, a clinical scoring protocol was created for assessment of the calves. Thereby, it was possible to establish distributional differences in certain age groups. For the second objective, three aggregated disease categories were created to classify if calves had more gastrointestinal, respiratory- or systemic disease, respectively, in herds with a recent incursion of *S.* Dublin compared to calves in herds without a recent incursion of *S.* Dublin. Mixed effects logistic regression models were used to determine associations between the outcomes and explanatory risk factors. Surprisingly, it was found that calves in Group C had significantly higher odds (OR = 8.0; 97.5% CI: 2.7 – 23.6) of having signs of gastrointestinal disease in newly *S.* Dublin test-positive herds compared to test-negative herds in a multivariable mixed effects model accounting for these other factors; ringworm, decreased cleanliness of the calf and a soiled environment. No other statistically significant associations were found between *S.* Dublin herd status and other disease categories, nor in other age-groups. In conclusion, this thesis found that a recent incursion of *S.* Dublin in naïve danish dairy herds had a propensity to cause enteric disease in calves older than most often described in literature. Simultaneously, a recent incursion of *S.* Dublin seems to lead to less additional disease in the calves in naïve danish dairy herds. This is useful knowledge to take into consideration in the future planning of the Danish *S.* Dublin surveillance and eradication programme. However, due to limited sample size in several of the data groups used for analysis, further research is necessary to establish more conclusive results regarding other disease categories and age-groups.

# Resumé

Der er stor variation i litteraturen, som beskriver kliniske manifestationer af *Salmonella enterica* subsp. *enterica* serovar Dublin (*S.* Dublin) i kalve, og det er uvist, hvilke af disse kliniske manifestationer, som er hyppigst forekommende i malkekvægsbesætninger under nuværende danske forhold. Formålet med dette studie var at forbedre den nuværende viden om klinisk sygdom i kalve med naturligt forekommende *S.* Dublin infektioner i naive malkekvægsbesætninger under danske forhold. Dette blev gjort ved at 1) beskrive fordelingen af kliniske tegn i kalve i mælkekvægsbesætninger med og uden nylig introduktion af *S.* Dublin, og 2) analysere associationerne mellem klinisk sygdom i kalve som udfald og forklarende risikofaktorer i kalve i malkekvægsbesætninger med og uden nylig introduktion af smitte med *S.* Dublin. Specialet anvendte data fra et forskningssamarbejde mellem Københavns Universitet og SEGES Innovation. Der blev inkluderet 67 besætninger, 29 nyligt *S.* Dublin test-positive besætninger og 38 *S.* Dublin test-negative besætninger. I alt blev 1799 kalve tilfældigt udvalgt og klinisk undersøgt. Kalvene blev inddelt i tre aldersgrupper (Gruppe A: 0–10 dage, Gruppe B: 14–28 dage og Gruppe C: 100–130 dage), og Gruppe A og C fik udtaget blodprøver. I forbindelse med det første delmål, blev der udarbejdet en klinisk scoreprotokol til at vurdere kalvene, som gjorde det muligt at fastslå forskelle i fordelinger i visse aldersgrupper. I forbindelse med det andet delmål, blev der oprettet tre sygdomskategorier for at klassificere syge kalve, som gastrointestinalt, respiratorisk eller systemisk syge i besætninger med og uden nylig introduktion af smitte med *S.* Dublin. Der blev anvendt mixed effects logistisk regression modeller til at fastslå associationer mellem udfaldene og de forklarende risikofaktorer. Det var muligt at fastslå, at kalve i Gruppe C havde signifikant højere odds (OR = 8.0; 97.5% CI: 2.7 – 23.6) for at have tegn på gastrointestinal sygdom i nylige *S.* Dublin test-positive besætninger sammenlignet med test-negative besætninger ved brug af en multivariabel mixed effects model. Modellen tog også højde for følgende faktorer; ringorm, nedsat renlighed af kalven og et beskidt miljø. Der kunne ikke påvises statistiske signifikante associationer mellem andre sygdomskategorier og besætningernes *S.* Dublin status, ej heller i andre aldersgrupper. Dette speciale konkluderede, at en nylig introduktion af *S.* Dublin i naive danske malkekvægsbesætninger medførte gastrointestinal sygdom hos ældre kalve, hvilket afgiver fra den gængse litteratur. Samtidig lader det til, at *S.* Dublin leder til mindre yderligere sygdom i kalvene. Dette er brugbar viden til fremtidig planlægning af det danske *S.* Dublin overvågnings- og udryddelsesprogram. Der er brug for yderligere forskning for at kunne opnå konkluderende resultater vedrørende de andre sygdomskategorier og aldersgrupper, da stikprøvestørrelsen i dette speciale var en begrænsende faktor for flere af analyserne.

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# 1. Introduction

*Salmonella enterica* subsp. *enterica* serovar Dublin (*S.* Dublin) is a rod-shaped, flagellated, aerobic, gram-negative pathogen (Pecoraro et al., 2017). *S.* Dublin is host-adapted to cattle and causes high morbidity and mortality in some affected herds (Hughes and Jones, 1973; Wray and Sojka, 1977). Another effect of the disease is decreased milk yield and abortion in adult cows (Hinton, 1974; Nielsen, 2012). In young stock, increased mortality is reported with varying clinical manifestations (Mohler et al., 2009; Rings, 1985; Smith and Jones, 1967). Therefore, it leads to economic losses among farmers, a reduced welfare in the affected cattle herds and increased veterinary expenses (Nielsen, 2012). These production and animal welfare effects, together with the fact that *S.* Dublin is a serious foodborne zoonosis (Statens Serum Institut, 2019), are the main motivations behind the ongoing Danish national surveillance and eradication programme for *S.* Dublin in cattle (Houe et al., 2014).

In literature it is described that onset and clinical signs of salmonellosis can vary and be non-specific with disease occurring from only sporadic up to major outbreaks with up to 100% morbidity and 50% mortality (Hughes and Jones, 1973). Faecal-oral transmission is the primary route of infection (Hardman et al., 1991). Others have reported routes to include the mucosa of the upper respiratory tract and conjunctiva (Ragione et al., 2013). Upon ingestion, *S.* Dublin bacteria colonize the intestinal tract and can invade the host through M-cells, enterocytes, and tonsillar lymphoid tissue. In lymphoid tissue, *S.* Dublin enter mononuclear phagocytes and are rapidly disseminated throughout the body (Ragione et al., 2013). Oral uptake of more than $10^6$ CFU of the pathogen often leads to clinical signs and/or to shedding of bacteria (Nazer and Osborne, 1977; Wray and Sojka, 1977). A large infectious dose will reproduce more clinical signs and consistent shedding of bacteria in faeces (Nielsen, 2013) and saliva (Richardson and Fawcett, 1973). Therefore, the pathogenesis in the calf depends on factors such as infectious dose, age at infection, passive transfer of specific immunoglobulins, immunity developed during previous infections, and the physiological state (Nielsen, 2013).

Rings (1985) reports that *S.* Dublin is clinically indistinguishable from *S.* Typhimurium and is characterized primarily by diarrhoea. However, *S.* Dublin has a much higher potential for systemic dissemination resulting in septicaemia (Carrique-Mas et al., 2010; Martin and Smith, 1984; McDonough et al., 1999). Septicaemic calves may often not present with diarrhoea (Carrique-Mas et al., 2010; Guizelini et al., 2020). Others report that bacteraemia and respiratory signs have been found to be predominating in *S.* Dublin infections (Mohler et al., 2009; Pecoraro et al., 2017). In

experimentally induced infections and in some naturally acquired outbreak studies, it is described that *S.* Dublin infection also occurs with clinical signs of respiratory disease (Gitter et al., 1978; Nazer and Osborne, 1977; Segall and Lindberg, 1991).

In naïve herds experiencing their first outbreak, the disease is primarily evident in calves after the first week of life with calves from 10 days until 3 months old being at the highest risk of developing disease (Guizelini et al., 2019). Summarising the knowledge of clinical signs, duration, and infectiousness of *S.* Dublin in naturally acquired and experimentally induced infections, calves can experience one or more of following clinical entities:

1) Peracute infection, in which the calf upon exposure can present lethargic, unable to stand and pyrexic (40.5 – 42 °C) due to septicaemia and endotoxic shock with sudden death occurring after 24-48h (Ragione et al., 2013). Often few to no clinical signs are exhibited prior to death often with no excretion of bacteria occurring (Nielsen, 2013).
2) Mild acute infection, often transient anorexia, and pyrexia with a duration of a few days. Often occurring in endemic infected herds in which calves excrete bacteria intermittently for around 17 days post infection (Nielsen et al., 2007).
3) Severe acute infection, where inappetence and depressed mentation are the first signs followed by pneumonia, laboured breathing, respiratory distress, and pyrexia (Mohler et al., 2009; Ragione et al., 2013). Dehydration and diarrhoea with faecal consistency ranging from watery, voluminous, and profuse to mucofibrinous haemorrhagic are also often evident 48 to 74h post-infection (Mohler et al., 2009). The invasiveness of *S.* Dublin can also result in septic arthritis in one or multiple joints, central nervous abnormalities due to meningoencephalitis and icteric mucous membranes following hepatitis (McDonough et al., 1999; Mohler et al., 2009; Ragione et al., 2013). Duration of one to three weeks which may extend to five or nine weeks. Large amounts from 1 to $10^8$ CFU/g may be shed in faeces and urine continuously or intermittently (Nielsen, 2013).
4) Chronic infection can manifest in calves surviving peracute and severe acute infection. A potential consequence is the development of dry gangrene in the extremities as the result of the immune-mediated process cold agglutination (Loeb et al., 2006). Calves surviving bronchopneumonia will failure to thrive and can have a continued cough (Rings, 1985). Chronic lameness can also be noted (Nielsen, 2013). Duration of this chronic stage can be months and the calf may or may not shed bacteria (Nielsen, 2013).

Pathological examinations of calves experimentally and naturally infected with *S.* Dublin include gross findings of enlarged livers, non-collapsed oedematous lungs, diphtheritic enteritis (Guizelini et al., 2020) and cervico-thoracic vertebral osteomyelitis (Healy et al., 1997).

Several risk factors may contribute to manifestation of disease in calves including failure of passive transfer (FPT), keeping calves of different ages in the same environment, exposing young calves with low colostral immunity, providing *S.* Dublin faecal contaminated drinking water, and moving calf carriers inducing stress, which reactivates the bacteria and thereby shedding (Mohler et al., 2009; Nielsen, 2013).

The complexity of *S.* Dublin and scarce literature of natural occurring clinical manifestations in Danish dairy calves is a continuous challenge for adequate and timely diagnostic procedures, decision on prevention and treatment. Present literature largely derives knowledge from experimental studies with higher infection doses which are unlikely to bear relationship to the presumably smaller dosages calves are exposed to under natural conditions. To the authors' knowledge, no observational study has been carried out to investigate the clinical manifestations of naturally acquired *S.* Dublin infection in calves, when the infection is introduced into naïve dairy herds under current Danish conditions. The overall purpose of this study was therefore to increase knowledge of this subject by providing relevant information to aid veterinarians and farmers in reducing and eradicating *S.* Dublin in cattle in Denmark. The aim was to compare clinical manifestations in calves in newly infected *S.* Dublin herds with calves in herds with no signs of *S.* Dublin infection in the Danish surveillance programme. The specific objectives of this study were to:

1) describe the distribution of clinical signs in calves in dairy herds with and without recent incursion of *S.* Dublin; and

2) analyse associations between the outcome of clinical disease in calves and explanatory risk factors in calves in dairy herds with and without recent incursion of *S.* Dublin.

# 2. Materials and methods

## 2.1 Selection of study herds

The data used in this thesis originates from a field study performed in collaboration between KU-SUND and SEGES. Data gathered between 14/09/2021 – 14/01/2022 for the original purpose were included in this thesis. The study was a matched case-control study with data being collected in a 1:2 ratio from case/control herds. Hence, within the time frame of this sub-project, herds were not included in the ratio 1:2. All participating herds were anonymized, and herd status (case or control) was partially blinded during sampling. For conciseness, herds with a recent incursion of *S.* Dublin are referred to as case herds and herds without a recent incursion of *S.* Dublin are referred to as control herds hereafter.

In Denmark, 'The *Salmonella* control programme' was established with the aim to eradicate *S.* Dublin. The revised legislative order 'BEK nr 2416 of 14/12/2021' valid from 01/07/2021, classified all Danish cattle herds into two *S.* Dublin surveillance levels based on bulk tank milk (BTM) *Salmonella* serogroup-D LPS (O:1,9,12) ELISA results. Serogroup cross reactions can occur, mainly serogroup-B *S.* Typhimurium LPS (O:1,4,5,12) can cross-react with serogroup-D, as the two shares similar O-antigen polysaccharide side chains of the LPS plate antigen (Konrad et al., 1994; Smith et al., 1995). It was estimated from the available serological data, that 6.2 – 13% of the serogroup-D test-positive samples originates from herds infected with serogroup-B *Salmonella* (Toft-Petersen, 2016). The ELISA results are measured as 'ODC%', which is the percentage of the background-corrected optical density (OD) to a known positive reference milk sample. Antibody levels in BTM are measured every three months and herds with an average of ODC% <25 in the last four samples and no increase of ODC% >20 in the last sample compared to the average of the previous three samples are classified 'Level 1', which denotes 'most likely free of *Salmonella* Dublin'. Herds that do not stay below these cut-offs are classified 'Level 2', which denotes 'likely to be infected with *Salmonella* Dublin or unknown' status (Nielsen and Ersbøll, 2005). The case herds were selected based on results of the third and fourth surveillance rounds of 2021. If the first sample (BTM A-sample) results in a change from 'Level 1' to 'Level 2', a second sample (BTM B-sample) collected around 3 weeks later was analysed to increase the certainty of herd classifications before inclusion.

There were in total three criteria for inclusion as a case herd in this study. The three criteria are listed below in Table 1.

**Table 1.** The criteria for inclusion as a *S.* Dublin test-positive herd in this study.

| Category | Criteria for inclusion |
|---|---|
| Dairy herd | Registered BTM ODC% the previous four surveillance rounds |
| Newly infected | 'Level 1' the last 24 months until change of status |
| Infected neighbour | There must be an infected neighbour within 10 km with the criteria:<br>• Level 2 or 3[a] or unknown for minimum 182 days within the last 12 months<br>• Positive blood sample or BTM within the 12 months |

[a] *'Level 3' were from the previous legislative order (BEK nr. 1791 of 02/12/2020).*

For a herd to be included as a possible control herd, the herd must have had 'Level 1' status for the previous 24 months and remain negative in the third and fourth surveillance period of 2021. For each case a list with 20 control herds was generated matching in size and location. The control herds were sorted randomly and contacted from the top-down until two herds agreed to participate making the 1:2 ratio. The matching size of the control herd were based on the current number of 'cow-years' in the case herd. The cow-years represent the summed average number of cows that were present in the specific herd within the last 12 months divided by 365 (Enevoldsen et al., 1996). The number of cow-years were recalculated each month. The grouping was as follows: 0-100, 100-200, 200-300, 300-500, >500 cow-years. The control herd also had to have an infected neighbour within 10 km as specified in Table 1 to ensure that all herds had an equal risk of possible exposure to *S.* Dublin. Lastly, the control herds were generally visited later than case herds, to minimize the risk of including control herds acquiring *S.* Dublin within the timeframe of the project.

## 2.2 Selection of calves for sampling

In advance to every herd visit a list from Dairy Management System (DMS) with all calves aged 0 – 180 days was generated. Three predefined age groups A, B and C (see Table 2) were included in the sampling. If the herd had a larger population of calves than needed for the sample size, the calves were chosen randomly from the list for age group A and B. If the requirements for target sample size could not be met, age group B would be expanded to include calves up until the age of 42 days. For age group C, in larger herds with more than 15 calves between 100-130 days the required target sample size was calculated. The estimated proportion were 0.222 (0.3 * 0.74) as the expected within herd prevalence of seropositive calves are 30% and 0.74 is the lower limit of diagnostic Se in calves in the age of 100-299 days with cut-off set at ODC% 25 (Nielsen et al., 2004). The allowable error (L) was set to 0.1. If the requirements for target sample size of minimum 15 could not be met, age

group C would be expanded to include calves until the age of 180 days. Convenience sampling was used if the randomised a priori selected calves were absent upon arrival at the farm, difficult to catch or sold. This method was only used in the herds where the number of animals made it possible. Neonatal calves were upon herd visit added to age group A based on the farmers registrations of date of birth. For conciseness, the age groups are hereafter referred to as Group A, B and C.

**Table 2.** Target age group with age in days and sample size.

| Target age group | Age in days | Target sample size |
|---|---|---|
| Group A | 0 – 10 days | 10 |
| Group B | 14 – 28 days | 10 |
| Group C | 100 – 130 days | 15-26 |

## 2.3 The *Salmonella* Dublin protocol

For this project an existing standard protocol from another project, the so-called Robust Calves project (Brydensholt and Klompmaker, 2020) was used as an inspiration. The standard protocol was modified for this study to match specific known and suspected clinical parameters described in literature about calves with *S.* Dublin infections. The modifications were developed with assistance from participating veterinarians. The modified protocol is found in Appendix A.

In total, 'the *Salmonella* Dublin protocol' consisted of 19 clinical- and 6 environmental parameters and blood sampling in Group A and C. The protocol was applied to every visited case or control herd and systematically began with Group A, then Group B and lastly Group C with a frequent change of gloves. This was done to minimize risk of transferring disease within the herd.

## 2.4 Sample collection and diagnostics

Blood was drawn from the jugular vein on all animals or occasionally the coccygeal vein in Group C calves using a vacutainer, vacutainer needle and BD Vacutainer® CAT 10.0 mL tubes. The sample was marked individually with the herd ID, age group letter and calf number for traceability giving each calf in the study an individual 'calf ID'. After each herd visit samples from Group C were stored in separate Ziplock bags with the herd ID note and stored in a cooled Styrofoam box at environmental temperatures. Daily, upon return from herd visits, the samples were stored at 5 °C. The samples were delivered to Eurofins Laboratory (Brørup, Denmark) for analysis of *Salmonella* serogroup-D antibodies (ODC%). The laboratory results were sent to SEGES, who delivered the information in anonymized form to the authors.

Evaluating the serological response of calves to *S.* Dublin antigens provides an indirect indication of exposure. In calves above 3 months old, ELISA run on serum can be used to provide evidence of occurrence and localization of spread within BTM positive herds. The serum ELISA measures the level of immunoglobulins directed against *S.* Dublin LPS (O:1,9,12) (Smith et al., 1995) to evaluate the humoral immune response as an indicator of current or previous disease (Robertsson, 1984) in the range from 0 – 200 within the individual (SEGES Kvæg, 2021). Measuring the antibody response is valid after 11-12 weeks of life, because a sufficient response cannot be correctly measured as maternally derived antibodies complicate interpretation of the ELISA, and because the calves do not produce antibodies consistently until then (Roden et al., 1992). Hence why, only age Group C had blood drawn for this specific purpose. Antibody reactions are often highest at age 15-16 weeks, where there is a spread of disease amongst the calves. Infected calves above three months of age often seroconvert within two weeks of infection, but not all infected animals will respond equally, and some will produce no antibody reaction towards infection at all (SEGES Kvæg, 2021). Therefore, multiple animal groups must be tested, with amount of blood samples being calculated by herd size as featured in 2.3 'Selection of calves for sampling'.

## 2.5 Data management

### 2.5.1 Data preparation for analysis

Collection and processing of data was carried out with the statistical software R (version R i386 4.1.2) with RStudio (version RStudio 2021.09.1 Build 372) and Microsoft Excel 2010 (Microsoft Corp.). Data used for this thesis were handed over from SEGES Innovation in an anonymized form in Excel files.

All observations included in this study were carried out by Author 1 and Author 2. In total, 55 calves, which lacked several clinical observations or were absent (i.e., sold) when sampling were removed from the dataset. Calves with only a few missing observations that did not affect the ability to categorize the disease status of individuals in Table 3, were kept in the dataset.

Group A was only included for the first objective. For the second objective, the obtained sample size and number of observations of certain aspects of the protocol were inadequate for analysis. The authors chose to include Group B and C in analysis of association based on literature regarding clinical onset of disease in calves infected with *S.* Dublin.

Not all variables from the *S.* Dublin protocol were included in the purposes of this thesis. 'Girth measure' was excluded and 'Pain' were only featured in Table 4. The information both variables

provided were covered by the variables ‘Body condition’, ‘General condition’ and ‘Weightbearing, lameness’. No valuable information gained from these valuables were excluded. The variable ‘Body condition’ was reduced to contain the scores ‘0’ (normal) and ‘1’ (lean). Three calves from score ‘2’ (overweight) were placed in the score ‘0’. In the variable ‘Diarrhoea’ score ‘1’ (watery diarrhoea) and score ‘2’ (bloody diarrhoea) were collapsed, so that score ‘1’ included both loose watery diarrhoea and bloody diarrhoea for the purpose of analysis. No author observed signs of necrosis in the extremities of any calf, therefore the variable ‘Necrosis in pinnae and tail’ was not included in the disease categories in Table 3.

### 2.5.2 New variables

Two variables were created in R. In the period of collecting data, Author 1 observed a total of 45 herds and Author 1 and Author 2 observed 22 herds together. Therefore, the variable ‘Observer’ containing two scores ‘0’ (Author 1) and ‘1’ (Author 1 and Author 2) were created to assess if Author 2 had an impact on the scores observed by Author 1.

The study period 14/09/2021 – 14/01/2022 had variations in weather conditions with average temperatures ranging from 2.1 - 14.1 °C (The Danish Meteorological Institute, 2022). Neonate calves are susceptible to cold stress if ambient temperatures were <15°C and calves older than three weeks could begin to experience cold stress at ambient temperatures of <5.5°C (Noordhuizen, 2021). As herd visits were somewhat clustered with more case herds being visited earlier in the study period and more control herds later in the study period the variable 'Season' divided weather conditions into ‘Early Fall’ (14/09/2021 – 14/10/2021), ‘Late Fall’ (15/10/2021 – 30/11/2021) and ‘Winter’ (01/12/2021 – 14/01/2022) to take seasonal occurrence of diseases into account.

## 2.6 Statistical analysis

### 2.6.1 Distribution of clinical and environmental variables

Descriptive statistics were carried out by calculation of the distribution for each of the 18 clinical and six environmental variables subdivided into age group and herd status. The results are given in number and percentage for qualitative observations. For quantitative observations i.e., number of direct contacts and temperature a minimum, maximum, mean and median were calculated.

## 2.6.2 Disease categories

In this thesis, three binominal disease categories were created with inputs from participating veterinarians from KU-SUND and SEGES. The disease categories were 'Respiratory disease', 'Gastrointestinal disease' and 'Systemic disease' with the scores '0' (no) and '1' (yes). The three categories are listed in Table 3 showing a certain variable score or string of variable scores that had to be fulfilled for a calf to be deemed diseased in each category. In each of the three categories, the combination of variable scores were defined to match the described clinical sign of *S.* Dublin from literature. In R, the functions mutate() and ifelse() were used to create each category. For each category, number of calves and distribution were calculated again divided in age group and herds status.

**Table 3.** Categories of disease and which criteria must be met for each disease.

| Category | Criteria for disease |
|---|---|
| **Respiratory disease** | 'Cough 1' or 'Respiration type 1', or<br>'Nasal discharge 1' & 'Ocular discharge 2' & 'Temperature ≥ 39.3 °C', or<br>'Nasal discharge 2' & 'Ocular discharge 1' & 'Temperature ≥ 39.3 °C', or<br>'Nasal discharge 2' & 'Ocular discharge 2' & 'Temperature ≥ 39.3 °C', or<br>'Nasal discharge 1' & 'Ocular discharge 2' & 'Ear drop and/or head tilt 2', or<br>'Nasal discharge 2' & 'Ocular discharge 1' & 'Ear drop and/or head tilt 2', or<br>'Nasal discharge 2' & 'Ocular discharge 2' & 'Ear drop and/or head tilt 2', or<br>'Auscultation 1' & 'Temperature ≥ 39.3 °C' |
| **Gastrointestinal disease** | 'Diarrhoea 1' or 'Hairloss hind 1' |
| **Systemic disease** | 'General condition ≥ 1' or 'Lameness 2' or 'Temperature ≥ 40.0 °C' |

## 2.6.3 Serum serology

In R, the result of ODC% measurements from each calf in Group C were grouped according to herd ID and herd status (case or control). Each calf (animal-level) in case herds was also categorized as 'positive' (ODC > 0%) or 'negative' (ODC% = 0). Using the package ggplot2, a boxplot of ODC% measurements at herd-level were created using an ascending order of herds sorted by the median to visualize distribution of all herds with an average ODC% above 0. Case herds with an average ODC% of 0 and control herds with an average ODC% > 0 were identified as a possible exclusion parameter for test of model fits in section 2.7.5 'Multivariable analysis'.

### 2.6.4 Explanatory and outcome variables

The explanatory variable 'Case control' was a binomial variable describing whether a calf was housed in a herd classified as *S.* Dublin test-positive (case) or test-negative (control). The binomial variables 'Ringworm' (no, yes), 'Body condition' (normal, lean) and ranked ordinal variables 'Cleanliness' (clean, moderately soiled, severely soiled), 'Umbilical region' (normal, swelling, inflammation) were explanatory variables at calf level. Other explanatory variables were recorded for each calf to describe environmental factors, which could potentially affect the risk that the calf would get ill: rank-ordinal 'Hygiene bedding' (>75% clean, 50-75% clean, <50% clean), 'Hygiene water' (clean, signs of manure, clearly soiled), 'Hygiene feed' (clean, signs of manure, clearly soiled) and numeric discrete variables 'Calf number pen' (number of calves in a pen in the same pen as the calf), 'Direct contact' (number of calves that the calf could have direct nozzle contact with) and 'Indirect contact' (number of calves that the calf could have indirect nozzle contact). The explanatory variable 'Season' were a three-levelled nominal variable (Early Fall, Late Fall, and Winter) and 'Observer' was a categorical variable accounting for the potential differences in individuals' observations and scores. The categorical explanatory variable 'Herd ID' represented the herd identification number for the herd that the calf was in at the time of registration.

If a calf was given the score '1' (yes) in one of the three disease categories based on criteria from Table 3, it would then be considered diseased. Score '0' (no) would indicate a calf, not being sick based on the criteria. 'Respiratory disease', 'Gastrointestinal disease' and 'Systemic disease' were therefore binary outcome variables. The distribution of the outcome variables can be found in Table 4 in section 3.2 'Outcome of clinical disease'. A more elaborative description of each of the scores can be found in the 'The *Salmonella* Dublin protocol' in Appendix A.

### 2.6.5 Multivariable analysis

The nature of the data suggested mixed effects logistic regression as the best choice of analytical method. All models contained mixed-effects logistic regression with binomial outcome and logit link function using the glmer() function in the lme4 package in R. The mixed-effects regression adjusts for data that contain nested grouping structures (here calves within herds). Therefore, data with nested or clustered structure were adjusted for in all models at herd-level by including 'HerdID' as a random effect factor. As the second objective was to analyse association between clinical disease outcome in calves in dairy herds with and without recent incursion of *S.* Dublin, the fixed effect 'Case control'

was forced into all models. It was kept in, even when not statistically significant as to study the effect of *Salmonella* exposure.

The analysis of data was performed using backward elimination of explanatory variables. The criterion for variables to remain in the model was set at 5% significance level. Biological considerations were made when choosing which explanatory variables to test for best model fit. The variables 'Direct contact' and 'Indirect contact' were logarithmic transformed and scaled by 10 to lessen issues with outlier observations and tested for inclusion as to allow the effect (the slope) of the number of direct and indirect contacts on the odds of disease in the calves to differ between herds. All 'NA' in the dataset were filtered out using the function '!is.na'. All fixed effects were tested for correlations with the correlation() function and for interaction with the function anova() in R before being included in the models.

After initial reduction of the model, explanatory variables were reintroduced by forward selection to test for confounding. Confounding was considered, when comparing estimates of fixed effects variables. If the estimates varied by more than 25% (Houe et al., 2004), the fixed effect were determined as confounders and therefore included. Confounding explanatory variables were tested using the function lrtest() in R as was the variable level significance of variables with more than two levels.

Models were created for each of the disease outcome variables. The generalised mixed effects logarithm model is for the probability $p_i$ of the $i^{th}$ animal being diseased is (Dohoo et al., 2009):

$$logit(p_i) = a + A_i + B_i(\dots) + yx_i + G_j$$

$p_i$ is the probability for a calf *i* being diseased in one of the three disease groups given the explanatory variables

$a$ is the intercept

$A_i$ is the fixed effect of 'Case control', for calf *i*

$B_i$ is the fixed effects of other qualitative explanatory variables for calf *i*

$x_i$ is the fixed effect of quantitative explanatory variables for calf *i*

$y$ is the slope for continuous variable

$G_j$ is the random effect, 'herd ID', j = 1…122, for calf *i* in a herd *j*

For each estimate included in the models, odds ratio (OR) and 97.5% confidence interval (CI) were calculated. As the collection of data on animal-level could be regarded as a cross-sectional study, the probability (P) was furthermore calculated for the outcomes of the models.

# 3. Results

## 3.1 Descriptive statistics

The final dataset for this thesis included 1799 calves from a total of 67 Danish dairy herds, more precisely 29 case herds with a total of 804 calves and 38 control herds with a total of 995 calves. The obtained sample size of Group A included 346 calves, 162 calves from case herds and 184 control herds both with calves with a mean age of 5 days. For Group B, the obtained sample size were 541 calves, 227 calves from case herds and 314 from control herds. The mean age of Group B was 24 days for case herds and 23 days for control herds. The obtained sample size for Group C were 912 calves, 415 from case herds and 397 from control herds. The mean age for Group C were 125 days for case herds and 127 days for control herds. The study had no sex and breed criteria included. The dataset consisted of 1446 heifer calves and 353 bull calves made up by 1267 Holstein calves, 350 crossbreed calves, 114 Jersey calves and 68 Red Danish Dairy Cattle calves. In average case herds were visited by the authors 12 days after a positive B-sample. In the early Fall 11 case herds and 11 control herds were visited, six case herds and 21 control herds were visited in the late Fall and 12 case herds, and six control herds were visited in the Winter season periods.

In Table 4, general clinical signs indicative of respiratory disease, had a seemingly higher distribution in control herds. When the calves were auscultated, the distribution of rales, harsh or rhonchi lung sounds occurred more in Group A and B calves in case herds. Only a small proportion of the calves received the highest score ‘2’, and a higher proportion of the calves received the score ‘1’. The distributions showed that signs of diarrhoea and hair loss on the hind were more apparent in case herds than control herds across age groups. Similarly, a larger proportion of calves in case herds had an affected ‘General condition’. No necrosis of the extremities were observed, but for calves in Group B in case herds ‘Lameness’ scores ‘1’ and ‘2’ were noted. Calves scored ‘1’ had signs of physical injury or congenital deformities, while calves scored ‘2’ were found to have inflamed, swollen joints consistent with polyarthritis. These calves were also found to be depressed with diarrhoea and hair loss and fever consistent with *S.* Dublin.

**Table 4.** Clinical variables and scores divided in three age groups (A, B and C), herd status (case or control) with number and percentage of observations.

| **Target age group** | | **A** *n = 346* | | **B** *n = 541* | | **C** *n = 912* | |
|---|---|---|---|---|---|---|---|
| **Variable** | **Score** | **Case** *n = 162* | **Control** *n = 184* | **Case** *n = 227* | **Control** *n = 314* | **Case** *n = 415* | **Control** *n = 497* |
| **Ocular discharge** | 0 | 113 (69.8%) | 112 (60.9%) | 128 (56.4%) | 159 (50.6%) | 351 (84.6%) | 394 (79.3%) |
| | 1 | 46 (28.4%) | 70 (38.0%) | 90 (39.6%) | 149 (47.5%) | 55 (13.3%) | 100 (20.1%) |
| | 2 | 3 (1.9%) | 2 (1.1%) | 9 (4.0%) | 6 (1.9%) | 9 (2.2%) | 3 (0.6%) |
| **Nasal discharge** | 0 | 60 (37.0%) | 67 (36.4%) | 94 (41.4%) | 116 (36.9%) | 180 (43.4%) | 154 (31.0%) |
| | 1 | 89 (54.9%) | 98 (53.3%) | 109 (48.0%) | 163 (51.9%) | 202 (48.7%) | 275 (55.3%) |
| | 2 | 13 (8.0%) | 19 (10.3%) | 24 (10.6%) | 35 (11.1%) | 33 (8.0%) | 68 (13.7%) |
| **Eardrop and/or head tilt** | 0 | 153 (94.4%) | 177 (96.2%) | 208 (91.6%) | 296 (94.3%) | 401 (96.6%) | 477 (96.0%) |
| | 1 | 7 (4.3%) | 6 (3.3%) | 11 (4.8%) | 10 (3.2%) | 7 (1.7%) | 9 (1.8%) |
| | 2 | 2 (1.2%) | 1 (0.5%) | 8 (3.5%) | 8 (2.5%) | 7 (1.7%) | 11 (2.2%) |
| **Type of respiration** | 0 | 158 (97.5%) | 178 (96.7%) | 219 (96.5%) | 302 (96.2%) | 403 (97.1%) | 469 (94.4%) |
| | 1 | 4 (2.5%) | 6 (3.3%) | 8 (3.5%) | 12 (3.8%) | 12 (2.9%) | 28 (5.6%) |
| **Auscultation**[a] | 0 | 99 (61.1%) | 124 (67.4%) | 119 (52.4%) | 205 (65.3%) | 192 (46.3%) | 162 (32.6%) |
| | 1 | 63 (38.9%) | 60 (32.6%) | 108 (47.6%) | 108 (34.4%) | 223 (53.7%) | 334 (67.2%) |
| **Cough**[a] | 0 | 158 (97.5%) | 179 (97.3%) | 208 (91.6%) | 280 (89.2%) | 331 (79.8%) | 355 (71.4%) |
| | 1 | 4 (2.5%) | 5 (2.7%) | 19 (8.4%) | 34 (10.8%) | 84 (20.2%) | 141 (28.4%) |
| **Hairloss hind**[a] | 0 | 143 (88.3%) | 174 (94.6%) | 152 (67.0%) | 237 (75.5%) | 372 (89.6%) | 488 (98.2%) |
| | 1 | 19 (11.7%) | 9 (4.9%) | 75 (33.0%) | 77 (24.5%) | 42 (10.1%) | 9 (1.8%) |
| **Diarrhoea** | 0 | 142 (87.7%) | 164 (89.1%) | 207 (91.2%) | 304 (96.8%) | 405 (97.6%) | 493 (99.2%) |
| | 1 | 20 (12.3%) | 20 (10.9%) | 20 (8.8%) | 10 (3.2%) | 10 (2.4%) | 4 (0.8%) |
| **General condition** | 0 | 134 (82.7%) | 161 (87.5%) | 206 (90.7%) | 294 (93.6%) | 403 (97.1%) | 488 (98.2%) |
| | 1 | 24 (14.8%) | 19 (10.3%) | 19 (8.4%) | 19 (6.1%) | 12 (2.9%) | 9 (1.8%) |
| | 2 | 4 (2.5%) | 4 (2.2%) | 2 (0.9%) | 1 (0.3%) | 0 (0,0%) | 0 (0,0%) |
| **Lameness**[b] | 0 | 160 (98.8%) | 180 (97.8%) | 216 (95.2%) | 314 (100%) | 410 (98.8%) | 492 (99%) |
| | 1 | 0 (0,0%) | 1 (0.5%) | 7 (3.1%) | 0 (0,0%) | 5 (1.2%) | 5 (1.0%) |
| | 2 | 0 (0,0%) | 0 (0,0%) | 2 (0.9%) | 0 (0,0%) | 0 (0,0%) | 0 (0,0%) |
| **Umbilical region**[c] | 0 | 116 (71.6%) | 128 (69.6%) | 182 (80.2%) | 270 (86.0%) | 399 (96.1%) | 481 (96.8%) |
| | 1 | 31 (19.1%) | 36 (19.6%) | 35 (15.4%) | 33 (10.5%) | 15 (3.6%) | 15 (3.0%) |
| | 2 | 15 (9.3%) | 19 (10.3%) | 10 (4.4%) | 10 (3.2%) | 1 (0.2%) | 0 (0,0%) |
| **Necrosis of pinnae and tail** | 0 | 162 (100%) | 184 (100%) | 227 (100%) | 314 (100%) | 415 (100%) | 497 (100%) |

| Target age group | | A *n = 346* | | B *n = 541* | | C *n = 912* | |
|---|---|---|---|---|---|---|---|
| **Variable** | **Score** | **Case** *n = 162* | **Control** *n = 184* | **Case** *n = 227* | **Control** *n = 314* | **Case** *n = 415* | **Control** *n = 497* |
| **Pain** | 0 | 153 (94.4%) | 184 (100%) | 221 (97.4%) | 312 (99.4%) | 412 (99.3%) | 495 (99.6%) |
| | 1 | 9 (5.6%) | 0 (0.0%) | 6 (2.6%) | 2 (0.6%) | 3 (0.7%) | 2 (0.4%) |
| **Ringworm** | 0 | 162 (100%) | 181 (98.4%) | 222 (97.8%) | 309 (98.4%) | 311 (74.9%) | 399 (80.3%) |
| | 1 | 0 (0,0%) | 3 (1.6%) | 5 (2.2%) | 5 (1.6%) | 104 (25.1%) | 98 (19.7%) |
| **Hair coat** | 0 | 112 (69.1%) | 140 (76.1%) | 149 (65.6%) | 189 (60.2%) | 206 (49.6%) | 222 (44.7%) |
| | 1 | 50 (30.9%) | 44 (23.9%) | 78 (34.4%) | 125 (39.8%) | 209 (50.4%) | 275 (55.3%) |
| **Cleanliness**[a] | 0 | 70 (43.2%) | 84 (45.7%) | 113 (49.8%) | 138 (43.9%) | 223 (53.7%) | 318 (64%) |
| | 1 | 67 (41.4%) | 62 (33.7%) | 88 (38.8%) | 137 (43.6%) | 128 (30.8%) | 142 (28.6%) |
| | 2 | 25 (15.4%) | 37 (20.1%) | 26 (11.5%) | 39 (12.4%) | 63 (15.2%) | 37 (7.4%) |
| **Body condition** | 0 | 148 (91.4%) | 159 (86.4%) | 189 (83.3%) | 256 (81.5%) | 335 (80.7%) | 368 (74.0%) |
| | 1 | 14 (8.6%) | 25 (13.6%) | 38 (16.7%) | 58 (18.5%) | 80 (19.3%) | 129 (26.0%) |

*[a] Removal of one - two missing observations in each. [b] Removal of five observations with the score 99, which were calves unable to be evaluated due to general condition score 2. [c] Removal of two observations with the score 99, which were calves unable to be evaluated due to general condition score 2.*

In Table 5, across age groups A and B, case herds appeared to have a less clean bedding environment. This was also evident for Group C although control herds appeared to have had same less favourable bedding conditions. Case herds across all age groups had a considerably higher occurrence of soiled drinking water. 'Hygiene feed' could not be evaluated for Group A, as observations lacked, and many calves received the score '99'. For Group B and C only half of the case herds had access to clean feed without any contamination. The score '99' was included for all three variables. The score was used when the authors were unable to score according to the protocol, i.e., when calves were housed on slatted flooring rather than deep bedding or when milk fed calves had bowls for milk/water/grain removed after feeding. Across the three groups 86 calves lacked observations. The number of missing observations and the score '99' were not featured in the table although they are still calculated as part of the proportion.

In Table 6, it is evident that the variables 'Direct contact' and 'Indirect contact' contained noticeable variation of the herds and within the age groups of the herds. The number of calves housed in the same pen differed as expected across age group and herd status. Some case and control herd had calves housed in such fashion, that they had very high numbers (>150-200) of direct and indirect contacts. The mean and median were considerably lower (>50) for all herds. No differences of mean were apparent for the rectal temperatures measured across age groups and herd status.

**Table 5.** Environmental hygiene variables and scores divided by age group (A, B and C), herd status (case or control) with number and percentage.

| **Target age group** | | **A** *n = 346* | | **B** *n = 541* | | **C** *n = 912* | |
|---|---|---|---|---|---|---|---|
| **Variable** | **Score** | **Case** *n = 162* | **Control** *n = 184* | **Case** *n = 227* | **Control** *n = 314* | **Case** *n = 415* | **Control** *n = 497* |
| **Bedding** [a b] | 0 | 109 (67.3%) | 153 (83.2%) | 161 (70.9%) | 268 (85.4%) | 140 (33.7%) | 158 (31.8%) |
| | 1 | 28 (17.3%) | 21 (11.4%) | 30 (13.2%) | 32 (10.2%) | 141 (34%) | 132 (26.6%) |
| | 2 | 18 (11.1%) | 6 (3.3%) | 35 (15.4%) | 12 (3.8%) | 120 (28.9%) | 185 (37.2%) |
| **Water** [a c] | 0 | 114 (70.4%) | 126 (68.5%) | 130 (53.3%) | 239 (76.1%) | 144 (34.7%) | 279 (56.1%) |
| | 1 | 29 (17.9%) | 24 (13%) | 57 (25.1%) | 37 (11.8%) | 173 (41.7%) | 143 (28.8%) |
| | 2 | 2 (1.2%) | 3 (1.6%) | 23 (10.1%) | 19 (6.1%) | 95 (22.9%) | 57 (11.5%) |
| **Feed** [a d] | 0 | 50 (30.9%) | 79 (42.9%) | 115 (50.7%) | 211 (67.2%) | 228 (54.9%) | 370 (74.4%) |
| | 1 | 27 (16.7%) | 17 (9.2%) | 63 (27.8%) | 33 (10.5%) | 140 (33.7%) | 68 (13.7%) |
| | 2 | 1 (0.6%) | 4 (2.2%) | 23 (10.1%) | 33 (10.5%) | 47 (11.3%) | 42 (8.5%) |

*[a]Missing observations not in the table from the left for all three variables separately: A: 7 (4.3%), 4 (2.2%), B: 1 (0.4%), 2 (0.6%), C: 0 (0.0%), 17 (4.8%). [b]Score 99 not featured for bedding: Group C case 14 (3.4%), control 5 (1%). [c]Score 99 not featured for water from the left: 10 (6.2%), 27 (14.7%), 16 (7%), 17 (5.4%), 3 (0.7%), 1 (0.2%). [d]Score 99 not featured for feed from the left:77 (47.5%), 80 (43.5%), 25 (11%), 35 (11.1%), 0 (0.0%), 0 (0.0%).*

**Table 6.** Quantitative variables with statistical measures divided in age group (A, B, C), herd status (case or control) with minimum (min), maximum (max), mean, and median.

| **Target age group** | | **A** *n = 346* | | **B** *n = 541* | | **C** *n = 912* | |
|---|---|---|---|---|---|---|---|
| **Variable** | **Measures** | **Case** *n = 162* | **Control** *n = 184* | **Case** *n = 227* | **Control** *n = 314* | **Case** *n = 415* | **Control** *n = 497* |
| **Number in pen** | Min | 1 | 1 | 1 | 1 | 1 | 1 |
| | Max | 4 | 17 | 10 | 17 | 54 | 33 |
| | Mean | 1.2 | 1.6 | 3.6 | 3.7 | 12.5 | 10.0 |
| | Median | 1 | 1 | 2 | 2 | 10 | 8 |
| **Direct contact** | Min | 0 | 0 | 0 | 0 | 0 | 0 |
| | Max | 21 | 176 | 118 | 176 | 230 | 68 |
| | Mean | 2.3 | 6.2 | 7.9 | 10.2 | 30.4 | 21.0 |
| | Median | 2 | 2 | 4 | 3 | 23 | 18 |
| **Indirect contact** | Min | 1 | 1 | 1 | 1 | 4 | 1 |
| | Max | 214 | 177 | 214 | 177 | 231 | 203 |
| | Mean | 11.7 | 13.3 | 20.2 | 20.2 | 46.0 | 37.0 |
| | Median | 4 | 7 | 7 | 12 | 27 | 30 |
| **Temperature** (°C) | Min | 37.8 | 37.3 | 37.3 | 37.0 | 37.7 | 38.1 |
| | Max | 40.5 | 39.8 | 41.1 | 40.5 | 41.0 | 41.2 |
| | Mean | 38.8 | 38.8 | 38.9 | 38.8 | 39.0 | 39.1 |
| | Median | 38.8 | 38.8 | 38.9 | 38.8 | 39.0 | 39.1 |

## 3.2 Outcome of disease categories

Table 7 shows the distribution of 'Respiratory disease', 'Gastrointestinal disease' and 'Systemic disease' based on aggregation of the criteria in Table 3. There was no apparent difference in the occurrence of respiratory disease between cases and controls in Group A and B. However, control Group C appeared to have a higher occurrence of respiratory disease, at 33.4% compared to 22.9% in the case Group C. Furthermore, there appeared to be a noticeably larger proportion of calves with the score '1' in case herds across Group A, B and C compared to control herds. For 'Systemic disease' there also appeared to be noticeable differences in groups A and B in case herds compared to groups A and B control. For Group C the distribution of calves suffering from systemic disease appears similar. Several individuals had multiple different clinical signs and were therefore deemed diseased in more than one category.

**Table 7.** Categories of disease with calves divided by disease score, age group and herd status.

| **Target age group** | | **A** *n = 346* | | **B** *n = 541* | | **C** *n = 912* | |
|---|---|---|---|---|---|---|---|
| **Category** | **Score** | **Case** *n = 162* | **Control** *n = 184* | **Case** *n = 227* | **Control** *n = 314* | **Case** *n = 162* | **Control** *n = 184* |
| **Respiratory disease**[a] | 0 | 153 (94.94%) | 174 (94.6%) | 195 (85.9%) | 268 (85.4%) | 320 (77.1%) | 330 (66.4%) |
| | 1 | 9 (5.6%) | 10 (5.4%) | 32 (14.1%) | 45 (14.3%) | 95 (22.9%) | 166 (33.4%) |
| **Gastrointestinal disease**[b] | 0 | 125 (77.2%) | 156 (84.8%) | 140 (61.7%) | 230 (73.2%) | 364 (87.8%) | 484 (97.4%) |
| | 1 | 37 (22.8%) | 27 (14.7%) | 87 (38.3%) | 84 (26.8%) | 50 (12%) | 13 (2.6%) |
| **Systemic disease** | 0 | 132 (81.5%) | 161 (87.5%) | 196 (86.3%) | 290 (92.4%) | 396 (95.4%) | 475 (95.6%) |
| | 1 | 30 (18.5%) | 23 (12.5%) | 31 (13.7%) | 24 (7.6%) | 19 (4.6%) | 22 (4.4%) |

[a] *n = 1 removed from 'Respiratory disease' control Group B and C.* [b]*n = 1 removed from 'Gastrointestinal disease' control Group A and case Group C.*

## 3.3 Distribution of serum serology

In total 912 calves from Group C had serum evaluated for antibodies (ODC%) against *S.* Dublin at Eurofins Laboratory. Of those, 415 came from calves in case herds and 497 from control herds. In six out of 29 case herds none of the sampled animals had an ODC% above 0. On animal-level in case herds 246 calves were negative (ODC % 0) and 166 calves were positive (ODC% > 0). Figure 1 shows that two calves from control herds had an *S.* Dublin antibody reaction. In herd 35, one animal had an ODC% of 30 and in herd 64 one animal had an ODC% of 8. Among case herds with ODC% > 0, the boxplot displays considerable herd and within herd variation of *S.* Dublin antibody levels. The overall highest measurement of ODC% was 135.

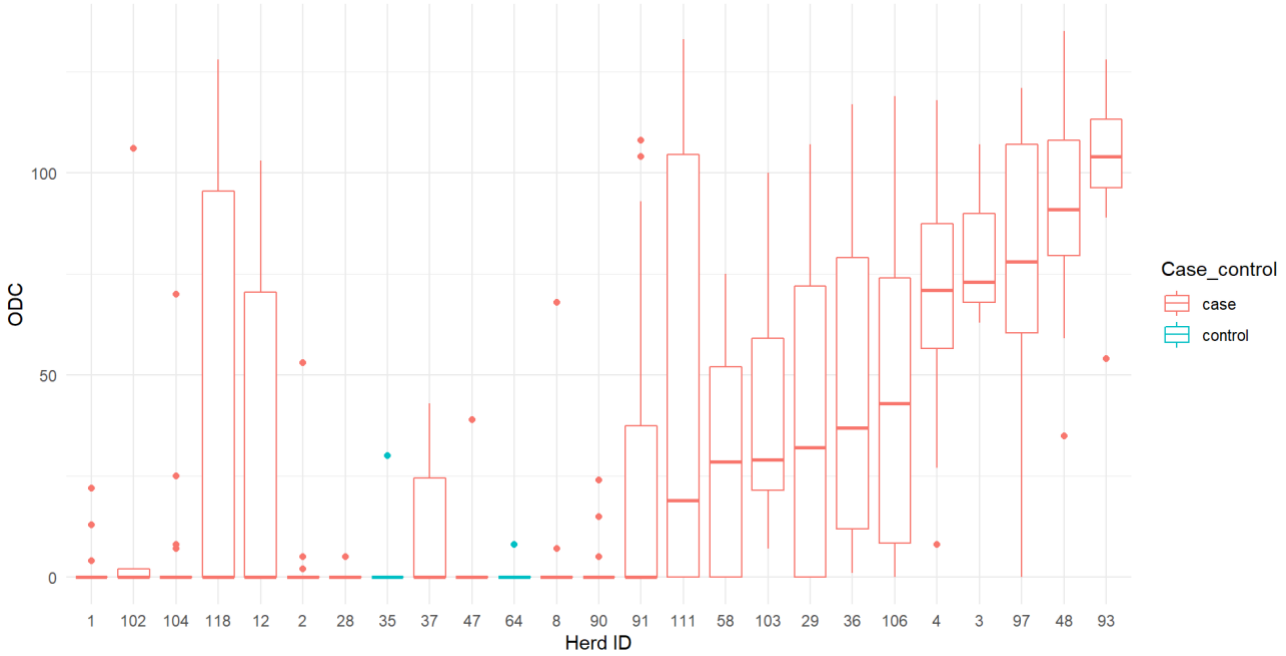



**Figure 1.** Boxplot of antibody test results (ODC%) in blood samples from calves in case (red) and control (blue) herds with *S.* Dublin antibody levels (ODC%) > 0. A box displays the five-number summary with the minimum, first quartile, median, third quartile, and maximum. Each dot displays an outlier calf located outside the whiskers of the box.

## 3.4 Multivariable analysis

The fixed effects 'Direct contact' and 'Indirect contact' were significantly correlated as $R^2 = 0.80$ and $P < 0.001$. No other fixed effects were significantly correlated. Nor was there any significant effect of using quantitative variables as random slope which would have allowed coefficients to vary between herds with different numbers of contacts in both models. Hence none are included in any final model with or without scaling and logarithmic transformation.

It was difficult to create a stable and meaningful mixed effects multivariable logistic regression model for Group B and C. Furthermore, 'Season', 'Observer' and 'Hygiene bedding' acted as confounders and markedly changed the fixed effect parameter of the 'Case control'-variable. Hence, no associations were found between 'Respiratory disease' or 'Systemic disease' and being calves in case versus control herds. The tentative models created for 'Respiratory disease' and 'Systemic disease' in the two age groups can be found in Appendix B.

## 3.4.1 Gastrointestinal disease model for Group B

**Table 9.** Results from the final logistic regressions model of the probability of having gastrointestinal disease as a Danish dairy herd calf in Group B in a case herd compared to a control herd and other different explanatory variables. The table includes log-transformed fixed effects estimates, standard error (SE), p-value of fixed effects, odds ratio (OR) with 97.5% confidence interval (CI). Variance ($\sigma^2$) and standard deviation (SD) were calculated of the random effect, Herd ID.

| Variables | | Estimate | SE | P | OR | 97.5% CI of OR | $\sigma^2$ | SD |
|---|---|---|---|---|---|---|---|---|
| *Fixed effects* | | | | | | | | |
| Intercept | | -1.17 | 0.37 | ** | | | | |
| Case control | | | | - | | | | |
| | Control | Ref | - | | - | - | | |
| | Case | 1.67 | 0.37 | - | 2.0 | (0.9-4.0) | | |
| Cleanliness | | | | ** | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | 0.65 | 0.25 | ** | 1.9 | (1.2-3.1) | | |
| | 2 | 1.34 | 0.37 | *** | 3.8 | (1.8-7.9) | | |
| Hygiene bedding | | | | ** | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | 0.87 | 0.39 | * | 2.4 | (1.1-5.1) | | |
| | 2 | -1.31 | 0.57 | * | 0.3 | (0.1-0.8) | | |
| Season | | | | - | | | | |
| | Early Fall | Ref | - | | - | - | | |
| | Late Fall | -1.02 | 0.42 | * | 0.4 | (0.2-0.8) | | |
| | Winter | -0.52 | 0.44 | - | 0.6 | (0.3-1.4) | | |
| *Random effect* | | | | | | | | |
| Herd ID | | | | | | | 1.00 | 1.00 |

** = p-value < .05, ** = p-value < .01, *** = p-value < .001*

In Table 9, there was no significant difference in the odds of having gastrointestinal disease in calves in the age of 14 – 28 (42) days in *S.* Dublin test-positive herds compared to test-negative herds. The variables 'Season' and 'Hygiene bedding' were found to confound with the fixed variable 'Case control'. Calves scoring '1' in 'Cleanliness' had increased odds (OR = 1.9; 97.5% CI: 1.2-3.1) of 'Gastrointestinal disease' compared to calves with score '0'. Moreover, calves scoring '2' in 'Cleanliness' had increased odds (OR = 3.8; 97.5% CI: 1.8-7.9) of 'Gastrointestinal disease' compared to score '0'. Similarly, calves scoring '1' in 'Hygiene bedding' had increased odds (OR = 2.4; 97.5% CI: 1.1-5.1) of having 'Gastrointestinal disease' between score '1' and '0'. Contra-intuitively, a calf scoring '2' in 'Hygiene bedding' had decreased odds (OR = 0.3; 97.5% CI: 0.1-0.8) of 'Gastrointestinal disease' between score '2' and score '0'. Calves examined in the 'Late Fall'

appeared to have significantly lower odds (OR = 0.4; 97.5% CI: 0.2-0.8) of 'Gastrointestinal disease' than calves examined in the 'Early Fall', whereas 'Winter' was not found different from 'Early Fall'. Estimates in the final model did not improve with exclusion of ODC% negative case herds and ODC% positive control herds.

The probability of disease in the reference group hereby a calf housed in a control herd with score '0' in 'Cleanliness' and 'Hygiene bedding' in 'Early Fall' were 23.7%. On the contrary, if housed in a case herd with the same scores the probability was 62.2%. The lowest probability of disease was 2.9%, when a calf housed in a control herd scored '0' in 'Cleanliness', '2' in 'Hygiene bedding' and in the 'Late Fall'. The highest probability of 'Gastrointestinal disease' was 93.8%, when a calf housed in a case herd scored '2' in 'Cleanliness', '1' in 'Hygiene bedding' in 'Early Fall'.

## 3.4.2 Gastrointestinal disease model for Group C

**Table 10.** Results from the final regressions model of the probability of having gastrointestinal disease as a Danish dairy herd calf in Group C in a case herd compared to a control herd and other different explanatory variables. The table includes log-transformed fixed effects estimates, standard error (SE), p-value of fixed effects, odds ratio (OR) with 97.5% confidence interval (CI). Variance ($\sigma^2$) and standard deviation (SD) were calculated of the random effect, 'Herd ID'.

| **Variables** | | **Estimate** | **SE** | **P** | **OR** | **97.5% CI of OR** | **$\sigma^2$** | **SD** |
|---|---|---|---|---|---|---|---|---|
| *Fixed effects* | | | | | | | | |
| Intercept | | -4.91 | 0.64 | *** | | | | |
| Case control | | | | *** | | | | |
| | Control | Ref | - | | - | - | | |
| | Case | 2.08 | 0.55 | *** | 8.0 | (2.7-23.6) | | |
| Ringworm | | | | * | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | 1.15 | 0.39 | ** | 3.2 | (1.5-6.8) | | |
| Cleanliness | | | | *** | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | 1.45 | 0.38 | *** | 4.3 | (2.0-9.0) | | |
| | 2 | 2.08 | 0.54 | *** | 8.0 | (2.8-22.8) | | |
| Season | | | | * | | | | |
| | Early Fall | Ref | - | | - | - | | |
| | Late Fall | -0.34 | 0.56 | . | 0.7 | (0.2-2.1) | | |
| | Winter | -2.12 | 0.68 | ** | 0.1 | (0.0-0.5) | | |
| *Random effect* | | | | | | | | |
| Herd ID | | | | | | | 1.34 | 1.16 |

* = *p*-value < .05, ** = *p*-value < .01, *** = *p*-value < .001

In Table 10, it is evident that calves in the age of 100 – 130 (180) days in *S.* Dublin test-positive herds were significantly associated (OR = 8.0; 97.5% CI: 2.7 – 23.6) with the increase in probability of having gastrointestinal disease compared to *S.* Dublin test-negative herds. Calves scored '1' in 'Ringworm' had increased odds (OR = 3.0) compared to score '0'. Calves scoring '1' in 'Cleanliness' had higher odds (OR = 4.3; 97.5% CI: 2.0-9.0) of disease than calves scoring '0'. Moreover, calves scoring '2' in 'Cleanliness' had increased odds (OR = 8.0; 97.5% CI: 2.8-22.8) of 'Gastrointestinal disease' compared to the reference '0'. Calves examined in 'Winter' appeared to have significantly less (OR = 0.1; 97.5% CI: 0.0-0.5) 'Gastrointestinal disease' than calves examined in the 'Early Fall'. No significant confounders or interactions were found for this age group. The final model did not change with exclusion of ODC% negative case herds and ODC% positive control herds.

The probability of disease in the reference group hereby a calf housed in a control herd with the score of '0' in 'Ringworm' and 'Cleanliness' in the 'Early Fall' was 1%. On the contrary, if the calf was housed in a case herd with the same scores, the probability of 'Gastrointestinal disease' was 6%. The lowest probability of 'Gastrointestinal disease' overall was 0.1%, when a calf was housed in a control herd with the score of '0' in 'Ringworm' and 'Cleanliness' in 'Winter'. The highest probability of 'Gastrointestinal disease' was 60%, when the calf was housed in a case herd scoring '1' in 'Ringworm', '2' in 'Cleanliness' and in 'Early Fall'.

# 4. Discussion

## 4.1 Main Findings

This thesis showed an overall association between gastrointestinal disease in calves and the incursion of *S.* Dublin in naïve dairy herds under Danish conditions. Specifically, Group C had significantly higher odds (OR = 8.0; 97.5% CI: 2.7 – 23.6) of having signs of gastrointestinal disease in *S.* Dublin test-positive herds compared to test-negative herds in a multivariable logistic mixed effects model accounting for these factors: ringworm, decreased cleanliness of the calf and a soiled environment. The association was a somewhat unexpected result as it shows that infection with *S.* Dublin caused an increased occurrence of gastrointestinal disease rather than respiratory disease in older calves housed in present Danish conditions.

Importantly, 84% of the calves in case herds were placed in the category gastrointestinal disease due to hair loss on the hind. The loss of hair on the hind occurs as a result of dermatitis caused by diarrhoea around three weeks after the onset (Nielsen et al., 2018). Therefore, hair loss of the hind could have been used as an indirect evaluation of the severity of diarrhoea, although the data did not

differentiate in the severity or stage of hair loss. The mean age of case Group C was 125 days and as previously mentioned, Guizelini et al. (2019) found that onset of clinical disease was possible at 3 months. Exact age of onset and duration of diarrhoea were unknown. Further analysis of data could have revealed, if the 16% calves found to have active diarrhoea were closer to 3 months of age rather than the upper age limit of this study at 180 days.

The immunity status of calves in the herds were likely to play a role in the susceptibility and outcome of *S.* Dublin infections as concurrent ringworm infection seemed to increase the occurrence of gastrointestinal disease. Roden et al. (1992) found that young calves (< 3 months) were more susceptible to infection and more at risk of experiencing clinical disease due to *S.* Dublin than older animals due to their reduced ability to produce antibodies. A concurrent ringworm infection compromising the immune status in the older animals, could decrease resistance to *S.* Dublin infection. The decreased resistance could increase the risk of experiencing clinical disease and potentially also prolong the persistence of infection.

The importance of hygiene was once again demonstrated to some extent by this study. Licking of coats contaminated by faeces of excretory calves could be an obvious mean of taking in large oral doses of organisms (Nazer and Osborne, 1977). As cleanliness of the calf decreased, the exposure to faecal matter increased and the odds of gastrointestinal disease increased similarly. Cleanliness of the calf was also considered as an indirect measure of environmental hygiene. Presence of moisture in the environment favours *Salmonella* proliferation and can result in environmental loads of $10^7$ *Salmonellae* per gram (Mohler et al., 2009). Calves in the Winter appeared to have significantly less gastrointestinal disease than calves in the early Fall. According to The Danish Meteorological Institute (2022), the months of September and October had a high occurrence of wet days and higher temperatures compared to the same months from 1991-2020 again providing warm and moist conditions favouring proliferation (Houe et al., 2014).

Calves in herds with a recent incursion of *S.* Dublin, had less clinical signs indicative of respiratory disease and systemic disease than expected based on literature. No other statistically significant associations were found between *S.* Dublin herd status and other disease categories, nor in other age-groups.

## 4.2 Clinical signs of disease in the study herds

In general, septicaemia is an important cause of death in neonatal calves and diarrhoea is the most important disease in calves less than 30 days of age. In calves over 30 days of age, pneumonia is

considered the most important problem (McGuirk, 2008). Although causal agents in control herds and case herds were unknown in this study, the test-negative status and negative *S.* Dublin antibody levels in the calves gave reason to exclude *S.* Dublin as causal agent in the control herds. The causal agents may change over time, depending on season of the year and population dynamics within the environmental site of exposure.

Assuming all other disease occurrences equal in case and control herds, thereby attributing the clinical signs observed in case herds to be influenced or a direct result of *S.* Dublin infection, less additional disease was found. Large oral dosages of $10^7$-$10^{10}$ CFU were used to reproduce salmonellosis in experimental studies (Nazer and Osborne, 1977; Smith and Jones, 1967). The dosages used to reproduce salmonellosis were unlikely to bear relationship to the presumably smaller dosages calves in case herds were exposed to under natural conditions. Wray and Sojka (1981) aimed to simulate natural infection with natural dosages the calf was likely to acquire. Here calves suckled on cows with a faecal excretion of $10^2$-$10^5$ CFU, drank *S.* Dublin contaminated water ($10^2$-$10^4$ organisms/ml) and were housed on *S.* Dublin contaminated bedding. None of the calves in this study were found to have clinical signs of salmonellosis.

However, at more than one occasion the authors had reason to suspect outbreak of clinical salmonellosis in the case herds. Suspicion rose when neonatal calves appeared severely depressed or comatose with forced breathing, signs of diarrhoea and had temperatures of 40.0 °C and above. This study found a slightly larger proportion of calves in case herds appeared depressed or comatose compared to calves in control herds. Other potential causes of a comatose state could have been *E. coli* septicaemia or metabolic acidosis and dehydration because of severe diarrhoea (Dillane et al., 2020; Ragione et al., 2013). In the case herds, two calves were found to have septic arthritis. None had signs of necrosis, which could have appeared in calves surviving peracute and severe acute infection with *S.* Dublin. The study was solely based on calves present in the herds on the day of the visit. Every so often calves selected for sampling were reported deceased by the farmer. Nielsen et al. (2010) found a high BTM *Salmonella* status was associated with increased risk of high calf mortality in dairy herds. The authors did not have the opportunity to routinely interview the farmers about calf mortality in their herds. It is possible that the calves suffering from septicaemia due to a recent incursion of *S.* Dublin already succumbed days or weeks before this study and thereby the number of calves could be underestimated. The possible mild, transient infections with *S.* Dublin resulting in transient anorexia and fever may also have been underestimated in this study.

Calves in control herds appeared to a higher distribution of clinical signs indicative of respiratory disease than calves in case herds. Only when auscultating the calves, distribution of rales, harsh or rhonchi lung sounds were more prevalent in case Group A and B. In Denmark, Bovine respiratory disease (BRD) also offers a challenge to health and welfare in calves (Fertner et al., 2016). BRD is caused by a mixture of viruses such as bovine respiratory syncytial virus (BRSV), bovine coronavirus (BCoV), bovine herpesvirus type 1 (BoHV-1), and bovine parainfluenza virus 3 (BpiV-3), bovine viral diarrhoea virus (BVD), and opportunistic bacteria belonging to *Pasteurellaceae* and *Mycoplasma bovis* (*M. bovis*) (Kudirkiene et al., 2021). Infection with *M. bovis* can also be associated with swollen joints, head tilt and eardrop caused by arthritis, meningitis, and otitis media (Dudek et al., 2020). Clinical signs of respiratory disease (i.e., nasal discharge, eye discharge and laboured breathing) observed in control herds could be attributed to BRD. In calves in case herds, it was possible that similar signs of respiratory disease were caused by the recent incursion of *S.* Dublin, but this thesis found no distributional differences indicative to support this.

The symptoms of gastrointestinal disease such as watery and bloody diarrhoea and hair loss on the hind were more apparent across all age groups in the case herds. In general, neonatal calves are susceptible to disease as exposure to numerous ubiquitous pathogens are inevitable. For younger calves, a substantial number of enteric pathogens could cause similar symptoms of disease in addition to *Salmonella* ssp. Not discussed as of yet, *S.* Typhimurium could give rise to some of the enteric symptoms observed in both case and control herds. Potential cross-reactivity are discussed in section 4.5 '*Salmonella* Dublin Herd Classification'. Amongst the enteric pathogens, *E. coli*, bovine rotavirus (BRV), bovine coronavirus (BcoV), *Cryptosporidium* ssp., *Clostridia* ssp. are known to cause disease (McGuirk, 2008). In the older calves, generally less susceptible to gastrointestinal disease coccidia (*Eimeria* spp.) could have been a prevalent causal agent when diarrhoea, elevated rectal temperature and chronic wasting were observed in the study herds.

Introduction and persistence of *S.* Dublin infection has been reported to be influenced by stress caused by concurrent infectious diseases. Vaessen et al. (1998) reported that herd infection with Fasciola hepatica were associated with infection with *S.* Dublin. Though few to no calves used in this study had access to grazing areas where such infection often occurs. Wray and Roeder (1987) reported that calves infected with bovine virus diarrhoea (BVD), virus showed more severe symptoms if they were also infected with S. Dublin in an experimental study. BVD is considered eradicated in Denmark (Houe et al., 2014), and therefore unlikely to play a role in calves in this study.

Rings (1985) found that clinical signs in affected calves could usually be associated with failure to thrive with undersized calves and scruffy haircoats, but no distributional differences between case and control herds were noted. From the *S.* Dublin protocol, repeated observations of weight in kilograms as a function of girth width measurements against sex, breed, and age of the calf (Heinrichs et al., 1992) could have provided valuable information about a possible retardation of growth rate in the calves affected with *S.* Dublin (Grønstøl et al., 1974). The *S.* Dublin protocol also included measurements of serum immunoglobulin G (IgG) of calves 0-10 days old to evaluate passive immunity status. The evaluations were carried out by using Brix refractometry (BRIX) measured as total solids or % (Buczinski et al., 2018). The results could have added valuable information regarding the overall immunity status of the calves in case and control herds, but unfortunately the sample size was inadequate and results potentially misleading i.e., dehydration could increase the relative number of total solids in serum (Buczinski et al., 2018). Increased risk of mortality, overall neonatal morbidity, as well as diarrhoea and respiratory disease have all been associated with lower IgG concentrations in calves (Raboisson et al., 2016). The authors were not able to pay regard to vaccination statuses in general, treatment protocols and management routines which could also affect the clinical observations in the study herds.

## 4.3 Statistical considerations and limitations

The data structure with information collected at two levels (herds and calves within herds) suggested mixed effects logistic regression as the best choice of analytical method as outcomes were dichotomized and allowed inclusion of the random effect 'Herd ID'. The inclusion of variables in the models were chosen for their assumed biological relevance. The statistically significant results were reasonably interpreted with corresponding biological processes to decide their biological significance. Robustness of the models did not differ with the exclusion of ODC% negative case herds and ODC% positive control herds. Some variable combinations led to low sample sizes in the cross-tabulations, which decreased the robustness of the models. As a result, several expected risk factors were not found associated with the outcomes. The effect of the *Salmonella* exposure variable was the primary focus; therefore, it was forcibly kept in all models. A larger dataset might provide for different results with more stable models. The variables 'Direct contact' and 'indirect contact' could not be included, neither as a fixed effects nor as random slope. This could be due to faulty created variables as they did not account for rate of infection within the herds. A naïve herd with a recent incursion of *S.* Dublin would presumably have a high rate of infection and infect more animals

in close contact. However, a high number of contacts does not necessitate a high prevalence of disease as the herd could be healthy with no to little disease and thereby a low rate of infection. For group C, 'Hygiene bedding' score '99' (calves housed on slatted floors) was removed due to a limited number of calves (n = 19 out of 912) even though it indicated a significant increase of disease. Contradictory, Martin and Smith (1984) suggested the use of slatted floors as a measure of control to minimize environmental contamination of *S.* Dublin. However, the result might also be due to younger calves moved into a more challenging environment with older animals rather than the composition of the floors.

The final model for gastrointestinal disease for Group B rendered the explanatory variable 'Case control' borderline significant (OR = 2.0; 97.5% CI: 0.9 - 4.0), thus in the end an association could not be proved. A calculation of the required sample size to render the variable significant proved inconclusive as the model contained many variables. However, the tentative model illustrated the importance of cleanliness again with odds of disease increasing with decreasing cleanliness of the calf. But surprisingly, the model suggested that calves housed in less favourable conditions (<50% dry bedding) were less likely to have gastrointestinal disease compared to calves housed in more favourable conditions (50-75% dry bedding). Though, the contradictory above-mentioned statistical association was derived from a small part of the dataset (n = 47).

It was not possible to create meaningful final models with respiratory disease as outcome for either Group B or C as shown in Appendix B. The distribution of clinical signs supported this conclusion, yet the overall result was somewhat unexpected when compared to literature of *S.* Dublin. This dataset could not reproduce the clinical signs found in experimentally derived studies with controlled environments and set oral dosages. However again, the infectious dosages a calf could acquire under natural conditions are thought to be less as discussed in section 4.2 'Clinical signs of disease in the study herds'. Both models had several confounders rendering them very unstable. Group housing, which have been associated with the greatest risk of BRD when comparing to other types of housing and a risk factor for *S.* Dublin (Losinger and Heinrichs, 1996; Nielsen, 2013) were found significant in the tentative model for Group B as an increasing number of calves in the same pen were associated with the risk of having respiratory disease. Tentative results of Group C indicated that the probability of having respiratory disease increased as weather conditions worsened, and the calves were exposed to colder temperatures, but also that a soiled feeding through had a protective effect.

The data did not support the possibility of creating meaningful models for systemic disease for Group B or C when comparing calves in case and control herds as shown in Appendix B. This could potentially be due to a 'faulty' category as the variables may not include the 'right' calves as pyrexic calves (≥ 40.0 °C) and calves appearing depressed ('General condition' score '1'), could have been consequences of severe gastrointestinal- or respiratory disease rather than a septicaemic effect.

The criteria of inclusion could potentially have been too broad as the tentative model for systemic disease in Group C had eight explanatory variables confounding with the fixed effect 'Case control' rendering a very unstable and not well explained model. The very uncertain model indicated that being thin or lean, housed in soiled bedding with a higher number of calves in the pen were associated with an increased risk of systemic disease. 'Observer' also had an effect as more systemic disease was prevalent with Author 2 present. But observations carried out including Author 2 were primarily within first weeks consisting of 80% case herds and the last weeks of the field-project with the seasonal effect of Winter. In Group B, severely soiled calves were associated with systemic disease. It was not established, if the depressed or comatose state of the calf led to a severely soiled appearance or the opposite.

## 4.4 Data Quality

The representativity of the Danish calf population were in general high as both organic and conventional herds, different breeds, management, and housing types were included. The study took place across Denmark but primarily in Jutland as the peninsula has the highest distribution of *S.* Dublin test-positive dairy herds (Houe et al., 2014). Differences in sex and breed were disregarded, though potential differences in disease resistance could occur.

The average cow-year herd size was 211. The smallest herd had 46 animals (cow-years) and the largest herd had 766 animals (cow-years). If all 67 herds had met the target sample size, Group A and B would have consisted of 670 calves each (actual size n = 346 and n = 541). Group C would have consisted of a minimum of 1005 calves (actual size n = 912). The age of the calves sampled relied solely on the farmers registrations of date of birth. Hence a bias could exist regarding the true age of the calves with some registrations being more accurate than others.

The authors were blinded to the status of the herd (case or control) until after observations were made to exclude any information which could subconsciously influence the scores. In some cases, when special precautions had to be taken or if a suspicion about salmonellosis rose, the authors would contact the veterinarian in charge and be informed of the status before completion of all observations.

To decrease observer bias, an ongoing calibration between authors took place as 22 out of 67 herds were scores collaboratively. To measure the inter-rater reliability and thereby improve data quality, a statistical measure of agreement would have been useful before, during and after data collection.

Herd visits were carried out twice a day in the data collection period with one herd visited in the morning and one herd visited in the afternoon. As each herd were only visited once at a 'random' point in time, it is therefore possible that the score given i.e., environmental scores do not reflect the general environment in the herd. To some extent, this were also true for the clinical signs observed. When scoring the calves, the authors found that interaction with the calves affected the scoring of variables. As possible, all observational data were gathered without disturbing the calves. In general, most observations were carried out before tying up the calf so as to not be disturbed by the rope i.e., 'Ear drop and/or Head tilt' and 'Type of respiration'. In Group C, larger pens with many calves often complicated the authors ability to score and therefore it was necessary to catch these individuals before assessment. When auscultating, the angle of the head and the general position of the calf were taking into evaluation. On occasions, when the larger calves proved difficult to catch, a slight increase of rectal temperature seemed to occur. However, this was rarely an issue.

When observing the variables 'Hair coat' and 'Umbilical region', the authors had the age group in mind. Calves less than 48 hours old with fresh umbilical regions and tousled hair coats, did not receive the scores '1' in either of the variables but were deemed as 'Normal'. For 'Weight bearing lameness', most calves were housed in deep bedding which could mask lameness' and hinder the correct observation. It is therefore possible that subtle lameness conditions were not discovered.

The variables 'Direct contact' and 'Indirect contact' were created to measure the likelihood of faecal-oral and aerosol transmission occurring. Solid walls were not considered as obstacles for 'Direct contact' if the calves were able to have nozzle contact through an opening, though the solid walls could lower the risk of faecal transmission between the pens.

## 4.5 *Salmonella* Dublin Herd Classification

The target population was chosen following the official Danish regulations regarding *S.* Dublin and the farmers willingness to participate. As mentioned, the ELISA-test are directed against serogroup-D: *S.* Dublin polysaccharide side chain of the LPS plate antigen (O:1,9,12). The test-prevalence of 'Level 2' herds was 10.9% as of March 2022 (SEGES Kvæg, 2022). The test may detect other *Salmonella* spp. since they share similar O-antigens. The cross-reaction mainly occurs with serogroup-B: *S.* Typhimurium LPS (O:1,4,5,12), but also with serogroup-A: *S.* Paratyphi (O:1,2,12)

(Smith et al., 1995). In Denmark, mainly *S.* Typhimurium cross-reacts (Nielsen et al., 2010) and 6.2 – 13% are estimated to be a result of cross-reactivity (Toft-Petersen, 2016). A misclassification could cause misinterpretations of the data used for this thesis as a smaller proportion of the clinical disease observed in case herds could be a result of *S.* Typhimurium or a concurrent infection with both *Salmonella* spp. within the herds.

The BTM status measured on lactating cows does not necessarily provide information of infection in young stock. In herds with separated barn areas for young stock and cows, the predictive value of the surveillance classifications based on BTM monitoring may therefore be low (Veling et al., 2002). As described in section 2.1 'Selection of study herds', the limit for new infection is set at ODC% > 25. 'Level 1' can therefore contain herds at a constant of ODC% i.e., 24, hence nominating herds with a chronic or subclinical infection of *S.* Dublin as 'most likely free of *Salmonella* Dublin'. If then, the BTM should rise above ODC% > 25, the same herds would then be categorized as herds with a new infection. They would then be included in this study with the presumption that clinical signs possibly observed to be of an acute nature. Furthermore, there are approximately 120 days in between each surveillance round in which introduction of disease is possible. The unknown time of introduction could also affect the nature of the clinical signs and serology measured in case herds in this study.

To further confirm the BTM status of the herds, serum ODC% for group C were measured. At animal-level 246 calves out of 412 calves did not have a measurable immunologic response. At herd-level, six case herds had no measurable immunologic response to *S.* Dublin (average ODC% of 0). Several reasons could explain why 1) an infection could be active among the sample group, but the infected calves had yet to serologically converted upon the day of the herd visit (Nielsen, 2013), 2) the infection had not yet reached the sample group potentially due to protective measures such as separate housing or adequate hygiene (Houe et al., 2014) or 3) the calf did not produce an antibody response (SEGES Kvæg, 2021). The two calves in control herds that had an ODC% >0 (8 and 30) were most likely due to potential contamination in the laboratory when analysing blood samples (Anonymous, 2021). However, if an infection did exist in the control herds, it could have occurred amongst the calves with no measurable ODC% in the BTM of the lactating cows or a dilution of the BTM to such an extent that it remained below detection at ODC% > 25.

An antibody reaction does not necessitate disease, but only proves *S.* Dublin exposure. A good immune status can give rise to a strong reaction and thereby high antibody levels (Roden et al., 1992). Paired samples within a shorter timeframe could have revealed if the antibody response were

increasing or decreasing. This could have provided valuable information of the infection and allowed for evaluation of calves potentially being cleared of visible clinical manifestations. There were no paired samples in this study, on the contrary disease groups were created to account for broader point in time. Hence, 'Auscultation' included in the respiratory disease group could identify pathological lung sounds as a result of both acute and chronic injury from a potentially recent *S.* Dublin infection. Nielsen et al., (2004) classified a calf test-positive, indicating *Salmonella*-exposure, if the antibody level was ≥50 ODC%. They estimated the Se of the serum ELISA a cut-off 50 ODC% for calves aged between 100 and 300 days to be approximately 0.77 and the Sp to 0.95. Lowering the cut-off increases the Se and lowers the Sp. As the Se is lower than the Sp, increasing the risk of false negatives, the true prevalence within the herds were likely underestimated.

The use of faecal sampling for bacterial culture was deliberately deselected as the diagnostic test of choice. Faecal sampling would provide a poor Se of 8-10% despite an Sp assumed at 1 (Nielsen et al., 2004) likely due to the intermittent shedding often in diluted concentrations (Houe et al., 2014). The advantage of faecal sampling, when not pooled, could have been the possibility to detect actively shedding individuals. However, the target condition for diagnostics used in this thesis were exposure and not testing for infectiousness.

Another possible diagnostic method could have been necropsy. As calves succumbing to infection were often bacteremic, isolation of *S.* Dublin from systemic sites could have provided evidence of causality. If calves were euthanized for necropsy during herd visits, it would have been best to sample calves during the acute stage of infection (Mohler et al., 2009)

# 5. Conclusion

A total of 1799 randomly selected individuals dispersed on 29 *S.* Dublin test-positive herds and 38 *S.* Dublin test-negative herds were subjected to a clinical assessment using a clinical score protocol including 19 clinical- and 6 environmental variables. They were separated into three groups (Group A: 0-10 days old, Group B: 14-28 days old and Group C: 100-130 days old). Group A and Group C were blood sampled. The distribution of clinical manifestations was illustrated in tables and from these, three disease categories were created to classify if either gastrointestinal, respiratory- or systemic disease occurred in calves in herds with a recent incursion of *S.* Dublin compared to calves in herds without a recent incursion of *S.* Dublin.

It was found that calves in Group C had significantly higher odds (OR = 8.0; 97.5% CI: 2.7 – 23.6) of having signs of gastrointestinal disease in *S.* Dublin test-positive herds compared to test-negative herds in a multivariable mixed effects model accounting for risk factors: ringworm, decreased cleanliness of the calf and a soiled environment. No other statistically significant associations were found between *S.* Dublin herd status and other disease categories, nor in other age-groups.

In conclusion, this thesis found a recent incursion of *S.* Dublin in naïve danish dairy herds had a propensity to cause enteric disease in calves older than most often described in literature. Simultaneously, less additional disease was found in the calves in naïve danish dairy herds with a recent incursion of *S.* Dublin. This is useful knowledge to take into account in the future planning of the control programme.

# 6. Perspectives

In this study calves in the age of 100-130 (180) days had higher odds (OR = 8.0; 97.5% CI: 2.7 – 23.6) of gastrointestinal disease in case herds compared to control herds when accounting for ringworm, decreased cleanliness of the calf and a soiled environment. No other associations were found with certainty between *S*. Dublin herd status and other disease categories, nor in other age-groups. These results can possibly aid farmers and veterinarians as the main findings of enteric disease differs from previous literature with varying clinical manifestations including signs of respiratory disease. The data can provide useful knowledge for use in the future planning of the '*Salmonella* control programme', although further research is needed to fully understand risk factors in naïve Danish dairy herds under danish conditions. Due to limitations of the sample size in this sub-project, it is recommendable to rerun the models once gathering of data is complete. A larger dataset might provide significant changes for the disease categories systemic and respiratory disease for all age groups.

One unmentioned challenge associated with the efforts to control and eradicate *S.* Dublin are the motivation of the farmers. Due to the variable clinical expression in infected herds and varying effects on farms, the producer's profitability incentive can be lacking (Houe et al., 2014). As this thesis found that *S.* Dublin had a propensity to cause enteric disease in calves older than first believed it could therefore be obvious to examine the consequences it would have on the growth rate of the calves. A stunted growth rate would negatively affect age at calving and first lactation milk yield.

The blood samples from this project can be used in further research to optimize the current surveillance of *S.* Dublin to help minimize cross-reactions when using ELISA as a diagnostic tool. Correct serogroup testing would aid in the customization of strategies for prevention and controlling disease as *S.* Dublin and *S.* Typhimurium differs in the persistence and survival within the herds.

# References


Anonymous, 2021. Section of Animal Welfare and Disease Control.

Brydensholt, M., Klompmaker, A., 2020. Diagnosing bovine respiratory disease in Danish calves less than four months old.

Buczinski, S., Gicquel, E., Fecteau, G., Takwoingi, Y., Chigerwe, M., Vandeweerd, J.M., 2018. Systematic Review and Meta-Analysis of Diagnostic Accuracy of Serum Refractometry and Brix Refractometry for the Diagnosis of Inadequate Transfer of Passive Immunity in Calves. Journal of Veterinary Internal Medicine. https://doi.org/10.1111/jvim.14893

Carrique-Mas, J.J., Willmington, J.A., Papadopoulou, C., Watson, E.N., Davies, R.H., 2010. Salmonella infection in cattle in Great Britain, 2003 to 2008. Veterinary Record 167, 560–565. https://doi.org/10.1136/vr.c4943

Department of Clinical Veterinary Medicine, U. of C., 2019. Infectious respiratory diseases (Medicine, Surgery and Reproduction).

Dillane, P., Krump, L., Kennedy, E., Sayers, R.G., Sayers, G.P., 2020. Determining the predictive capability of a Clinical Assessment Scoring Chart to differentiate severity of the clinical consequences of neonatal calf diarrhea relative to gold-standard blood gas analysis. PLoS ONE 15. https://doi.org/10.1371/journal.pone.0230708

Dohoo, I., Martin, W., Stryhn, H., 2009. Veterinary epidemiologic research , 2nd Edition. ed. VER Inc.

Dudek, K., Nicholas, R.A.J., Szacawa, E., Bednarek, D., 2020. Mycoplasma bovis Infections—Occurrence, Diagnosis and Control. Pathogens 9, 640. https://doi.org/10.3390/pathogens9080640

Enevoldsen, C., Hindhede, J., Kristensen, T., 1996. Dairy Herd Management Types Assessed from Indicators of Health, Reproduction, Replacement Milk Production. Journal of Dairy Science 79, 1221–1236. https://doi.org/10.3168/jds.S0022-0302(96)76475-5

Fertner, M., Toft, N., Martin, H.L., Boklund, A., 2016. A register-based study of the antimicrobial usage in Danish veal calves and young bulls. Preventive Veterinary Medicine 131, 41–47. https://doi.org/10.1016/j.prevetmed.2016.07.004

Gitter, M., Wray, C., Richardson, C., Pepper, R.T., 1978. Chronic Salmonella dublin infection in calves. The British veterinary journal 134, 113–114. https://doi.org/10.1016/S0007-1935(17)33535-2

Godden, S.M., Lombard, J.E., Woolums, A.R., 2019. Colostrum Management for Dairy Calves. Veterinary Clinics of North America - Food Animal Practice. https://doi.org/10.1016/j.cvfa.2019.07.005

Grønstøl, H., Osborne, A.D., Pethiyagoda, S., 1974. Experimental Salmonella Infection in Calves. 2. Virulence and the Spread of Infection, Source: The Journal of Hygiene.

Guizelini, C.C., Pupin, R.C., Leal, C.R.B., Ramos, C.A.N., Pavarini, S.P., Gomes, D.C., Martins, T.B., Lemos, R.A.A., 2019. Salmonellosis in calves without intestinal lesions. Pesquisa Veterinaria Brasileira 39, 580–586. https://doi.org/10.1590/1678-5150-PVB-6328

Guizelini, C.C., Tutija, J.F., Morais, D.R., Bacha, F.B., Ramos, C.A.N., Leal, C.R.B., Zaquetti, M.E., Lemos, R.A.A., 2020. Outbreak investigation of septicemic salmonellosis in calves. Journal of Infection in Developing Countries 14, 104–108. https://doi.org/10.3855/jidc.12087

Hardman, P., Wathes, C., Wray, C., 1991. Transmission of salmonellae among calves penned individually. Veterinary Record 129, 327–329. https://doi.org/10.1136/vr.129.15.327

Healy, A.M., Doherty, M.L., Monaghan, M.L., McAllister, H., 1997. Cervico-thoracic vertebral osteomyelitis in 14 calves. The Veterinary Journal 154, 227–232. https://doi.org/10.1016/S1090-0233(97)80027-5

Heinrichs, A., Rogers, G., Cooper, J., 1992. Predicting body weight and wither height in holstein heifers using body measurements. Journal of Dairy Science 3576–3581.

Hinton, M., 1974. Salmonella dublin abortion in cattle: studies on the clinical aspects of the condition. British Veterinary Journal 130, 556–563. https://doi.org/10.1016/S0007-1935(17)35742-1

Houe, H., Ersbøll, K.A., Toft, N., 2004. Introduction to Veterinary Epidemiology, 1. Edition. ed. Biofolia.

Houe, H., Rosenbaum Nielsen, L., Saxmose Nielsen, S., 2014. Control and Eradication of Endemic Infectious Diseases in Cattle. College Publications.

Hughes, K.L., Jones, T.E., 1973. Salmonella Dublin from cattle in Victoria. Australian Veterinary Journal 49, 175–176. https://doi.org/10.1111/j.1751-0813.1973.tb06779.x

Konrad, H., Smith, B.P., Dilling, G.W., House, J.K., 1994. Production of Salmonella serogroup D (O9)-specific enzyme-linked immunosorbent assay antigen. American Journal of Veterinary Researcg 55, 1647–1651.

Kudirkiene, E., Aagaard, A.K., Schmidt, L.M.B., Pansri, P., Krogh, K.M., Olsen, J.E., 2021. Occurrence of major and minor pathogens in calves diagnosed with bovine respiratory disease. Veterinary Microbiology 259. https://doi.org/10.1016/j.vetmic.2021.109135

Loeb, E., Toussaint, M.J.M., Rutten, V.P.M.G., Koeman, J.P., 2006. Dry Gangrene of the Extremities in Calves Associated with Salmonella dublin Infection; a Possible Immune-mediated Reaction. Journal of Comparative Pathology 134, 366–369. https://doi.org/10.1016/j.jcpa.2006.01.005

Losinger, W.C., Heinrichs, J.A., 1996. Management variables associated with high mortality rates attributable to respiratory tract problems in female calves prior to weaning. Article in Journal of the American Veterinary Medical Association 209, 1756–1759.

Martin, P.A.J., Smith, B.P., 1984. Control of salmonellosis in dairy calves, in: Proceedings of the International Symposium on Salmonella. pp. 194–198.

McDonough, P.L., Fogelman, D., Shin, S.J., Brunner, M.A., Lein, D.H., 1999. Salmonella enterica Serotype Dublin Infection: an Emerging Infectious Disease for the Northeastern United States. Journal of Clinical Microbiology 37, 2418–2427.

McGuirk, S.M., 2008. Disease Management of Dairy Calves and Heifers. Veterinary Clinics of North America - Food Animal Practice. https://doi.org/10.1016/j.cvfa.2007.10.003

Mohler, V.L., Izzo, M.M., House, J.K., 2009. Salmonella in Calves. Veterinary Clinics of North America - Food Animal Practice. https://doi.org/10.1016/j.cvfa.2008.10.009

Nazer, A.H.K., Osborne, A.D., 1977. Experimental Salmonella Dublin Infection in Calves. British Veterinary Journal 133, 388–398. https://doi.org/10.1016/S0007-1935(17)34040-X

Nielsen, B.H., Spelth, P., Martin, H.L., Petersen, B.M., 2018. Robuste kalve - Klinisk protokol.

Nielsen, L.R., 2013. Review of pathogenesis and diagnostic methods of immediate relevance for epidemiology and control of Salmonella Dublin in cattle. Veterinary Microbiology. https://doi.org/10.1016/j.vetmic.2012.08.003

Nielsen, L.R., Ersbøll, A.K., 2005. Factors associated with variation in bulk-tank-milk Salmonella Dublin ELISA ODC% in dairy herds. Preventive Veterinary Medicine 68, 165–179. https://doi.org/10.1016/j.prevetmed.2004.12.006

Nielsen, L.R., Toft, N., Ersbøll, A.K., 2004. Evaluation of an indirect serum ELISA and a bacteriological faecal culture test for diagnosis of Salmonella serotype Dublin in cattle using latent class models. Journal of Applied Microbiology 96, 311–319. https://doi.org/10.1046/j.1365-2672.2004.02151.x

Nielsen, L.R., van den Borne, B., van Schaik, G., 2007. Salmonella Dublin infection in young dairy calves: Transmission parameters estimated from field data and an SIR-model. Preventive Veterinary Medicine 79, 46–58. https://doi.org/10.1016/j.prevetmed.2006.11.006

Nielsen, T.D., 2012. Consequences of Salmonella Dublin on health and economy in Danish dairy cattle herds : PhD thesis. Faculty of Health Sciences, University of Copenhagen.

Nielsen, T.D., Nielsen, L.R., Toft, N., Houe, H., 2010. Association between bulk-tank milk Salmonella antibody level and high calf mortality in Danish dairy herds. Journal of Dairy Science 93, 304–310. https://doi.org/10.3168/jds.2009-2528

Noordhuizen, J., 2021. Managing Cold Stress in Young Dairy Calves: a Review. Archives of Veterinary and Animal Sciences 3, 1–5.

Pecoraro, H.L., Thompson, B., Duhamel, G.E., 2017. Histopathology case definition of naturally acquired Salmonella enterica serovar Dublin infection in young Holstein cattle in the northeastern United States. Journal of Veterinary Diagnostic Investigation 29, 860–864. https://doi.org/10.1177/1040638717712757

Raboisson, D., Trillat, P., Cahuzac, C., 2016. Failure of passive immune transfer in calves: A meta-analysis on the consequences and assessment of the economic impact. PLoS ONE 11. https://doi.org/10.1371/journal.pone.0150452

Ragione, R. la, Metcalfe, H.J., Villareal-Ramos, B., Werling, D., 2013. Salmonella in Domestic Animals, 2nd Edition. ed. CAB International.

Richardson, A., Fawcett, A.R., 1973. Salmonella dublin infection in calves: The value of rectal swabs in diagnosis and epidemiological studies, Br. vet.

Rings, M.D., 1985. Salmonellosis in Calves. Veterinary Clinics of North America: Food Animal Practice 1, 529–539. https://doi.org/10.1016/S0749-0720(15)31301-3

Robertsson, J.Å., 1984. Humoral antibody responses to exmerimental and spontaneous Salmonella infections in cattle measured by ELISA. Zentralblatt für Veterinärmedizin 367–380.

Roden, L. da, Smith, B.P., Spier, S.J., Dilling, G.W., 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 10, 1895–1899.

Segall, T., Lindberg, A.A., 1991. Experimental Oral Salmonella dublin Infection in Calves: A Bacteriological and Pathological Study. Journal of Veterinary Medicine 169–185.

SEGES Kvæg, 2022. Key Figures regarding Salmonella Dublin [WWW Document]. URL https://kvaegvet.dk/Dublin/AASduHist.html (accessed 3.4.22).

SEGES Kvæg, 2021. For veterinarians: How to use diagnostic tests in the fight against Salmonella Dublin [WWW Document]. URL https://www.landbrugsinfo.dk/public/b/0/5/sundhed_velfard_dyrlager_test_kvagbesatninger_salmonella (accessed 3.24.22).

Smith, B.P., Dilling, G.W., House, J.K., Konrad, H., Moore, N., 1995. Enzyme-linked immunosorbent assay for Salmonella serology using lipopolysaccharide antigen, J Vet Diagn Invest.

Smith, W.H., Jones, J.E.T., 1967. Observations on experimental oral infection with Salmonella dublin in calves andSalmonella choleraesuis in pigs. The Journal of Pathology and Bacteriology 93, 141–156. https://doi.org/10.1002/path.1700930114

Statens Serum Institut, 2019. Infection with Salmonella [WWW Document]. URL https://www.ssi.dk/sygdomme-beredskab-og-forskning/sygdomsleksikon/s/salmonellainfektion (accessed 3.21.22).

The Danish Meteorological Institute, 2022. Text, maps and key figures [WWW Document]. URL https://www.dmi.dk/vejrarkiv/manedens-sasonens-og-arets-vejr/tekst-kort-og-nogletal-maned/ (accessed 2.1.22).

Toft-Petersen, E.E., 2016. Misclassification of dairy herds in the Danish Salmonella Dublin surveillance. Copenhagen.

Tschoner, T., 2021. Methods for pain assessment in calves and their use for the evaluation of pain during different procedures—a review. Animals. https://doi.org/10.3390/ani11051235

Vaessen, M.A., Veling, J., Frankena, K., Graat, E.A.M., Klunder, T., 1998. Risk factors for Salmonella dublin infection on dairy farms. Veterinary Quarterly 20, 97–99. https://doi.org/10.1080/01652176.1998.9694848

Veling, J., Barkema, H.W., van der Schans, J., van Zijderveld, F., Verhoeff, J., 2002. Herd-level diagnosis for Salmonella enterica subsp. enterica serovar Dublin infection in bovine dairy herds. Preventive Veterinary Medicine 53, 31–42. https://doi.org/10.1016/S0167-5877(01)00276-8

Welfare Quality, 2009. Welfare Quality ® Assessment protocol for cattle.

Wray, C., Roeder, P.L., 1987. Effect of bovine virus diarrhoea—mucosal disease virus infection on salmonella infection in calves. Research in Veterinary Science 42, 213–218. https://doi.org/10.1016/S0034-5288(18)30688-X

Wray, C., Sojka, W.J., 1981. Salmonella dublin Infection of Calves: Use of Small Doses to Simulate Natural Infection on the Farm, Source: The Journal of Hygiene.

Wray, C., Sojka, W.J., 1977. Reviews of the progress of Dairy Science: bovine salmonellosis. Journal of Dairy Science 44, 383–425.

# Appendix

## Appendix A – The Salmonella Dublin protocol

| Observation | Number | Type | Age group | | |
|---|---|---|---|---|---|
| | | | Group A<br>0-10 days | Group B<br>14-28 (42) days | Group C<br>100-130 (180) days |
| **Eye discharge** | 1 | Clinical | x | x | x |
| **Nasal discharge** | 2 | Clinical | x | x | x |
| **Eardrop and/or head tilt** | 3 | Clinical | x | x | x |
| **Hair coat** | 4 | Clinical | x | x | x |
| **Cleanliness** | 5 | Clinical | x | x | x |
| **Hair loss, hindquarter** | 6 | Clinical | x | x | x |
| **Ringworm** | 7 | Clinical | x | x | x |
| **Body condition** | 8 | Clinical | x | x | x |
| **General condition** | 9 | Clinical | x | x | x |
| **Weightbearing, lameness** | 10 | Clinical | x | x | x |
| **Respiration type** | 11 | Clinical | x | x | x |
| **Auscultation** | 12 | Clinical | x | x | x |
| **Umbilical region** | 13 | Clinical | x | x | x |
| **Girth measure** | 14 | Clinical | x | x | x |
| **Rectal temperature** | 15 | Clinical | x | x | x |
| **Coughing** | 16 | Clinical | x | x | x |
| **Diarrhoea** | 17 | Clinical | x | x | x |
| **Necrosis pinnae** | 18 | Clinical | x | x | x |

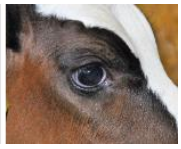
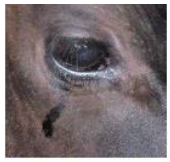
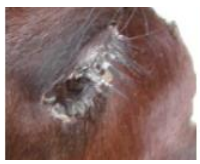
| | | | | | |
|---|---|---|---|---|---|
| **Pain** | 19 | Clinical | x | x | x |
| **Hygiene, bedding** | 20 | Environment | x | x | x |
| **Hygiene, water** | 21 | Environment | x | x | x |
| **Hygiene, feed** | 22 | Environment | x | x | x |
| **Contact, direct** | 23 | Environment | x | x | x |
| **Contact, indirect** | 24 | Environment | x | x | x |
| **Number of calves in pen** | 25 | Environment | x | x | x |
| **Blood sample** | 26 | Sample | x | | x |
| **Brix%** | 27 | Sample | x | | |

Pictures used in the protocol are from referenced articles, the Robust calves project and from herd visits with acceptance of the respective farmer.

## Clinical observations

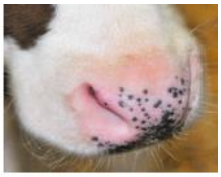
### 1. **Eye discharge** (Welfare Quality, 2009)

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

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>Normal, no discharge, dry eye surroundings. |   |
| **1** | **Serous**<br>Serous discharge (transparent, thin). |   |
| **2** | **Mucopurulent / purulent**<br>Mucopurulent or purulent discharge. Often stuck in eyelashes. Fresh pus and/or dry crusts. |   |

**2. Nasal discharge** (Welfare Quality, 2009)

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

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>Normal, no signs of fluid, exudate, or pus in the nostrils |   |
| **1** | **Serous**<br>Clear, serous fluid/exudate in one or both nostrils. No signs of mucopurulent or purulent exudate. |   |
| **2** | **Mucopurulent / purulent**<br>Cloudy (mucopurulent) or coupious in one or both nostrils. Fresh pus and/or dry crusts. |   |

**3. Eardrop and/or head tilt** (Welfare Quality, 2009)

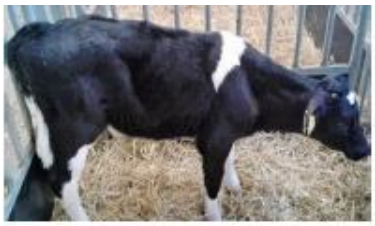
Look at the calf from the front. Examine the head and ears. Are the ears held equally high (at or above a horizontal line between them)? Is the head tilted (are the eyes at a horizontal line)?

| Score | Description | Example |
|---|---|---|
| **0** | **Normal positioned ears and head**<br>Normal positioned ears and head. Horizontal lines between ears and between eyes. |  |
| **1** | **Unilateral eardrop**<br>One ear positioned lower than the other. |  |

| | | |
|---|---|---|
| **2** | **Bilateral eardrop or head tilted**<br>Both ears are below a horizontal line drawn through the forehead or the head is tilted. |  <br>  |

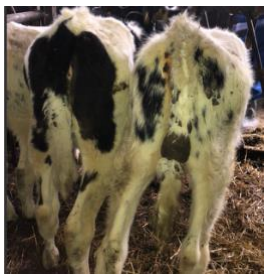
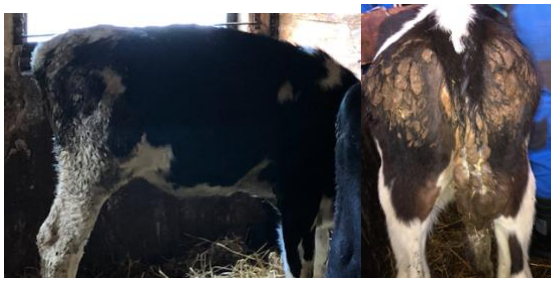
**4. Hair coat** (Nielsen et al., 2018)

Look at the calf from both sides and down the back. Assess the hair coat: is it glossy or dull? Is it looking ruffled?

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>Normal, glossy, healthy coat appearance, appropriate to season |  |
| **1** | **Rough looking, dull**<br>Rough looking, dull haircoat. Seems ruffled. Seem too long for the season. |  |

**5. Cleanliness** (Nielsen et al., 2018)

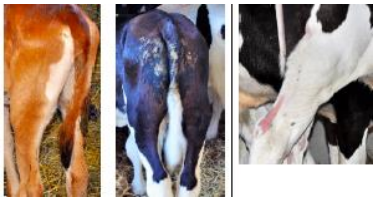
Calf must be standing. All of the body is examined except from the head and the legs from and below the hocks/knees. Soiled means fresh and/or dried cakes/stenches and/or moisture on shoulders, abdomen, sides and/or hindquarter/tail. The total area of all soiled areas is scored. Calves with blankets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

| Score | Description | Example |
|---|---|---|
| **0** | **Clean**<br>Less than the area of 2 palms soiled in total. |  |
| **1** | **Moderately soiled**<br>Area of in total 2 palms up to 25% soiled. |  |
| **2** | **Severely soiled**<br>At least 25% of the calf's surface is soiled. |  |

**6. Hairloss, hind** (Nielsen et al., 2018)

Look at the calf from behind. Examine the hair coat from the perianal area down towards the hocks. Hair loss is defined as areas equal or bigger than a palm (without fingers)

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>No signs of hair loss. |  |

| | | |
|---|---|---|
| **1** | **Hair loss**<br>Hair loss or hair regrowing after hair loss. |  |

**7. Ringworm** (Nielsen et al., 2018)

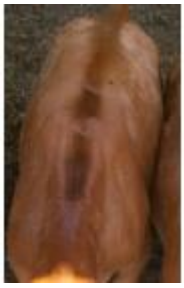
During the clinical examination of the calf, the skin is observed for signs of ringworm. All of the calf is observed.

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>Normal skin, no signs of ringworm. |  |
| **1** | **Ringworm**<br>Circular, scaling lesions. Can be found on the entire body. Typically non-itching. |  |

**8. Body Condition** (Welfare Quality, 2009)

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

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>Normal body condition - tuber coxae visible and rounded. Spine and transverse processes are distinguishable but rounded. |  |
| **1** | **Lean / very lean**<br>Protruding tuber coxae and spinal processes. Ends of transverse processes are distinguishable. Angular hooks and pins. |  |

| | | |
|---|---|---|
| **2** | **Overweight / fat**<br>Tuber coxae and ends of transverse processes not/almost not distinguishable. |  |

## 9. General condition (Dillane et al., 2020)

General condition is observed and evaluated when there is work in and around the pen. How is the calf responding upon activity outside the pen and again at entry of the pen?

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>'Bright, alert and responsive'. Calf is curious when there is activity around and in the pen. |  <br>  |
| **1** | **Depressed**<br>Depressed, less curious about activity in and around the pen. Only stands, when neared. |  <br>  |

| | | |
|---|---|---|
| **2** | **Comatose**<br>Minimal reaction to stimuli. Does not stand or is unable to stand, when neared in pen. |  |

**10. Weight bearing, lameness** (Nielsen et al., 2018)

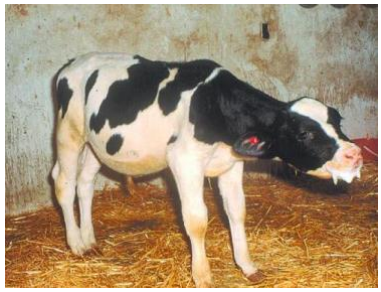
During the clinical examination of the standing calf, assess the weight bearing – is the calf putting equal weight on all four legs or is it relieving one or more legs. If the calf is found to be lame, it is then assessed by the criteria in score 1 and 2.

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>Normal weight bearing, equally on all four legs. No lameness. | |
| **1** | **Lameness**<br>One or more legs are relieved - or the calf stands reluctantly. Physical injury, congenital disease. No signs of inflammation. | |
| **2** | **Arthritis**<br>Lameness due to inflammation in one or more joints (swollen, warm joints). | |

**11. Respiration type** (Department of Clinical Veterinary Medicine, 2019)

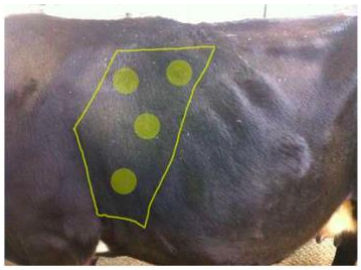
The calf is observed, and it is noted whether the respiration is predominantly thoracoabdominal or it is a ragged, forced, or laboured abdominal respiration.

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>Thoracoabdominal respiration. Minimal effort. | |

| | | |
|---|---|---|
| **1** | **Abnormal**<br>Mostly abdominal respiration, troubled or forced breathing. Open mouth breathing, extensive neck, unwilling to lie down. |  |

**12. Auscultation** (Department of Clinical Veterinary Medicine, 2019)

The calf is auscultated on the right and left side of thorax within the picture of the example.

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>Normal, vesicular lung sounds. |  |
| **1** | **Abnormal**<br>Rales, harsh, rhonci lung sounds. | |

**13. Umbilical region** (Nielsen et al., 2018)

During the clinical examination, palpate the umbilical region for swellings and signs of inflammation (warmth, pain). The examination is performed on a standing calf.

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>No swelling or warmth. | |
| **1** | **Swelling**<br>Swelling, but no signs of inflammation. | |
| **2** | **Inflammation**<br>Swelling with warmth and/or pain. | |

**14. Girth measure** (Nielsen et al., 2018)

Girth measurement is taken using a flexible tape measure. Measured in cm. The calf stands erect with the weight evenly distributed on both front legs. The measurement is taken at the level of the largest circumference of the calf. The maximal girth is not always obvious, and the tape may need to be moved up and down to find the point of maximum circumference.

**15. Rectal temperature** (Nielsen et al., 2018)

Body temperature measured rectally. Numeric value with one digit.

**16. Coughing** (Nielsen et al., 2018)

During the clinical examination of the calf, it is observed whether the calf coughs spontaneously. Coughing is evaluated during the examination or when an observer is present in the stable and can identify the calf.

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>No coughing. | |
| **1** | **Abnormal**<br>The calf coughs one or several times. | |

**17. Diarrhoea** (Mohler et al., 2009)

It is noted whether the calf has watery or bloody diarrhoea during the clinical examination. Faeces can be visually evaluated during the rectal temperature procedure. The evaluation of diarrhoea is only visual and obtained in the time lapse where the calf and the surroundings are evaluated.

| Score | Description | Example |
|---|---|---|

| | | |
|---|---|---|
| **0** | **Normal**<br>No diarrhoea, dry and clean at hindquarters. Faeces has a pasty-like consistency, moldable. |  |
| **1** | **Watery diarrhoea**<br>The calf is not clean at hindquarters, watery, very little texture, runny between fingers if unavoidable. |  |
| **2** | **Bloody diarrhoea**<br>The calf is not clean at hindquarters, watery, very little texture, runny between fingers if unavoidable with fresh or coagulated blood. | |

**18. Necrosis of pinnae and tail** (Loeb et al., 2006)

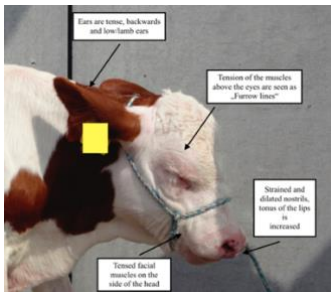
It is noted whether necrosis of pinnae or tail is present during the examination of the calves. In case of such findings, pictures will be taken to be used in the master thesis.

| Score | Description | Example |
|---|---|---|
| **0** | **Normal**<br>No necrosis of pinnae or tail. | |
| **1** | **Necrosis**<br>Necrotic areas are found on the pinnae and/or tail. Healed areas are included. |  |

**19. Pain** (Tschoner, 2021)

If the calf is in pain with or without obvious cause this score is used. Usually in cases of scoring of general condition.

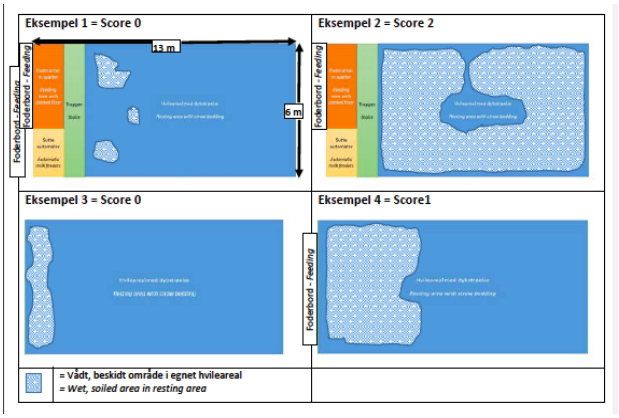
| Score | Description | Example |
|---|---|---|

| | | |
|---|---|---|
| **0** | **Normal**<br>No clear sign of pain. | |
| **1** | **Pain**<br>Presence of one or more of the following parameters: abdominal cramping and/or lordose and/or unwillingness to move, and/or vocalisation and/or pain face. |  |

## Environmental parameters

**20. Hygiene, bedding** (Nielsen et al., 2018)

Assess how much of the total area available to the calf is dry, clean, and suitable for resting. Stairs, slatted feeding area and slanted areas are measured as part of the total area but are not deemed suitable for resting. Therefore, measure how much of the total area has a dry, clean surface suitable for resting. Areas around automatic feeders are not considered in either total area or resting area.

| Score | Description | Example |
|---|---|---|
| **0** | >75% dry/clean, sufficient amount |  |
| **1** | 50-75% dry/clean, less sufficient | |
| **2** | <50% dry/clean, non-sufficient amount | |

**21. Hygiene, water** (Welfare Quality, 2009)

Examine the water points in the calf pen (trough, reservoir, bowl or alike) and visually score the cleanliness. Presence of old and/or fresh dirt/food residues and manure as well as staining of water.

| Score | Description | Example |
|---|---|---|

| | | |
|---|---|---|
| **0** | **Clean**<br>No or small amount of fresh food residues in the trough/water. Milk mixed with milk or electrolytes |  |
| **1** | **Sign of dirty/manure/unclear water**<br>Signs of water and/or slimy/greasy coating and/or biofilm/manure. Might be specks, smaller area on environment. |  |
| **2** | **Clearly dirty/manure/unclear water**<br>The water is obviously contaminated with slimy/greasy coating and/or biofilm/manure |  |

**22. Hygiene, feed** (Welfare Quality, 2009)

Examine the feeding troughs in the calf pen (trough, reservoir, bowl or alike) and visually score the cleanliness. Presence of old and/or fresh dirt/feed residues and manure.

| Score | Description | Example |
|---|---|---|
| **0** | **Clean**<br>Clean trough or trough with dry food residues. |  |
| **1** | **Signs of dirt**<br>The through is slightly dirty, or there are signs of manure. Might be specks of rotting food residues. |  |
| **2** | **Clearly dirty/manure**<br>Severely dirty – moldy, rotting food residues and/or manure. | |

**23. Contact, direct** (Anonymous, 2021)

Contact directly indicates the number a calf can have direct nozzle contact with in the pen or through accessible fencing. The number is noted for each calf. The calf itself is not included in the number.

| | |
|---|---|
| **Number, numeric** | |

**24. Contact, indirect** (Anonymous, 2021)

This indicates the number of calves who potentially can have nozzle contact through one another. It determines the chain of calves who can transfer infection through contact. Ex. younger calves housed in rows with only wire mesh between them. Ex. the calf at the end of the line of 8 will have indirect contact with 8, but only direct contact to one calf.

| | |
|---|---|
| **Number, numeric** | |

**25. Number of calves in pen** (Nielsen et al., 2018)

Number of calves in the pen including itself. 1 indicates the calf is housed in a single pen.

| | |
|---|---|
| **Number, numeric** | |

**26. Blood samples** (Nielsen et al., 2018)

Blood samples can be drawn from the jugular vein or from the coccygeal vein. Tubes need to be filled at least 2/3. Identification of samples: The sample is marked with herd ID number, group, and the number of the calf. Ex. 1_A_19 which is noted on the side of the sample. The number of the calf matches the registrations from the herd.

**27. Brix%** (Nielsen et al., 2018)

Blood samples are drawn from the jugular vein for the purpose of measuring Brix%. Store the samples after collection at environmental temperature for 1-2 hours. Next the samples are spun in a Eickemeyer PLC-02 centrifuge at location at the setting 'HIGH' (4500 rounds pr minute) for a total of 10 minutes. The samples are hereafter pipetted and Brix% are evaluated on two different Atago-PAL-1. Note results individually and calculate a mean. The remaining serum are stored in serum tubes marked with the calf ID for traceability. The cut-off for 'sufficient' brix% is >8.4 and for 'poor' quality <8.4. (Godden et al., 2019).

# Appendix B – Tentative disease models of respiratory and systemic disease

## A.B.1 Respiratory disease model for Group B

**Table 11.** Results from the final logistic regressions model of the probability of having respiratory disease as a Danish dairy herd calf Group B in a case compared to control herd and other different explanatory variables. The table includes parameter estimate, standard error (SE), p-value of fixed effects, odds ratio (OR) with 97.5% confidence interval (CI) and variance and standard deviation (SD) of the *random effect*.

| **Variables** | | **Estimate** | **SE** | **P** | **OR** | **97.5% CI of OR** | $\sigma^2$ | **SD** |
|---|---|---|---|---|---|---|---|---|
| *Fixed effects* | | | | | | | | |
| Intercept | | -2.33 | 0.92 | * | | | | |
| Case control | | | | - | | | | |
| | Control | Ref | - | | - | - | | |
| | Case | -0.09 | 0.56 | - | 0.9 | (0.3-2.7) | | |
| Body Condition | | | | - | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | -1.09 | 0.57 | - | 0.3 | (0.1-1.0) | | |
| Hygiene bedding | | | | - | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | 0.46 | 0.63 | - | 1.6 | (0.5-5.5) | | |
| | 2 | -1.25 | 1.04 | - | 0.3 | (0.04-2.2) | | |
| Hygiene feed | | | | - | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | 0.18 | 0.78 | - | 1.2 | (0.3-5.5) | | |
| | 2 | 1.39 | 0.96 | - | 4.0 | (0.6-26.3) | | |
| Hygiene water | | | | - | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | 0.97 | 0.67 | - | 2.6 | (0.7-9.7) | | |
| | 2 | 0.49 | 0.93 | - | 1.6 | (0.3-10.1) | | |
| Calf no pen | | | | * | | | | |
| | | 0.16 | 0.07 | * | 1.2 | (1.0-1.4) | | |
| Season | | | | - | | | | |
| | Early Fall | Ref | - | | | | | |
| | Late Fall | -1.16 | 0.66 | - | 0.3 | (0.1-1.1) | | |
| | Winter | -1.27 | 0.98 | - | 0.3 | (0.04-1.9) | | |
| Observer | | | | * | | | | |
| | Author 1+2 | Ref | - | | - | - | | |
| | Author 1 | -0.31 | 0.79 | - | 0.7 | (0.2-3.5) | | |
| *Random effect* | | | | | | | | |
| Herd ID | | | | | | | 1.88 | 1.37 |

* = *p*-value < .05, ** = *p*-value < .01, *** = *p*-value < .001

The results of the explanatory variables from the tentative mixed effects logistic regression model respiratory disease for Group B are shown in Table 11. There appeared to be no significant association between 'Respiratory disease' and case or control herds. In this model the variables 'Body condition', 'Hygiene bedding', 'Hygiene water', Hygiene feed', 'Season' and 'Observer' were confounding explanatory variables. There were no significant interactions in this model. Explanatory variables 'Calf no pen' and 'Observer' were significant. 'Calf no pen' seemed to be associated with 'Respiratory disease'.

## A.B.2 Respiratory disease model for Group C

**Table 12.** Results from the final logistic regressions model of the probability of having respiratory disease as a Danish dairy herd calf Group C in a case compared to a control herd and other different explanatory variables. The table includes parameter estimate, standard error (SE), p-value of fixed effects, odds ratio (OR) with 97.5% confidence interval (CI) and variance and standard deviation (SD) of the *random effect*.

| **Variables** | | **Estimate** | **SE** | **P** | **OR** | **97.5% CI of OR** | **$\sigma^2$** | **SD** |
|---|---|---|---|---|---|---|---|---|
| *Fixed effects* | | | | | | | | |
| Intercept | | -1.07 | 0.52 | * | | | | |
| Case control | | | | * | | | | |
| | Control | Ref | - | | - | - | | |
| | Case | -0.34 | 0.31 | - | 0.7 | (0.4-1.3) | | |
| Hygiene feed | | | | ** | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | -1.38 | 0.38 | *** | 0.3 | (0.1-0.5) | | |
| | 2 | -1.74 | 0.52 | *** | 0.2 | (0.1-0.5) | | |
| Season | | | | - | | | | |
| | Early Fall | Ref | - | | - | - | | |
| | Late Fall | 0.65 | 0.35 | - | 1.9 | (1.0-3.8) | | |
| | Winter | 1.58 | 0.52 | ** | 4.9 | (1.8-13.5) | | |
| Observer | | | | - | | | | |
| | Author 1+2 | Ref | - | | - | - | | |
| | Author 1 | 0.08 | 0.44 | - | 1.1 | (0.5-2.6) | | |
| Contact indirect | | | | - | | | | |
| | | -0.04 | 0.03 | - | 1.0 | (0.9-1.0) | | |
| *Random effect* | | | | | | | | |
| Herd ID | | | | | | | 0.71 | 0.84 |

* = *p*-value < .05, ** = *p*-value < .01, *** = *p*-value < .001

The results of the explanatory variables from the tentative mixed effects logistic regression model 'Respiratory disease' for Group C are shown in Table 12. The variables 'Case control' and 'Hygiene feed' seemed significant. However, there were' no significant difference between case herds and control herds. On the opposite this tentative model suggested if the herds had a score of 'Hygiene feed' '1' or '2' it was less associated with 'Respiratory disease'. In this model 'Contact indirect' (scaled), 'Season' and 'Observer' appeared as confounders. The score 'Winter' was associated with more 'Respiratory disease' compared to 'Early Fall'. Furthermore, the score 'Late Fall' was borderline significant (OR = 1.9; 97.5% CI: 1.0-3.8).

### A.B.3 Systemic disease model for Group B

**Table 13:** Results from the final logistic regressions model of the probability of having systemic disease as a Danish dairy herd calf Group B in a case compared to control herd and other different explanatory variables. The table includes parameter estimate, standard error (SE), p-value of fixed effects, odds ratio (OR) with 97.5% confidence interval (CI) and variance and standard deviation (SD) of the *random effect*.

| **Variables** | | **Estimate** | **SE** | **P** | **OR** | **97.5% CI of OR** | $\sigma^2$ | **SD** |
|---|---|---|---|---|---|---|---|---|
| *Fixed effects* | | | | | | | | |
| Intercept | | -3.73 | 0.52 | *** | | | | |
| Case control | | | | - | | | | |
| | Control | Ref | - | | - | - | | |
| | Case | 1.00 | 0.52 | - | 2.7 | (1.0-7.5) | | |
| Cleanliness | | | | - | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | 0.57 | 0.37 | - | 1.8 | (0.9-3.7) | | |
| | 2 | 1.16 | 0.51 | * | 3.2 | (1.2-8.6) | | |
| *Random effect* | | | | | | | | |
| Herd ID | | | | | | | 1.84 | 1.36 |

* = *p*-value < .05, ** = *p*-value < .01, *** = *p*-value < .001

The results of the explanatory variables from the tentative mixed effect logistic regression systemic disease model for Group B are shown in Table 13. There appeared to be no significant association between 'Systemic disease' and case or control herds. There were no significant explanatory variables, confounders, or interactions. 'Cleanliness' score '2' appeared as the only significant variable in this tentative model. For 'Cleanliness' score '2' the odds increased (OR = 3.2; 97.5% CI: 1.2-8.6).

## A.B.4 Systemic disease model for Group C

**Table 14:** Results from the final logistic regressions model of the probability of having respiratory disease as a Danish dairy herd calf Group C in a case compared to a control herd and other different explanatory variables. The table includes parameter estimate, standard error (SE), p-value of fixed effects, odds ratio (OR) with 97.5% confidence interval (CI) and variance and standard deviation (SD) of the *random effect*.

| **Variables** | | **Estimate** | **SE** | **P** | **OR** | **97.5% CI of OR** | $\sigma^2$ | **SD** |
|---|---|---|---|---|---|---|---|---|
| *Fixed effects* | | | | | | | | |
| Intercept | | -2.81 | 0.78 | *** | | | | |
| Case control | | | | - | | | | |
| | Control | Ref | - | | - | - | | |
| | Case | -0.36 | 0.50 | - | 0.7 | (0.3-1.9) | | |
| Ringworm | | | | - | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | 0.36 | 0.44 | - | 1.4 | (0.6-3.4) | | |
| Body Condition | | | | *** | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | 1.25 | 0.42 | ** | 3.5 | (1.5-7.9) | | |
| Cleanliness | | | | - | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | -0.43 | 0.46 | - | 0.7 | (0.3-1.6) | | |
| | 2 | -0.17 | 0.69 | - | 0.8 | (0.2-3.3) | | |
| Hygiene bedding | | | | * | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | 0.80 | 0.64 | - | 2.2 | (0.6-7.8) | | |
| | 2 | 0.94 | 0.60 | - | 2.6 | (0.8-8.2) | | |
| Hygiene feed | | | | - | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | -0.65 | 0.73 | - | 0.5 | (0.1-2.2) | | |
| | 2 | -0.18 | 1.06 | - | 0.8 | (0.1-6.7) | | |
| Hygiene water | | | | - | | | | |
| | 0 | Ref | - | | - | - | | |
| | 1 | -0.41 | 0.45 | - | 0.7 | (0.3-1.6) | | |
| | 2 | -1.32 | 0.80 | - | 0.3 | (0.1-1.3) | | |
| Calf no pen | | | | * | | | | |
| | | 0.06 | 0.02 | ** | 1.1 | (1.0-1.1) | | |
| Season | | | | - | | | | |
| | Early Fall | Ref | - | | - | - | | |
| | Late Fall | -0.40 | 0.60 | - | 0.7 | (0.2-2.2) | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Winter | -0.92 | 0.91 | - | 0.4 | (0.1-2.4) | | |
| Observer | | | | - | | | | |
| | Author 1+2 | Ref | - | | - | - | | |
| | Author 1 | -1.77 | 0.72 | ** | 0.2 | (0.04-0.7) | | |
| *Random effect* | | | | | | | | |
| Herd ID | | | | | | | 0.30 | 0.55 |

* = *p*-value < .05, ** = *p*-value < .01, *** = *p*-value < .001

The results of the explanatory variables from the tentative mixed effect logistic regression systemic disease model for Group C are shown in Table 14. There appeared to be no significant association between systemic disease and case or control herds. In this tentative model ‘Ringworm’, ‘Body condition’, ‘Cleanliness’, ‘Hygiene bedding’, ‘Hygiene feed’, ‘Hygiene water’, ‘Calf no pen’, ‘Season’ and ‘Observer’ were confounding with ‘Case control’. The variables ‘Body condition’, ‘Hygiene bedding’ and ‘Calf no pen’ were significantly associated with the probability of ‘Systemic disease’. Should the calf be scored ‘Body condition’ ‘1’, there was a higher probability of having systemic disease. Should ‘Hygiene bedding’ be scored ‘2’ the probability of ‘Systemic disease’ increases. Furthermore, if the calf was housed in a pen with several housing mates, the probability of disease was higher.